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Original article

Analysis on Isozyme and Diterpene Lactones Variability during Ontogenesis of *Andrographis paniculata*

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ABSTRACT

Objective To understand the relationship between isozyme activities and diterpene lactone biosynthesis of *Andrographis paniculata*. **Methods** Plants were collected during ontogeny from seeding to seed maturity, and separated into leaves and stems for determination. Morphological and yield parameters were used to describe plant growing states. Isozyme changes were tested by polyacrylamide gel electrophoresis. HPLC was used to develop the fingerprints as well as to determine the diterpene lactone content. **Results** Significant increases were observed in the activities of isozymes, such as aspartate aminotransferase (AST), malate dehydrogenase (MDH), peroxidase (POD), and catalase (CAT), around the early stage of bud in leaves, and the activities of these four kinds of isozymes increased gradually as time progressed in stems. The content changes of diterpene lactones in leaves and stem were various. In the leaves, andrographolide (**1**) was recorded the highest [(23.63 ± 1.06) mg/g] at the early stage of bud, whereas deoxyandrographolide (**2**) was the lowest [(6.78 ± 0.27) mg/g] at this period and it reached the highest level at the seeding stage [(26.05 ± 1.04) mg/g]. Dehydroandrographolide (**3**) and neoandrographolide (**4**) fluctuated during growing stages. Meanwhile, the HPLC fingerprint showed that the content changes of two unknown compounds were related to that of dehydroandrographolide in leaves. In stems, andrographolide had increased gradually until the bud stage [(8.26 ± 0.33) mg/g], and other three diterpene lactones showed a trend of fluctuation. The yield of total diterpene lactones in aerial part reached the highest at the first flowering stage (806.71 mg/plant). **Conclusion** These results lay the foundation for the future research on the relationship of isozymes and diterpene lactones, and for determining the most favorable time for harvesting *A. paniculata*.

Key words

Andrographis paniculata; diterpene lactones; isozymes; plant ontogeny

1. Introduction

Andrographis paniculata (Burm. F.) Nees, commonly known as "King of Bitter" (Gomathinayagam et al, 2009; Kumar et al, 2011), is an important traditional medicine in China, India, Thailand, and Scandinavia. *A. paniculata* has various medicinal properties, such as antibacterial, antiviral and anti-HIV, anti-inflammatory, anti-platelet aggregation,

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anti-thrombogenic, hepatoprotective and antimalarial (Calabrese et al, 2000; Otake et al, 1995; Shen et al, 2002; Thisoda et al, 2006; Kapil et al, 1993; Misra et al, 1992; Jin et al, 2014). These pharmacological activities are mainly contributed to diterpene lactones including andrographolide (**1**), dehydroandrographolide (**3**), neoandrographolide (**4**), and deoxyandrographolide (**2**). Moreover, each of these active compounds possesses some different pharmacological activities from the others. Andrographolide showed higher activities of anti-inflammation and anti-cancer (Abu-Ghefreh et al, 2009; Kumar et al, 2004); Dehydroandrographolide produced a potent hypotensive effect (Yoopan et al, 2007) and higher antiplatelet activity (Thisoda et al, 2006). Neoandrographolide displayed greater activity against malaria (Misra et al, 1992) and could scavenge free radicals (Kamdem et al, 2002); Deoxyandrographolide showed anti-hypertensive activity (Zhang and Tan, 1999). The different potencies of these four diterpene lactones indicate that the content control of active compounds in *A. paniculata* is very important to the curative effect. Previous studies (Pholphana et al, 2013) reported that in *A. paniculata* leaves, the highest levels of andrographolide were at the vegetative stage and seed-forming stage in field and greenhouse experiments respectively, and dehydroandrographolide was at the transfer stage, while deoxyandrographolide and neoandrographolide contents were low at all growing stages. The relationship among andrographolide, neoandrographolide, and deoxyandrographolide on different harvesting dates was also studied (Bhan et al, 2006). Zhu and Mo (2004) gave the result that the content of main chemical compounds showed cumulative process as the extension of growing time. The content of dehydroandrographolide increased during the storage of crude powders and extracts, which may be due to the degradation of andrographolide (Pholphana et al, 2004; Thisoda et al, 2006). Since the demand of *A. paniculata* is increasing day by day, now it is commercially cultivated (Chauhan et al, 2009). However, the quality and quantity of this herb in cultivation are unstable due to environmental conditions, uneven harvesting time and genetic variation (Bhan et al, 2006).

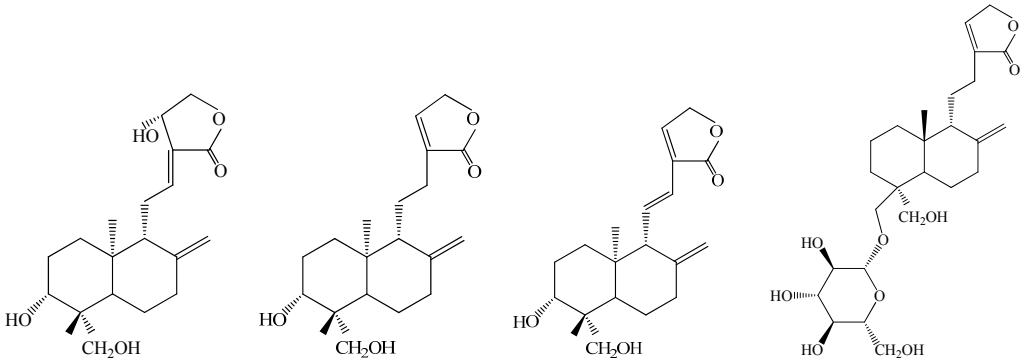

Figure 1 Chemical structures of four active diterpene lactones in *A. paniculata*

Isozymes are related to the biosynthesis of secondary metabolites in many species (Ji et al, 2012). Rutin-degrading isozymes were the key enzymes responsible for the conversion of rutin to quercetin (Li et al, 2013). In the suspension cells of *Scutellaria baicalensis* Georgi, peroxidases could hydrolyze baicalin into baicalein (Morimoto et al, 1998). The POD-dependent reactions were important in the accumulation of lignin and phenolics in the thick root of sweet potato (Kim et al, 2015). The biosynthesis of terpenoids in plants can be conceptually divided into three discrete processes: the formation of terpene precursors, the construction of terpene skeletons, followed by the complex post-modifications, according to that the isozymes may be classified as the third process (Li et al, 2009). The relative technologies are also being applied to the studies of *A. paniculata*. The correlation between active compounds and *ent*-copalyl diphosphate synthase (CPS) at various growing periods was analyzed after the CPS gene was cloned (Chen et al, 2014; Yao et al, 2012). The activities of antioxidant enzymes were increased by abscisic acid (ABA) and gibberellic acid (GA_3) treatments as well as the content of andrographolide (Anuradha et al, 2010).

In this study, the changes in the isozyme activities of aspartate aminotransferase (AST), malate dehydrogenase (MDH), peroxidase (POD), catalase (CAT), and the diterpene lactone content at different growing stages in *A. paniculata* were examined. The aims were to explore the relationship between isozyme activities and diterpene lactone biosynthesis at different growing stages in *A. paniculata*, and also to determine the most favorable harvesting time.

2. Materials and methods

2.1 Plant materials

The seeds of *A. paniculata* were sown in the cultivation base at Qingyuan, China, on April 17th, 2014. The fresh plants were collected at nine different growing stages, viz., No. 1: seedling stage; No. 2−4: fast growing stages; No. 5: early bud stage; No. 6: bud stage; No. 7: first flowering stage; No. 8: full bloom stage; No. 9: seed maturity stage, and separated into leaves and stems for determination. Samples were collected on the same land to ensure the same growing environment. Fresh leaves and stems of the plants were used

for isozymes analysis, and those dried in shade for isolation of diterpene lactones.

2.2 Analysis of morphological values and yield parameters

Morphological values and yield parameters: plant height (PH), number of branches (NB), middle leaf length (LL), middle leaf width (LW), leaf dry weight (LDW), stem dry weight (SDW), and plant dry weight (PDW) were selected to describe plant growing at different stages. Fresh plants were used to measure PH, NB, LL, and LW. Plants dried until a stable weight was reached were used to determine LDW, SDW, and PDW.

2.3 Isozyme analysis

The first fully developed leaves and young stems from three plants were picked and then mixed to extract isozymes separately. Leaves or stems (0.5 g) was weighed and ground into fine powder in liquid nitrogen. The powder was gently homogenized with 1 mL extraction buffer, 0.1 mol/L Tris-HCl buffer (pH 7.5) containing 0.2% β-mercaptoethanol for AST and MDH and 1 mol/L Tris-HCl buffer (pH 8.0) containing 0.2% β-mercaptoethanol for POD and CAT, using mortar and pestle in an ice bath. The extracts were centrifuged at 12 000 r/min for 20 min at 4 ^{o}C. The supernatants were stored at −80 ^{o}C. Supernatants (100 μL) was then mixed with an equal volume of 40% sucrose and 1/2 volume of 0.05% bromophenol blue. Discontinuous native PAGE was used for resolving the isozymes. Sample (20 μL) was used to electrophorese at 10 mA in stacking gel and 25 mA in separating gel with a slab gel of 10 × 10 × 0.1 cm. The electrophoretic separations were performed in Tris-Glycine buffer (pH 8.3) at 4 ^{o}C. The electrophoretic systems of AST, MDH, POD, and CAT were as following: 7.5% separating gel and 4% stacking gel were selected for AST. The electrophoretic systems of MDH and POD were 10% separating gel and 4% stacking gel. The CAT electrophoretic systems were 7% separating gel and 4% stacking gel. Gels were stained by the methods as described earlier (Siciliano and Shaw, 1980; He and Zhang, 1999) with slight modifications. After electrophoresis, AST gel was submerged in staining buffer [0.2 mol/L Tris-HCl (120 mL pH 8.0), *L*-aspartic acid (120 mg), ketoglutaric acid (240 mg), pyridoxal-5-phosphate (3 mg)] for 15 min, and then 240 mg fast blue BB was added and stained for 15 min at room temperature. MDH isozymes were detected in a solution with 5 mL substrate solutions containing 10.15 g Na_2CO_3 and 13.4 g *L*-malic acid per 100 mL water, 25 mg nicotinamide adenine dinucleotide (NAD), 15 mg nitrotetrazolium blue chloride (NBT), 1 mg phenazine methosulfate (PMS), 10 mL 0.2 mol/L Tris-HCl (pH 8.0), and 35 mL water for 15 min. POD activity was stained with 1% benzidine and 3% H_2O_2. CAT gel was incubated in 0.9 mmol/L H_2O_2 for 10 min. After that, the CAT gel was rapidly rinsed twice and submerged in a mixed solution of 2% $K_3[Fe(CN)_6]$ and 2% $FeCl_3$ until the bands appeared clearly.

2.4 Analysis of diterpene lactones

The shade dried leaves and stems were milled to 65 mesh powder as extraction samples separately. The samples were extracted using the method described earlier (Nie and Luo, 2006) with slight modifications. Sample (0.2 g) was accurately weighed and extracted with 10 mL of 70 % ethanol in an ultrasonic bath at room temperature for 30 min. The supernatants were filtered through a 0.22 μm filter membrane to obtain the test solution. HPLC analyses were carried out on PerkinElmer series 200 system (USA). The separation was performed with a Unimicro Technologies Ins C_{18} column (250 mm × 4.6 mm, 5 μm) with the mobile phases of (A) acetonitrile and (B) 0.4 % phosphoric acid in water. The flow rate was 0.8 mL/min at 30 ^{o}C. The UV detector monitored at wavelength of 226 nm and the injection volume was 20 μL for all samples and standards. Retention time of andrographolide, neoandrographolide, deoxyandrographolide, and dehydroandrographolide reference substances was used to identify peaks in the fingerprints. The validation of the analytical method was done by linearity, precision, and accuracy. A mixed standard stock solution with concentration of 1 mg/mL for andrographolide and deoxy-andrographolide and 0.5 mg/mL for neoandro-grapholide and dehydroandrographolide was prepared in methanol and diluted to six different concentration. Three injections per concentration were performed to generate calibration curves. Standard solution (0.206 mg/mL) of dehydroandrographolide was used to determine the intra- and inter-day precision. The precision of the method was examined by analyzing for six times repeatedly within 1 d on three consecutive days. The precision was expressed in terms of relative standard deviation (RSD). The accuracy of the method was evaluated by standard addition with pre-analyzed sample solution. Three different concentration of standard mixtures were added to the sample and extracted with the developed method. Each sample was replicated in triplicate. The retention time, regression equation, and correlation coefficient of each analyte were summarized in Table 1. The RSD of intra-day precision was 3.29% and the inter-day was 4.06%. The recovery was between 95%−105%.

Table 1 Retention time, regression equations, and correlation coefficient of four compounds from *A. panaculata*

Analytes	Retention time / min	Regression equations	R^2
andrographolide	29.90	y = 25 217 939.68 *x* + 4370.66	0.9995
neoandrographolide	51.52	y = 5 239 063.21 *x* − 1453.14	0.9992
deoxyandrographolide	56.24	y = 6 794 318.47 *x* − 206 256.72	0.9951
dehydroandrographolide	56.96	y = 14 434 363.39 *x* − 119.28	0.9995

2.5 Statistical analysis

Experiments were carried out in triplicate, and values were expressed as $\bar{x} \pm s$. All statistical analyses were performed using software SPSS 17.0.

3. Results

3.1 Morphological values and yield parameters

The collection time of samples and corresponding mean values of morphological and yield parameters were presented in Table 2. The plants were at seedling stage on June 18th (No. 1), and then they got into the fast growing period until August 13rd (No. 5) when a few buds appeared. Since then, the reproductive growth was coming. August 25th, September 5th, September 20th, and October 10th were in the stages of bud, first flowering, full bloom, and seed maturity, respectively (No. 6−9). PH and NB increased continually with the growing time prolonging and almost reached the maxima after the stage of first flowering [No. 7, (54.00 ± 4.51) and (28 ± 4) cm]. LL and LW kept a high level after fast growing stage (No. 2), whereas it began to decrease obviously after the bud stage [(No. 6, (9.80 ± 1.03) and (2.93 ± 0.43) cm. Similarly, LDW, SDW, and PDW reached the highest at the first flowering stage [No. 7, (15.55 ± 2.14), (13.92 ± 0.67), and (29.47 ± 1.74) g/plant]. Data showed that almost all the parameters were found to be maximum at the first flowering stage.

3.2 Changes of isozyme activities

The activities of isozymes can be measured through bands numbers and their color intensity based on PAGE (Peng et al, 2013), as shown in Figure 2. In the leaves, the activities of AST, MDH, POD, and CAT (Figures 2A−2D) increased obviously around the early bud stage (No. 5). There were three bands in the AST isozymes patterns through all growing stages. The main difference was the deepening of the bands from fast growing period to the bud (No. 4−6). The bands of the MDH were divided into two parts, A and B. The first band of part A and all the bands of part B were high levels of enzyme activity. The difference was the same as that of AST. The POD bands mainly emerged at part A. There was one highly active band at part A and no special band appeared. The activity of POD at part a enhanced at the early bud and bud stages (No. 5 and 6), meanwhile a new weak band emerged at part B. The activity of CAT increased from fast growing period to the bud stage (No. 3−6) and two more bands appeared at July 31st (No 4). The PAGE results of AST, MDH, POD, and CAT in stems at different growing stages are presented in Figures 2E−2H. As the growing stages went on, AST, MDH, POD, and CAT activities increased gradually, especially after the bud stage (No. 6).

Table 2 Morphological values and yield parameters during different growing stages ($\bar{x} \pm s$, $n = 7$)

No.	Collection time	Morphological and yield parameters						
		PH / cm	NB	LL / cm	LW / cm	LDW / (g·plant^{-1})	SDW / (g·plant^{-1})	PDW / (g·plant^{-1})
1	Jun. 18th, 2014	10.50 ± 0.85	0	4.85 ± 0.74	2.15 ± 0.33	0.31 ± 0.15	0.06 ± 0.02	0.37 ± 0.17
2	Jul. 4th, 2014	17.40 ± 3.13	7 ± 3	9.07 ± 0.80	3.35 ± 0.37	1.48 ± 0.54	0.36 ± 0.14	1.85 ± 0.66
3	Jul. 18th, 2014	22.77 ± 1.32	11 ± 1	10.07 ± 0.50	3.78 ± 0.32	2.73 ± 0.45	1.15 ± 0.23	3.88 ± 0.64
4	Jul. 31st, 2014	33.00 ± 3.56	15 ± 3	9.25 ± 1.26	3.08 ± 0.96	5.29 ± 1.82	3.49 ± 1.51	8.78 ± 3.31
5	Aug. 13rd, 2014	46.00 ± 4.30	22 ± 5	9.30 ± 1.15	2.90 ± 0.22	6.38 ± 2.29	5.15 ± 2.28	11.52 ± 4.54
6	Aug. 25th, 2014	47.25 ± 3.86	24 ± 2	9.80 ± 1.03	2.93 ± 0.43	8.65 ± 1.26	6.69 ± 1.26	15.34 ± 2.45
7	Sept. 5th, 2014	54.00 ± 4.51	28 ± 4	8.64 ± 0.94	2.63 ± 0.34	15.55 ± 2.14	13.92 ± 0.67	29.47 ± 1.74
8	Sept. 20th, 2014	56.80 ± 5.63	28 ± 5	8.19 ± 1.41	2.33 ± 0.58	10.44 ± 3.88	12.23 ± 0.55	20.94 ± 1.70
9	Oct. 10th, 2014	57.00 ± 1.44	27 ± 1	6.69 ± 0.25	1.76 ± 0.24	8.33 ± 1.50	12.97 ± 1.50	21.30 ± 1.77

3.3 Changes of diterpene lactone content

The HPLC fingerprints of the extracts from *A. paniculata* leaves and stems extracts were given in Figure 3. Analysis showed that the characteristic peaks of *A. paniculata* leaves and stems at different growing stages exhibited high similarity, but the content of characteristic compounds in leaves was almost three times higher than those in stems. Content changes of those four diterpene lactones in leaves and stems under different growing stages wre given in Figure 4. In leaves, andrographolide was found to vary from (14.54 ± 0.69) to (23.63 ± 1.06) mg/g. Andrographolide was recorded lowest at the seedling stage [No. 1, (14.54 ± 0.69) mg/g], and then it began to increase until the early stage of bud [No. 5, (23.63 ± 1.06) mg/g], whereas the content decreased with plant maturity (No. 5−9). Deoxyandrographolide was at the highest level at the seedling stage [No. 1, (26.05 ± 1.04) mg/g] with a decrease tendency before the early stage of bud [No. 5, (6.78 ± 0.27) mg/g], and then the content stayed low at reproductive growth stage. The highest levels of these two compounds were consistent with the record by Pholphana et al (2013). Dehydroandrographolide and neoandrographolide had a state of fluctuation during growing stages in leaves. In stems, andrographolide changed from (2.57 ± 0.06) to (8.26 ± 0.33) mg/g, which represented the stages of seedling (No. 1) and bud (No. 6), respectively, and then it remained the highest at reproductive growth stage. Deoxyandrographolide, dehydroandrographolide, and neoandrographolide fluctuated in stems. Meanwhile, the fingerprints of *A. paniculata* occurring in all samples appeared two high-content peaks, X1 and X2, with unclear structures. The HPLC results in

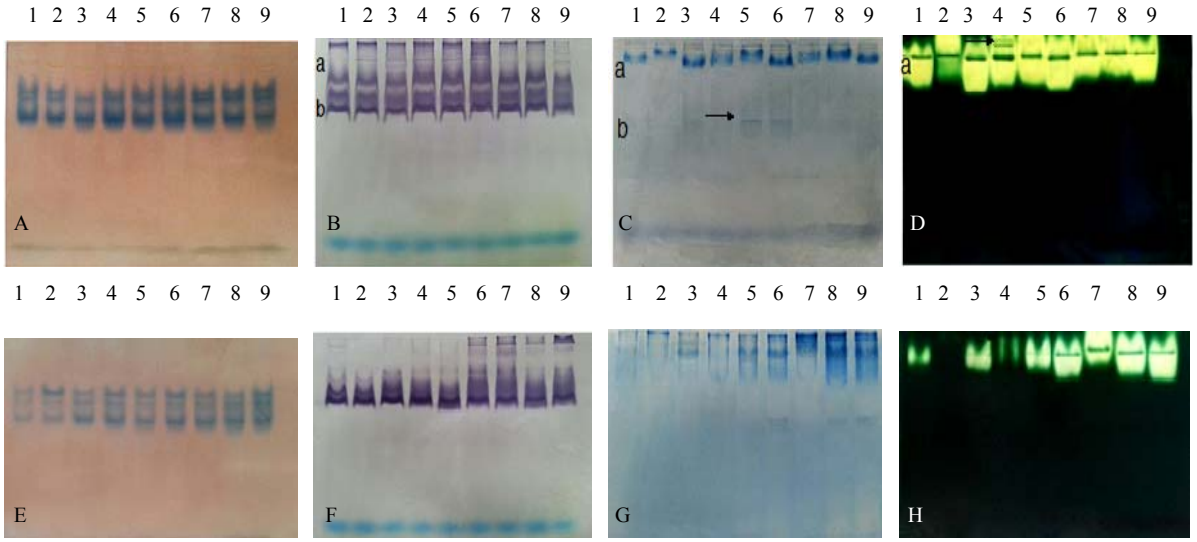

Figure 2 Isozyme electrophoretograms of AST, MDH, POD, and CAT by PAGE

(A) AST, (B) MDH, (C) POD, and (D) CAT of *A. paniculata* leaves; (E) AST, (F) MDH, (G) POD, and (H) CAT of *A. paniculata* stems

1-Jun. 18th, 2014; 2-Jul. 4th, 2014; 3-Jul. 18th, 2014; 4-Jul. 31st, 2014; 5-Aug. 13rd, 2014; 6-Aug. 25th, 2014; 7-Sept. 5th, 2014; 8-Sept. 20th, 2014; 9-Oct. 10th, 2014

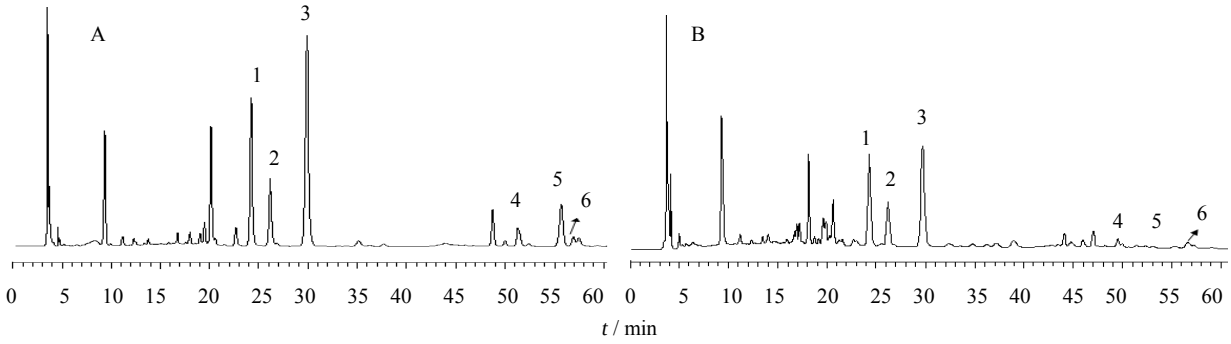

Figure 3 HPLC fingerprints of *A. paniculata* leaves (A) and stems (B)

1-X1; 2-X2; 3-andrographolide; 4-neoandrographolide; 5-deoxyandrographolide; 6-dehydroandrographolide

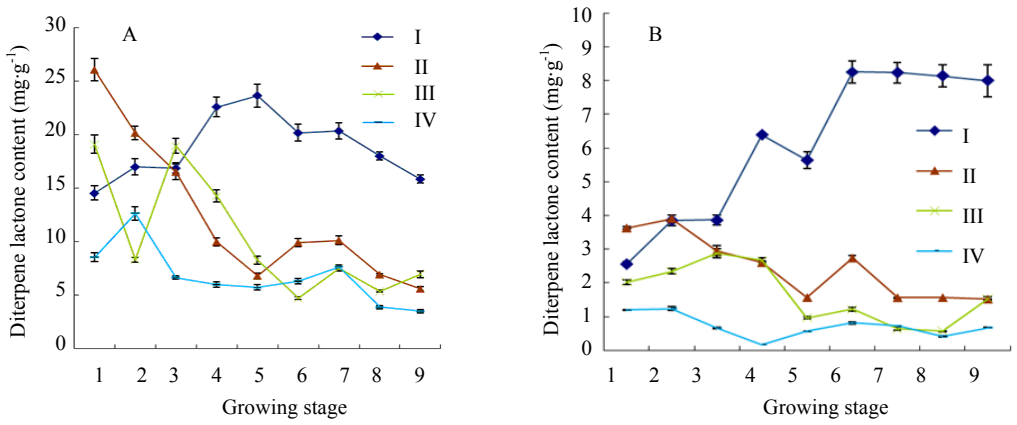

Figure 4 Changes of four active diterpene lactones in *A. paniculata* leaves (A) and stems (B) at different growing stages ($\bar{x} \pm s$, $n = 3$)

1- Jun. 18th, 2014; 2- Jul. 4th, 2014; 3- Jul. 18th, 2014; 4- Jul. 31st, 2014; 5- Aug. 13rd, 2014;

6- Aug. 25th, 2014; 7- Sept. 5th, 2014; 8- Sept. 20th, 2014; 9- Oct. 10th, 2014; same as below

I-andrographolide; II-deoxyandrographolide; III-dehydroandrographolide; IV-neoandrographolide

leaves showed that there was a similarity between the content changes of X1 and X2, and furthermore the changes were on the contrary with that of dehydroandrographolide (Figure 5). The yield changes of total diterpene lactones, constituted of andrographolide, deoxyandrographolide, dehydroandrographolide, and neoandrographolide are showed in Figure 6. The highest yields of total diterpene lactones in leaves and aerial part were 639.22 and 806.71 mg per plant at the first flowering stage (No 7), respectively.

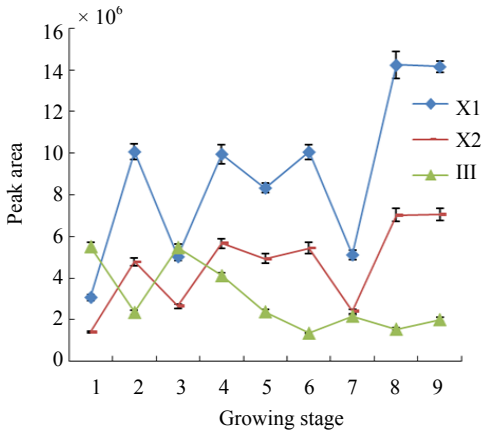

Figure 5 Content changes of X1, X2, III, and in leaves ($\bar{x} \pm s$, $n = 3$)

III-dehydroandrographolide

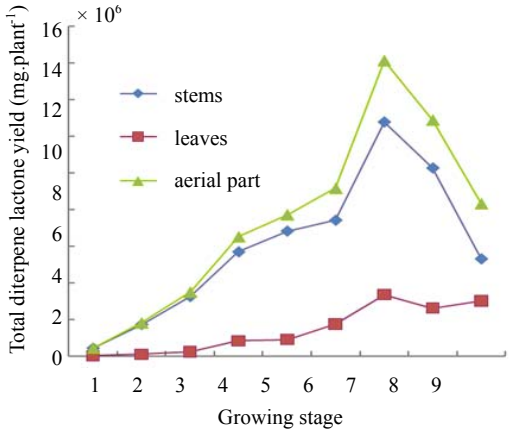

Figure 6 Yield changes of total diterpene lactones in *A. paniculata* leaves, stems, and aerial part at different growing stages

4. Discussion

In this study, the changes in isozyme activities and diterpene lactone accumulation of *A. paniculata* leaves and stems at different growing stages were evaluated. The activities of AST, MDH, POD, and CAT increased obviously around the early stage of bud in the leaves, whereas they increased gradually as time progressed in stems. The levels of andrographolide reached the highest at the early stage of bud in the leaves and had increased in the stems until the bud stage. Significant correlation was observed between the activities of AST, MDH, POD, and CAT and the accumulation of andrographolide. The results indicate that these isozymes may play certain role in the pathway of andrographolide biosynthesis. Furthermore, the deduced protein encoding cloned CPS gene from *A. paniculata*, a key limiting enzyme of andrographolide biosynthesis, included conserved aspartic acid-rich motif of plant terpene synthases (Yao et al, 2012). AST can catalytic oxaloacetic acid and glutamic acid into aspartic acid (Wang et al, 2002). The appearance of conserved aspartic acid-rich motif gives us further evidence that AST may contribute to andrographolide biosynthesis. MDH is the key enzyme of malic acid metabolism, and it is also the important enzyme in the processes of tricarboxylic acid cycle, glycolytic pathway and pentose phosphate pathway, making sure that enough energy and precursors of biosynthesis, such as nucleotides and amino acids are provided (Sweetman et al, 2009; Wang et al, 2002; Zhao et al, 2012). It has been suggested that reactive oxygen species (ROS), remaining at a stable level by the coordination of SOD, POD, CAT, etc. (Larkindale and Knight 2002), might be signaling molecules involved in the process of terpenoid biosynthesis (Zhai et al, 2011; Ma et al, 2015). Therefore, we conclude that the isozyme activities of AST, MDH, POD and CAT are highly correlated to the accumulation of andrographolide, and they may be the enzymes involved in andrographolide biosynthesis. The conclusion needs to be further proved at genetic level.

HPLC analyses showed the content variety of diterpene lactones in *A. paniculata* at different growing stages. The highest content of andrographolide in the leaves and stems was at the stages of early bud and bud, respectively, corresponding to calendar months of August, which were similar to those reported by Mo et al (2003) and Pholphana et al (2013). This was probably due to the accumulation of andrographolide being continuous as plant growing, and after maturity it slows down and andrographolide is dehydrated to dehydroandrographolide (Bhan et al, 2006). The similarity between the report of Pholphnana (2013) and the present study also exists in that the content of deoxyandrographolide was the highest at the seedling stage in leaves. The opposite tendency of X1 and X2 to dehydroandrographolide indicates that there may be transformation among the three compounds. The supporting results were also observed earlier that dehydroandrographolide was the main degradation product of andrographolide and there were other compounds that could degrade to dehydroandrographolide (Pholphana et al, 2004). Since dehydroandrographolide is presented in small amounts in this plant, it will be valuable to enhance the transformation of X1 and X2 to increase the content of dehydroandrographolide. In the process of the experiment, we also found that the crude extracts by methanol were unstable and the instability was mainly existed in andrographolide, X1 and X2. What's more, andrographolide sharply increased, whereas X1 and X2 decreased quickly even within a few hours. Those make us to believe that X1 and X2 may play an important role in the transformation of

diterpene lactones. Proper method needs to be created to promote the transformation toward the direction that is helpful to improve the clinical effects or the quality of *A. paniculata.* The harvesting time is recommended around first flowering stage when the yield of total diterpene lactones per plant was the highest. The result is consistent with that from Zeng et al (2007). The dry leaf weight per plant was almost 50% and the leaves contributed most to the biomass of total diterpene lactones at the first flowering stage. The leaf ratio at this stage was higher than that required in *Chinese Pharmacopoeia* 2010. Thus, the leaves can be considered as the medicinal parts of *A. paniculata.*

5. Conclusion

In conclusion, the changes of AST, MDH, POD and CAT isozymes and the accumulation of diterpene lactones in *A. paniculata* leaves and stems at different growing stages are determined. The results show that both the isozyme activities and andrographolide accumulation could reach the highest around the early bud stage in leaves, whereas the highest data are reached around the bud stage in the stems. The consistency of isozyme activities and andrographolide accumulation may indicate that these four isozymes may participate in the andrographolide biosynthesis and it will lay the foundation for future study of diterpene lactone biosynthesis. Therefore, further study of isozyme gene expression at different growing stages in *A. paniculata* will be useful for exploring the mechanism of diterpene lactone biosynthesis. The yield of PDW and total diterpene lactones is the highest at the first flowering stage and this result provides the scientific evidence for determining the best harvesting time.

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Conflict of interest statement

The authors declare no conflict of interest.

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