The Localization and Dynamic Change of Saponin in Vegetative Organs of *Polygala tenuifolia* Willd

Hong-Mei Teng 1, 2, Min-Feng Fang 1, Xia Cai 1, Zheng-Hai Hu 1*

(1 Key Laboratory of Resource Biology and Biotechnology in West China
(Northwest University), Ministry of Education, Xi’an 710069, China
2 YunCheng College, ShanXi 044000, China)

Abstract: Anatomical, histochemical and phytochemical methods were used to investigate the structure, localization and dynamic changes of total saponin and segenenin of vegetative organs in *Polygala tenuifolia* Willd. Histochemical localization results showed that saponin accumulated mainly in parenchyma cells of vegetative organs. The phytochemical results also showed that the saponin accumulated in the vegetative organs of *P. tenuifolia*, with higher content in roots and lower content in the aerial parts that included stems and leaves. The saponin content and dry weight of the vegetative organs of *P. tenuifolia* had dynamic variance at the developmental stages and all reached highest level in post-fruit period. So the roots and aerial parts should be gathered at August to make full use of the plant. As the root was the main medicinal organ of *P. tenuifolia*, the content of total saponin and segenenin of different-year-old and different parts of root were determined. The content of total saponin and segenenin exhibited a sustained decreasing trend with the root age increasing, therefore the annual roots had high quality. The content of total saponin and segenenin in different parts of root differed obviously. The content in the “skin areas” was much higher than that of xylem. The results offer theoretical basis for determining the appropriate harvesting stage and a reasonable harvest of *Polygala tenuifolia*.

Key words: *Polygala tenuifolia*; vegetative organs; structure; saponin; segenenin; localization; dynamic change

*Polygala tenuifolia* Willd, which belongs to *Polygala* in Polygalaceae, is a perennial herbaceous plant mainly distributing in Shan Xi, Shaan Xi, He Nan provinces of China. As a well-known traditional Chinese herb, its root has many medical functions, such as eliminating phlegm,

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*Author for correspondence. E-mail: zhenghaihu@sina.com. Tel:029-88302684.
detumescence, smoothing the nerves and enhancing intelligence (Chinese pharmacopoeia 2005). Extensive phytochemical and pharmacological studies on this plant proved the saponin to be the main bioactive principles (Fu and Zhang 2006). Specially, in recent years, researches had indicated that the saponin also can decrease blood sugar, enhance immunity, and bringing down the blood pressure (Nagai and Suzuki 2001; Sun 2005). As far as medicinal parts are concerned, Chinese Pharmacopoeia stipulates the root of Polygala tenuifolia Willd. as crude drug (2005). At present, there have had many studies on P. tenuifolia, most of them have focused on the chemical structure and identification of saponin (Sakuma and Shoji 1981a; 1981b), pharmacology (Chen et al. 2004), clinical application (Jung et al. 2007), content determination (Yang and Sun 2007; Liu 2001) as well as the root developmental and anatomy (Teng and Hu 2008). However, studies on the localization and accumulation of saponin in vegetative organs of P. tenuifolia had not been reported. P. tenuifolia was mainly wild in nature. But, in recent years, the wild drugs were nearly extinct because of over-digging. At same time, the cultivated crude drug mainly in Shan Xi, Shaan Xi could not meet the market because of limited amounts. The aim of this study was to analyze the structure characteristics of vegetative organs, and ultimately investigate localization and dynamic change of saponin of P. tenuifolia at different developmental stages, so as to offer theoretical basis for applying and developing P. tenuifolia resource rationally.

**Results**

**The secondary structure of root and histochemical Localization of saponin**

Roots at different age of growth of P. tenuifolia were collected from the middle part of the taproots (the primary structure occurs at the tip of root). The root secondary structure of P. tenuifolia consisted of periderm and secondary vascular tissue crosswise (Figure 1A), and the structures of different-year-old root were similar. Periderm consisted of phellem, phellogen and phelloderm. With suberized wall, the 7-9 layers cell of phellem were closely arranged. Phellogen had 1 layer of cells, and phelloderm had 2-3 layers of parenchyma cells (Figure 1B). Secondary vascular tissue consisted of secondary phloem, vascular cambium and secondary xylem. Root cross-section was mainly composed of secondary phloem whose thickness made up about 3/5-2/3 of the root diameter. Phloem parenchyma cells were round or nearly round in shape, large or small
in size, formed the majority of secondary phloem (Figure 1A, B). There were rare sieve vessels and companion cells among phloem parenchyma cells, and phloem fiber was not been found. In comparison with secondary phloem, secondary xylem made up 2/5-1/3 of the whole diameter of taproot and was composed of woody fibers, vessels, a few xylem parenchyma cells and xylem rays (Figure 1A, C). Vessels had different diameters and scattered with higher frequency. Woody fibers were distributed in a clumped manner. Most xylem rays comprised a row of cells, and few comprised two rows of cells (Figure 1A, C).

Histochemical localization results showed that, in the root having secondary structure, phellose displayed purplish red, secondary phloem red, xylem ray and xylem parenchyma pale red, while other cells no color (Figure 1 G, H).

The structure of stem and histochemical localization of saponin

The stem of *P. tenuifolia* composed of epidermis, cortex and vascular bundle from outside to inside (Figure 1D, E). The epidermis cells were square or rectangle in shape, small or big in size, arranged closely. There were stomatal apparatus among epidermis cells (Figure 1D). In cortex, there were 4-5 layers of big parenchyma cells which were nearly round and arranged loosely. There were chloroplasts in the outer 2-3 layers of parenchyma cells, and the inner 1-2 layers parenchyma cells were big and dark-dyed (Figure 1D,E). The central cylinder consisted of vascular bundle and pith, and the vascular bundle connected into a round. In vascular bundle, the phloem consisted of sieve tubes, companion cells and phloem parenchyma cells. The cells of phloem lined closely. Vascular cambium that lied between phloem and xylem was not obvious. Xylem consisted of large number of xylem fibers, vessels and few xylem parenchyma cells. Xylem fibers lined radially, and vessels had wider diameter and arranged irregularly. Xylem rays were obvious, most of them comprised a row of cells, and few comprised two rows of cells (Figure 1E). There were well-developed pith in the center of stem, and the pith was composed of big parenchyma cells which were nearly round (Figure 1D). Histochemical localization results showed that, in stem, cortex displayed purplish red, epidermis and secondary phloem pale red, while the other tissues no color (Figure 1 I, J).

The structure of leaf and histochemical Localization of saponin

The leaf of *P. tenuifolia* was typically bifacial one. The lamina of *P. tenuifolia* consisted of epidermis, mesophyll and leaf vein. Two sides of leaf superficies comprised a layer of epidermic cells, and the cells were mainly rectangle or square, arranged irregularly and closely. There were
stomatal apparatus among the epidermic cells of the upper and lower epidermis. Stomatal apparatus had bigger substomatal chamber. The mesophyll composed of distinguished palisade tissue and spongy tissue. The palisade tissue cells arranged orderly. The spongy tissue composed of 3-4 layers of cells that arranged loosely and had obvious interspace (Figure 1F). Histochemical localization results showed that palisade tissue displayed red, epidermis and spongy tissue pale red (Figure 1K).

In the control experiment, the sections of different vegetative organs that had been dealt with FAA (formalin-aceticacid-70% alcohol) could not react with 5% vanillin-glacial acetic acid-perchloric acid solution to produce characteristic color (Figure 1L). The above results indicated that saponin accumulated in root, stem, and leaf of *P. tenuifolia*, and mainly in parenchymal cells of vegetative organs.

**The content change of total saponin and dry weight of vegetative organs of *P. tenuifolia* at different developmental stages**

The results of histochemical localization had preliminarily proved that the saponin was distributed in vegetative organs of *P. tenuifolia*. We determined the content of total saponin at different developmental stages in vegetative organs of annual plant of *P. tenuifolia* to proof the above results further. The results showed that the saponin accumulated in the vegetative organs of *P. tenuifolia*, with higher content in roots and lower content in the aerial parts that included stems and leaves (Figure 2). The content in the aerial parts was about 1/5 as much as that in roots on average at the whole developmental stages, and was about a quarter of that in roots at post-fruit period. The statistical analysis demonstrated that the total saponin content of vegetative organs at the four stages showed significant differences (*P*<0.05). At the same time, the total saponin content of vegetative organs showed a dynamic variance trend during the whole growth period from April to October. At the pre-blossom period, the content in root was high. With the efflorescence and fruits forming, accumulation of saponins decreased. After blossom-fruit period, the content increased comparatively quickly, reaching the highest level of 5.176% at post-fruit period. With the withering period coming, the content decreased again, and came to the lowest level during the whole growth period. On the other hand, the saponin content in aerial parts increased with the plant development before post-fruit period, and also reached the highest level of 1.296% at post-fruit period. Afterward, the content decreased and then was close to the level of pre-blossom period.
Figure 2. The dynamic variation of saponin content in annual roots and aerial parts at different stages (bars are mean ± SE).

Figure 3. The dynamic variation of dry weight in annual roots and aerial parts at different stages (bars are mean ± SE).

Note: Different letters among the different materials of the same item means significant difference at 0.05 level. The below is same.
Figure 3 showed the dynamic variance trend of dry weight of vegetative organs during the whole growth period. It could be seen that the dry weight of roots and aerial parts increased from blossom-fruit period to post-fruit period, and reached the crescendo at post-fruit period. After post-fruit period the dry weight began to decrease. From Figure 2 and Figure 3, it could be found that the saponin content and dry weight in annual roots and aerial parts all were at highest-level at post-fruit period.

The content change of total saponin and segenenin in different years roots of \textit{P. tenuifolia}

Practically, the crude drugs of roots of \textit{P. tenuifolia} usually were gathered at autumn of second or third growth year. We determined the content of total saponin and segenenin in different-year-old roots of \textit{P. tenuifolia}. The result showed that the content of total saponin and segenenin content of annual roots were higher than that of biennial roots, and content of biennial root were higher than that of triennial roots (Figure 4). The content of total saponin and segenenin of different-year-old roots showed the significant difference ($P<0.05$).

Figure 4. The content change of total saponin and segenenin in different-year-old roots of \textit{P. tenuifolia} (bars are mean $\pm$ SE).

The content change of total saponin and segenenin in different parts of roots of \textit{P. tenuifolia}
The results of histochemical localization had preliminarily proved that the saponin was mainly distributed in “skin areas” of root of *P. tenuifolia*, less distributed in xylem. The roots of *P. tenuifolia* collected in August of the first, second, and third growth year were manipulated by hands into two parts: the xylem and the “skin areas” which included periderm and phloem, both for determination of total saponin and senegenin content. The results indicated that the content of total saponin and senegenin in different parts of root differed obviously. On average, the content of total saponins and senegenin of the “skin areas” of different-year-old roots were about 13.51 and 14.25 times respectively as much as that in the xylem at the whole developmental stages, indicating that the “skin areas” was the main storage locus. At the same time, the content of total saponin and senegenin of the “skin areas” of roots decreased with the growth year increasing, the content of total saponin and senegenin of the xylem of different-year-old roots were lower and near. It could be found that the quality of annual root was high (Figure 5).

![Graph showing content change of total saponin and senegenin in different parts of roots of *P. tenuifolia*](image)

1、“skin areas” of annual roots  2、“skin areas” of biennial roots  3、“skin areas” of triennial roots  4、“xylem” of annual roots  5、“xylem” of biennial roots  6、“xylem” of triennial roots

Figure 5. The content change of total saponin and senegenin in different parts of roots of *P. tenuifolia* (bars are mean ± SE).
Discussion

The study results showed that the structure of vegetative organs of *P. tenuifolia* resembled those of usual dicots. However, in the root, the secondary phloem took up a great part of secondary vascular tissue, and contained parenchyma mainly. This characteristic was consistent with the storing function of the root of *P. tenuifolia*.

The histochemical localization of saponin had been carried out on many plants, including *Panax, Gynostemma pentaphyllum,* and *Bupleurum chinense* DC (Kubo 1980; Liu et al. 2005; Tan et al. 2007). The studies indicated saponin can react with vanillin-glacial acetic acid-perchloric acid solution to produce characteristic colors, which is from pale red to red to purplish red, and the accumulation of saponin was positively related to different shades of red. The saponin in *P. tenuifolia* was pentacyclic triterpenoid that belong to the type of oleanane, so we judge the localization and accumulation of saponin by the above method. Through histochemical experiments, saponin in *P. tenuifolia* was found accumulating in root, stem and leaf. In root, phelloderm and secondary phloem showed purplish red and red, while xylem ray cells and xylem parenchyma cells showed pale red. Therefore saponin mainly accumulated in phelloderm, secondary phloem, rarely in xylem ray cells and xylem parenchyma cells. In stem, saponin was distributed mainly in cortex, less in epidermis and secondary phloem. While in leaf, saponin was mainly distributed in palisade tissue, less in epidermis and spongy tissue. Thus, it can be drawn that saponin accumulated mainly in parenchyma of vegetative organs. The result was as same as the one in *Gynostemma pentaphyllum* that ginsenoside was regarded distributing mainly in the parenchyma cells of vegetative organ (Liu et al. 2005).

Through ultraviolet spectrophotometer determination, we found that there was saponin accumulating in roots and aerial parts of *P. tenuifolia*, this result was consistent with the findings obtained through the histochemistry experiment. At the same time, the total saponin content in roots was higher than that in aerial parts at whole developmental stages. The content in the aerial parts was about 1/5 as much as that in roots on average at whole developmental stages, and was about a quarter of that in roots at post-fruit period. Based on these results, we advocate utilize the aerial parts of *P. tenuifolia* facing the shortage of *P. tenuifolia* resource today.

The total saponin content in vegetative organs of *P. tenuifolia* at whole developmental stages
showed dynamic variance trends. The trends displayed that the total saponin content was low before June. Because vegetative growth and reproductive growth were exuberant in this period, great amount of photosynthesis products were consumed, the total saponin accumulation was low. After middle ten days of June, accompanied with the reproductive growth nearly finishing, the nutrient consuming decreased, so the content of saponin increased comparatively quickly from blossom-fruit period, and reached the crescendo in post-fruit period. Afterward, with the withering of stems and leaves, the saponin content in roots and aerial parts decreased because of the amount of photosynthesis product decreasing.

The trend of dynamic variance of dry weight of vegetative organs showed that the dry weight both in roots and aerial parts reached the crescendo at post-fruit period, this trend was consistent with that of the saponin contents. Therefore, *P. tenuifolia* should be harvested at the post-fruit period for obtaining more saponin. We should collect the stems and leaves before they withering in order to make full use of *P. tenuifolia*.

Judging from the content of total saponin and segenenin, we could found that the content of annual roots was higher than that of the biennial roots and triennial roots, so the annual roots had better quality. We guessed that triterpenoid in *P. tenuifolia* may be chemical defenses based on the fact that *P. tenuifolia* grow in barren and drought land, and not susceptible to plant diseases and insect pests. From the former research, some triterpenoids had been confirmed to be chemical defenses (Govindachari et al. 2000). For example, the triterpenoids of plant in Meliaceae have repellent and poison effects to some insects (Champagne et al. 1992; Zhao et al. 1984; Zhang and Zhao 1983), and have broad-spectrum insecticidal and bactericidal effects (Govindachari et al. 1999; Locke 1995). The annual plants was small, and its resistance was low, so they produce more saponin to resist diseases, insects and environmental stress. As the plant growing, the resistance of the plant enhanced and the synthesis of saponin decreased. Therefore, the content of total saponin and segenenin of annual roots was higher than that of the biennial roots and triennial roots.

In general, we took the dynamic accumulation of active components and developmental stages of plant as two criterions to determine the best collecting period (Han et al. 2003). Teng and Hu observed the status of different-year-old roots, they found the diameters the annual roots, biennial roots and triennial roots were 2.5-3.0 mm, 4.0-5.0 mm and 7.0-8.0 mm respectively (Teng
and Hu 2008). Therefore, the annual roots and the biennial roots have lower biologic yield, the triennial roots have the higher one. Judging from the total yield of saponin, we thought it was appropriate to harvest at the third planting year.

Analysing from the content of total saponin and segenenin of “skin areas” and xylem of three different-year-old roots, the content of total saponin and segenenin in the “skin areas” was about 13.51 and 14.25 times respectively as much as that in the xylem on average at the whole developmental stages, showing significant difference. Thus, the secondary phloem was the main storage locus for saponin. The result was consisted with the histochemical localization. Accordingly, the crude drug that has thick “skin areas” and thin xylems was of top grade.

Material and Methods

Plant material

Materials of *P. tenuifolia* were collected from the planting base of ZhengBeiZhuang Village of Yuncheng City (Shanxi, RP. China) from April to October, 2007. All materials were identified by Prof. Zheng-hai Hu from Northwest University.

Anatomical method

Materials of vegetative organs of *P. tenuifolia* at different developmental stages were separately taken from the plants in pre-blossom period (April 20-30, 2007), blossom-fruit period (June 10-20, 2007), post-fruit period (August 10-20, 2007) and withering period (October 10-20, 2007). The fresh materials were taken at random as ten samples for anatomical and histochemical study, thirty samples for phytochemical study. Afterward the samples for phytochemical study were dried to constant weight in an oven at 60°C. The fresh materials were cut into small blocks and fixed in formalin-acetic acid-alcohol (FAA) at room temperature, then dehydrated in a graded alcohol series and embedded in paraffin. The thickness of sections was 8-10μm, cutting with a Leica microtome (RM 2135), and subsequently sections were doubly stained with Safranin O and Fast Green FeF, observed and photographed under the light microscope of Leica-DMLB.

Histochemical method

Sections of fresh roots at different developmental stages were cut by hand or by using a
Leica-CM 1850 cryostat microtome at -19°, and stained with 5% vanillin-glacial acetic acid-perchloric acid solution. Then sections were observed and photographed under the light microscope of Leica-DMLB.

**Determination of saponin by ultraviolet-visible spectrophotometry**

**Instruments and standards**

Instruments: KS-80D supersonic extractor, DR-HW-1 thermostat-controlled water-bath, MA 200 electric balance, 752C ultraviolet-visible spectrophotometer.

Standard: Authentic senegenin standard were obtained from China National Institute for the Control of Pharmaceutical and Biological Products (No. 111572-200301).

**Standard curve**

The senegenin standard (0.2 mg) was put into a 1 mL volumetric flask and dissolved in methanol adequately. Then the methanol was added carefully and the volume was fixed to 1ml. We moved separately 100, 200, 300, 400, 500 μL of the sample solution and put them into five vials and evaporated them in a thermostat-controlled water-bath. After which 200 μL of 5% vanillin-glacial acetic acid solution and 800 μL perchloric acid were added, the five vials were heated for 15 min in a thermostat-controlled water-bath at 70°C, and finally being taken out and cooled with cool water, afterward, 5 mL of glacial acetic acid was added. Finally, the saponins content of above-mentioned standard solutions was determined by 752C ultraviolet-visible spectrophotometer. The detective wavelength was 570nm. The regression equation between absorbance (Y) and the quantity of senegenin (X) was: Y= 0.1363 X +0.0069, r = 0.9992.

**Determination of the total content of saponins**

Dried powder (1.0 g) material of vegetative organs was put into a 50 mL conical flask. After 25 mL of methanol was added, it was extracted with ultrasonic for 0.5 h at room temperature, and then kept for a night. Then 5 mL of the upper clear liquid was moved and 15 mL ether was added. After settle formed utterly, upper clear liquid was moved. Then the settle was dissolved in methanol at a 25 mL volumetric flask and made homogenous by shake after adding methanol to its scale. At last, 300 μL of the sample solution was used to react according to the method of making standard curve and then the content of saponins was determined.

**Determination of segenenin content by HPLC**
Instruments and conditions


Conditions: VP-ODS Column (150 mm×4.6 mm, 5.0 μm); mobile phase: acetonitrile-0.05% phosphoric acid solution (40:60); flow rate: 1 mL / min; injection volume: 20 μL; detection wavelength: 205nm. Column temperature: 25°C.

Standard curve

The senegenin standard was diluted into 0.01 mg/mL, 0.05 mg/mL, 0.1mg/mL, 0.2mg/mL, 0.3mg/mL, 0.4mg/mL, and 0.5 mg/mL solutions with methanol. The standard curve for senegenin was constructed by separate injection of 20 μL of the above-mentioned standard solutions. The regression equation between peak area (Y) and the quantity of senegenin (X) was: Y=7232006X+15124, r=0.9990.

Determination of senegenin content

One gram of the dried root powder was put into a 25 mL conical flask. Then 10 mL of methanol was added for 30minute’s ultrasonic extraction at room temperature, standing over night afterwards. After 2 mL of the clear supernatant fluid was removed, three times that volume of ether was added. When precipitation appeared, the supernatant fluid was decanted. The precipitate was redissolved with 10 mL of 10% hydrochloric acid solution, then was transferred little by little to round flask and was refluxed for 2 h in a thermostat-controlled water-bath at 100°C, cooled to room temperature and filtered. The brown disposition on the filter paper was redissolved with methanol and transferred to a 10 mL volumetric flask and was shaked vigorously after adding methanol to its scale. The sample solutions were filtered through 0.45 μm filters prior to HPLC analysis.

Acknowledgements

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References:


**Figure captions:**

Figure 1A-L. Structure of the vegetative organs and the localization of saponin in them of *P. tenuifolia*

A. Portion of cross-section of biennial taproot, showing each part of secondary structure. P. Periderm; SP. Secondary phloem; SX. Secondary xylem. Scale Bars, 200μm.

B. Portion of cross-section of biennial taproot, showing the secondary phloem and periderm. P. Periderm; SP. Secondary phloem. Scale Bars, 100μm.

C. Portion of cross section of biennial taproot, showing the vessels, wood fibers and xylem rays of secondary xylem. V. Vessel; XR. Xylem ray; XP. Xylem parenchyma cell; WF. Wood fiber. Scale Bars, 25μm.

D. Portion of cross-section of stem, showing the epidermis, cortex, vascular cylinder and stoma. Epiderm; C. Cortex; St. Stoma; Pi. Pith. Scale Bars, 50μm.

E. Portion of cross-section of stem, showing the characteristics of cells in each part. Scale Bars, 25μm.

F. Portion of cross-section of leaf, showing the portion of every part, the epidermis, mesophyll tissue, stoma and substomatal chamber. St. Stoma. Scale Bars, 50μm.

G. Cross-section of root having secondary structure, showing secondary phloem displayed red and phelloderm displayed purplish red. Pd. Phelloderm. Scale Bars, 100μm.

H. Cross-section of root having secondary structure, showing xylem rays and xylem parenchyma cells displayed pale red. XR. Xylem ray; XP. Xylem parenchyma cell. Scale Bars, 25μm.

I. Cross-section of stem, showing cortex displayed purplish red, epidermis and secondary phloem displayed pale red. Scale Bars, 200μm.

J. Showing the partial magnification of the Figure 1I. Scale Bars, 100μm.

K. Cross-section of leaf, showing palisade tissue displayed red and epidermis as well as spongy
tissue displayed pale red. Scale Bars, 50μm.

L. Cross-section of root having secondary structure, showing the root sections could not produce characteristic colour in the control experiment. Scale Bars, 100μm.