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ORIGINAL ARTICLE

Analysis and determination of diterpenoids in unprocessed and processed *Euphorbia lathyris* seeds by HPLC–ESI-MS

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Euphorbia lathyris;
Diterpenoids

Abstract *Euphorbia lathyris* (Caper spurge) is a toxic and potent Chinese materia medica (T/PCMM). This study sought a method for identifying five diterpenoids (Euphorbia factors L_1−L_3, L_{7a} and L_8) with the spectra of UV and mass, quantifying three diterpenoids L_1, L_2, and L_8 in crude extracts of unprocessed and processed *E. lathyris* seeds by liquid chromatography/electrospray ionization mass spectrometry (LC–ESI-MS). The analysis was achieved on an Agilent Eclipse XDB-C18 column (4.6 mm × 150 mm i.d., 5 μm) with an isocratic elution with a mobile phase consisting of water and acetonitrile at a flow rate of 0.25 mL/min at column temperature of 30 °C and UV detection was set at 272 nm. An ESI source was used with a positive ionization mode. The calibration curve was linear in the ranges of 9.9–79 μg/mL for Euphorbia factor L_1, 3.8–30.5 μg/mL for Euphorbia factor L_2, and 1.0–20.6 μg/mL for Euphorbia factor L_8. The average recoveries ($n=6$) of three diterpenoids were 98.39%, 91.10% and 96.94%, respectively, with RSD of 2.5%, 2.4% and 2.1%, respectively. The contents of the three diterpenoids in processed *E. lathyris* seeds were 3.435, 1.367 and 0.286 mg/g, respectively, which decreased more sharply than those in unprocessed *E. lathyris* seeds which were 4.915, 1.944 and 0.425 mg/g, respectively. The method is simple, accurate, reliable and reproducible, and it can be applied to control the quality of unprocessed and processed *E. lathyris* seeds.

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1. Introduction

Toxic and potent Chinese materia medica (T/PCMM) has become a hot and sensitive topic as more and more people around the world are turning to herbal medicine for treatment. With over 2000 years' history of clinical use, their unique therapeutic effects in curing some formidable diseases make them widely used [1–4]. However, they can easily cause serious problems because of their intrinsic toxicity [5]. In view of their

toxic side effects, accurate and reliable authentication is indispensable to ensure their safe use.

Euphorbia lathyris (Caper spurge) is a toxic and potent Chinese materia medica which has been used for remedying hydropsy, ascites, coprostasis, anuresis, amenorrhea, venous stasis, terminal schistosomiasis, scabies, and snakebite [6], and it also has toxicological effects similar to those of croton oil. According to the regulations and the practice of Chinese medicine stipulated by the State Food and Drug Administration of China, only processed seeds of *E. lathyris* can be used legally [7], because the processed *E. lathyris* seeds have lower toxicity. *E. lathyris* seeds contain a series of diterpenoids known as Euphorbia factors L_1–L_{11} [8–16]. It is possible that diterpenoids have undesired toxic side effects [17]. Moreover, only qualitative identification by TLC exists for the seeds of *E. lathyris L.* in Chinese Pharmacopoeia. In the past, quality control of Semen Euphorbiae was limited to the quantitative determination of this polyol in hydrolyzed homeopathic mother tinctures from various spurges by high-performance liquid chromatography (HPLC) with ODS as the stationary phase [18]. HPLC-UV and HPLC-positive–ESI-MS methods were employed to analyze the diterpenoid fraction of caper spurge seed oil before and after selective hydrolysis products [19]. HPLC-UV method was developed and applied to the simultaneous determination of four lathyrane diterpenoids in the seeds of *E. lathyris*, but the detection time was a little long [20]. There was no report on identification and quantification of diterpenoids in unprocessed and processed *E. lathyris* seeds by high-performance liquid chromatography/electrospray ionization mass spectrometry (HPLC–ESI-MS).

In this study, a rapid on-line HPLC–ESI-MS method was established for the analysis of five diterpenoids in processed and unprocessed *E. lathyris* seeds. All diterpenoids (Euphorbia factors L_1–L_3, L_{7a} and L_8) were identified according to their UV spectra and ESI–TOF–MS mass spectra, and three diterpenoids (Euphorbia factors L_1, L_2 and L_8) were quantified. The structures of the five diterpenoids studied in this research are shown in Fig. 1.

2. Experimental

2.1. Materials

The standard herbs of unprocessed seeds of *E. lathyris* were purchased from the market of Chinese Materia Medica (Yulin, Guangxi, China), and authenticated by Prof. Hai-Bo Bai of Zhejiang University, Hangzhou, P.R. China. The processed seeds of *E. lathyris* were prepared according to the standard method [6].

2.2. Chemicals and reagents

Euphorbia factors L_1, L_2 and L_8 were isolated from the seeds of *E. lathyris* and corroborated by comparison of their spectroscopic data with those reported in the literature [14,15]. Their purities were above 95%, as determined by HPLC analysis. HPLC-grade acetonitrile (Merck, Darmstadt, Germany) was utilized for the HPLC analysis. Deionized water was purified by Milli-Q water purification system (Millipore, Bedford, MA, USA). All the other chemicals and solvents were of analytical grade. All solvents and samples were filtered through a millipore filter (0.45 μm) before injection.

2.3. Sample preparation

The unprocessed and processed seeds of *E. lathyris* were powdered to a homogeneous size (80 mesh). An accurately weighed 0.1 g sample was extracted with 10 mL methanol in an ultrasonic bath for 30 min and filtered. This extraction was repeated twice. The combined filtrate was evaporated to dryness *in vacuo*. The residue was then dissolved in methanol and diluted to 10 mL in a volumetric flask and filtered through a 0.45 μm filter membrane before analysis. Aliquots (20 μL) of samples were automatically injected into the HPLC system.

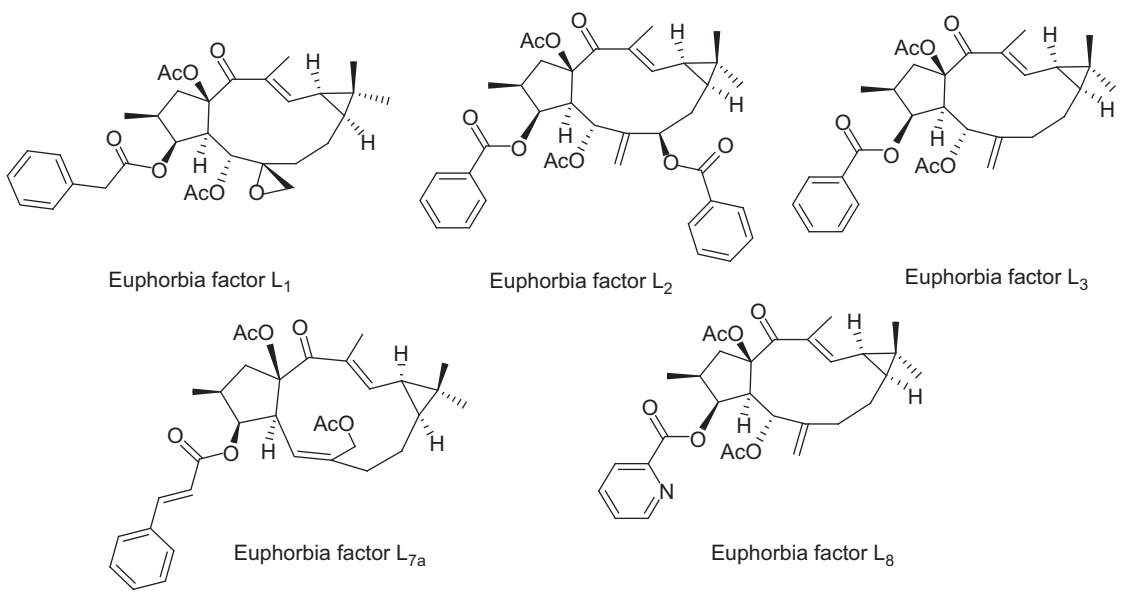

Fig. 1 The structures of five diterpenoids from the seeds of *Euphorbia lathyris*.

2.4. HPLC–ESI-MS analysis

2.4.1. Standard solutions

The standard samples of Euphorbia factors L_1, L_2 and L_8 were accurately weighed and then dissolved in methanol to prepare stock standard solutions. These working solutions were prepared by appropriate dilution of the stock solutions with methanol. Stock and working standard solutions were stored at 4 °C. Calibration curves were established based on eight concentrations within the ranges of 9.9–79 μg/mL for Euphorbia factor L_1, 3.8–30.5 μg/mL for Euphorbia factor L_2, and 1.0–20.6 μg/mL for Euphorbia factor L_8 by diluting the stock solution in series.

2.4.2. Apparatus and HPLC conditions

The separation of diterpenoids was performed on an Agilent 1200 series LC system, consisting of quaternary pump, on-line

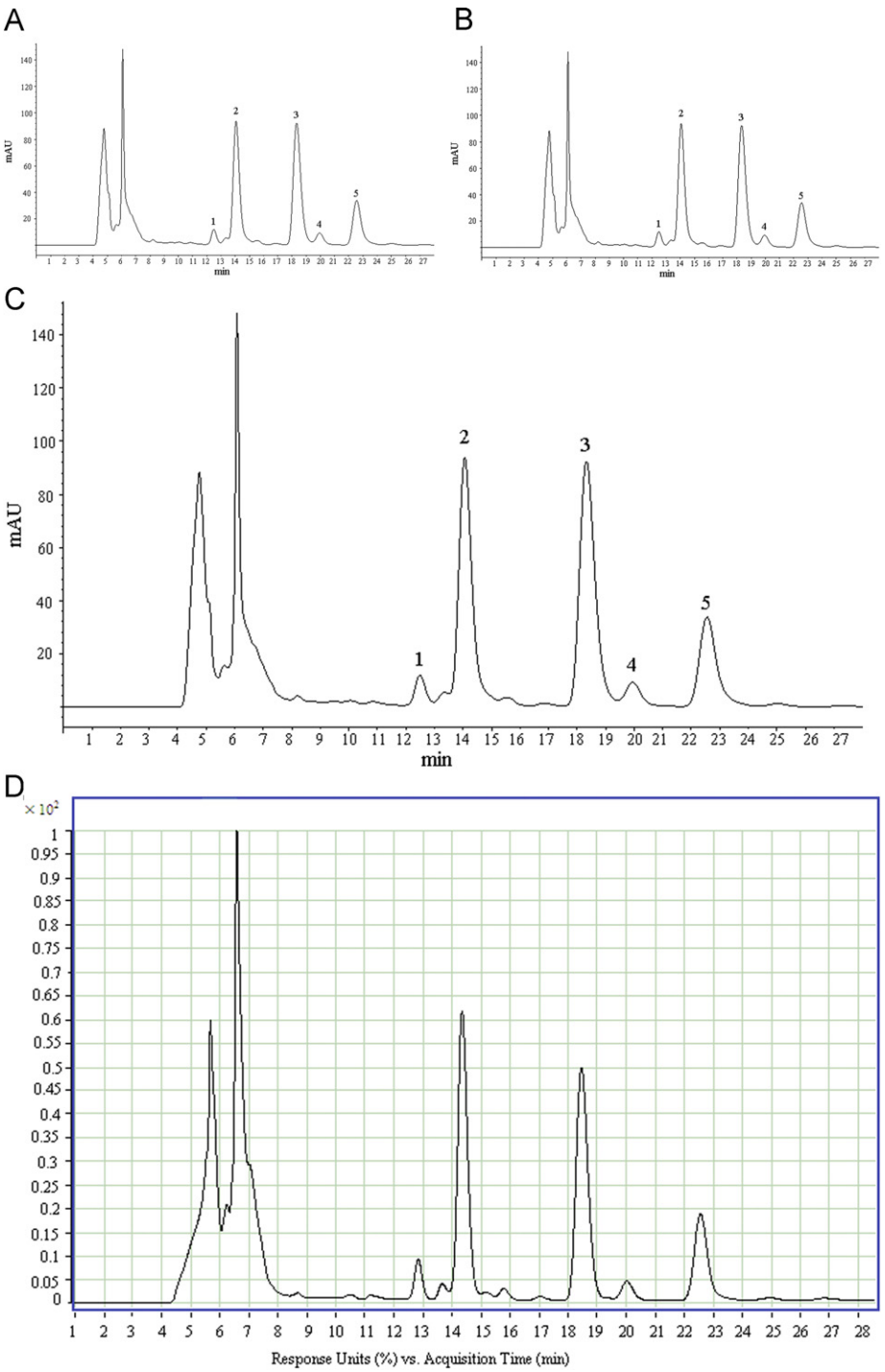

Fig. 2 HPLC-chromatogram of unprocessed and processed *E. lathyris* seeds: (A) unprocessed *E. lathyris* seeds; (B) processed *E. lathyris* seeds; (C) combined unprocessed and processed *E. lathyris* seeds; (D) HPLC–ESI-MS chromatogram of processed *E. lathyris* seeds. Peaks: 1, Euphorbia factor L_8; 2, Euphorbia factor L_1; 3, Euphorbia factor L_3; 4, Euphorbia factor L_{7a}; and 5, Euphorbia factor L_2.

degasser, well-plate autosampler, thermostatic column compartment and UV detector. An Agilent Eclipse XDB-C_{18} column (4.6 mm × 150 mm i.d., 5 μm) was employed and the column temperature was 30 °C. The mobile phase consisted of acetonitrile:water (85:15, v/v) at a flow rate of 0.25 m/min, and the wavelength was 272 nm. The injected volume was 20 μL.

2.4.3. *Mass spectrometry*

ESI-MS analysis was performed with an Agilent (MA, USA) 6210 TOF LC/MS system. The mass detector was operated in the positive mode with nitrogen as the nebulization and drying gas under the following condition: nebulization pressure, 50 psi; drying gas temperature, 325 °C; drying gas flow rate, 10 L/min; capillary voltage, 3500 V; fragmentation voltage, 175 V; skimmer, 65 V; OCT1RFVpp, 250 V; acquisition range, 100–1000*m*/*z*.

3. Results and discussion

3.1. *Separation of diterpenoids by HPLC*

The HPLC chromatogram (Fig. 2) demonstrated the retention time of different components in unprocessed and processed *E. lathyris* seeds within a short time. Compared to systems with methanol, systems with acetonitrile had a better resolution and a smoother baseline. Different ratios of water to acetonitrile were further tried, and a satisfactory separation within a suitable period of time was obtained. Of the optimal parameters, the organic modifier percentage had the greatest effect on the separation, peak shape and detection sensitivity of diterpenoids.

Diterpenoids were best separated using a mobile phase of water (15%) and acetonitrile (85%). By comparing the LC chromatograms of the herbs recorded at wavelengths from 200 to 500 nm and the corresponding UV absorption maximum for each chemical standard, it was found that a wavelength of 272 nm could represent the profile of the major constituents in *E. lathyris* seeds.

3.2. *Identification of diterpenoids by MS*

The HPLC–ESI-MS chromatogram of the extract of processed *E. lathyris* seeds is shown in Fig. 2D. In LC/MS method, using positive ion detection, five diterpenoids were detected without interferences. As shown in Fig. 3, the ESI-MS spectrum of peak 1 exhibited mass ion signal at *m*/*z* 524.3 $[M+H]^+$; by comparing the mass spectrum and LC spectrum with Euphorbia factor L_8 standard, peak 1 was positively identified as Euphorbia factor L_8. The ESI-MS spectrum of peak 2 exhibited mass ion signal at *m*/*z* 553.3 $[M+H]^+$; by comparing the mass spectrum and LC spectrum with Euphorbia factor L_1 standard, peak 2 was

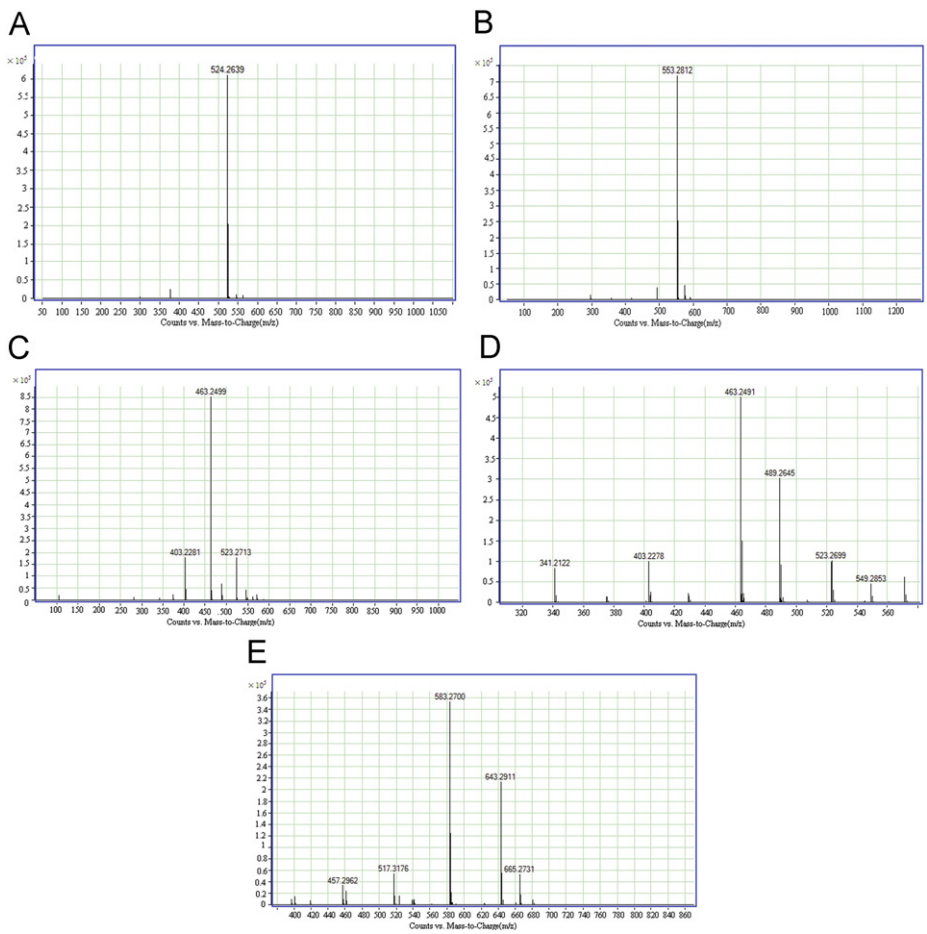

Fig. 3 Positive ESI-MS profiles of diterpenoids: (A) Euphorbia factor L_8; (B) Euphorbia factor L_1; (C) Euphorbia factor L_3; (D) Euphorbia factor L_{7a}; and (E) Euphorbia factor L_2.

Table 1 HPLC–ESI-MS measurements of diterpenoids in unprocessed and processed *Euphorbia lathyris* seeds.

Peak	Identification	Retention time (min)	*m/z* ESI/MS	UV spectra (λ_{max})
1	Euphorbia factor L_8	12.2	524.3	272
2	Euphorbia factor L_1	13.8	553.3	272
3	Euphorbia factor L_3	17.8	523.3	272
4	Euphorbia factor L_{7a}	18.1	549.3	272
5	Euphorbia factor L_2	21.8	643.3	272

Table 2 HPLC–TOF-MS measurements of diterpenoids in processed *Euphorbia lathyris* seeds.

Peak	t_R (min)	Observed mass	Calculated mass	Identification
1	12.2	524.2639 $[M+H]^+$	524.2648	Euphorbia factor L_8 ($C_{30}H_{37}NO_7$)
2	13.8	553.2812 $[M+H]^+$	554.2801	Euphorbia factor L_1 ($C_{32}H_{40}O_8$)
3	17.8	523.2713 $[M+H]^+$	523.2696	Euphorbia factor L_3($C_{31}H_{38}O_7$)
4	18.1	549.2853 $[M+H]^+$	549.2852	Euphorbia factor L_{7a} ($C_{33}H_{63}O_7$)
5	21.8	643.2911 $[M+H]^+$	643.2907	Euphorbia factor L_2 ($C_{38}H_{42}O_9$)

positively identified as Euphorbia factor L_1. The ESI-MS spectrum of peak 3 exhibited mass ion signals at *m/z* 523.3 $[M+H]^+$, 463.3 $[M–59]^+$ and 403.3 $[M–119]^+$. We deduced *m/z* 463.3 as possible $(M+H-CH_3COOH)^+$ and *m/z* 403.3 as possible $[M+H-2CH_3COOH]^+$. By comparing the mass spectrum with literature data, peak 3 was preliminary identified as Euphorbia factor L_3. The ESI-MS spectrum of peak 4 exhibited mass ion signal at *m/z* 549.3 $[M+H]^+$ and 489.3 $[M–59]^+$, so we deduced *m/z* 489.3 as possible $[M+H-CH_3COOH]^+$; by comparing the mass spectrum with literature data, peak 4 was preliminary identified as Euphorbia factor L_{7a}. The ESI-MS spectrum of peak 5 exhibited mass ion signal at *m/z* 643.3 $[M+H]^+$ and 583.3 $[M+H-CH_3COOH]^+$. By comparing the mass spectrum and LC spectrum with Euphorbia factor L_2 standard, peak 5 was preliminarily identified as Euphorbia factor L_2. The HPLC–ESI-MS data of diterpenoids in unprocessed and processed *E. lathyris* seeds are listed in Table 1. In order to confirm these diterpenoids, HPLC–TOF–MS (Agilent 6210 TOF LC/MS) analysis was employed to detect the corresponding molecular weight information of components (Tables 1 and 2).

3.3. *Quantification of Euphorbia factors L_1, L_2 and L_8*

3.3.1. *Linearity*

The calibration curves of the individual standards were constructed at eight concentrations by plotting the peak areas against the concentration of the compounds. The calibration curves indicated good linearity ($r>0.999$): $Y=112.37x-8.0571$ (0.9999) in the range of 9.9–79 μg/mL for Euphorbia factor L_1, $Y=131.36x-0.5959$ (0.9999) in the range of 3.8–30.5 μg/mL for Euphorbia factor L_2, $Y=122.49x-19.507$(0.9997) in the range of 1.0–20.6 μg/mL for Euphorbia factor L_8.

Table 3 The contents of three diterpenoids in unprocessed and processed *Euphorbia lathyris* seeds (μg/g).

Compound	Unprocessed drug		Processed drug	
	Content (μg/g, mean±SD)	RSD (%)	Content (μg/g, mean±SD)	RSD (%)
Euphorbia factor L_1	4.915±0.097	2.0	3.435±0.053	1.5
Euphorbia factor L_2	1.944±0.042	2.2	1.367±0.024	1.8
Euphorbia factor L_8	0.425±0.011	2.6	0.286±0.006	2.0

3.3.2. *Precision*

The precision of the method was validated by determination of intra- and inter-day variance. The intra-day precision was determined with five replications prepared from the *E. lathyris* seeds sample within one day, while the inter-day precision was determined over three consecutive days. The quantity of each ingredient contained in the *E. lathyris* seeds sample was determined from the corresponding calibration curve. The relative standard deviation (RSD) was taken as a measure of precision. The intra-day precisions (RSD) of three diterpenoids were 0.26%, 0.33% and 0.23%, respectively, while inter-day precisions (RSD) of the investigated components were 0.83%, 1.0% and 1.3%, respectively. The results indicated that the method is precise for simultaneous determination of three diterpenoids.

3.3.3. *Repeatability*

In order to test the repeatability, six sample solutions of unprocessed *E. lathyris* seeds were prepared. The contents of three diterpenoids were 4.915, 1.945 and 0.425 mg/g, respectively, and the RSDs were 2.0%, 2.2% and 2.6%, respectively.

Over 6 days, the mean contents of three components in the unprocessed *E. lathyris* seeds were 4.885, 1.923 and 0.424 mg/g, respectively. It was indicated that the sample is stable in the experimental conditions.

3.3.4. *Recovery (accuracy)*

Recovery was determined by adding the accurate volumes of three standard solutions to approximate 0.1 g unprocessed *E. lathyris* seeds, which were treated according to the procedure described above. The recovery of each compound was calculated as the percentage of the net amount of each compound obtained after extraction from what had been added prior to the extraction. The mean recoveries of the three markers were 98.39%, 91.10% and 96.94%, respectively, and the RSDs were 3.1%, 2.4% and 2.1%, respectively. The RSDs of intra-day and inter-day were less than 5.0%. It was indicated that the extraction method is efficient enough for the determination of three diterpenes in *E. lathyris* seeds.

3.4. Application

The sample solutions obtained from unprocessed and processed *E. lathyris* seeds were injected into the instrument and the peaks in the chromatograms were identified by comparing retention time and on-line UV spectra with those of the standards. The amounts of the three compounds in the samples were calculated. The results are listed in Table 3. It can be seen that the contents of the three diterpenoids in processed *E. lathyris* seeds decreased more sharply than in unprocessed *E. lathyris* seeds.

4. Conclusion

In this paper, the diterpenoids in the extracts of unprocessed and processed *E. lathyris* seeds were identified by HPLC–ESI-MS. Using mobile phase consisting of water (15%), and acetonitrile (85%), a very simple and fast isocratic LC–ESI-MS method is presented. The described LC–ESI-MS method involving the use of the $[M+H]^+$ allows us to determine five diterpenoids in *E. lathyris* seeds with good selectively and sensitivity. The good linearity over a wide range of concentrations, precision and accuracy were obtained with this method. In comparison to previously reported methods, the newly proposed method was simpler in its extraction technique with a high extraction efficiency. The contents of the three diterpenoids in processed *E. lathyris* seeds decreased more sharply than in unprocessed *E. lathyris* seeds. The results indicate that the processed *E. lathyris* seeds have lower toxicity. The developed method is reliable and suitable for the quality control of unprocessed and processed *E. lathyris* seeds.

Acknowledgments

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