Evaluation of the Best Procurement Time for Ashwagandha [Withania somnifera (L.) Dunal] Root by Liquid Chromatographic and Pharmacognostical Studies

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ABSTRACT

Aim: The present study was undertaken to establish the best procurement time for collection of Ashwagandha [Withania somnifera (L.) Dunal] root by analyzing the variation in bioactive secondary metabolite during six seasons by quantitative high performance liquid chromatography (HPLC) and comparative pharmacognosy by taking into consideration the Ayurvedic literature. In Ayurveda, Sharad ritu has been prescribed for procurement of crude drugs. But the description for procurement of useful part of individual plants is not mentioned anywhere in the literature. Therefore, to establish the best time for collection, the present study has been taken up.

Materials and methods: To establish the best time for collection, Ashwagandha root was collected from the same habitat in all six seasons described in Ayurveda: i.e., Shishir (January-February), Vasant (March-April), Grishm (May-June), Varsha (July-August), Sharad (September-October), and Hemant (November-December). Authentication of the source of collected plant was carried out in the herbarium NVARI, Jhansi. Identification and authentication of the collected plant material through macro-, micro-, and powder microscopic studies were carried out besides extraction and its quantitative HPLC evaluation for the best procurement time for all six seasons.

Results: The present study showed that internal microscopical features remain the same throughout the year, although a variation was observed in extractive values and selective marker compound. Withanolide A was taken as a selected marker compound and was quantified in samples collected in each season by HPLC. An extractive value was found to be maximum at 0.77 g in the sample collected in Sharad ritu for methanol extract. HPLC estimation showed that the abundance of Withanolide A is more up to 0.183% in the Hemant ritu sample among all seasons.

Keywords: Ashwagandha, Authentication and pharmacognosy, Best procurement time, HPLC, Seasonal variation, Withanolide A.


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Conflict of interest: None

INTRODUCTION

Ashwagandha [Withania somnifera (L.) Dunal] is a bushy evergreen herb common in dried parts of India. It is mentioned as an important drug in Ayurvedic literature.1,2 The drug consists of the roots, which are prescribed as somniferous, thermogenic, stimulant, aphrodisiac, and tonic. They are useful in nervous disorders, insomnia, lumbar pain, asthma, cardiac disorders, and senile debility. Leaves are recommended for fever, painful swelling, tumors, etc. Seeds are reported to be diuretic and hypnogenic.3,4 The drug processed with milk is used as a nerve tonic.5 Decoction with milk and ghee has been mentioned in sterility.6 Root powder mixed with sugar and ghee alleviates insomnia and brings sleep quickly.7 It occurs as a weed under a partial shade throughout semi-arid regions of India. It flowers during July-September and fruit ripens in the autumn season.8 The crop is cultivated in 5-6 months, sown during September and harvested in February-March.

It is mentioned that it is a very useful Ayurvedic drug efficacious in nervous disorders, pain management, dyspnea, and general nerve tonic. Therefore, the establishment of harvesting techniques under good agricultural practices is necessary. In Ayurvedic texts, there is no reference available regarding the best suitable procurement time, particularly, for Ashwagandha [Withania somnifera (L.) Dunal]. Therefore, scientific validation for the best suitable procurement time is necessary (Table 1).

Ayurveda advocates that a particular part of the plant collected in a particular season will possess more active principles: roots in summer or in the late winter, leaves and
branches in rainy and spring (early summer) seasons as the leaves and branches are at their fresh and healthy state and contain an optimum of the products of the plant metabolism and one can obtain the most desirable therapeutic action. Flowers and fruits should be collected in the spring season or when they are ripe or full bloom. The bark, stem, and latex were said to be the best in early winter (Sharad ritu). Economically important part of Ashwagandha is root. The main phytoconstituents isolated from W. somnifera included alkaloids and steroidal lactones namely Withaferins and Withanolides. The major chemical constituents are Withaferin-A, Withanosides I-VII, Withanolides A, B, and D, 17-hydroxy-27 deoxywithaferin A, and Withanone. In the present study, Withanolide A (Fig. 8) is taken as a selected marker compound. Withanolide A was quantified by HPLC in the sample procured in six different seasons procured from the same habitat. The variations in the morphological and microscopical characters of Withania somnifera (L.) Dunal were recorded in all the six seasons. The material was subjected to shade drying for about 4 weeks. The shade-dried materials were further crushed to a powder, sieved through the mesh 22, and stored in airtight containers for further analysis. This process was repeated for each season.

Macroscopic Study

Macroscopic features were studied and photographs were taken with Nikon DSLR Camera in each season (Figs 1 to 5).

### MATERIALS AND METHODS

#### Collection of Plant Material and Authentication

The plant material (root) was collected from the medicinal plant garden of Nvari, Jhansi, and authenticated at Herbarium NVARI, Jhansi with Accession no. 23599 (15 September 2014). The plant samples collected in triplicate were washed under running fresh tap water to remove adherent soil and dirt. The useful plant part, i.e., the root of Ashwagandha [Withania somnifera (L.) Dunal] was preserved in 70% alcohol for histological studies. This process was repeated for each season.

#### Drying of Plant Material

The plant was subjected to shade drying for about 4 weeks. The shade-dried materials were further crushed to a powder, sieved through the mesh 22, and stored in airtight containers for further analysis. This process was repeated for each season.

### Macroscopic and Microscopic Analyses

#### Macroscopic Study

Macroscopic features were studied and photographs were taken with Nikon DSLR Camera in each season (Figs 1 to 5).

<table>
<thead>
<tr>
<th>Shishir</th>
<th>Vasant</th>
<th>Grishm</th>
<th>Varsha</th>
<th>Sharad</th>
<th>Hemant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit (unripe) (Fig. 1)</td>
<td>Fruit (unripe) (Fig. 2)</td>
<td>Fruit (ripe) (Fig. 3)</td>
<td>Flowering (Fig. 4)</td>
<td>Flowering and fruiting (unripe) (Fig. 5)</td>
<td>Fruit (Fig. 5)</td>
</tr>
</tbody>
</table>

### Table 1: Macro- and microscopical features of Ashwagandha (Withania somnifera (L.) Dunal) in different seasons

Macroscopic: Dried root is conical to cylindrical, about 20–30 cm in length and 1–2.5 cm in diameter, rough, longitudinally wrinkled, at places exhibit lenticels and wiry brittle rootlets or scars left by their removal; fracture short and starchy; externally buff, internally white. Taste slightly sweet; odor characteristic (Fig. 6)

Microscopic: Diagrammatic TS of root is circular in outline and shows a wide central xylem occupying the major area of the section, encircled by narrow parenchymatous phloem, cortex and a narrow band of peripheral cork.

Detailed TS shows outermost 4–8 rows of suberized cells of the cortex, occasionally at places interrupted by lenticels; followed by 15–20 layers of cortical parenchyma, loaded with simple, compound starch grains and microsphenoidal crystals of calcium oxalate; narrow parenchymatous phloem containing starch grains; unis- to multiseriate medullary rays, in continuation with xylem lies underneath this, cambium is distinct, xylem is wide consisting of isolated rarely groups of 2–3 vessels embedded in thin-walled fibers occupying the major area of the wood, parenchyma are vesicentric and paratracheal and medullary rays are bent at places especially when run adjacent to the vessel (Figs 7A and B).

Powder: Shows abundant, simple and compound, spherical, oval or cup shaped starch grains with slit like or stellate hilum, scattered as such throughout or embedded in the parenchymatous cells of the cortex; microsphenoidal crystals of calcium oxalate embedded in the parenchymatous cells of the cortex; fragments of suberized cork in transverse and surface view; fragments of longitudinally cut and horse-shoe-shaped pitted vessels; radially cut medullary rays crossing the vessels and fibers; thin-walled xylem fibers (Fig. 7C).
Macroscopic characters were studied with the help of published floras. A comparison of macroscopic characters for different seasons has been carried out.

**Morphology**

Collected root samples were observed carefully for its identical morphological characters. Morphological characters like color, shape, and size were noted down. Characters of plant parts were studied per taxonomy and measurements were taken by a scale through the naked eye.

**Microscopic Analysis**

Microscopic examinations of the root were studied according to the standard method. Transverse sections of the Ashwagandha root was prepared and stained with Safranin and Fast Green per procedure. The same procedure was followed for powder microscopy. The microphotographs were taken by a bright-field microscope with a digital camera (Fig. 7).

**HPLC Methodology/Quantitative Analysis**

*Extraction:* About 5 g of each root-powdered sample of *Withania somnifera* (L.) Dunal collected in six different seasons were taken and extracted in Soxhlet with solvents such as methanol, ethanol, and aq. alcohol for 24 hours. The extracts were evaporated to dryness under reduced
pressure and the obtained residues (Table 2) were stored in sample vial in a refrigerator at 2–8°C.

HPLC Estimation of Withanolide A: Test Solution: The residues obtained from methanol, ethanol, and hydro alcohol extracts of each six seasons were weighed in triplicate and dissolved in methanol using a 10-mL volumetric flask, filtered through a 0.22-μm membrane filter and used for HPLC analysis.

Standard solution: About 1 mg of Withanolide A was dissolved in HPLC grade methanol in a 10-mL volumetric flask and made up to the volume.

Chromatographic conditions: Instruments—Agilent 1200 series with a manual sampler
Column—C₁₈ Eclipse, XBD, 4.6 mm × 150 mm
Detection—VWD detector at 227 nm
Mobile phase—acetonitrile:water (65:35)
Flow rate—1 mL/min
Injection volume—10 μL

Calibration curve: About 1 mg of Withanolide A was accurately weighed and added to a 10 mL volumetric flask, dissolved in HPLC grade methanol and the volume was made up to 10 mL to obtain 0.1 mg/mL. This solution was appropriately diluted further to

Figs 7A to C: TS *Withania somnifera* root. (A) 4×; (B) 10×, ck, cork; ct; mr, medullary rays; ph, phloem; xvy, xylem vessels; mscr, microsphenoidal crystals of calcium oxalate; sg, starch grains; cam, cambium; (C) Power microscopy of *Withania somnifera* root. (a) Pitted xylem vessel; (b) Cortical parenchyma with starch grains and microsphenoidal crytals of calcium oxalate; (c) Radically cut medullary rays crossing the vessel and fibre; (d) Fibre; (e) Cork in surface view; (f) Cork with cortex in transverse view; (g) Starch grains
get a concentration of 0.1, 0.05, and 0.025 mg/mL of Withanolide A. Each of the standard solutions was run through the HPLC and the calibration curve was established for peak area vs concentration of Withanolide A applied (Figs 9 and 10).

**Estimation of Withanolide A in the Drug**

About 10 μL of each test solution was injected into the HPLC system to record the chromatogram and determine the area of the peak corresponding to that of Withanolide A as described above in the calibration curve. The amount of Withanolide A was calculated in the residue extracted in various solvents such as hydro-alcohol, ethanol, and methanol each test samples obtained from the various seasons of *Withania somnifera* (L.) Dunal as given in Table 3.

**RESULTS AND DISCUSSION**

**Morphology**

*Withania somnifera* (L.) Dunal is a bushy evergreen herb, around 1 m high, with broadly ovate, sub-acute leaves, greenish-yellow flowers born on sub-umbellate auxiliary cymes, and globular fruits enclosed in an enlarged calyx (Figs 1 to 3).

**Quantitative Evaluation of Withanolide A**

*Selected Chemical Constituent*

**Table 2: Extractive values**

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Seasonal name (g</th>
<th>Residue weight</th>
<th>Methanol extract (g)</th>
<th>Ethanol extract (g)</th>
<th>Hydro-alcohol extract (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Shishir ritu S1 (5)</td>
<td>0.3960</td>
<td>0.2950</td>
<td>0.2720</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Basant ritu S2 (5)</td>
<td>0.7601</td>
<td>0.4229</td>
<td>0.5506</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Grishma ritu S3 (5)</td>
<td>0.7105</td>
<td>0.3533</td>
<td>0.5344</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Varsha ritu S4 (5)</td>
<td>0.5608</td>
<td>0.3498</td>
<td>0.5748</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sharad ritu S5 (5)</td>
<td>0.7687</td>
<td>0.3519</td>
<td>0.5723</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Hemant ritu S6 (5)</td>
<td>0.5850</td>
<td>0.2990</td>
<td>0.4330</td>
<td></td>
</tr>
</tbody>
</table>

**Chromatographic Conditions**

- **Instruments**—Agilent 1200 series with a manual sampler
- **Column**—C18 Eclipse, XBD, 4.6 mm × 150 mm
- **Detection**—VWD detector at 227 nm
- **Mobile phase**—acetonitrile:water (65:35)
- **Flow rate**—1 mL/min
- **Injection volume**—10 μL

**DISCUSSION**

Literature review indicates that *Ashwagandha* has high medicinal value and used in various Ayurvedic formulations. To increase the efficacy of these formulations, it is very important to procure the root of this plant in a suitable season when secondary metabolites are in the highest concentration. This study was aimed to standardize the drug in all six seasons, i.e., *Shishir* (January-February), *Vasant* (March-April), *Grishm* (May-June), *Varsha* (July-August), *Sharad* (September-October), and *Hemant* (November-December) microscopically to document internal changes in the histology of the root. The present study showed that internal microscopical features remain the same throughout the year. The useful part of *Withania somnifera* (L.) Dunal is root which is procured from the same habitat (RARI garden) in all the six seasons. Moreover, the drug is an evergreen herb; therefore, the drug shows the same characters in powder microscopy. Though it was seen in different fields in different seasons, the same type of structures has been observed in all seasons. The drug is a bushy evergreen herb and the Ashwagandha procured for the pharmacognostical study was neither sowed nor harvested in the respective seasons. A selected marker compound Withanolide A was quantified in each season by HPLC. The extractive value was found to be maximum, i.e., 0.77 g of the *Sharad ritu* sample for methanol extract (Table 1). HPLC estimation showed that abundance of Withanolide A is more up to 0.183 % in the *Hemant ritu* sample among all seasons (Table 3).

In the *Hemant ritu*, *Withania somnifera* (L.) Dunal will be in the flowering fruiting stage (Fig. 6), and, with these observations, it can be concluded that the best suitable procurement time of collection of a wild
Evaluation of the Best Procurement Time for Ashwagandha Root

Table 3: HPLC estimation of Withanolide A in different seasons

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Seasonal name</th>
<th>Withanolide A (%) w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Shishir ritu</td>
<td>0.0356–0.0665</td>
</tr>
<tr>
<td>2</td>
<td>Vasant ritu</td>
<td>0.0712–0.1154</td>
</tr>
<tr>
<td>3</td>
<td>Grishma ritu</td>
<td>0.0037–0.0047</td>
</tr>
<tr>
<td>4</td>
<td>Varsha ritu</td>
<td>0.0007–0.0056</td>
</tr>
<tr>
<td>5</td>
<td>Sharad ritu</td>
<td>0.0379–0.0654</td>
</tr>
<tr>
<td>6</td>
<td>Hemant ritu</td>
<td>0.0562–0.1835</td>
</tr>
</tbody>
</table>

*Range of results was given from the means of triplicates of optimized three solvents of hydro-alcohol, ethanol, and methanol.

From the above table, it was observed that the abundance of Withanolide A is more in Hemant ritu samples followed by the Vasant ritu sample for the source collected at National Vrshayurveda Research Institute, Jhansi, Uttar Pradesh, India.

Variety of *Withania somnifera* (L.) Dunal is the Hemant ritu (November-December).

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हिंदी सारांश

लिक्युइड क्रोमेटोग्राफिक और भेषजगुणविज्ञानीय अध्ययनों द्वारा अश्वगंधा (विधानिया सोमनिफेरा) (एल.) जड़ के लिए उत्तम- प्राप्तण समय का मूल्यांकन

उद्देश्य: आयुर्विदिक सहायता को ध्यान में रखते हुए, वर्तमान अध्ययन गुणवत्तात्मक उच्च निष्पादन वाली लिक्युइड क्रोमेटोग्राफिक (एचपीएलसी) और तुलनात्मक भेषजगुण विज्ञान द्वारा छह ऋतुओं के दौरान उच्चस्तरीय सैकड़ी मेडिकॉलैंड में आई भिन्नताओं के विश्लेषण के माध्यम से अश्वगंधा (विधानिया सोमनिफेरा (एल.) जड़) के एक्त्रण हेतु उत्तम- प्राप्तण समय को जानने के लिए किया गया। आयुर्विद में, अपरिष्कृत औषधियों के प्राप्तण के लिए शरद ऋतु को उचित समय निर्धारित किया गया है। परंतु अलग-अलग पादपों के उपयोगी भाग के प्राप्तण का विवरण सहायता में कहीं भी नहीं दिया गया है। अतः एक्त्रण के लिए उचित समय के निर्धारण हेतु, वर्तमान अध्ययन किया गया है।

सामग्री और पद्धति: एक्त्रण के लिए उचित समय निर्धारित करने हेतु, आयुर्विद में वर्णित सभी छह ऋतुओं अर्थात शिरिर (जनवरी-फरवरी), वसंत (मार्च-अप्रेल), शीत (मई-जून), वर्षा (जुलाई-अगस्त), शरद (सितंबर-अक्टूबर), हेमंत (नवंबर-दिसंबर) में एक ही स्थान से अश्वगंधा जड़ एक्त्रित की गई।

परिणाम: वर्तमान अध्ययन दर्शाता है कि अंतरिक जायक्रोकोस्मिकल विशेषताओं वर्ष के दौरान समान रहती हैं, यद्यपि निष्कर्ष मूल्यों और चयनित मार्कर कंपून्ड में भिन्नता देखी गई। विधानोलाइड ए का चयन मार्कर कंपून्ड के रूप में किया गया और एचपीएलसी द्वारा पत्रक ऋतु में एक्त्रित नमूनों में इसकी मात्रा निर्धारित की गई। मेधानोल सल्ट हेतु शरद ऋतु में एक्त्रित नमूने में निष्कर्ष मूल्य अधिकतम 0.77 ग्राम पाया गया। एचपीएलसी अनुमान दर्शाता है कि सभी ऋतुओं में से हेमंत ऋतु नमूने में विधानोलाइड ए की अधिकता 0.183% तक है।

मुख्य शब्द: अश्वगंधा, प्राप्तणकरण और भेषजगुण विज्ञान, उत्तम प्राप्तण समय, एचपीएलसी, ऋतु-संबंधी भिन्नता, विधानोलाइड ए।