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Full Length Research Paper

Variation in contents of major bioactive compounds in *Glechoma longituba* related to harvesting time and geographic distribution

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Glechoma longituba **(Nakai) Kupr. (Labiatae) is an important medicinal plant with various pharmacological activities. Current study was carried out to establish the variation pattern in the contents of ethanol-soluble extractive, total flavonoids, ursolic acid (UA) and oleanolic acid (OA) in *G. longituba* in relation to harvesting time and geographical origin. *G. longituba* was harvested at one month interval during ontogenetic development phases and twenty-nine populations were collected from different distribution areas in China. The results showed that the contents of ethanol-soluble extractive, total flavonoids, UA and OA in *G. longituba* were related to harvesting time and distribution origin. The optimum harvesting time should be in mid-April in terms of the contents of ethanol-soluble extractive and total flavonoids, and be in mid-August or in mid-March in terms of the contents of UA and OA. Populations of *G. longituba* showed remarkable differences in chemical composition depending on the provenance of plants. And the differences among 29 populations are likely to be genetically controlled since all populations were grown under uniform conditions. Our results on seasonal and environmental factors will be useful for commercial producers of *G. longituba* in determining the optimum harvesting time and the most appropriate plants for germplasm evaluation and breeding.**

Key words: *Glechoma longituba* (Nakai) Kupr., harvesting time, inter-populational variability, total flavonoids, ursolic acid, oleanolic acid.

INTRODUCTION

Glechoma longituba (Nakai) Kupr. (Labiatae) is an herbaceous medicinal plant that is widespread in China, Russia and North Korea (Flora of China, 1977). *Glechomae herba*, the dried aerial part of *G. longituba,* is a standard medicinal material in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010) and is a well-known Chinese medicinal herb used for the treatment of diuretic, cholagogue, lithagogue, heat-clearing, detoxicating, swelling and pain caused by traumatic injury, eliminating concretion and anti-diarrhea. Additionally, it is also used as main ingredient in many formulas such as a famous Chinese patent medicine named Lithagogue Granules (Paishi Keli in Chinese) (Ni et al., 2010, 2011; Zhang et al., 2011).

A number of bioactive constituents, including triterpenoids (for example, ursolic acid and oleanolic acid), phenolic acids, flavonoids and essential oil have been isolated from *G. herba* and identified (Ni et al., 2011). Both oleanolic acid (OA) and ursolic acid (UA) have many important pharmacological effects, such as anti-inflammatory, hepatoprotective, antitumour, anti-HIV, antimicrobial, antifungal, gastroprotective, hypoglycemic and antihyperlipidemic properties (Janicsák et al., 2006). In addition, total flavonoids have been reported to exhibit a wide range of biological effects, including antiviral, anti-inflammatory, antibacterial, vasodilatory and antiallergic

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actions (Cook and Samman, 1996), and the content of total flavonoids are regarded as the important criteria for determining *G. longituba* quality (Cheng et al., 2005). The content of ethanol-soluble extractive is a necessary criteria to evaluate *G. longituba* quality in Chinese Pharmacopoeia, which should be not less than 25.0% (Chinese Pharmacopoeia Commission, 2010).

Medicinal plant production is largely dependant on genetic background and environmental conditions, and contents of the major bioactive compounds varied according to different environments due to the responses to different physical, chemical and biotic elicitors (He et al., 2010). For example, Zhang et al. (2011) investigated the effects of water deficiency on the growth, physiological characteristics and total flavonoid content of *G. longituba* and suggested that 80 to 85% of field capacity is the most suitable watering treatment for the growth of *G. longituba*. In addition, the content of hypericin in *Hypericum perforatum* varied significantly in plants collected from different locations and depended on plant development stage. And the authors suggested that populations of *H. perforatum* in geographically separated regions synthesize different amounts of the major bioactive compounds, probably, due to the specific environments and/or genetic variations of the plants (Bagdonaite et al., 2010).

The determination of harvesting time is an important aspect of *G. longituba* production to maximize yields and content of desirable compounds. In the Chinese Pharmacopoeia 2010 edition, the wide period from spring to autumn is taken as the harvesting time (Chinese Pharmacopoeia Commission, 2010). Nevertheless, the previous studies on the accumulation dynamics of bioactive compounds in the aerial part of *G. longituba* showed conflicting results (Huang and Chen, 2004; Xin et al., 2009).

So far, there are few reports on interpopulational variability of the contents of some bioactive compounds in the aerial part of *G. longituba.* Wang et al. (2006) found that the flavonoids content in *G. longituba* plants from two locations showed marked difference, and no significant difference was detected in the wild-growing and cultivated samples *G. longituba* plants from same locations. In the case of OA and UA, the wild-grown plants had higher contents than cultivated ones. Additionally, Ni et al. (2011) established analytical method for simultaneous determination of nine bioactive phenolic compounds, and applied this method for the quality control of twenty batches of *G. longituba* from nine provinces of China. And they found that variations in the content of the different markers in different samples were independent of the producing area.

It was therefore necessary to study the variations in content of major bioactive compounds in the aerial part of *G. longituba* in relation to harvesting time and geographical origin. In the present study, the accumulation dynamic of ethanol-soluble extractive, total flavonoids, UA and OA in *G. longituba* was investigated. In addition, the chemical polymorphisms of 29 *G. longituba* populations were studied by being collected at different locations and planted at the same location under identical conditions. The main objective of this work was to establish the variation pattern in the contents of ethanol-soluble extractive, total flavonoids, UA and OA in *G. longituba* in relation to harvesting time and geographical origin.

MATERIALS AND METHODS

Plant material and growth conditions

The aerial parts of *G. longituba* were collected with different harvesting time at one month interval from 15th January to 15th December 2009 at stationary location at Zijin Mountain, Nanjing, Jiangsu Province, P.R China. Additionally, we collected 29 *G. longituba* populations from different distribution areas in China and planted them in April 2010 in research field of the Institute of Chinese Medicinal Materials, Nanjing Agricultural University, Nanjing, P.R China using conventional commercial cultivation methods under identical conditions. Each plot measured 1.5 × 2 m (3 m^2). The plots were arranged in a completely randomized block design with three replications. Aerial parts of the plants were collected in October 2010. The plant material was washed by distilled water, air-dried, and ground to pass through a 0.25 mm sieve. For the determining of dry mass, the material (1.00 g) was kept in an oven at 105°C for 3 h, and stored at a room temperature under light-protected and humidity-proof conditions until it is used for chemical analysis.

Determination of the ethanol-soluble extractive content

The content of ethanol-soluble extractive was determined according to the method of Chinese Pharmacopoeia (2010). Dried *G. longituba* powder (2.0 g) was extracted by 100 ml 55% ethanol in a conical flask after keeping at room temperature for 1 h and reflux extraction for 1 h. Then the extractive was filtered. Twenty five milliliter of the filtrate was dried on water-bath, and weighed after oven-dried at 105°C for 3 h.

Determination of UA and OA content

Ursolic acid and oleanolic acid content was determined using an HPLC system consisting of an LC-20AT liquid chromatograph (Shimadzu, Kyoto, Japan). The methods for determining those two bioactive components have been described previously (Zhang et al., 2007; Wang et al., 2008).

Determination of total flavonoids content

The flavonoid extraction method was carried out according to the method of Lee et al. (2005) with slight modification. Dried *G. longituba* powder was dissolved in methanol and left at room temperature for 4 h, and then in an ultrasonic bath for 1 h. Total flavonoids content was determined with a spectrophotometric method. One milliliter of the methanol extract was mixed with 5 ml of distilled water, followed by the addition of 1 ml of 5% (w/w) $NaNO_2$ solution. After 6 and 12 min, 1 and 10 ml, respectively, of 10% (w/w) $Al(NO_3)_3$ solution and 1 M NaOH were added. The mixture was brought to 25 ml with distilled water and determined at 510 nm using a spectrophotometer. The content of total flavonoids was calculated as rutin equivalents.

Statistical analysis

The data was subjected to a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). Statistical analyses were conducted using the statistical software package SPSS 13.0 for Windows. A level of $P<0.05$ was used as the criterion for statistical significance.

RESULTS

Determination of the optimum harvesting time

The contents of ethanol-soluble extractive, total flavonoids, UA and OA in *G. longituba* aerial part varied significantly with harvesting time, that is, with ontogenetic development phases (Figure 1).

There were three accumulation peaks of total flavonoids within a year, that is, January, April and October (Figure 1b). The highest total flavonoids content was found on 15th April (13.543%), while the lowest content was exhibited on 15th November (3.302%). Additionally, there are no significant difference between total flavonoids on 15th April and 15th May. The variation tendency of ethanol-soluble extractive content was similar to total flavonoids content (Figure 1a), except that there was no significant difference in the ethanol-soluble extractive content between 15th November and 15th January.

The variation of UA and OA contents fluctuated during the ontogenetic development phases of *G. longituba* (Figure 1c and d). And a significant linear correlation ($p<0.01$) was found between UA and OA content during the developmental stages (Figure 2). The highest values of UA and OA contents were observed on 15th August (0.166% for UA and 0.031% for OA) followed by 15th March (0.163% for UA and 0.030% for OA), and there was no difference between those two harvesting time. While the minimal UA and OA contents were obtained on 15th April. The content of UA harvested on 15th August was 4.26 folds of that harvested on 15th April, while the corresponding value was 7.75 folds.

Geographical distribution of the populations

G. longituba plants of different populations showed significant variation in contents of ethanol-soluble extractive, total flavonoids, UA and OA in aerial parts when grown under the same growth conditions (Table 1). The geographical distribution of the populations was shown in Figure 3.

The content of ethanol-soluble extractive of different populations ranged from 22.258 to 34.414%, with the average content 28.022%. The highest value was found in the *G. longituba* population from G11 (Jiaxing, Zhejiang Province, 34.414%) followed by G01 (Dandong, Liaoning Province, 34.284%), while the lowest value was observed in the population from G21 (Nanchang, Jiangxi Province, 22.258%). The general variation trend of ethanol-soluble extractive content decreased as the latitude decreased from Northeast China to South China. Nevertheless, the average ethanol-soluble extractive content in the population from Jianghuai Hilly region (consisting of 11 samples, G07 to G17) and Yunnan-Guizhou Plateau (consisting of G22 and G26) were 29.578 and 28.523%, respectively, which were higher significantly than other populations except for G01.

The total flavonoids content showed decreased with the increasing latitude. The highest total flavonoids content was found in G26 (Kunming, Yunnan Province, 10.053%) and the lowest value was observed in G02 (Beijing, 2.941%), with the average value of 6.387%. In case of distribution zone, the populations from Jiangnan Hilly (G03, G11, and G14) and Yunnan-Guizhou Plateau (G22 and G26) had higher relatively total flavonoids content than other zones, with the value of 9.818 and 9.499%, respectively.

Among the 29 populations, the one from Northeast China (G01) showed the highest oleanolic acid content (0.145%), which was higher significantly than other population. The one from Beijing (G02) showed the lowest value (0.008%). And the average oleanolic acid content of 29 populations was 0.088%. Except for G01, the oleanolic acid content decreased with the decreasing latitude.

The population from Jiaxing (Zhejiang province, G11) showed the highest ursolic acid content (0.762%), while the one from Beijing (G02) showed the lowest value (0.043%). In generally, the populations from the area of the downstream Yangtze River and Jianghuai Hilly region (G3 to G5, G8 to G11, G14 to G17) and Southwest China (G22 to G28) had higher ursolic acid content than those from other area (G01, G02, and G27 etc).

DISCUSSION

In the present research, the highest contents of ethanol-soluble extractive and total flavonoids were observed in mid-April, while the contents of UA and OA peaked in mid-August followed by mid-March. These results disagreed with the previous works of Huang and Chen (2004) and Xin et al. (2009). Huang and Chen (2004) found that the highest total flavonoids content was found in July to August in wild *G. longituba* on Guangdong Province, while in the works of Xin et al. (2009), the highest total flavonoids content in the wild *G. longituba* collected on Hubei Province was found in August to September. This may be explained that the materials used in those studies were collected from different regions under varying climate and soil conditions. Therefore, the optimum harvesting time should be determined according to the accumulation dynamics of certain bioactive compounds, namely, *G. longituba* being harvested in mid-April in terms of the contents of ethanol-soluble extractive and total flavonoids, and being

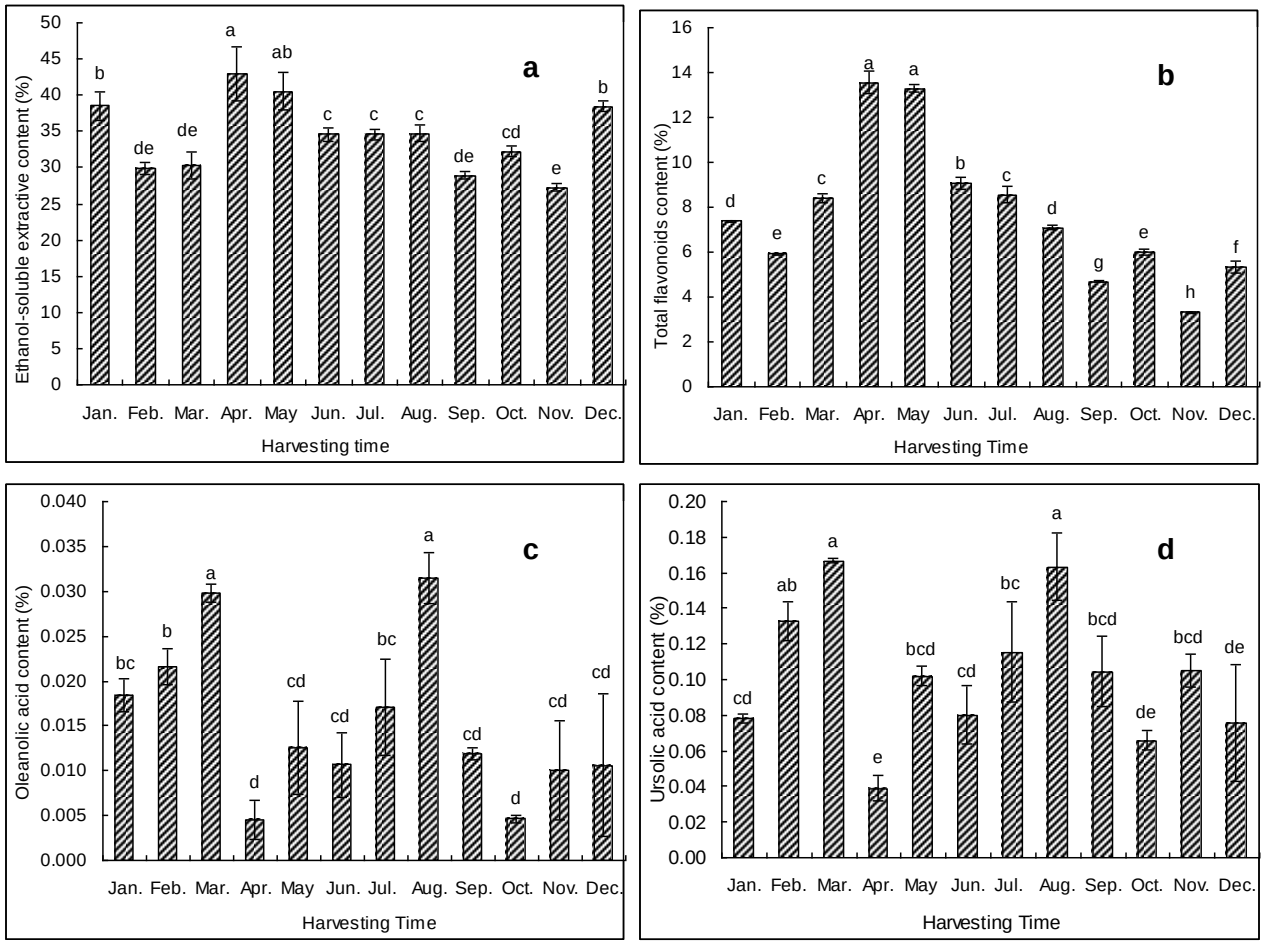

Figure 1. Accumulation dynamics of ethanol-soluble extractive (a), total flavonoids (b), ursolic acid (c) and oleanolic acid (d) in aerial part of *G. longituba*. The letters above the columns indicate significant differences at $P<0.05$.

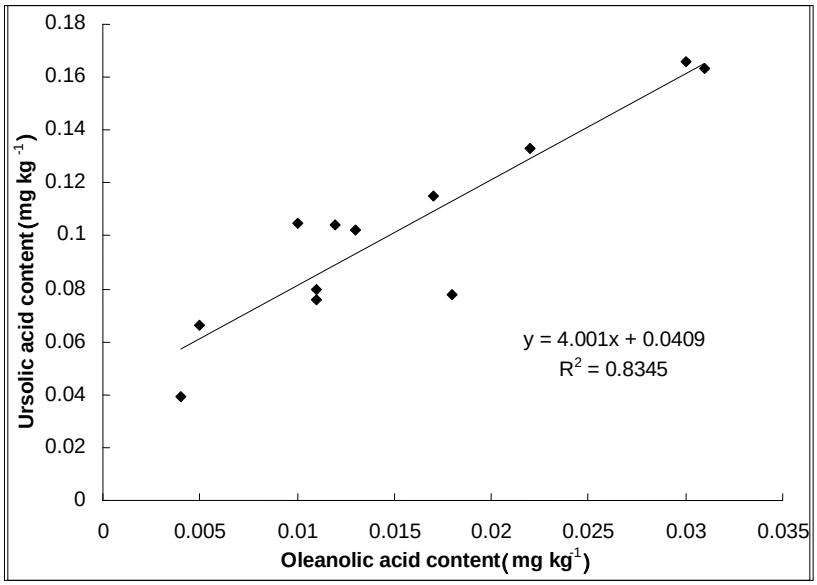

Figure 2. Relationship between ursolic acid and oleanolic acid contents in aerial parts of *G. longituba* plants during ontogenetic development phases.

Table 1. Ethanol-soluble extractive, total flavonoids, UA and OA content in 29 *G. longituba* populations from different Chinese regions.

Code	Ethanol-soluble extractive content (%)	Total flavonoids content (%)	Oleanolic acid content (%)	Ursolic acid content (%)
G01	34.287±4.419	5.137±0.035	0.145±0.033	0.385±0.018
G02	28.220±0.725	2.941±0.073	0.008±0.001	0.043±0.005
G03	29.189±0.563	9.933±0.391	0.100±0.009	0.344±0.027
G04	25.659±1.508	6.702±0.122	0.096±0.012	0.330±0.015
G05	23.368±0.637	7.477±0.331	0.033±0.004	0.386±0.037
G06	24.396±0.751	3.557±0.204	0.079±0.006	0.228±0.012
G07	28.512±0.545	4.697±0.132	0.106±0.003	0.295±0.016
G08	32.178±2.588	6.496±0.144	0.057±0.007	0.394±0.049
G09	30.378±0.694	3.862±0.142	0.060±0.002	0.548±0.040
G10	23.648±0.612	4.054±0.104	0.052±0.003	0.398±0.006
G11	34.414±0.906	9.692±0.183	0.082±0.011	0.762±0.002
G12	23.617±0.710	3.513±0.020	0.097±0.008	0.291±0.044
G13	31.675±2.711	4.074±0.076	0.069±0.002	0.206±0.016
G14	30.270±0.797	9.830±0.176	0.091±0.022	0.393±0.024
G15	29.227±0.443	5.061±0.184	0.080±0.007	0.392±0.029
G16	30.517±0.682	8.941±0.414	0.092±0.001	0.335±0.026
G17	30.925±1.796	6.611±0.225	0.077±0.009	0.368±0.051
G18	26.419±0.800	7.852±0.167	0.074±0.003	0.325±0.058
G19	25.590±4.719	6.802±0.191	0.074±0.007	0.376±0.027
G20	27.222±0.702	5.623±0.011	0.068±0.007	0.223±0.024
G21	22.258±0.147	4.639±0.161	0.075±0.002	0.392±0.007
G22	27.178±0.416	8.922±0.947	0.056±0.005	0.307±0.002
G23	23.698±6.785	7.372±0.161	0.119±0.008	0.543±0.008
G24	30.100±0.180	8.510±0.133	0.046±0.008	0.330±0.021
G25	25.060±0.220	4.177±0.145	0.055±0.006	0.522±0.043
G26	29.868±0.434	10.053±1.276	0.061±0.007	0.429±0.012
G27	28.732±3.987	6.324±0.185	0.501±0.005	0.316±0.017
G28	27.121±0.658	6.229±0.316	0.060±0.005	0.501±0.016
G29	28.922±0.269	6.143±0.100	0.050±0.006	0.274±0.017

Values were means±SD. G01-G29 represented the 29 *G. longituba* populations from different Chinese regions.

harvested in mid-August or in mid-March in terms of the contents of UA and OA.

Chemical variation between populations can be attributed to genetic and/or environmental factors. Wide intraspecific variation in essential oil composition has been described in several aromatic and medicinal plants (Echeverrigaray, 2003). Similarly, a high chemical variability was observed in *G. longituba* populations collected from different distribution zones. The populations from Jianghuai Hilly region, the area of the downstream Yangtze River, and Yunnan-Guizhou Plateau rich in ethanol-soluble extractive, total flavonoids, UA and OA. In contrast, the population from Beijing was poor in total flavonoids, UA and OA content than other samples. The variation of bioactive compounds in *G. longituba* plants may represent an adaptive strategy in relation to environmental variation of geographical origin. As the plants examined were cultivated under the same conditions, the variations observed may be under genetic rather than environmental control (Agostini et al., 2010; He et al., 2010; Bagdonaite et al., 2010).

Genetic material with high content of target compounds could serve as an excellent genetic infrastructure for studying the genetic factors controlling biochemical pathways (Sheng et al., 2008; Beharav et al., 2010). And phytochemical analysis of *G. longituba* populations plays an increasingly important role in the management and utilization of plant genetic resources. Hence, it is necessary to evaluate the wild populations of *G. longituba* and to look for the wild genotype rich in certain bioactive compounds. Based on the results of this study, the high-quality *G. longituba* populations that are rich in ethanol-soluble extractive, total flavonoids, UA and OA, such as the population from Jianghuai Hilly region and

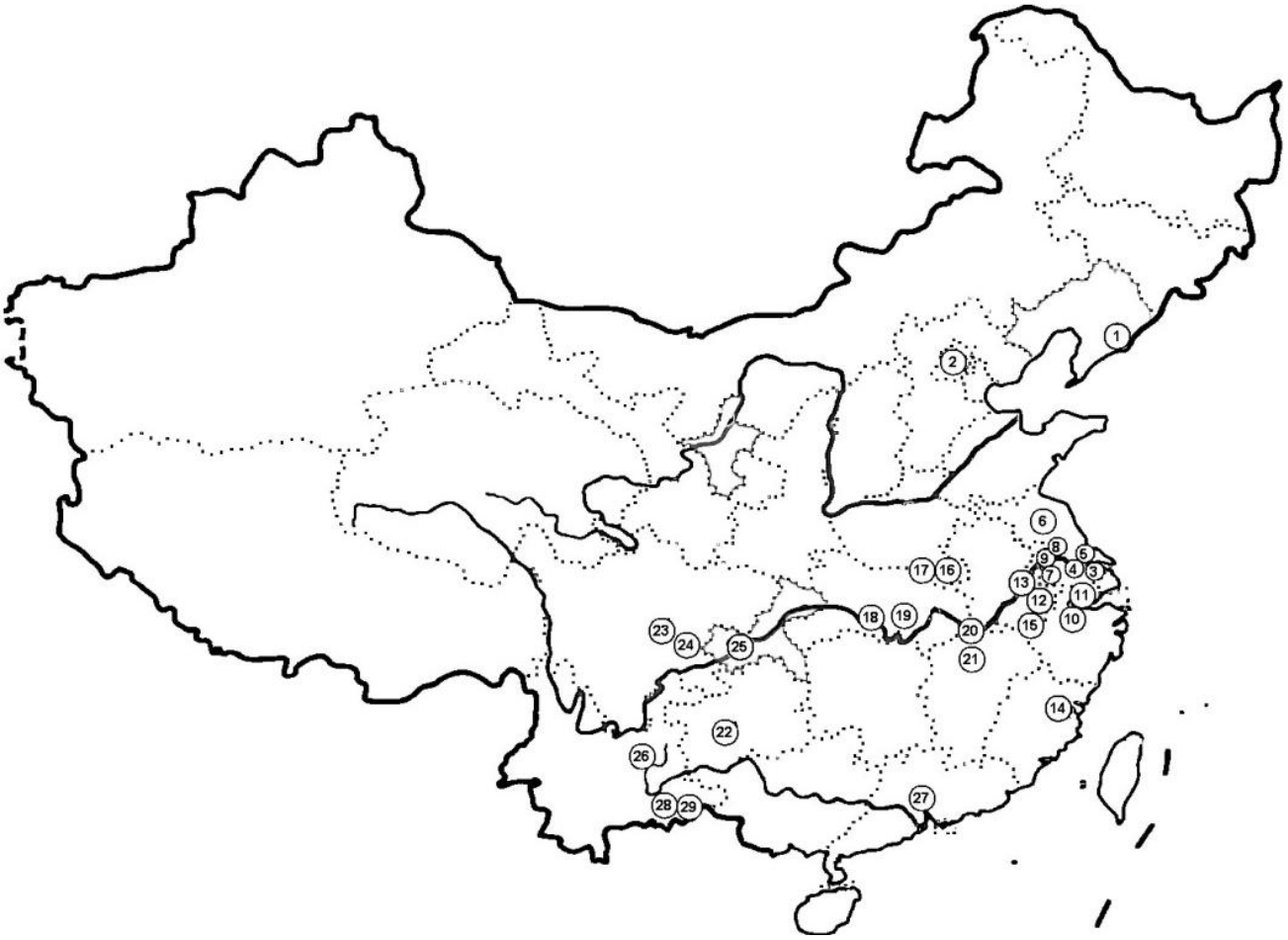

Figure 3. Geographical origins of the 29 populations of *G. longituba* analyzed.

Yunnan-Guizhou Plateau, should be interesting to candidates for future research aiming to elevate major bioactive compounds levels in cultivated *G. longituba* material.

Conclusions

The results showed that the contents of ethanol-soluble extractive, total flavonoids, UA and OA in *G. longituba* were related to harvesting time and distribution origin. The optimum harvesting time of *G. longituba* should be determined according to the accumulation dynamics of certain bioactive compounds. The best harvesting time should be in mid-April in terms of the contents of ethanol-soluble extractive and total flavonoids, and be in mid-August or in mid-March in terms of the contents of UA and OA. Additionally, the high-quality *G. longituba* populations that rich in ethanol-soluble extractive, total flavonoids, UA and OA, such as the populations from Jianghuai Hilly region and Yunnan-Guizhou Plateau, had considerable potential for the domestication and genetic improvement of *G. longituba*.

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