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ANALYSIS OF PROTEIN FRACTION CONTENT AND PEPTIDE ACTIVITY OF *COIX LACRYMA-JOBI* L. VAR. *MA-YUEN* STAPF (POACEAE) FROM DIFFERENT REGIONS OF CHINA

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Abstract

Coix lacryma-jobi L. var. *ma-yuen* Stapf has been used for the treatment of edema, dysuria, and diarrhea in traditional Chinese medicine for many years. Main components and the protein fractions content, including albumin, globulin, prolamin, and glutelin, of 11 batches of this plant collected from different regions of China were studied. Small molecular peptides were generated by hydrolyzing glutelin with pepsin. Angiotensin converting enzyme (ACE) inhibitory activity of the peptides and triolein, the main active pharmaceutical ingredient, were evaluated by UV-HPLC and ELSD-HPLC, respectively. In the protein fractions, glutelin content ranged from 35.48% to 40.81% with significant differences among batches. Diversity in ACE inhibitory activity was also detected. HCA results showed that samples Y1 from Liaoning Muren, Y5 from Yunnan Qujing, and Y6 from Yunnan Lancang manifested great antihypertensive potential. The data generated from this study revealed the characteristic diversity of *Coix* collected from different locations, which will facilitate the utilization of antihypertensive peptides from glutelin.

Key words: *Coix*; Angiotensin converting enzyme; Glutelin; Triolei.

Introduction

Coix lacryma-jobi L. var. *ma-yuen* Stapf of the family Poaceae, commonly known as adlay or Job's tears, is a vital medicine and food worldwide. It is used for the treatment of edema, dysuria, and diarrhea in clinical traditional Chinese medicine based on the Chinese Pharmacopoeia Commission (2020). *Coix* has diverse pharmacological functions, such as anticancer (Chang *et al.*, 2003), antioxidant (Wang *et al.*, 2015a), hypolipidemic (Ha *et al.*, 2010) and hypoglycemic (Lin *et al.*, 2010). Notably, Kanglaite, made from *Coix* seed oil, applied in the treatment of multiple cancers is approved by the Food and Drug Administration to undergo clinical trials in the United States (Normile & Dennis, 2003). Thus, there is increasing demand for the *Coix* seeds in a medical setting. However, previous studies were mainly focused on *Coix* agronomic characteristics (Zhang *et al.*, 2019) and chemical components (Zhu, 2016). The bioactivity of *Coix* mainly depends on the plant variety. Genetic diversity has been evaluated in *Coix lacryma-jobi* mainly originating from Guangxi, Guizhou, and Yunnan Provinces in southern China (Li *et al.*, 2001; Ma *et al.*, 2010). Moreover, *Coix* is widely distributed in most Chinese provinces, except Qinghai and Ningxia. Meanwhile, the quality of medicinal materials is mainly related with the environment of the production area. Owing to the great differences in the geographical environment and cultivation conditions, *Coix* germplasm resources in China are extremely rich and diverse. Furthermore, the differences in the protein fraction distribution of *Coix* batches from different regions are still not fully understood.

Angiotensin converting enzyme (ACE) plays an important role in the renin-angiotensin-aldosterone system, which catalyzes the conversion of angiotensin I to the potent vasoconstrictor octapeptide angiotensin II (Wysocki *et al.*, 2006). ACE inhibitors (captopril, enalapril, ramipril etc.) are recommended as first-line therapy for patients with cardiovascular disease. Therefore, increasing attention has been focused on identifying ACE inhibitory peptides from natural plant and animal proteins, including maize (Wang *et al.*, 2015b), wheat (Zou *et al.*, 2020), rice (Pinciroli *et al.*, 2019), and sorghum (Kamath *et al.*, 2007). Most of these ACE inhibitory peptides can be obtained via the enzymatic release from precursor proteins *in vitro* or *in vivo*. A novel remarkable antihypertensive peptide derived from *Coix* glutelin enzymatic hydrolysate was obtained previously by our research group (Li *et al.*, 2017a).

The objectives of this study were as follows: (1) to determine the main components content of 11 *Coix lacryma-jobi* var. *ma-yuen* batches from different regions of China; (2) to compare the diversity of protein fractions and triolein (one major active pharmaceutical ingredient) content in different batches; (3) to evaluate the ACE inhibitory activities of peptides from *Coix* glutelin and provide a foundation for parent selection in *Coix* breeding, especially with respect to the treatment of hypertension. Our data will provide a solid basis for scientific research and clinical application of *C. lacryma-jobi*.

Materials and Methods

Plant material and chemicals: Eleven batches of *C. lacryma-jobi* var. *ma-yuen* seed samples (Fig. 1) were collected from different areas in China and authenticated by Prof. Lingzhi Wang. The voucher specimens were deposited at Beijing University of Chinese Medicine with reference numbers, and detailed information was listed in Table 1. Pepsin, ACE, hippuryl-L-histidyl-L-leucine (HHL) and hippuric acid (HA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Captopril was purchased from Beijing Shuguang Pharmaceutical Co., Ltd (Beijing, China). Acetonitrile and isopropanol (HPLC grade) were supplied by Fisher Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid (MS grade) was purchased from Merck KGaA (Darmstadt, Germany). All other chemicals and reagents were of analytical grade.

Fig. 1. *Coix* seed samples.

Table 1. Collection information of 11 batches samples of *Coix*.

No.	Source area	Specimen No.
Y1	Muren, Liaoning Province, PRC	NO. 20180901
Y2	Xianyou, Fujian Province, PRC	NO. 20180902
Y3	Xingren, Guizhou Province, PRC	NO. 20180903
Y4	Xingren, Guizhou Province, PRC	NO. 20180904
Y5	Qujing, Yunnan Province, PRC	NO. 20180905
Y6	Lancang, Yunnan Province, PRC	NO. 20180906
Y7	Anguo, Hebei Province, PRC	NO. 20180907
Y8	Anguo, Hebei Province, PRC	NO. 20180908
Y9	Jinchun, Hubei Province, PRC	NO. 20180909
Y10	Guizhou Province, PRC	NO. 20181001
Y11	Guizhou Province, PRC	NO. 20181002

Methods

Determination of main component content in *Coix* seeds: The *C. lacryma-jobi* var. *ma-yuen* seeds were powdered and filtered through a 40-mesh sieve. The moisture and ash content of the sample was measured by the drying method and muffle furnace method described in the Pharmacopoeia of the People's Republic of China (2020). Starch content was determined according to the Ewers polarimetric method-ISO 10520:1997. Fat content was determined by the Soxhlet extraction method (Li *et al.*, 2000). Total protein was determined by the Kjeldahl method (Sapan & Lundblad, 2015).

Extraction and determination of *Coix* seed protein: The seed powder was defatted in cooled petroleum ether and dried overnight. Albumin, globulin, prolamin, and glutelin were sequentially extracted according to the method reported by Yuan *et al.*, (2014). Bradford method was used to quantify the albumin and globulin contents. Contents of prolamin, glutenin, and the residue were determined by the Kjeldahl method with three replicates.

Preparation of glutelin peptides of *Coix* seeds: To produce bioactive peptides from *Coix* glutelin, the enzymatic hydrolysis method was applied (Li *et al.*, 2017a). Glutenin powder was hydrolyzed by pepsin. The hydrolysis was performed at 37℃ for 48 h with 2% (w/v) substrate concentration and an enzyme-to-substrate ratio of 1:10 (w/w). Then the supernatant was transferred to the centrifugal ultrafiltration device (Amicon Ultra-15, MIllipore) with a 3 kDa molecular weight cutoff and then centrifuged at 5,000 rpm for 10 minutes at 4℃. The filtrate was collected to obtain peptides with a molecular weight less than 3 kDa.

Determination of ACE inhibitory activity *In vitro*: The peptide concentration was determined by the Lowry method. The ACE inhibitory activity of the samples was determined by monitoring the formation of HA generated from the substrate HHL (Chen *et al.*, 2020), with captopril used as the positive control and borate buffer solution as the blank control.

Characterization of *Coix* seed oil by thin-layer chromatography (TLC): The identification of *Coix* seed oil was conducted with reference to the Pharmacopoeia of the People's Republic of China (2020). The reference substance was dissolved in petroleum ether (60~90℃) to prepare a standard solution at a concentration of 4 mg/mL. The samples were extracted with the appropriate amount of petroleum ether (60~90℃). After ultrasonic treatment for 15 min at room temperature, the clear filtrate was collected for further analysis. Then, 2 μL of sample was spotted on the silica gel TLC plate and developed in the mobile phase containing petroleum ether-ethyl acetate-acetic acid (25:5:1). The chromatograms were stained with 5% vanillin/sulfuric acid-ethanol solution.

Determination of triolein content: Exactly 3.0 g of dried powder was dissolved in 30 mL of acetonitrile-isopropanol (51:49, v/v), soaked for 2 h, and then subjected to ultrasonic treatment for 30 min. The solution was filtered with a 0.45 μm membrane and kept at 4℃ for analysis. The triolein content was determined by HPLC-ELSD method as described by He et al. with slight modification (He *et al.*, 2020). The chromatographic separation was performed using an Anilent 1100 HPLC (Agilent, USA) equipped with a Shimadzu ELSD-LT II detector (Shimadzu, Japan). The analytes were separated using a C18 column (250 × 4.6 mm, 5 μm, Kromasil) with acetonitrile-isopropanol (51:49, v/v) as the mobile phase for isocratic elution. The column temperature was set 30℃, and the flow rate was 1.0 mL/min with an injection volume of 10 μL. The flow rate of the nebulizer gas was 2.0 L/min with a drift tube temperature of 70℃. Stock standard solution was also prepared with the mobile phase and diluted to a working standard concentration of 50 - 2,000 μg/mL. Quantification was conducted from peak areas of the samples by the standard graph.

Table 2. Main component content of *Coix* seeds from different batches.

No.	Moisture (%)	Ash (%)	Fat (%)	Starch (%)	Triolein (%)	Total protein (mg/100mg)
Y1	10.80 ± 0.02^{d}	1.54 ± 0.02^{d}	4.50 ± 0.10^{f}	72.37 ± 2.86^{bc}	0.50 ± 0.02^{f}	12.72 ± 0.08^{d}
Y2	10.98 ± 0.02^{c}	0.49 ± 0.03^{f}	1.17 ± 0.04^{i}	82.06 ± 0.57^{a}	0.31 ± 0.00^{g}	12.54 ± 0.08^{d}
Y3	10.20 ± 0.04^{e}	1.75 ± 0.01^{c}	7.74 ± 0.12^{b}	68.99 ± 1.49^{c}	0.56 ± 0.038^{e}	14.26 ± 0.07^{b}
Y4	10.23 ± 0.06^{e}	1.52 ± 0.01^{de}	2.62 ± 0.10^{h}	71.83 ± 0.36^{bc}	0.68 ± 0.02^{cd}	13.81 ± 0.05^{bc}
Y5	11.88 ± 0.04^{a}	1.73 ± 0.10^{c}	6.60 ± 0.04^{c}	72.46 ± 2.00^{bc}	0.68 ± 0.03^{cd}	12.88 ± 0.01^{d}
Y6	11.00 ± 0.03^{c}	1.52 ± 0.01^{de}	5.17 ± 0.20^{e}	72.06 ± 2.76^{bc}	0.68 ± 0.03^{cd}	12.80 ± 0.08^{d}
Y7	11.21 ± 0.02^{b}	1.49 ± 0.07^{de}	5.75 ± 0.18^{d}	69.50 ± 1.98^{c}	0.95 ± 0.05^{b}	14.23 ± 0.27^{b}
Y8	10.81 ± 0.05^{d}	2.03 ± 0.01^{a}	8.30 ± 0.16^{a}	70.40 ± 2.00^{c}	1.33 ± 0.06^{a}	14.72 ± 0.32^{a}
Y9	10.75 ± 0.02^{d}	1.42 ± 0.01^{e}	3.92 ± 0.15^{g}	76.03 ± 1.63^{b}	0.64 ± 0.01^{d}	14.15 ± 0.34^{b}
Y10	10.97 ± 0.05^{c}	1.87 ± 0.01^{b}	5.17 ± 0.20^{e}	75.93 ± 0.30^{b}	0.74 ± 0.02^{c}	12.72 ± 0.11^{d}
Y11	9.84 ± 0.04^{f}	1.89 ± 0.01^{b}	4.73 ± 0.10^{f}	69.86 ± 1.73^{c}	0.67 ± 0.01^{cd}	13.69 ± 0.09^{c}

Note: Different letters of indicated having significantly different (p<0.05)

Table 3. Protein fraction content of *Coix* seeds from different batches.

No.	Albumin	Globulin	Prolamin	Glutelin	Residue
	Percent of total protein (%)				
Y1	1.36 ± 0.04^{c}	3.95 ± 0.07^{cd}	52.62 ± 0.77^{a}	38.35 ± 1.02^{bc}	5.16 ± 0.60^{a}
Y2	1.62 ± 0.03^{b}	3.54 ± 0.06^{e}	50.70 ± 0.66^{a}	38.14 ± 0.66^{bcd}	2.62 ± 0.76^{c}
Y3	1.78 ± 0.06^{a}	4.55 ± 0.08^{a}	46.63 ± 1.00^{bc}	39.48 ± 0.29^{ab}	3.99 ± 0.69^{abc}
Y4	1.21 ± 0.06^{e}	3.50 ± 0.06^{e}	40.89 ± 1.03^{bc}	35.48 ± 1.03^{d}	3.96 ± 0.69^{abc}
Y5	1.32 ± 0.01^{c}	4.32 ± 0.11^{b}	50.61 ± 1.13^{a}	35.67 ± 1.22^{cd}	4.25 ± 0.29^{ab}
Y6	1.13 ± 0.05^{e}	3.62 ± 0.11^{e}	52.86 ± 1.71^{a}	39.19 ± 0.64^{ab}	3.93 ± 0.30^{abc}
Y7	1.33 ± 0.04^{c}	3.20 ± 0.17^{f}	47.96 ± 1.00^{b}	36.07 ± 1.53^{cd}	4.46 ± 0.51^{ab}
Y8	1.76 ± 0.06^{a}	4.10 ± 0.10^{c}	44.19 ± 0.74^{c}	38.04 ± 0.97^{bcd}	3.12 ± 0.49^{bc}
Y9	1.02 ± 0.02^{f}	2.74 ± 0.11^{g}	46.38 ± 1.34^{bc}	40.81 ± 1.01^{a}	4.48 ± 0.51^{ab}
Y10	1.26 ± 0.05^{cd}	3.85 ± 0.11^{d}	45.23 ± 1.02^{bc}	38.01 ± 0.57^{bcd}	3.61 ± 0.57^{abc}
Y11	1.16 ± 0.02^{e}	3.44 ± 0.07^{e}	42.02 ± 0.53^{d}	36.11 ± 0.71^{cd}	3.99 ± 0.70^{abc}

Note: Different letters of indicated having significantly different (p<0.05)

Statistical analysis

All data were obtained from at least three independent experiments at the same time and place, and were expressed as the mean ± standard deviation. SAS 8.2 was used to conduct hierarchical cluster analysis (HCA) and multiple comparison analysis when values of p<0.05 were considered significant. Ward's method was applied for analyzing variance between clusters and Squared Euclidean distance was selected as a measurement parameter.

Results

Main component analysis of *Coix* seed samples from different batches: As with most cereal seeds, starch accounted for above 50% (Table 2) of the kernel weight and significant differences among the batches were detected. The highest content was 82.06% in Y2. Maximum moisture content (11.88%) was observed in sample Y5 while and the minimum (9.84%) in sample Y11. The ash content ranged from 2.03% (Y8) to 0.49% (Y2) with significant differences among the samples. Thus, all the samples conformed to the requirement of the Chinese Pharmacopoeia (2020), with moisture and ash contents no higher than 15.0% and 3.0%, respectively. Distinct variation was also detected in fat content among the batches, varying from 8.30% to 1.17%. Content of kernel protein, the main nutritional component, significantly differed among the samples. The highest was observed in Y8 (14.72 mg/100 mg total weight) and the lowest in Y2 (12.54 mg/100 mg total weight).

Protein fraction distribution of *Coix* seeds from different batches: Protein fraction distribution in *Coix* kernels was shown in (Table 3). There was considerable variability in the four protein-fraction contents among the batches from different areas. Overall, albumin was the lowest fraction and accounted for less than 2% of the total protein. Globulin content ranged from 2.74% (Y9) to 4.55% (Y3). Prolamin accounted for the highest fraction in the kernel (approximately 50% of total protein) which was 52.86% (Y6) to 42.02% (Y11). Moreover, obvious variation was also detected in glutelin content, with the maximum value in Y9 (40.81%) and the minimum value in Y4 (35.48%). Insoluble protein accounted for 2.62-5.16% of the total protein, indicating high extraction efficiency.

ACE inhibitory activity of glutelin peptides: The results of *in vitro* ACE inhibitory activity of *Coix* glutelin peptides (≤ 3 kDa) estimated by RP-HPLC were shown in (Fig. 2). The retention times of HA and HHL were 5.33 and 8.82 min, respectively. The resolution between HA and HHL in the reaction system was ideal (R > 1.5). The peptides from *Coix* glutelin had different inhibitory activities at the final concentration of 0.01 mg/mL. Y5 showed the highest inhibitory percentage of 17.41%, followed by Y1 at 15.17%, and Y2 had the lowest inhibitory activity of 10.87%. The positive control captopril manifested an ideal inhibitory rate of 90.93% at a concentration of 2×10^{-8} mol/L. These findings indicate that peptides from *Coix* glutelin possessed ACE inhibitory potential at some extent.

Characterization of *Coix* seed oil: The results of *Coix* seed oil analyzed by TLC revealed that all samples from different origins had similar chromatographic mobility to that of the reference (Fig. 3a).

Triolein content of *Coix* seeds: The triolein content in all batches of *Coix* was determined by the ELSD-HPLC method. The retention time of the reference standard was 40.69 min (Fig. 3b), and the spectrum of the samples (Fig. 3c) showed that a satisfactory separation was achieved using the conditions described. A calibration curve was obtained with the regression equation being y = 690,886x − 187,936 (r^2=0.996) under a linear range of 2.5 – 20 μg. The triolein contents in the 11 batches were shown in Table 2. The Chinese Pharmacopoeia (2020) stipulates that the content of triolein in *Coix* seed should be no less than 0.50%. Thus, all the samples, except Y2 (0.31%), complied with the pharmacopoeial standards.

Hierarchical clustering analysis: HCA was performed on total protein and protein fraction content of 11 batches of *Coix* seed samples. The results were shown in (Fig. 4). The 11 batches of *Coix* Seed were clustered into 3 groups. Y3 and Y8 were group III. The total protein and protein fraction content of the samples in this group were higher than those from other producing areas, and could be used as high-protein candidate breeding parents in the near future. Y10 and Y11 were classified into group II which had relatively low protein content.

To evaluate the antihypertensive potential of 11 batches, HCA was also performed based on three indexes (total protein, glutelin content and ACE inhibitory activity). The results were shown in (Fig. 5). All samples were classified into 4 group, Y1, Y5, and Y6 were clustered into group I charactered high ACE inhibition activity. These three samples could be used as candidate breeding material for antihypertension purpose. Y2 and Y10 were clustered into group II, and the 3 indexes of which were relatively low.

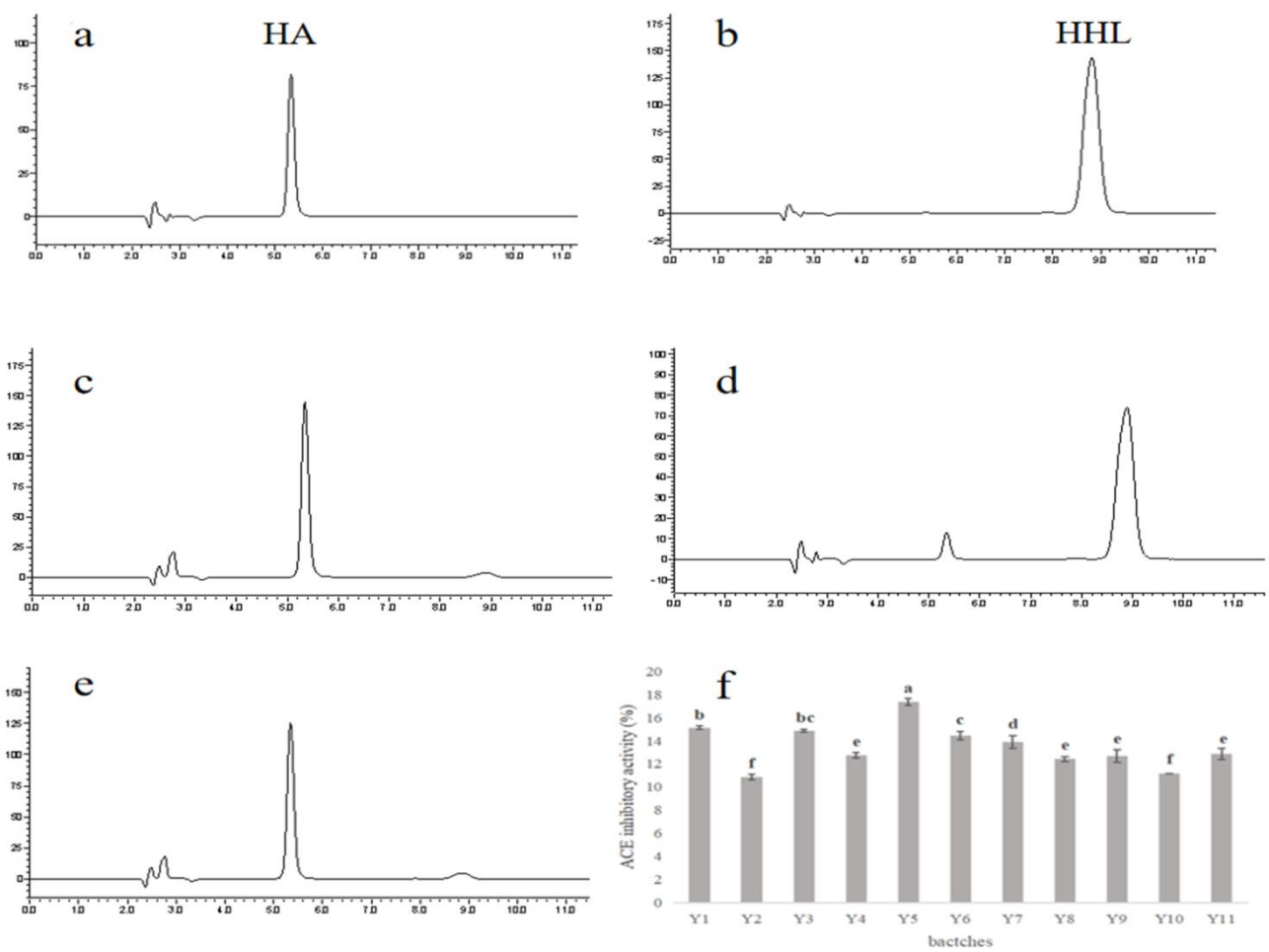

Fig. 2. HPLC chromatograms of (a) HA reference substance, (b) HHL reference substance, (c) blank control, (d) positive control (captopril, 2×10^{-8} mol/L), (e) glutelin peptides (sample Y10, 2×10^{-3} mg/mL), (f) ACE inhibitory rate (%) of *Coix* glutelin peptides (≤ 3 kDa) from different producing areas.

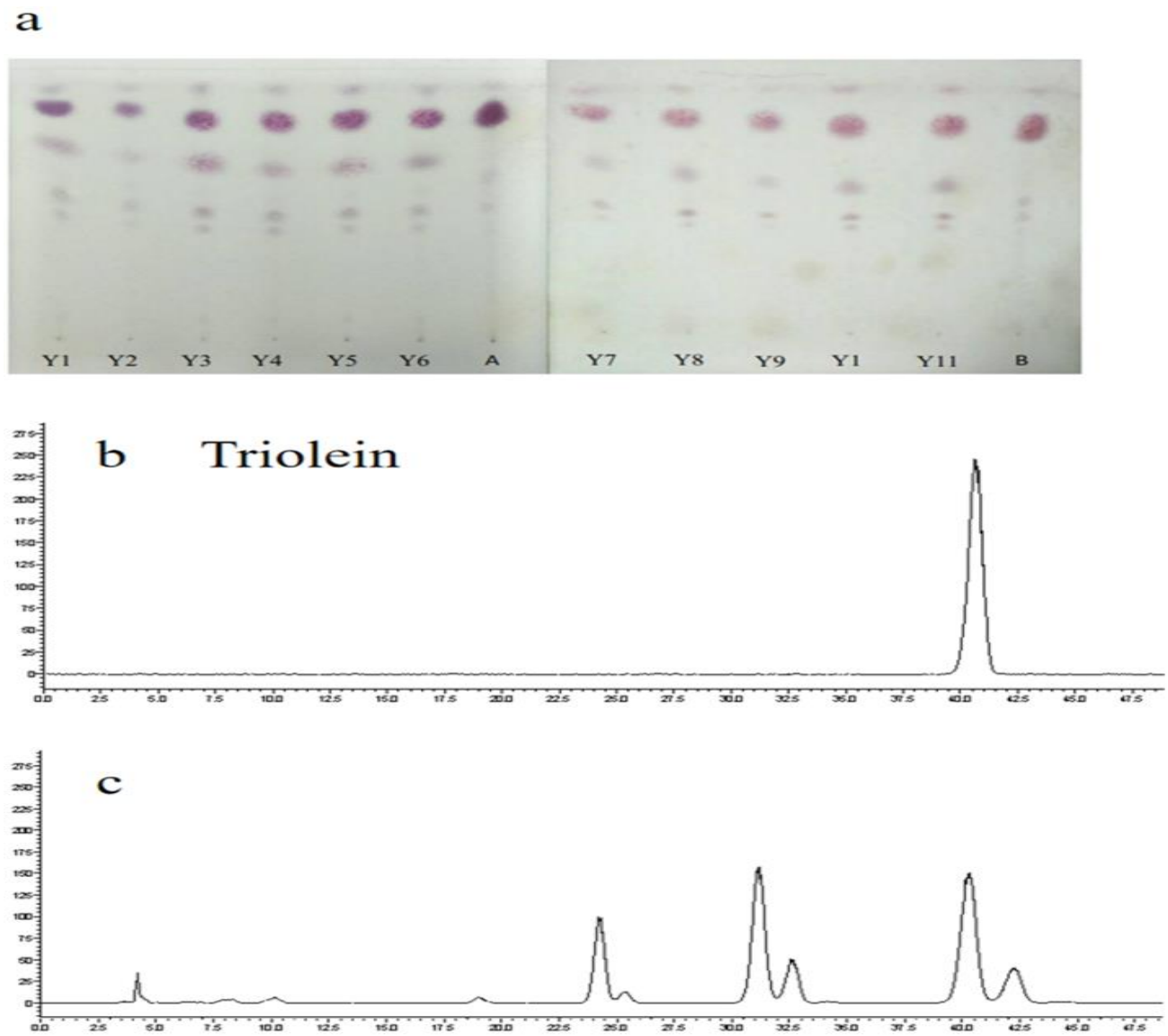

Fig. 3 (a). TLC results of *Coix* seed oil. A: reference substance, B: Y1-11 *Coix* seed sample, ELSD-HPLC chromatogram of triolein (b) reference standard and (c) *Coix* seed sample.

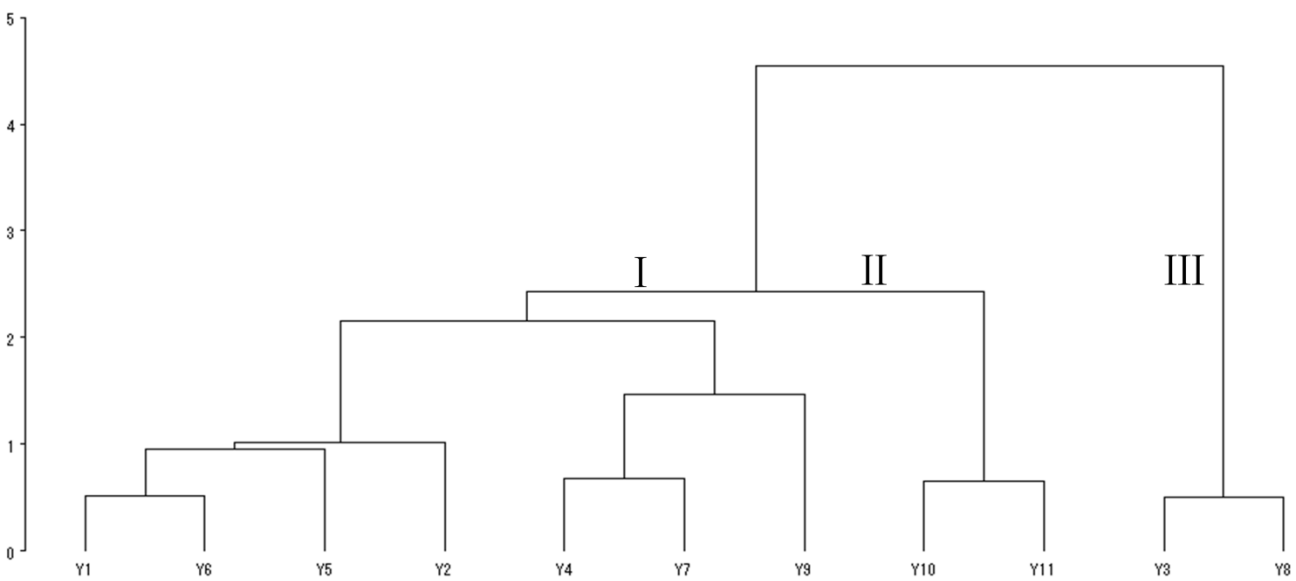

Fig. 4. Hierarchical cluster analysis (HCA) dendrogram of *Coix* seed protein content.

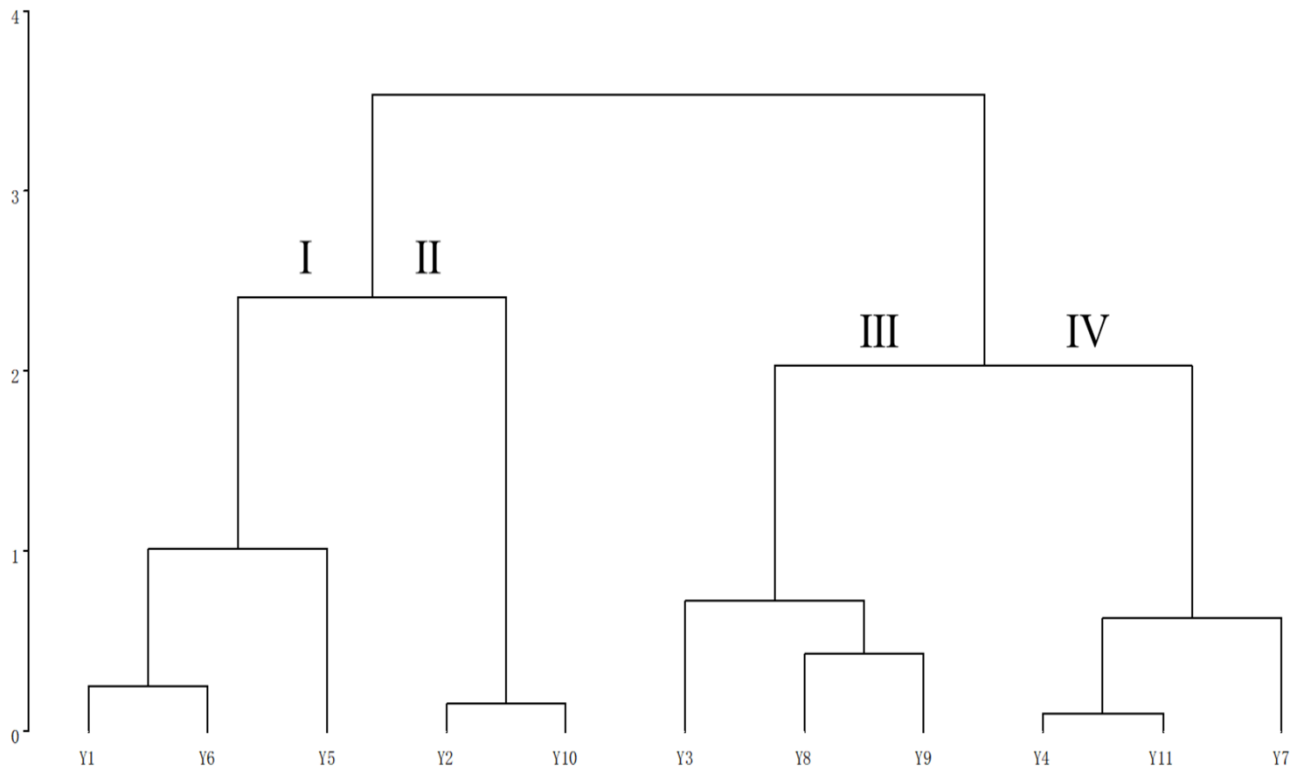

Fig. 5. HCA dendrogram of *Coix* seed antihypertensive potential based on total protein, glutelin content and ACE inhibitory activity.

Discussion

According to the Chinese Pharmacopoeia (2020), triolein is the major quality control index, which is no lower than 0.50%. Therefore, most of the collections qualified, except for batch Y2. Moreover, batch Y8, which was collected from Hebei Province could be the optimum candidate for the consideration of triolein content. As the main herb used in Traditional Chinese Medicine, it is significant to explore novel quality control indexes. *Coix* seed has the highest protein content among cereal crops and has attracted wide attention (Ottoboni *et al.*, 1990). The proteins of cereal grains are classically divided into albumin, globulin, prolamin, and glutelin based on their solubility in different solvents. In maize and teosinte, zein (prolamin) accounts for approximately half or more of total kernel crude protein, followed by glutelin, whereas albumin and globulin contents are less than 10% (Wang *et al.*, 2008). Similar data were reported in *Sorghum* (Afify *et al.*, 2012) and barley (Zhao *et al.*, 2011) protein fraction distributions. Our data regarding protein fractions of *Coix* were consistent with these research results, and all those crops were close relatives of Poaceae. Meanwhile, there was a great difference in protein fractions among different batches. HCA performed on total protein and protein fraction content of 11 batches showed that Y3 (from Guizhou Provinces) and Y8 (from Hebei Provinces) samples had higher protein content, which was superior to other production areas and could be used as high-protein candidate breeding parents. Glutelin is a kind of protein that readily soluble under alkaline (pH>10) conditions. Its emulsifying and foaming properties make it a main food ingredient (Amagliani *et al.*, 2017). Meanwhile, glutelin molecules have abundant intra and intermolecular disulfide bonds and are hydrophobic. Thus, this unsatisfactory solubility in an aqueous system has limited its application as a functional ingredient (Zheng *et al.*, 2015). Improving its solubility and increasing physiological properties have been performed in many studies. The deamidation of barley glutelin improved the structure, aggregation, and emulsifying properties (Zhao *et al.*, 2011). Enzymatic hydrolysis of barley glutelin generated antioxidant peptides and alcalase hydrolysates with significantly higher activity than those treated by flavourzyme (Xia *et al.*, 2012). Hydrolysis decreased the molecular weight and disulfide bond content and increased the solubility of corn glutelin, whereas the hydrolysates exhibit excellent antioxidant properties (Zheng *et al.*, 2015). Enzymatic proteolysis for modifying the functional properties of *Coix* protein is significant for its health and pharmaceutical applications. In our previous research, the immunomodulatory activity was detected from small molecular peptides of *Coix* glutelin (Li *et al.*, 2017b) and a novel antihypertensive peptide was derived from it (Li *et al.*, 2017a).

In this study, the *In vitro* ACE inhibitory activity of *Coix* glutelin peptides and the content of triolein in different batches were determined. The results showed that there were significant differences among different collections. Triolein, an important active pharmaceutical ingredient of *Coix*, was used to evaluate the quality of adlay seeds according to the Chinese Pharmacopoeia (2020). The samples Y5, Y6 (from Yunnan Provinces) and Y7 (from Hebei Provinces) had comparatively high ACE inhibitory activity and triolein content, thus could

be selected as “double high” parent material for future breeding. Glutelin hydrolysate from sample Y5 collected from Yunnan Province showed the highest ACE inhibitory activity of 17.41%, followed by that of Y1 and Y3 from Liaoning and Guizhou Provinces, respectively. HCA performed besed on total protein and glutelin content and ACE inhibitory activity indicated that3 samples manifested great antihypertensive potential. Furthermore, the HCA results also showed that the accessions clustered into one group were not from the same production area suggesting that *C. lacryma-jobi* var. *ma yuen* antihypertensive potential, protein content were not strongly related to the production location. This might be attributed to differences in growing conditions, such as the light, moisture, climate, and soil of the producing area.

Conclusion

The protein fractions, active pharmaceutical ingredient, and ACE inhibitory activity of glutelin peptide of 11 *Coix lacryma-jobi* var. *ma yuen* batches collected from China were evaluated. These data will contribute to the understanding of *Coix* protein related characters to promote the *Coix* breeding for medicinal purposes.

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Author Contributions

Lingzhi Wang and Zujun Chen designed the experiments. Yuqing Ouyang and Haoyu Shi performed the experiments. Yi Zhao and Yanyan Yin analyzed the data. Yuqing Ouyang, Fuzhen Zhu, and Lingzhi Wang wrote the paper. All authors read and approved the final manuscript.

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