

QUANTITATIVE EVOLUTION OF WITHANOLIDES CONTENT OF EGYPTIAN *WITHANIA SOMNIFERA* (L.) ROOTS.

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ABSTRACT:

Withanolides are important secondary metabolite in *Withania somnifera* (L.), which have high medicinal values and possess potent anti-tumor and antioxidant properties. A simple, rapid and specific reversed-phase HPLC method for the simultaneous analysis of nine structurally similar withanolides, namely, Withanolides A, B, IV, V, withanone, 27-hydroxywithanone, withaferin A, withastramonolide and physagulin D of root tissues of *Withania somnifera*, collected from Egypt, has been developed using a linear binary gradient solvent system comprising of (A) 0.14 g of potassium dihydrogen phosphate in 900 mL of water, 0.5 mL of phosphoric acid, dilute with water to 1000 mL, and (B) acetonitrile were used to profile the extract compositions and to quantify the withanolides therein. Homogeneity and purity of each peak was ascertained by comparative evaluation of the on-line UV spectra of the eluted compounds with those of the reference compounds. The method described can be easily and reliably applied to the quantitative analyses of withanolides in roots of *Withania somnifera* L.

1- INTRODUCTION

The Solanaceae family is comprised of 84 genera that include about 3,000 species, scattered throughout the world. Members of this family are generally annual shrubs. The genus play an important role in the indigenous medicine of South East Asia, e.g. in the Unani and Ayurvedic systems. The twenty-three known *Withania* species are widely distributed in the drier parts of tropical and subtropical zones, ranging from the Canary Islands, the Mediterranean region and Northern Africa to Southwest Asia (McGuffin, *et al.*, 2000; Hawkes, *et al.*, 1991; Warriar, *et al.*, 1996; USDA, 2013). Among them, only two species, *W. somnifera* and *W. coagulans*, are economically and medicinally significant, being used and cultivated in several regions (Mishra, *et al.*, 2000; Singh and Kummar, 1998; Dhar, *et al.*, 2006). *W. somnifera* (L.) Dunal, commonly known as Ashwagandha, is an important medicinal plant that has been used in Ayurvedic and indigenous medicine for over 3,000 years (CSIR, 1976). In view of its varied therapeutic potential, it has also been the subject of considerable modern scientific attention. Ashwagandha roots are a constituent of over 200 formulations in Ayurvedha, Siddha and Unani medicine, which are used in the treatment of various physiological disorders (Asthana and Raina, 1989; Singh and Kumar, 1998). In Ayurveda, *Withania* is widely claimed to have potent aphrodisiac, sedative, rejuvenative and life prolonging properties. It is also used as a general energy-enhancing tonic known as Medharasayana, which promotes learning and a good memory' and in geriatric problems Mohammad, *et al.*, 2009; Nadkarni, 1976). The plant was traditionally used to promote youthful vigor, endurance, strength, and health, nurturing the time elements of the body and increasing the production of vital fluids, muscle fat, lymph, and semen cells. The similarity between these restorative properties and those of ginseng roots has led to Ashwagandha roots being called Indian ginseng [10]. It also helps counteract chronic fatigue, dehydration, bone weakness, loose teeth, thirst, impotency, premature ageing, emaciation, debility, and muscle tension. The leaves of the plant are bitter in taste

and used as an antihelmantic. The infusion is given in fever. Bruised leaves and fruits are locally applied to tumors and tubercular glands, carbuncles and ulcers (**Kapoor, 2001**). The roots are used as a health restorative in pregnant women and old people. The decoction of the root boiled with milk and ghee is recommended for curing sterility in women. The roots are also used in constipation, senile debility, rheumatism, general debility, nervous exhaustion, loss of memory, loss of muscular energy and spermatorrhoea **Singh and Kumar, 1998**).

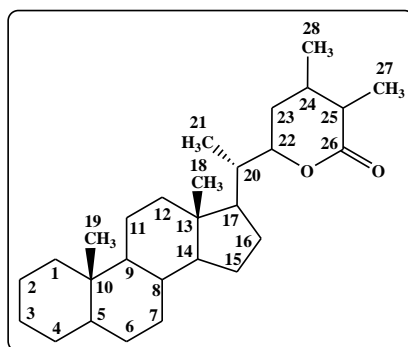
Much of ashwagandha's pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D (**Mishra, et al., 2000**). Standard monographs of *Withania somnifera* (L.) Dunal are present in official pharmacopeias of the United States (United States Pharmacopeia-National Formulary, *USP-NF*) (**USP, 2012**), the Indian Pharmacopeia (**Indian Pharmacopeia, 2007**), the (**Ayurvedic Pharmacopoeia of India, 2001**), and the (**British Pharmacopoeia, 2012**).

The chemistry of *Withania* species has been extensively studied and several groups of chemical

constituents such as steroidal lactones, alkaloids, flavonoids and tannins have been extracted and identified (**Atta, et al., 1991; Atta, et al., 1993; Choudary, et al., 1996; Bandyopadhyay, et al., 2007; Tursunova, et al., 1977; Christen, 1986; Glotter, 1991**).

Withanolides

More than 12 steroidal alkaloids, 40 withanolides, and several sitoindosides (a withanolide containing a glucose molecule at carbon 27) have been isolated and reported from aerial parts, roots and berries of *Withania* species (**Alfonso, et al., 1993; Alfonso, et al., 1994; Kirson, et al., 1971; El-Olemy and Kadry, 1984**). The major chemical constituents of these plants, withanolides, are mainly localized in leaves, and their concentration usually ranges from 0.001 to 0.5% dry weight (**Atta, et al., 1991; Atta, et al., 1993; Choudary, et al., 1996; Bandyopadhyay, et al., 2007; Tursunova, et al., 1977; Christen, 1986; Glotter, 1991**). The withanolides are a group of naturally occurring C28-steroidal lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring. The basic structure (**Figure 1**) is designated as the withanolide skeleton (**Alfonso, et al., 1993; Alfonso, et al., 1994; Kirson, et al., 1971; El-Olemy and Kadry, 1984**).



(Figure 1) The basic structure of withanolides.

The withanolide skeleton may be defined as a 22-hydroxyergostan-26-oic acid-26,22-lactone. There are many novel structural variants of withanolides with modifications either of the carbocyclic skeleton or the side chain and these have often been described as modified

withanolides or ergostan type steroids related to withanolides. These compounds are generally polyoxygenated and it is believed that plants elaborating them possess an enzyme system capable of oxidizing all carbon atoms in a steroid nucleus. The characteristic feature of withanolides and ergosane-type steroids is one C8 or C9-side chain with a lactone or lactol ring but the lactone ring may be either six-membered or five membered and may be fused with the carbocyclic part of the molecule through a carbon-carbon bond or through an oxygen bridge. Appropriate oxygen substituents may lead to bond scission, formation of new bonds, aromatization of rings and many other kinds of rearrangements resulting in compounds with novel structures (Alfonso, *et al.*, 1993; Alfonso, *et al.*, 1994; Kirson, *et al.*, 1971; El-Olemy and Kadry, 1984). The major withanolides of ashwagandha are represented in (Figure 2).

The number of analytical reports for the determination of Withanolides is comparatively small. Besides a TLC method for the quantification of some Withanolides such as Withaferin-A (Bessalle and Lavie, 1987) a few HPLC methods are described in literature. Most of them showed disadvantages either, the acetylation of some Withanolides are required prior to analysis (Hunter, *et al.*, 1979), the separation time is long (Banerjee, *et al.*, 1994) or the compounds are not baseline separated and elute, more or less with the injection peak (Banerjee, *et al.*, 1994).

There are no systematic efforts on quantification of withanolides content of *Withania somnifera* growing wild in Egypt. However, quantification of such active compounds from various organs is very valuable for the proper standardization of herbs and formulations thereof. In virtue of the important medical effect, it is very important to know the distribution of these compounds in order to choose the right organs and to obtain good resources for extraction.

In the present study, a HPLC method suitable for direct determination of withanolides content of *Withania somnifera* (WS) roots, growing wild in Egypt is discussed.

2- EXPERIMENTAL

2.1. Plant Materials

Withania somnifera (WS) samples growing wild in Egypt were collected from Mounfia and Qalubia Governorates, Egypt. The root samples (Table 1) were washed in water, dried at 40°C in an oven, and pulverized.

(Table 1) Plant materials collected from different study areas of Egypt.

Sample	Description	Source	Collection Time
WS 1Q	WS roots	Qalubia Governorate, Egypt	August 2011
WS 2Q	WS roots	Qalubia Governorate, Egypt	August 2012
WS 3M	WS roots	Mounfia Governorate, Egypt	July 2011
WS 4M	WS roots	Mounfia Governorate, Egypt	July 2012

2.2. Chemicals and reagents

Acetonitrile of HPLC grade was used. Water was prepared by a Mill-Q purification system from Millipore (Milford, MA, USA). Potassium dihydrogen phosphate, phosphoric acid, and other chemicals were analytical grade. Withanolides A, B, IV, V, withanone, 27-hydroxywithanone, withaferin A, withastramonolide and physagulin D were kindly provided from Dr. Maged Hussein Sharaf, United States Pharmacopeial Convention, Rockville, Maryland, USA.

2.3. Sample preparations

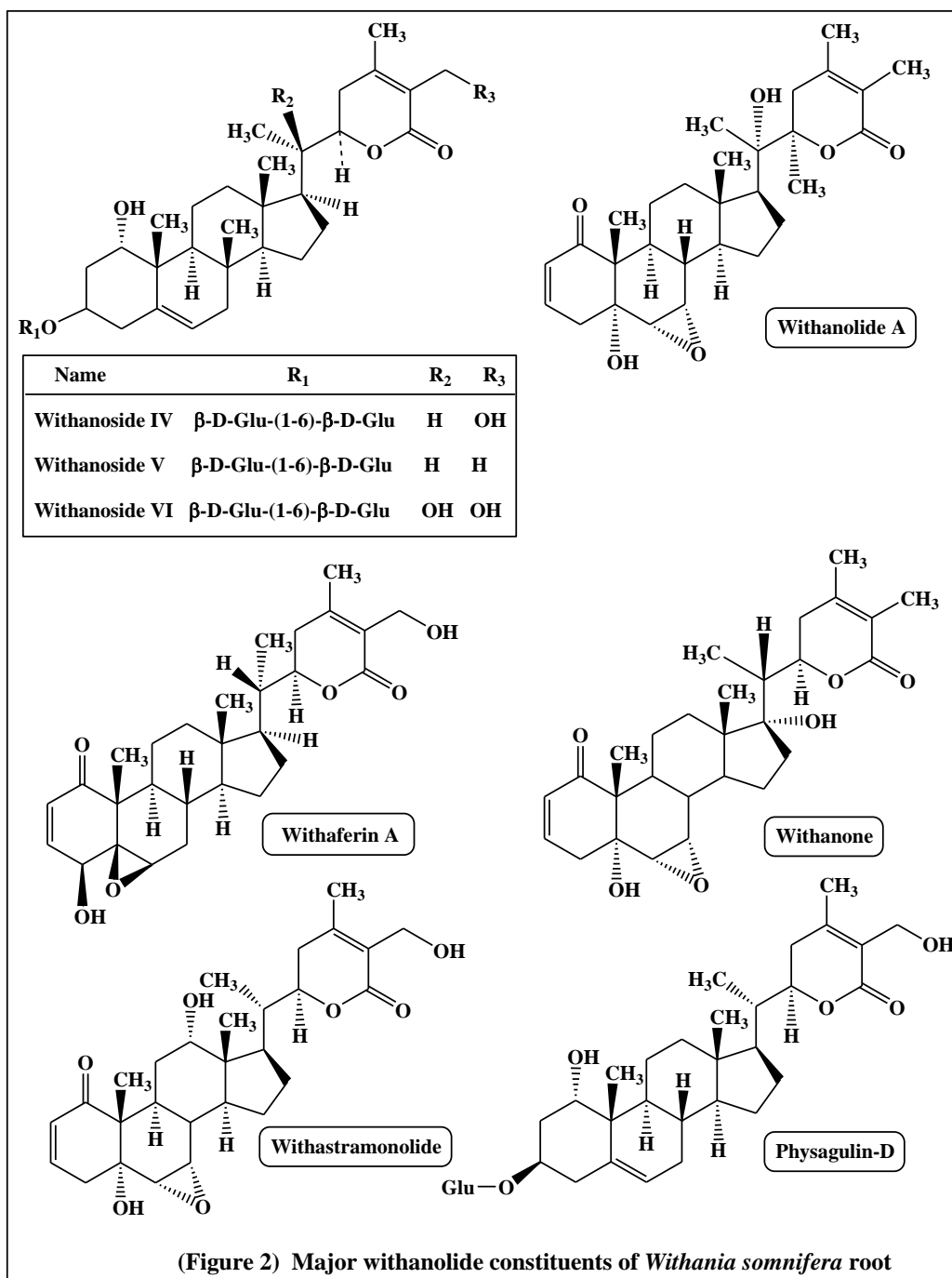
The air dried (25-30 °C) of each plant sample of *W. somnifera* L. (5 g) was transfer to a separate 250-mL flask fitted with a reflux condenser, add 50 ml of methanol, reflux on a water bath for 10–15 min, cool to room temperature, and decant the supernatant. The residue was re-extracted three times till exhaustion with 50 mL of methanol. The combined extracts were concentrated to dryness under *vacuum* at 45 °C and dissolved in 5 mL methanol (HPLC grade). The sample was clarified using Millipore filters (0.22 µm), degassed for one minute and subjected to HPLC analysis.

2.4. Chromatography

The analytical HPLC analysis was carried out according to the method in the monograph published in the United States Pharmacoeia (**USP, 2012**). Quantitative analyses were performed on an Agilent 1100 HPLC system equipped with variable wavelength detector operating at 227 nm and Phenomenex Luna C₁₈(2) (4.6-mm × 25-cm, end-capped, 5 µm). The mobile phase consisted of (**A**) 0.14 g of potassium dihydrogen phosphate in 900 mL of water, add 0.5 mL of phosphoric acid, dilute with water to 1000 mL, and (**B**) acetonitrile with a gradient elution of 95–55–20–20–95–95% of **A** and 5–45–80–80–5–5% of **B** at 0–18–25–28–30–40 min. The column was thermostatically maintained at 27°C, the flow rate was 1.5 mL/min. Validation of quantitative method was performed with samples for five injections of 20 µl each.

2.5. Standard preparations

0.1 mg/ml of USP withanolides A, B, IV, V, withanone, 27-hydroxywithanone, withaferin A, withastramonolide and physagulin in 0.2 ml methanol (HPLC grade).



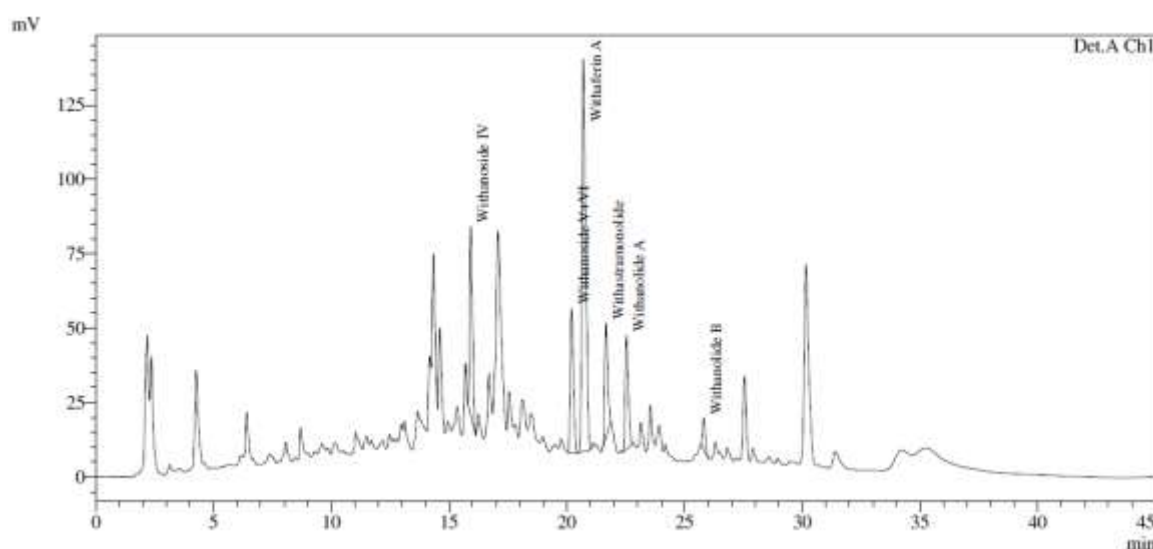
RESULTS AND DISCUSSION

Suitability of the LC analytical system was established following the requirements listed in the monograph published in the United States Pharmacopeia (USP, 2012). The chromatogram obtained for sample solution was similar to the reference chromatogram provided with the powdered *W. somnifera* L. extract used. Resolution between the withanolide A and withanone peaks was not less than 1.0, and not less than 3.0 between the withaferin A and co-eluting withanoside V and withanoside VI peaks.

Standard solution A, was not more than 1.5. The retention times of the peaks corresponding to the various withanolides were identified in chromatograms of sample preparations. A representative chromatogram of the content of withanolides is provided in (Figure 3). The approximate relative retention times of the different withanolides are provided in (Table 2).

(Table 2) Relative retention times of the different withanolides content of *Withania somnifera* growing wild in Egypt

Analyte	Relative retention times
Withanoside IV	0.70
Physagulin D	0.75
27-hydroxywithanone	0.80
Withanoside V	0.89
Withanoside VI	0.89
Withaferin A	0.92
Withastramonolide	0.96
Withanolide A	1.00
Withanone	1.01
Withanolide B	1.14



(Figure 3) Representative chromatogram of content of withanolides in *Withania somnifera* roots growing wild in Egypt.

The tested *Withania somnifera* (WS) root samples did not meet the acceptance criteria listed in the monograph of the United States Pharmacopeia [14] for the content of withanolides. The monograph requires the presence of not less than 0.3% of total withanolides. The tested samples collected from Qalubia Governorate contained 0.03 and 0.02% (w/w). On the other hand, the collected roots from Mounfia Governorate, contained 0.47% and 0.60% of total withanolides. Results of analysis are summarized in (Table 3).

(Table 3) Percentage (%) of withanolides in roots samples of *W. somnifera* L. collected from Egypt.

Sample	Withanosiide IV (%)	Withanosiide V (%)	Withaferin A (%)	Withastromnolide (%)	Withanolide A (%)	Withanolide B (%)	Sum (% w/w)
WS 1Q	0.021	0.00	0.01	0.00	0.00	0.00	0.03
WS 2Q	0.012	0.00	0.01	0.00	0.00	0.00	0.02
WS 3M	0.032	0.00	0.01	0.54	0.00	0.015	0.60
WS 4M	0.036	0.00	0.01	0.41	0.00	0.017	0.47

CONCLUSION

A validated method has been developed for the estimation of withanolides of *Withania somnifera* (L.) growing in Egypt. Proposed method is simple, accurate and precise. The method is suitable for routine analysis of withanolides in extracts. The simplicity of this method allows its application in laboratories that lack sophisticated analytical instruments such as HPTLC, LC-MS. Detection and quantification limit achieved, describe the method is very sensitive, accurate and precise. Hence, the method is recommended for routine quality control analysis of withanolides.

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التقييم الكمي لمحتوى الوزانولويد لجذور نبات ويزانيا سومينفرا (سم الفراخ) المصرى

سعيد قطب

قسم العقاقير - كلية الصيدلة - جامعة الأزهر

فى هذا البحث تم التوصل الى طريقة سهلة وسريعة ومتطورة باستخدام جهاز كروماتوجرافيا السائل فائق الجودة لتقييم كمية الوزانولويدز الشبيهة التركيب مثل ويزانولويد **A, B, V, IV** ويزانون و ٢٦ هيدروكسى ويزانولن ، ويزانيفرين **A** وفياجولين مقارنة بمركبات قياسية كما يمكن استخدام هذه الطريقة لتقييم المادة الخام وكذلك المستحضرات والمنتجات المحتوية على اليزانيا سومينفرا أو مستخلصاتها .