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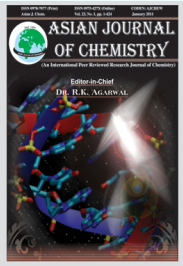

Determination of Triterpenoid Saponins (*Quillaja saponaria* Molina) from Roots of *Withania somnifera* (L.) Dunal by High Performance Liquid Chromatography

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Saponins are a class of chemicals found in natural sources with soapy character due to their surfactant properties. Saponins are often referred to as a "natural detergents" because of their foamy texture. On hydrolysis they yield a variety of sugar detergents. In *Withania somnifera* saponins contain an additional acyl group (sitoindosides VII and VIII) and exhibit characteristics such as strong foaming power in aqueous solutions from which the name 'saponins' has been derived. Saponin rich extracts of the Chilean indigenous tree *Quillaja saponaria* Molina are widely used as natural foaming agents in foods and beverage, food emulsifiers, photographic emulsions, vaccine adjuvants, *etc*. This work describes the determination of the Chilean quillaja tree (*Quillaja saponaria* Molina) as alternative low cost surfactant. This present study was carried out to evaluate determination of *Quillaja saponaria* Molina triterpenoid saponins from the roots of *Withania somnifera*. The reverse phase-high performance liquid chromatography (RP-HPLC) method was successfully applied to determine triterpenoid saponins content in the two samples of roots of *Withania somnifera* from two different places, namely Ujjain, Madhya Pradesh, India and Bhopal, Madhya Pradesh, India which provide a new basis of overall assessment on quality of *Withania somnifera*.

Key Words: *Withania somnifera*, *Quillaja saponaria* Molina saponins, Triterpenoid, HPLC.

INTRODUCTION

Withania somnifera (WS) is popularly known as ashwagandha or winter cherry[1]. It is a green shrub (family solanaceae)[2] found throughout the drier parts of India, Baluchistan, Pakistan, Afghanistan, Sri Lanka, Egypt, South Africa and Jordon. In India it is widely grown in the provinces of Madhya Pradesh, Uttar Pradesh, plains of Punjab and north western parts of India like Gujarat and Rajasthan[3]. The practitioners of the traditional system of medicine in India regard *Withania somnifera* as the "Indian ginseng"[4]. Various parts of the plants have been used for centuries to treat variety of ailments[5-7]. Many pharmacological studies have been carried out to describe multiple biological properties of *Withania somnifera*[8]. These studies have shown that the plant preparation has antiinflammatory[9], anticancer[10,11], antistress and immunomodulatory[12-15], adaptogenic[16], central nervous system[7,17-21], endocrine[22] and cardiovascular[8,23] activities, respectively. Its roots are the main part of use according to Indian pharmacopoeia (1955). Now a days, more than 100 Ayurvedic formulations in which ashwagandha is present alone or in combination with other herbal drugs, are being sold in the market under different trade names *viz*., stresscom, ashwagandharist, *etc*.[24,25]. Duke[26] has stated that it has value as abortificient, amoebicide, anodyne, bactericide, contraceptive, diuretic, emenagogue, fungicide, narcotic and sedative. It was also reported as antiinflammatory, hypnotic, nervine tonic, aphrodisiac and also effective in cases of leucoderma, bronchitis and asthma[27-30]. It is also well known that pharmacological activities of various parts of this plant are ascribed to the presence of various alkaloids and withanolides. Many compounds have been isolated by various researchers and majority of these compounds belong to alkaloid group and steroidal lactones[31-38]. Saponins are natural surface active compounds that give stable foams in aqueous solutions. Chemically they are high molecular weight glycosides, consisting of a sugar moiety linked to a triterpene or steroidal aglycone[39]. Quillaja extracts are among the most important industrial sources of triterpenoid saponins. These extracts are approved for human consumption in the US (FDA) and Japan and have been used for over 100 years as foaming agent in beverages (*e.g.*, root beer), production of slush-type drinks, blood analysis and photographic emulsions[40]. Quillaja extracts are derived from the aqueous extraction of quillaja biomass (wood and bark). It would be interesting to determine the content of triterpenoid saponins (*Quillaja saponaria* Molina) in the two

samples of roots of *Withania somnifera* grown in different places used as the internal standard. A potential surfactant alternative can be derived from the extracts of the Chilean Quillaja tree (*Quillaja saponaria* Molina). These extracts contain saponins, which are natural non-ionic surfactants in food and beverages. Due to their particular chemical properties[41-45], saponins are natural surface active compounds that give stable foams in aqueous solutions. Chemically they are high molecular weight glycosides, consisting of a sugar molecule linked to a triterpene or steroidal aglycone[46]. The bark of *Quillaja saponaria* Molina is one of the major sources of industrial triterpenoid saponins. Quillaja extracts are normally commercialized as liquid concentrates. Non-refined extracts contain all water soluble Quillaja solids, such as saponins, polyphenols and sugars. Refined extracts contain primarily Quillaja saponins. The saponins content of commercial Quillaja extracts can be determined by reverse phase HPLC[47]. The aim of our present study is to determine the content of commercial saponins from Quillaja bark in different root samples of *Withania somnifera*. Based on the above, the aim of this work is to extend the use of RP-HPLC technique to determine Quillaja saponins in commercial products used in food and beverages, cosmetics, *etc*. HPLC is a powerful separation and determination tool since 1970. HPLC and related methods are the first choice in the assay of saponins and have been used in the most analytical studies. With regard to the assay conditions, columns, event programs and detectors are the key factors in the HPLC analysis. Since saponins from *Withania somnifera* are polar molecules, they can be separated with a reverse-phase HPLC column. For the mobile phase, the aqueous acetonitrile system was usually utilized. The separations are performed usually on normal (silica gel) and reversed phase (C_8, C_{18}) columns, of which C_{18} has been definitely preferred.

EXPERIMENTAL

HPLC grade methanol and acetonitrile used were from Ranbaxy (Fine chemicals limited). Standard was purchased from across organics (2440, Geel) Belgium.

The roots of *Withania somnifera* were collected from the field in and around Ujjain and Bhopal (M.P.), India in the month of December 2007. Plant materials were oven dried at 50 ºC for 48 h. These plant materials were grinded in a Crompton and Grieves mixer grinder.

Equal volume of soil from each pot of same depth from the root zone area was collected. The samples collected were air dried and sieved through 2 mm size sieve and their further evaluation was done in terms of physical properties.

Method of characterization

Physical properties of soil samples: Bulk density and particle density were measured following Veighmeyer and Henderielson (1946) method. Porosity was calculated empirically using particle density and bulk density, following Bodman (1942) method. Water holding capacity was measured by saturated soil pest technique. Conductivity was measured by conductivity meter in 1:2 soil water suspension and pH was determined by using pH meter.

Preparation of standard: (0.5 mg/mL) weighed saponin standard was prepared in 70 % (v/v) aqueous methanol.

Sample preparation for HPLC analysis: 10 mL of 70 % aqueous methanol was added to 1 g of powdered sample. The suspension was ultrasonically extracted for 20 min and filtered. This extraction was repeated twice. The combined filtrate was evaporated at 40 ºC. The residue was then dissolved in 5 mL of 70 % aqueous methanol (v/v). Prior to HPLC analysis, the solution was filtered through a 0.45 µm membrane filter.

Chromatographic determination: HPLC analysis was conducted on a Thermo Finnigen HPLC system. (Cromquest, class v.p.), consisting of a binary pump and a photodiode array detector (PDA) and equipped with a 4.6 mm × 250 mm C_{18} column (Thermohypersil, Germany), packed with 5 µm diameter particles. In this chromatographic determination, the saponin standard solution was filtered (0.45 µm) and then 20 µL sample was applied to a 4.6 mm × 250 mm C_{18} column equilibrated with 50:50 acetonitrile-water (v/v). The solvent was delivered to the column at a flow rate of 0.5 mL/min at room temperature and the injection volume was 50 µL. The ultraviolet detection wavelength was set at 203 nm and a photodiode array scanning was from 190-400 nm wavelength. Chromatographic determination was achieved by measuring to peak height[48].

RESULTS AND DISCUSSION

In this study, the physical properties of Ujjain soil and Bhopal soil were compared and the result shows that, the pH of the Ujjain soil and Bhopal soil varies from 7.9-8.1 which is slightly alkaline in nature. pH plays an important role in improving the soil fertility. The electrical conductivity (EC) values of Ujjain soil and Bhopal soil are 0.378 and 0.414 mS/cm, respectively, which are invariably low. It indicates that the presence of both anion and cation are slightly higher in Bhopal soil as compared to Ujjain soil. The bulk densities of both soils are 1.23 and 1.28 g/cc. Ujjain soil having relatively less bulk density as compared to the Bhopal soil. The Ujjain soil (45.23) has higher porosity. Porosity of the Bhopal soil being low. This allows relatively slow air and water movement despite of the large amount of total space available. Porosity of Ujjain soil was found higher which is beneficial for low porosity of clay soils, for ideal condition of aeration, permeability and water retention. The water holding capacity (WHC) of Ujjain soil and Bhopal soil were observed as 54.36 and 56.26 %, respectively (Table-1).

TABLE-1
PHYSICAL PROPERTIES OF SOIL SAMPLES

Parameters	Ujjain soil sample	Bhopal soil sample
pH	7.90	8.1
Electrical conductivity (mS/cm)	0.378	0.414
Bulk density(g/cc)	1.23	1.28
Porosity (%)	45.23	44.62
Water holding capacity (%)	54.36	56.26

HPLC Method development: To study the general chromatographic behaviour of saponins, methanol extracts of plant material were analyzed by HPLC on a C_{18} stationary phase using mobile phase of aqueous acetonitrile. The chromatographic conditions were developed and optimized using saponin standard and root samples of *Withania somnifera*. All

the saponin peaks are well resolved from each other. The sample chromatograms contain more peaks than those of the standard, indicating the presence of the unknown components in different root samples of *Withania somnifera*. In order to obtain extraction, variables involved in the procedure such as solvent and extraction time were optimized. Pure and aqueous methanol solutions were tried as the extraction solvent. The best solvent was found to be 70 % aqueous methanol that allowed complete extraction of all the saponins in high yield. It was found that the ultrasonic extraction was simpler and more effective for extraction of saponins with little impurity. The influence of the extraction time on the efficiency of the extraction was also investigated, in which powdered samples were extracted with 70 % aqueous methanol for 1 h, respectively. Preliminary study reveals that three ultrasonic extractions (20 min each) are needed to completely extract the saponins of interest. Methanol and aqueous methanol were commonly used for the extraction of saponins while studying the *Withania somnifera*.

Determination of triterpenoid saponins in the *Withania somnifera*: The amount of quillaja triterpenoid (*Quillaja saponaria* Molina) was determine using HPLC. Fig. 1 illustrates a typical chromatogram obtained when the quillaja Molina triterpenoid saponins standard was analyzed by HPLC for its total triterpenoid content. The peak represents the total saponin content of the Quillaja Molina saponins standard. As demonstrated in Fig. 1 *ca.* 6 min are required per chromatographic run, with the saponins being resolved and eluted in *ca.* 4 min. When the peak height expressed in mm is plotted against the triterpenoid saponins concentration expressed as mg/mL, the data indicate that the method is linear over at least a concentration range of 40-1200 µg/mL. In Table-2 data are presented for the triterpenoid saponins analyzed with HPLC. The percentage of recovery from the starting material of the saponins in *Withania somnifera* ranged from 0.8-1.8 % of the total amount of starting material. Fig. 2 show the separation by HPLC of the samples in *Withania somnifera*.

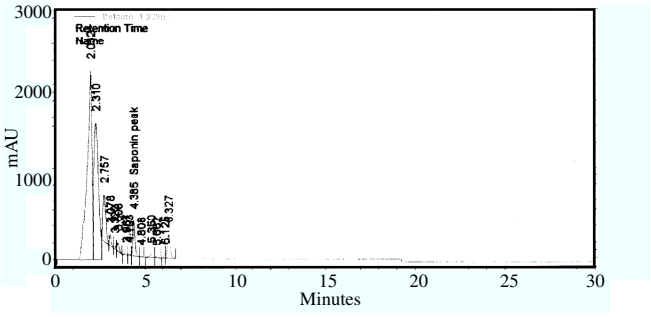

Fig. 1. HPLC of the *Quillaja saponaria* Molina triterpenoid saponins standard. Chromatographic conditions: column: thermohypersil, Germany C_{18} (4.6 mm × 250 mm, 5 µm), mobile phase: acetonitrile/water (50/50 v/v), flow rate: 0.5 mL min^{-1}, wave length: 230 mm, injection volume: 10 µL.

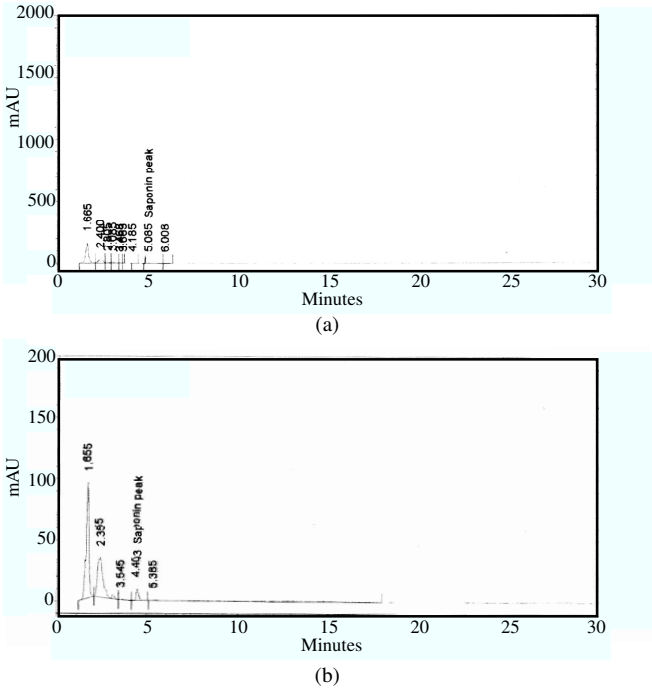

Fig. 2. Chromatograms represent the separation of Quillaja triterpenoid saponins in the Ujjain roots (a) and Bhopal roots (b) sample of *Withania somnifera*. Chromatographic conditions: column: C_{18} (4.6 mm × 250 mm, 5 µm), thermohypersil, Germany. elution solvent: acetonitrile in water gradient, flow rate: 0.5 mL min^{-1}, wave length: 230 nm, injection volume: 10 µL.

Conclusion

The result of present percentage of recovery shows that the samples contain very minute content of triterpenoid saponins (*Quillaja saponaria* Molina), (Ujjain root sample 0.8 % and Bhopal root sample 1.8 %) because in Bhopal soil sample concentration of all soil parameters such as pH, EC, BD, WHC is higher than Ujjain soil sample and results show that the fertility of Bhopal soil is high as compared to Ujjain soil. On the basis of above results of soil parameters it is concluded that in the Bhopal soil, plant growth rate is higher than in Ujjain soil. During observation of these studies it is observed that the advantage of HPLC method besides its sensitivity and speed is non-destructive and the injected sample can be recovered for subsequent chemical determination. In other words, we can say that this study describes a simple and efficient extraction procedure to isolate saponins in *Withania*

TABLE-2

Sample	Area (µM/L)	Starting concentration of triterpenoid ds (mg/mL^{-1})[a]	Method	Mean value (mg/mL^{-1}) ± SD[b], n[c]	Percentage of recovery (%)[d]
Withania somnifera root sample (grown in Ujjain)	11118	1.0	HPLC	0.7 ± 0.1, n = 2	0.8
Withania somnifera root sample (grown in Bhopal)	25170	1.3	HPLC	1.0 ± 0, n = 2	1.8

[a]Amount of triterpenoid saponins standard used for preparing *Withania somnifera* root sample (grown in Ujjain) and *Withania somnifera* root sample (grown in Bhopal). [b]SD: Standard deviation. [c]n: number of determinations. [d]Recovery was calculated in the final product as per cent of the starting material.

somnifera allowing the determination and characterization of these components with a variety of methods. We believe that the methods described in this study are of value for routine analysis of triterpenoid saponins.

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