Accumulation and Dynamic Trends of Triterpenoid Saponin in Vegetative Organs of Achyranthus bidentata

Jinting Li1,2 and Zhenghai Hu1∗
(1Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, Northwest University, Xi’an 710069, China; 2College of Life Sciences, Henan Normal University, Xinxiang 453007, China)

Abstract

The relationship between structural features of various vegetative organs and triterpenoid saponin accumulation in Achyranthus bidentata Blume was investigated using anatomy, histochemistry and phytochemistry. The results showed that the primary and secondary structures of roots, and the structures of stems and leaves of A. bidentata, were similar to those of ordinary dicotyledonous plants. The enlargement of its roots, however, was primarily associated with growth and differentiation of tertiary structures. There were collateral medullary vascular bundles in addition to the normal vascular bundles in the stem. The tertiary structure was not only main parts in the roots of A. bidentata, but also important storage region of triterpenoid saponin in its growth and development. The stem may be the essential transport organ of triterpenoid saponin, while palisade parenchyma may be the primary synthesis location. In November, the total quantity of triterpenoid saponin and overall biomass in the roots reach a maximum level. This was the best time, therefore, to harvest the roots and corresponded to the traditional harvest period. Despite the withered appearance of leaves, stems also contained substantial amounts of triterpenoid saponin, and it was recommended that the stems of A. bidentata should be used.

Key words: Achyranthus bidentata; anatomy; oleanolic acid; triterpenoid saponin.


Available online at www.jipb.net

Achyranthus bidentata, a herb of the Amaranthaceae family, has become one of the most important Chinese traditional medicinal plants. In China this species is mainly distributed in the Guhuaiqiu area of Henan Province. Its dried roots contain several secondary metabolites such as saponin, ecdysterone, polysaccharide, and betaine. Several saponins have been separated and identified from A. bidentata, and include triterpenoid saponin with oleanolic acid glycon, which is a traditional indicator of quality according to the Chinese Pharmacopoeia (China Pharmacopoeia Committee 2005). Triterpenoid saponin is widely distributed in higher plants and is an important secondary metabolite. Not only does it possess antibiosis and pest resistance characteristics (Papadopoulou et al. 1999; Haralampidis et al. 2002), but it also may reduce cholesterol levels and have anti-cancer, anti-inflammatory and analgesic properties (Gao et al. 2003; Hu et al. 2005; Huang et al. 2006a). Several studies have reported on the separation, identification, chemical structure and biological activities of saponins (Wang and Zhu 1996; Meng et al. 2002; Sparg et al. 2004; Khamidullina et al. 2006). The few reports that are available mainly focus on root development (Zhang and Hu 1988; Wei et al. 1997; Li and Hu 2006). However, there are no studies on stem and leaf structures. The relationship between plant structure and the accumulation of active substances has been explored in other species, including Aloe arborescens Mill. and Lamium album (Savchenko et al. 2001; Liao et al. 2006). This dissertation investigated the relationship between organ structure and accumulation of triterpenoid saponin in A. bidentata. Data obtained in this research can be used to determine the most appropriate harvesting stage and parts and contribute to standardization of
cultivation techniques, thus improving output and quality of the medicinal plant.

Results

Structures and histochemical observation of roots

The developmental anatomy of *A. bidentata* roots has been reported (Li and Hu 2006). In addition to the primary and secondary structures of normal dicotyledonous plants, its roots also have tertiary structures formed by the differentiation of extra cambium (Figure 1A–C). The tertiary vascular bundles are neatly arranged in a concentric ring, separated by parenchyma cells.

In the primary structures of roots, cells of the epidermis, cortex and primary xylem did not show any color reaction, whereas a light pink color appeared in the pericycle, primary phloem, and parenchyma cells between primary phloem and primary xylem (Figure 2A). In the secondary root structures, the secondary phloem and the parenchyma cells of the phelloderm appeared a claret colour (Figure 2B,D). With the further development of roots, claret appeared in extra cambium cells formed by the dedifferentiation of parenchyma cells outside of the secondary phloem and close to the pericycle (Figure 2B); the cells of the tertiary phloem formed by differentiation of extra cambium cells also appear claret (Figure 2C).

Structures and histochemical observation of stems

The stems of *A. bidentata* were tetragonal and consisted of, from the outside to the inside, epidermis, cortex and a vascular cylinder (Figure 1D). There was one layer of epidermal cells, long or somewhat rectangular, neatly arranged in a dense pattern, with some protuberance in the external walls, and non-glandular hair. There were three to five rows of cortex parenchyma cells with chloroplasts; some cells contained brownish-yellow substances. Fully developed collenchyma cells were present at the edges and corners. The vascular cylinder consisted of vascular bundles, pith and pith ray. Vascular bundles were collateral, with the primary phloem fibers being relatively developed in the corners of the stems. The phloem was narrow and consisted of sieve tubes, companion cells and phloem parenchyma cells; xylem consisted of vessels, parenchyma cells and wood fibers (Figure 1E). Vessels in the xylem congregated as a group with wood fiber around the vessel cluster. There were several layers of vascular cambium cells between xylem and phloem. In the center of the stems were fully developed pith, which consisted of a large number of parenchyma cells, and there were two collateral medullary vascular bundles close to the center (Figure 1D,F). Phloem cells of the normal vascular bundles and medullary vascular bundles appeared a claret colour, whereas no color reaction was observed in other tissues (Figure 2E).

HPLC determination results of oleanolic acid in *A. bidentata* structures

The histochemical results indicated that the roots, stems and leaves of *A. bidentata* contained triterpenoid saponin. For further authentication, we used high-performance liquid chromatography (HPLC) to determine the contents of triterpenoid saponin in various vegetative organs at different developmental stages, using oleanolic acid as an evaluation index. The results showed that various vegetative organs contained different levels of oleanolic acid; chromatograms are shown in Figure 3.

There was dynamic variation of oleanolic acid contents in various vegetative organs at different developmental stages (Table 1; Figure 4). During the whole growth period, oleanolic acid in the roots revealed a “high-low-high” pattern. In early August, when *A. bidentata* was in a vegetative growth phase, the aerial parts grew vigorously, and the amount of oleanolic acid in the roots was the highest (7.76%). Between 15 and 20 August, a period of reproductive growth occurred, when inflorescence and flowers developed. At that time, oleanolic acid levels decreased quickly in the roots and reached the lowest annual level (1.03%) in September. With fruits maturation, however, the amount of oleanolic acid in roots increased gradually again and tended...
Accumulation of Saponins in *Achyranthus bidentata*

**Figure 1.** Structure of root, stem and leaf of *Achyranthus bidentata*.

(A) Primary structure of root.
(B) Secondary structure of root.
(C) Tertiary vascular in portion of root.
(D) Secondary structure of stem.
(E) Secondary structure of stem.
(F) Medullary bundle in portion of stem.
(G) Structure of leaf.
(H) Structure of the medial vein in blade.

AB, tertiary vascular bundles; C, cortex; CC, cork cambium; E, epidermis; MB, medullary bundle; Pe, pericycle; PP, primary phloem; PX, primary xylem; SX, secondary xylem; VB, vascular bundle.
Figure 2. Histochemical analysis of root, stem and leaf of Achyranthus bidentata.

(A) Cells of the primary and the pericycle of root (light red).
(B) Secondary phloem, supernumerary cambium and phelloderm of root (purple).
(C) Secondary phloem and phloem of tertiary vascular bundles of root (purple).
(D) Cells of phelloderm in root (purple).
(E) Phloem cells of the vascular bundle and medullary bundle in stem (purple).
(F–G) Cells of palisade and phloem of the medial vein in leaf (purple).
(H) Mesophyll cells in old leaf without red staining.
Accumulation of Saponins in *Achyranthus bidentata*

**Figure 3.** Chromatograms of oleanolic acid reference substance and different organs samples of *Achyranthus bidentata*.

(A) Oleanolic acid reference substance.
(B) Root.
(C) Stem.
(D) Leaf.

1, oleanolic acid.

**Table 1.** Oleanolic acid (OA) contents in different vegetative organs of *Achyranthus bidentata*

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Root (%)</th>
<th>Stem (%)</th>
<th>Leaf (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early August</td>
<td>7.76</td>
<td>4.09</td>
<td>4.13</td>
</tr>
<tr>
<td>Early September</td>
<td>1.03</td>
<td>2.76</td>
<td>3.37</td>
</tr>
<tr>
<td>Early October</td>
<td>2.95</td>
<td>1.17</td>
<td>0.25</td>
</tr>
<tr>
<td>Early November</td>
<td>2.90</td>
<td>1.09</td>
<td>0.06</td>
</tr>
<tr>
<td>Early December</td>
<td>2.59</td>
<td>1.47</td>
<td></td>
</tr>
</tbody>
</table>

The amount of oleanolic acid in both stems and leaves was highest in August, and then began to decrease gradually. In October, oleanolic acid levels in stems tended to stabilize, but the levels in leaves continued to decline. After November, when the roots of *A. bidentata* were fully developed, the aerial parts had withered and oleanolic acid levels in the leaves were extremely low (0.06%). At that time, the relative levels of oleanolic acid were roots > stems > leaves.

**Discussion**

Triterpenoid saponin is the important medicinal ingredient of *A. bidentata*, which is a traditional medicinal plant used to treat a variety of conditions. Saponins are widely distributed in higher plants, yet their specific form and distribution in different plant organs are highly diverse (Cao et al. 2004; Liu et al. 2005). Histochemical analysis of *A. bidentata* vegetative organs indicated that, in the root primary structures, saponins were mainly distributed in the pericycle, primary phloem and parenchyma cells between primary phloem and primary xylem. In the secondary root structures, saponins were mainly distributed in secondary phloem and parenchyma cells of the phelloderm. As roots developed, extra cambium was formed by the dedifferentiation of parenchyma cells outside of the
secondary phloem and close to the pericycle, and the tertiary vascular bundles were formed by the differentiation of the extra cambium. Saponins accumulated in the extra cambium cells, as well as in the phloem cells of tertiary vascular bundles. Saponins were mainly found in the phloem cells of normal vascular bundles and medullary vascular bundles of stems, and in palisade tissue and phloem cells of the main vein vascular bundles in leaves. Because the enlargement of roots in the growth process was primarily related to the occurrence and differentiation of tertiary structures, and tertiary structures were dominant in mature roots of *A. bidentata*, the tertiary structures were the main storage sites of triterpenoid saponin. The red substances in the stems were present mainly in the phloem of vascular bundles. In leaves, however, they were found mainly in the palisade tissue cells and the phloem of the vein vascular bundles. It was speculated, therefore, that saponins in *A. bidentata* were first synthesized in the leaves and then transported to the roots for storage via the phloem of veins and stem vascular bundles. The disappearance of saponins from leaves when they withered also supports the speculation that the leaves acted primarily as synthesis, and not storage locations. An additional further investigation will be needed to clarify the roles of these various structures in the synthesis and transfer process.

The accumulation patterns of triterpenoid saponins in various vegetative organs were quantified by using HPLC. The amount of oleanolic acid changed with the developmental stage of vegetative organs. The observed variability was similar to the oleanolic acid distribution patterns observed in such medicinal plants as *Abrus mollis* Hance and *Herba sambuci chinensis* (Zou and Chen 2005; Huang et al. 2006b). In early August, the proportions of oleanolic acid in the roots, stems and leaves of *A. bidentata* were 7.76%, 4.09% and 4.13%, respectively, which were the maximum measured levels. Here, *A. bidentata* was in the vegetative growth stage, and aerial parts grew vigorously. Large amount of anabolites were produced and accumulated and then transported to roots, which also grew quickly. Tertiary structures in the roots differentiated at a rapid rate. Levels of triterpenoid saponin in roots and aerial parts increased rapidly. This pattern of growth and saponin accumulation suggests that fertilizer application in early August may be appropriate in order to improve the biomass and quality of roots.

Between 15 and 20 August, *A. bidentata* entered into a reproductive phase. At that time, the amount of oleanolic acid quickly dropped, reaching the annual lowest level (1.03%) in September (full fruits period). When that occurred, the proportion of oleanolic acid in the stems decreased dramatically (from 4.09% to 2.76%) but levels in the leaves were unchangeable. This difference may be due to rapid consumption of nutrients by the developing reproductive organs, thus causing rapid decrease of triterpenoid saponin content in the roots. We determined that the oleanolic acid level in mature *A. bidentata* fruits was 7.73%. This indicated that the decrease of triterpenoid saponin content in roots and stem is closely related to reproductive growth of *A. bidentata*, which was not beneficial to the accumulation of saponins in roots. The result is in agreement with the standpoint of others (Cao et al. 2004). Hence, flowers should be removed or other measures taken to inhibit the growth and development of reproductive organs, and consumption of nutrients, in order to maximize the quality and quantity of *A. bidentata* roots.

With the development and ripening of fruits, the consumption of nutrients decreased relatively and the oleanolic acid contents in roots began to increase again, reaching a relatively stable 2.95% in October. At the same time, the amount of oleanolic acid in stems decreased to 1.17% and also began to stabilize. The contents of oleanolic acid in leaves decreased rapidly with the increase in the number of withered leaves. After November, the roots of *A. bidentata* were fully developed and mature, and the aerial parts of the plants began to die back and wither, resulting in an extremely low quantity (0.06%) of oleanolic acid. The proportions of oleanolic acid in various vegetative organs of *A. bidentata*, from highest to lowest, were roots > stems > leaves.

The accumulation pattern of active ingredients, and plant growth and development, are two important indicators that can help determine the most suitable harvest periods for (root) medicinal plants (Han et al. 2003). The determination of the best harvest period should be based not solely on the maximum level of triterpenoid saponin in the roots in a phenological phase, but on multiple factors such as total biomass of roots and extraction ratio of triterpenoid saponin, etc. Although maximum triterpenoid saponin levels in *A. bidentata* roots were reached in August, the biomass was low. In October, saponin levels tended to stabilize, while root biomass was increasing. By November, the aerial parts of plants were beginning to die back and wither. Roots, on the other hand, achieved maximum biomass and high triterpenoid saponin levels following a rapid growth period. This time in November, therefore, would be the ideal period to harvest *A. bidentata* roots in order to obtain the highest biomasses and concentration of saponin.

**Materials and Methods**

**Plant materials**

Seeds of *Achyranthus bidentata* Blume were sown in the Botanical Garden of Northwest University in China on 1 July in 2006 and emerged on 7 July. All plant material used in this study was harvested at each developmental stage from August to December 2006.

**Anatomical experiment**

The root, stem and leaf from each developmental stage were fixed in a formalin-acetic acid-alcohol (FAA) solution immediately. Samples were embedded in paraffin and sectioned into the 8–10 μm slices by a Leica microtome. The sections were
stained with safranino and pastgreen as previously described (Li and Hu 2006), observed and imaged under Leica-DMLB microscope (Solms, Germany).

**Histochemistry experiment**

Histochemical analysis was carried out according to the Liu Shibiao method (Liu and Hu 2005) with modification. Frozen sections (30–40 μm) were cut with Leica-CM 1850 cryostat, mounted on poly-L-lysine-coated slides, and soaked in a 5% lead acetate solution for 10 min. This process resulted in the precipitation of saponins in tissues. The sections were then placed in a mixture of 5% vanillic aldehyde-glacial acetic acid and perchloric acid for 5–10 min for color development. The control materials were soaked for 20 d in FAA fixing solution (prepared using 50% ethanol) to remove saponins. The prepared sections were observed under a Leica-DMLB microscope and imaged.

**Phytochemical analysis**

**Sample preparation**

The roots, stems and leaves were harvested in August, September, October, November and December, followed by baking dry at 60 °C, crushing with a minitype grinder (Model FW 80, Beijing Kewei Yongxing Apparatus Co., Beijing, China), and sieving through a 40-mesh screen. Because of the low temperature in December, all leaves were near and fell out, and the oleanolic acid content of the leaves at this stage was not measured. A 0.5 g sample was placed in 10 mL of methanol, followed by ultrasonic extraction for 40 min and concentration using a rotary vacuum evaporator (Model SENCOR R204B, Shanghai Shenke Technique Co., Shanghai, China). To the concentrated samples 10 mL of 4 mol/L hydrochloric acid was added for hydrolyzing at 85 °C for 1 h. After being allowed to cool, 10 mL of chloroform were added to the sample, followed by two countercurrent extractions at 60 °C, each extraction being 15 min. The bottom liquid was harvested and evaporated under reduced pressure. The residue was dissolved in methanol (3 mL), filtered through a Millipore filter (0.22 μm) (Tianjing Puxiang Co., Tianjing, China) and subjected to HPLC analysis.

**High-performance liquid chromatography analyses**

High-performance liquid chromatography was used to determine oleanolic acid in *A. bidentata* as described previously (Flores-Sánchez et al. 2002). The HPLC system consisted of a Shimadzu LC-10Atvp HPLC apparatus (Kyoto, Japan), diode array detector (SPD-M10Avp), class VP-LC workstation and a Shim-pack VP-ODS column (150.0 mm × 4.6 mm). The assay conditions were mobile phase: methanol-water-glacial acetic acid (90:10:0.05); flow rate: 0.9 mL/min; column temperature: 30 °C; detection wavelength: 210 nm. An oleanolic acid standard was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Quantification was based on calibration curves obtained from the samples to which standards of different concentrations had been added over the linear range of 0.04–8.00 μg. The reproducibility of the method was controlled by analyzing a standard five times (20 μL). The relative standard deviation (RSD) of peak area was 1.156%. Recovery efficiency was determined under the same operating conditions. The mean recovery rate was 97.83%. For a stability test, the samples were analyzed by HPLC after 0, 2, 4, 6, 8, 10 h under room temperature. Oleanolic acid was stable under room temperature for at least 10 h in methanol, with the RSD being 2.644%.

**References**


(Handling editor: Ning Li)