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Original article

Changes of Secondary Metabolites and Trace Elements in *Gentiana macrophylla* Flowers: A Potential Medicinal Plant Part

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ABSTRACT

Objective To search for the potential medicinal plant part of *Gentiana macrophylla* based on changes of secondary metabolites and trace elements in the flowers of *G. macrophylla*. **Methods** HPLC was used to detect the changes of the active constituents (longanic acid, sweroside, gentiopicroside, and swertiamarin) and ICP-AES was used for mineral nutrients in *G. macrophylla* during flower development. And soluble sugar, starch, crude protein, hemicelluloses, cellulose, and lignin were determined. **Results** Biomass of flower in full bloom (D2) phase was considerable during flower development, in which the contents of longanic acid and gentiopicroside were at the highest levels with 2.65 and 2.88 times higher than those recorded in *Chinese Pharmacopoeia 2010*, sweroside and swertiamarin in the flowers were reaching 6.06 and 1.25 times higher than those in roots. Florescence is the most valuable stage during flower development. The concentration of Fe, Mg, K, P, and B was higher in the flowers than that in roots. The accumulation of active constituents in the plant was influenced by the contents of metabolically linked carbon and nitrogen compounds. **Conclusion** The secondary metabolites, mineral nutrients, and physicochemical indicators are tightly regulated by flower organ development, D2 is an important stage for both biomass and extraction of active constituents such as longanic acid. The flowers of *G. macrophylla* could be used as a potential medicinal plant part for longanic acid at a high level.

Key words

flower development; *Gentiana macrophylla*; Gentianaceae; gentiopicroside; longanic acid; trace elements

1. Introduction

Gentiana macrophylla Pall., commonly known as *Qinjiao* in Chinese and mainly distributed in China and Siberia, is a medicinal plant of the family Gentianaceae. It has been used in traditional Chinese medicine (TCM) for over 2000 years. The dominant active constituents in the roots of *G. macrophylla* are loganic acid, gentiopicroside, and swertiamarin, with the bioactivities of analgesic, anti-inflammation, anti-pyretic, antirheumatic, diuretic, febrifuge, and hypoglycaemic, for treating hypotensive rheumatic pains, fevers, and allergic inflammations (Skrzypczak et al, 1993; Zhang et al, 1999; Yu et al, 2004). *G. macrophylla* is generally reproduced by seed, but the seed is too small and exhibits an after-ripening phenomenon. In addition, low and irregular germination seriously restricted the propagation of *G. macrophylla*. The

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natural ecology of this plant with a long growth period and special habitat is now in short supply because of the over-exploitation of the wild plant. As a result, it has been placed under key protection, thus creating a wide gap between supply and demand in China (Cao et al, 2005). It is therefore important to carry out the investigations on non-medicinal parts to understand their physiological processes, in order to provide the evidence reports for producing its active constituents to meet pharmaceutical demands.

Flowers of plants in *Gentiana* L. (Kim et al, 2008; Zheng et al, 2011) have been little studied in metabolic changes during flower development and medicinal usage, because only the root was considered and the flower was removed during opening. Plants in *Gentiana* L. are perennial, so flower could be harvested every year and production should be considerable. Zhao et al (2010) indicated that flower is also an important medicinal part of *Gentiana* L., and the contents of gentiopicroside in the stems, leaves, or flowers of *G. lhassica* Burk. and *G. waltonii* Burk. were higher than those in the roots according to their primary chemical analyses. Since seldom studies were carried out on *G. macrophylla*, it is important to better understand the metabolic changes during flower development and the relationship between flowers and roots. In addition, the relationship among microelement, illnesses, pharmacological effects, and theory of TCM has gained great attention. Modern scientific research has shown that elements under specific conditions are necessary for sustained health and prevention of diseases (Warren, 1989).

In this study, four active ingredients and various micro- and macro-elements of *G. macrophylla* were analyzed throughout the period of flower development to understand the inherent dynamics, beyond which we could find the most valuable period and then compare this period of flower with root, so as to evaluate the worth that may enlarge the resources of medicinal part and help in balancing the supply and demand of *G. macrophylla*. The carbohydrate, crude protein, and cell wall composition were also determined throughout the development of flower organ. These data would help us better understand the physiological processes.

2. Materials and methods

2.1 Plant materials

The flowers and roots of *G. macrophylla* were used in this study, indentified by Porf. Xue-hui Dong, numbered by sampling time, and deposited in the College of Agricultural and Biotechnology, China Agricultural University, Beijing, China. Samples were collected from biennial and triennial growing plants at the experimental site of China Agricultural University in Shanxi province, China in 2011. Flowers were collected during the period of flower development from June to August, and the entire flower development period was divided into four stages such as D1, flower bud; D2, full bloom; D3, initial stage of seed-producing period of flower organ; and D4, seed maturity and withering of flower organ. (Figure 1). R1 refers to roots separated from the plant at the same time during the four stages; R2 referes to other root samples collected at plant maturity when the above ground part withers in September.

2.2 Weight ratio of flower to roots

At each stage of development, nine samples from three repeat groups were collected, and the weights of flower and roots of individual plants were determined after drying (at 80 ^{o}C for 48 h in electrical oven) to a constant weight. The weight ratio of flower organ and root (R1) was then calculated. At each stage of the development, three samples in the same group were mixed, milled, screened via a 0.3 mm sieve, and prepared for the following tests. The determination results in each index repeated for three times in each group.

2.3 Determination of longanic acid, sweroside, gentiopicroside, and swertiamarin

Four reference substances were purchased from The National Standard Material Nets (Beijing, China). The four standard materials were accurately weighed, dissolved in methanol-water solution, and diluted to appropriate concentration. The mixture solutions were used for HPLC (Agilent 1200LC, USA) preparation of calibration curves after filtration through 0.45 μm filters. Plant materials (0.2 g) were subjected to ultrasound-assisted extraction with methanol-water (1:1, 10 mL) solution. The flower and root (R2) samples were used for HPLC analysis after filtration through 0.45 μm filters. The separation was performed on Agilent Eclipse Plus C_{18} column (250 mm × 4.6 mm, 5 μm), operated at normal temperature by two mobile phases of water (including 0.3% formic acid) and methanol at a flow rate of 1 mL/min. The detection wavelength was set at 240 nm by ultraviolet detector.

D1 D2 D3 D4

Figure 1 Development stages of *G. macrophylla* flower

2.4 Determination of macro- and micro-elements

The flower and root (R2) samples (0.2 g) were digested with nitric acid (10 mL) in polytetrafluoroethylene tubes according to standard procedures. by Microwave Digestion (CEM Co., Mars, USA). Under maximum power of 1200 W and utilization of 100%, the retention time was 5 and 10 min, temperatures were 120 and 180 ^{o}C for the procedures 1 and 2, respectively. After digestion, the solution was transferred into 25 mL of volumetric flask and diluted to volume with double-distilled water. Blank samples with only HNO_3 were prepared similarly as the samples. The solutions were used to determine the content of macro- and micro-elements by Inductively Coupled Plasma-Atomic Emission Spectrometry (Optima 3300DV, USA). The element standard solutions were purchased from The Center for National Standard Substances (Beijing, China).

2.5 Determination of soluble sugar, starch, crude protein, hemicelluloses, cellulose, and lignin

The soluble sugar and starch were determined using the anthrone-sulfonic acid method; The content of crude protein was determined using Kjeldahl method with a conversion factor of 6.25. The hemicelluloses, cellulose, and lignin were determined using Van Soest method.

3. Results and discussion

3.1 Weight ratio of flowers to roots

The flowers of *G. macrophylla* are bloomed at the second year, and the roots are reaped at the third or fourth year. The ratios of flowers of biennial and triennial plants to the roots (R1) varied similarly, increasing before stage D3 and decreasing at stage D4 (Figure 2). At the early stage of flowering (D1), the flower dry weight of the whole plant was only 15.8% (biennial) and 8.5% (triennial) of roots. The ratio of biennial flowers to roots by dry weight of whole plant increased to 95.1% at stage D2, subsequently exceeded 100% to 244.0% at stage D3, and then decreased to 152.0% at stage D4. Relative to triennial plant flowers, the highest ratios observed at stages D2 and D3 were 44.7% and 48.0%, respectively, without significant difference.

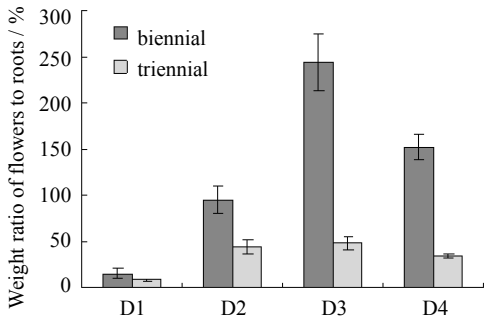

Figure 2 Changes in weight ratios of flowers to roots during flower development ($\bar{x} \pm s$, $n = 9$)

The data in Figure 2 showed that the flower weight of a single biennial plant reached the weight of roots (R2) at stages D2, D3, and D4, and the weight of flowers of a single triennial plant was nearly 50% of roots at stages D2 and D3. This indicated that the production of flower organ (D2) was considerable and it was wasteful to discard it at opening.

3.2 Active ingredients changes in flower and their comparison between flower (D2) and root (R2) stages

Variation in the concentration of longanic acid, sweroside, gentiopicroside, and sewertiamarin during flower development is shown in Figure 3. There were common trends between biennial and triennial plants. The content of longanic acid decreased gradually, reaching the lowest level upon full flower opening (D2), and subsequently increased until the flowers withered. The content of sweroside increased throughout floral development with a sharp decrease at flower senescence. Compared with longanic acid, gentiopicroside showed an opposite trend with a peak at anthesis (D2), with steady decline at the end. Changes in the content of swertiamarin during flower development were consistent with those of sweroside. In conclusion, the content changes in the four chemical ingredients were much significant at stages D2 and D3, and those peaks varied with different plants. In some flowers the peak values occurred at flower opening (Cooper et al, 2009; Salem et al, 2011; Ammar et al, 2012). In other reports, maximum contents occurred before flower opening and subsequently decreased with flower development (Douglas et al, 1996; Joshi et al, 2011). Robertson et al (1995) found that the levels of green leaf volatiles declined and the monoterpenes camphere and limonene increased as the inflorescences of red raspberry matured. This may be related to changes in primary metabolic substrate and biosynthetic pathway of iridoids (Grassmann, 2005). Aharoni and Galili (2011) reported that the contents of metabolically linked carbon and nitrogen compounds had a major quantitative impact on the extent of production of secondary metabolites in plants. The carbon-nitrogen balance hypothesis (Coley et al, 1985) indicated that under the conditions of limited nitrogen availability, secondary metabolism was directed toward producing carbon-rich metabolites and vice versa. For the dominant active constituents in the flowers of *G. macrophylla*, this may indicate that flowers in stages D2 and D3 have the potential to become the valuable part during flower development.

Among the four active ingredients, the amount of gentiopicroside (approximately 87 mg/g) was much higher than others at flower opening, while the contents of longanic acid, sweroside, and swertiamarin were all lower than 10 mg/g during flower development. Furthermore, the amounts of the ingredients except longanic acid in the biennial plants were lower than those in the triennial plants at different stages of flower development. The accumulation of secondary metabolites in plants may be related to these results. Derita et al (2009) indicated that the contents of the active constituents in *Polygonum acuminatum* Kunth. were influenced by plant

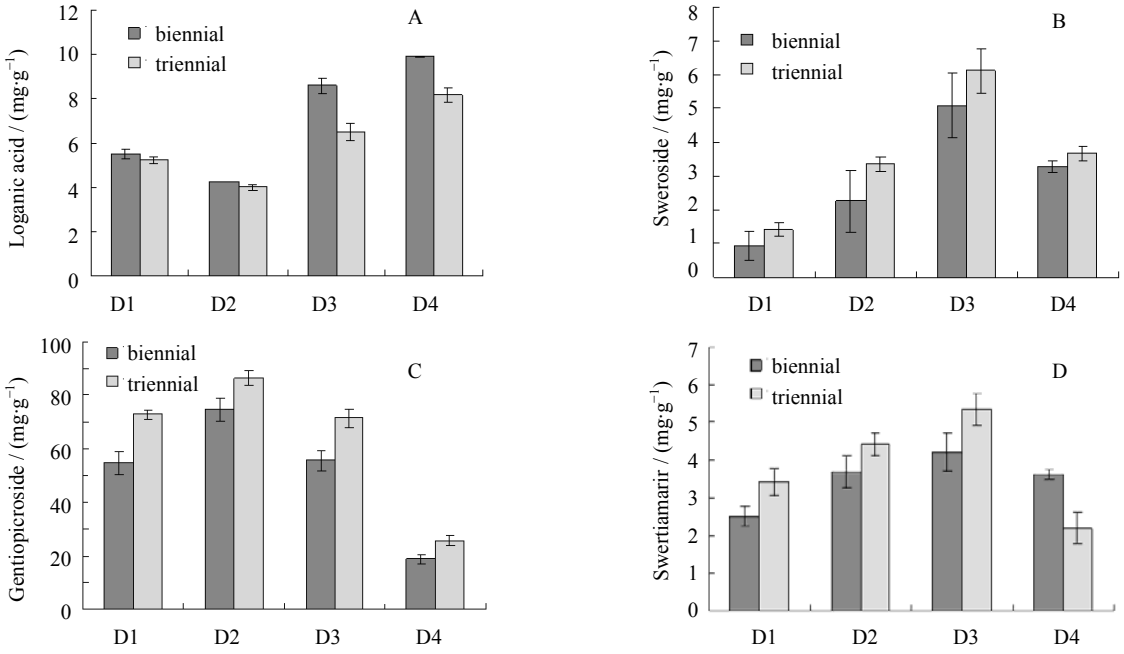

Figure 3 Changes in contents of longanic acid (A), sweroside(B), gentiopicroside (C), and swertiamarin (D) during flower development of *G. macrophylla* ($\bar{x} \pm s$, $n = 9$)

part and collection season. No matter in biennial or triennial flowers, total contents of loganic acid and gentiopicroside have met the standard of *Chinese Pharmacopoeia 2010*, and are higher than 2.5% (25 mg/g) when calculated on dry weight at stages D2 or D3. Taking the higher total contents into consideration, D2 may be the better candidate for the medicinal purpose during flower development.

Compared with the contents in the roots (R2), the contents of sweroside and swertiamarin at flower blooming (D2) stage were 6.06 and 1.25 times higher; Gentiopicroside almost took up 65%; Loganic acid of biennial and triennial plants were 87% and 42% higher (Table 1), respectively. Based on the *Chinese Pharmacopoeia 2010* recommendation, the total contents of loganic acid and gentiopicroside must be higher than 2.5% (25 mg/g) when calculated on dry weight and the flowers have met the standard. At the moment in China, the cases of negligence of some potential part of medicinal plants are common because the focus has always been on medicinal parts obtained at harvesting. Niu et al (2012) showed that contents of chlorogenic and caffeic acids in the fresh leaves of plants in *Lonicera* Linn. were 2.572 and 1.498 higher than those cited in *Chinese Pharmacopoeia 2010*, indicating the necessity for further studies on the development of the fresh leaves in *Lonicera japonica* Thunb. Production and composition of stage D2 of flower were comprehensively evaluated, and the flowers in this stage had a chance to be a potential medicinal part and the further researches still need to be conducted.

Table 1 Contents of active constituents in flowers (D2) and roots (R2) of biennial and triennial *G. macrophylla* ($\bar{x} \pm s$, $n = 9$)

Plant age	Plant parts	Ingredients			
		Loganic acid	Sweroside	Gentiopicroside	Swertiamarin
biennial	flowers	4.27 ± 0	2.26 ± 0.92	74.7 ± 4.3	3.68 ± 0.41
	roots	4.91 ± 0.63	0.32 ± 0.11	112.8 ± 8.5	1.85 ± 0.39
triennial	flowers	4.01 ± 0.14	3.35 ± 0.21	75.4 ± 9.9	4.42 ± 0.31
	roots	9.46 ± 0.63	0.78 ± 0.21	120.1 ± 3.0	1.96 ± 0.42

3.3 Macro- and micro-elements changes in flowers and comparation between flowers (D2) and roots (R2)

Data for the concentration of macro- (P, K, Ca, Mg, and Na) and micro-elements (Fe, Mn, Cu, Zn, and B) in biennial and triennial plants during flower development are shown in Table 2. The concentration of P in biennial flowers decreased sharply (54.8%) from flower bud to senescence; The concentration in triennial plants decreased slightly (13%) between flower budding and senescence. The concentration of K in the biennial flowers showed no significant changes throughout flower development, however in the triennial plants, there was a slightly decrease at flower opening and subsequent increase at the next the period. The concentration of Ca in the biennial and triennial flowers varied similarly with marked decline at flower opening and almost no fluctuation at other periods. The concentration of Mg and Na peaked at flower opening and decreased to the initial content

Table 2 Changes in macro- and micro-elements during flower development of *G. macrophylla* ($\bar{x} \pm s$, $n = 4$)

Plant ages	Stages	Contents of macro- and micro-elements									
		P / ($mg \cdot g^{-1}$)	K / ($mg \cdot g^{-1}$)	Ca / ($mg \cdot g^{-1}$)	Mg / ($mg \cdot g^{-1}$)	Na / ($\mu g \cdot g^{-1}$)	Fe / ($mg \cdot g^{-1}$)	Mn / ($\mu g \cdot g^{-1}$)	Zn / ($\mu g \cdot g^{-1}$)	B / ($\mu g \cdot g^{-1}$)	Cu / ($\mu g \cdot g^{-1}$)
biennial	D1	2.49 ± 0.07	21.6 ± 0.5	6.68 ± 0.10	2.31 ± 0.04	269 ± 10	1.04 ± 0.03	50.7 ± 0.7	35.0 ± 0.3	13.0 ± 0.4	8.24 ± 0.10
	D2	1.98 ± 0.02	21.8 ± 0.2	3.99 ± 0.01	1.60 ± 0.01	176 ± 3	0.59 ± 0.00	34.0 ± 0.5	27.2 ± 0.4	10.4 ± 0.3	7.51 ± 0.05
	D3	1.34 ± 0.02	21.8 ± 0.1	4.02 ± 0.00	2.07 ± 0.02	224 ± 3	3.53 ± 0.01	99.5 ± 0.5	23.3 ± 0.1	12.6 ± 0.2	6.96 ± 0.10
	D4	1.13 ± 0.01	20.5 ± 0.5	4.05 ± 0.05	2.24 ± 0.01	271 ± 2	4.20 ± 0.01	124.0 ± 2.0	25.3 ± 0.6	17.6 ± 0.1	7.97 ± 0.26
triennial	D1	2.17 ± 0.03	20.8 ± 0.1	6.74 ± 0.03	2.04 ± 0.03	244 ± 1	0.57 ± 0.00	36.0 ± 0.6	37.0 ± 0.5	11.4 ± 0.3	6.97 ± 0.06
	D2	1.74 ± 0.02	19.2 ± 0	4.94 ± 0.02	1.54 ± 0.01	149 ± 4	0.44 ± 0.00	24.6 ± 0.2	24.2 ± 0.8	14.3 ± 0.3	5.09 ± 0.14
	D3	1.74 ± 0.02	24.5 ± 0	4.55 ± 0.01	1.73 ± 0.01	178 ± 4	1.37 ± 0.03	46.2 ± 0.4	24.9 ± 0.3	19.7 ± 0.5	6.62 ± 0.12
	D4	1.87 ± 0.04	24.1 ± 0.3	5.13 ± 0.08	1.68 ± 0.02	220 ± 2	1.24 ± 0.02	48.8 ± 0.5	26.4 ± 0.6	17.1 ± 0.3	6.88 ± 0.14

(D1) at the senescence with a similar trend for the biennial and triennial flowers. The general order of abundance of macro-elements in flowers was K > Ca > P and Mg > Na, in accordance with Storey and Treeby (2000), and this order was unrelated to growth age and growth period.

The concentration of Fe and Mn in the biennial and triennial plants decreased from D1, reached the lowest level at D2, and then increased gradually to D4. In the biennial plants, the increase from D3 to D4 was very sharp and the concentration at D4 was 3 and 1.45 times higher than D1, respectively. The concentration of Zn during flower development varied in parallel with that of Ca, while the changes in the content of B were similar to those of Mg and Na. The concentration of Cu in the biennial plants showed no considerable change throughout flower development, however in triennial plants, it decreased at D2 and increased at D3 and D4 to nearly the initial content (D1). The general order of abundance of micro-elements in the flowers was Fe > Mn > Zn > B > Cu, similar to Tokalıoğlu (2012), and this order was also not affected by growth year and growth period.

The levels of almost all the elements in the flowers (Table 3) decreased compared with D1, and subsequently varied to different extents. These decrease may be induced by elements incorporated in the structures of proteins, enzymes, and complex carbohydrates to participate in biochemical reaction (Abugassa et al, 2008; Kolasani et al, 2011), which was supported by the fact that longanic acid and gentiopicroside peaked at D2. Compared with the contents in the roots (R2) at the same growth year, the elemental concentration at flower opening (D2) was all lower except for K and B in the biennial plants and Fe, Mg, K, P, and B in triennial plants. This reduction was remarkable for Na where the floral concentration in biennial and triennial plants was only 37.3% and 35.0% of that in the roots. This indicated that the distribution of elements was affected by parts of the plant as shown previously (Fircks, 2001). The orders of macro- and micro-element in the flowers existed in the roots at the same time.

Table 3 Comparison on elemental contents in flowers (D2) and roots of biennial and triennial *G. macrophylla* ($\bar{x} \pm s$, $n = 4$)

Plant ages	Plant parts	Contents of macro- and micro-elements									
		P / ($mg \cdot g^{-1}$)	K / ($mg \cdot g^{-1}$)	Ca / ($mg \cdot g^{-1}$)	Mg / ($mg \cdot g^{-1}$)	Na / ($\mu g \cdot g^{-1}$)	Fe / ($mg \cdot g^{-1}$)	Mn / ($\mu g \cdot g^{-1}$)	Zn / ($\mu g \cdot g^{-1}$)	B / ($\mu g \cdot g^{-1}$)	Cu / ($\mu g \cdot g^{-1}$)
biennial	flowers	1.97 ± 0.02	21.8 ± 0.2	3.99 ± 0.01	1.60 ± 0.01	175 ± 3	0.584 ± 0.001	34.0 ± 0.5	27.2 ± 0.4	10.4 ± 0.3	7.5 ± 0.1
	roots	2.43 ± 0.02	13.0 ± 0.0	5.24 ± 0.03	1.72 ± 0.01	471 ± 2	1.855 ± 0.023	58.7 ± 0.4	29.7 ± 0.2	6.8 ± 0.1	13.4 ± 0.1
triennial	flowers	1.74 ± 0.02	19.2 ± 0.0	4.95 ± 0.02	1.54 ± 0.01	149 ± 4.	0.439 ± 0.003	24.6 ± 0.2	24.2 ± 0.8	14.3 ± 0.3	5.1 ± 0.1
	roots	1.67 ± 0.03	10.9 ± 0.0	5.60 ± 0.06	1.22 ± 0.01	427 ± 10	0.380 ± 0.012	24.9 ± 0.3	26.8 ± 0.6	4.2 ± 0.09	8.0 ± 0.1

3.4 Changes of carbohydrate, crude protein, and cell wall composition

The biennial and triennial plants showed similar changes (Figure 4). The amount of soluble sugars (Figure 4A) increased during early stages of floral bud development (by approximately 17.3% and 21.3% for the biennial and triennial plants), peaked at flower opening (29.1% and 26.5%, respectively), and decreased to the lowest levels at senescence (6.6% and 9.8%, respectively). In most plants, accumulation of soluble sugars is tightly linked to the flower development and increased drastically at full bloom (Yap et al, 2008; Sood et al, 2006). The role of sugars in the flower development may be multifunctional. They act as energy source (Moalem-Beno et al, 1997), osmotic regulators (Bieleski, 1993), and precursors for metabolic processes as substantiated by the present study in which changes in active ingredients were in accordance with those in soluble sugars. The crude protein (Figure 4B) concentration of the biennial plants decreased throughout flower development (from 16.2% to 10.9%); There were no obvious changes in the triennial plants but fluctuation still existed. A loss of crude protein has been reported during the flower development in daylily petals (Lay-Yee et al, 1992) and tea flowers (Joshi et al, 2011). The amount of starch (Figure 4C) showed a similar trend with that of soluble sugars; It increased during early stages of floral bud development (approximately 4.7% and 5.3%, respectively), peaked at flower opening (6.7% and 6.6%,

respectively), and decreased to the lowest level at senescence (2.4 and 3.2%, respectively). While in some flowers, variations in starch content were inverse to those of soluble sugars (Yap et al, 2008). As a result, carbohydrate metabolism was found to regulate the flower development as observed in species such as creeping bellflower (Vergauwen et al, 2000) and *Dendrobium crumenatum* Sw. (Yap et al, 2008).

Hemicelluloses play the important structural roles in the cross-linking of cellulose in the cell walls and the breakdown of hemicellulose may contribute to the alterations in primary cell wall structure (Brummell, 2006). In our study, The hemicellulose (Figure 4D) levels declined from about 6.2% (averaged for the biennial and triennial plants) to 4.7% as floral buds developed to maturity and increased to 11.4% during senescence. Cellulose (Figure 4E) and lignin (Figure 4F) concentration increased steadily throughout from 10.5% to 15.1% and 16.0% to 27.3% respectively. Li et al (2006) also found that cellulose increased during the development of the outer pericarp tissues of kiwifruit.

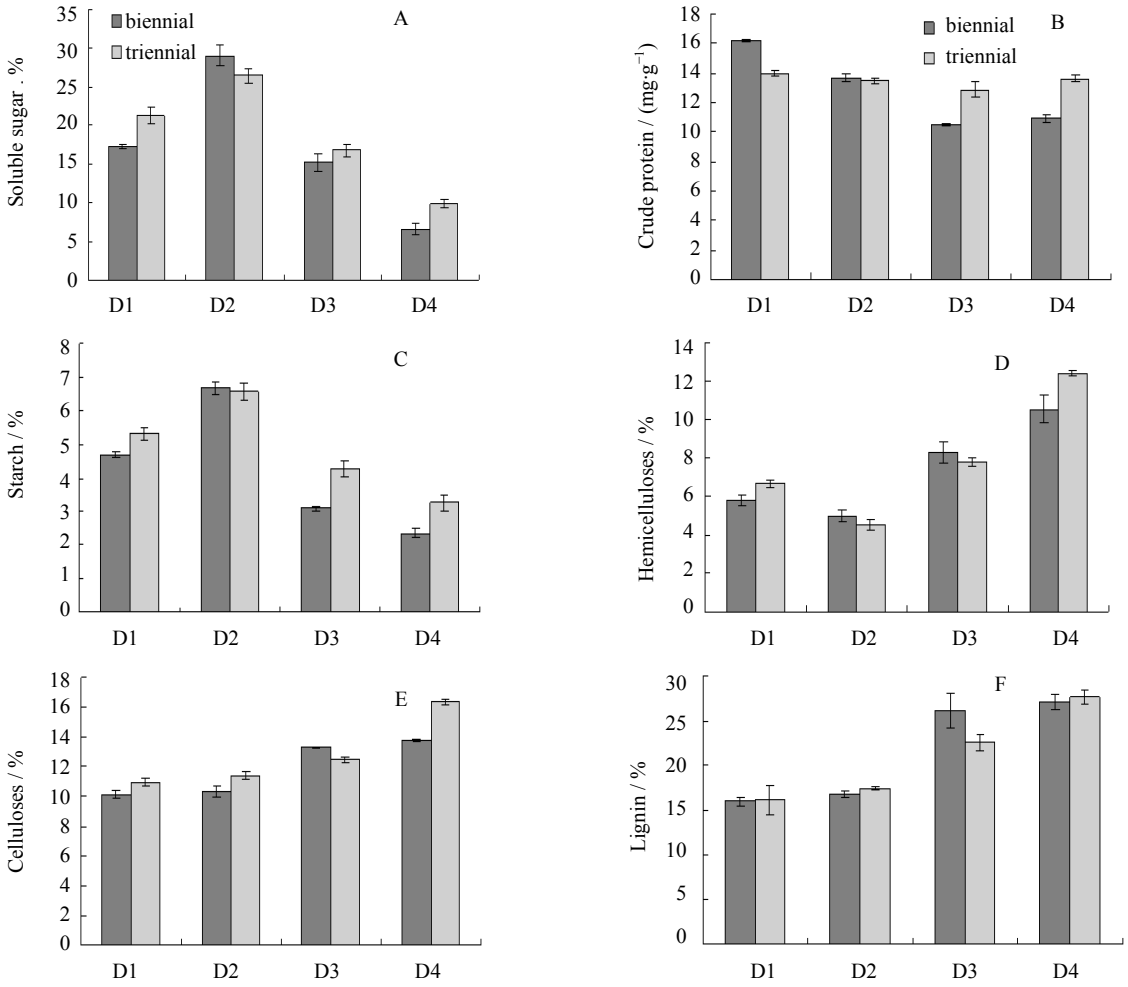

Figure 4 Changes in carbohydrate (A: soluble sugar; C: starch), crude protein (B), and cell wall composition (D: hemicellulose; E: cellulose; F: lignin) during flower development of *G. macrophylla* ($\bar{x} \pm s$, $n = 9$)

4. Conclusion

Changes in the active constituents, mineral nutrients, and physiological indices are tightly regulated at the flower development and show the some common features during the flower senescence. Compared with the roots, the contents of the active constituents and mineral nutrients in the flowers (D2) show that the flowers of *G. macrophylla* are a potential medicinal part.

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