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Research Article

ISOLATION AND CHARACTERIZATION OF WITHAFERIN-A FROM THE *WITHANIA SOMNIFERA* (ASHWAGANDHA)

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ABSTRACT

The research work is related to an improved process of isolation & characterization of Withaferin-A from *withania somnifera* (Sanskrit: Ashawagandha, English: Winter cherry). *Withania somnifera*, commonly known as Ashwagandha, is a valued herb in Ayurvedic medicine. Roots, leaves and preparations of the plant are traditionally used as tonic, hypnotic, sedative and diuretic. *W. somnifera* mainly contains withanolides including withaferin-A which are specific to the Solanaceae family. Withanolides are biologically active secondary metabolites present in roots and leaves of *W.somnifera*. In the present study, we have standardized the protocol for the isolation of Withaferin-A from the Ashwagandha punchang. Withaferin A possess anti-inflammatory and anti-stress properties. This study contains newer and conventional method of isolation of Withaferin-A from *Withania somnifera* as well Quantitative and qualitative techniques involved in the purification of the compound was followed throughout this research work. In this study, we have taken different trials based on hydro-alcoholic solvent composition. Based on different solvent extraction process pure 98% Withaferin-A was successfully isolated.

Keywords: Withania somnifera, Withaferin-A, Isolation, TLC, HPLC.

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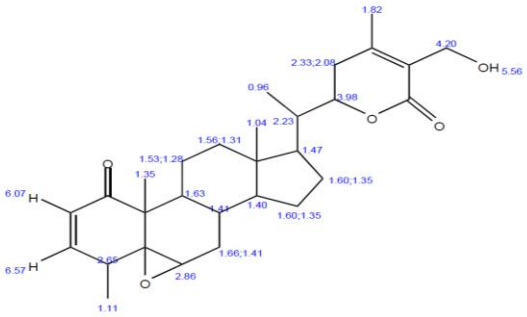
INTRODUCTION:

Since ancient times, medicinal plants have played a key role in the management of some health problems and in the discovery of new drugs[1]

Modern herbal medicinal science is concerned with using leading-edge technology to develop plant-derived drugs through chemistry & Analytical techniques. This is unique way in that it combines the study of herbal medicinal science with a strong emphasis on the underlying chemistry. Medicinal chemistry is disciplines at the intersection of chemistry, especially synthetic organic chemistry, pharmacology and various other natural plants, where it is involved with design, chemical synthesis and development for market of pharmaceuticals agents.[2,3]

Medicinal plants are a tremendous source of raw material for the modern drug industry. Since time immemorial, plants have been extensively exploited for their therapeutic property.

The isolation of active principles of the medicinal plant is necessary for the benefits of human being.[4] Whole plant or parts of plants were the main components of folk or ethanomedicine, practiced in India and other parts of the world like China, Middle East Africa and South America. *Withania somnifera*, also known as Ashwagandha, Indian ginseng is an important medicinal plant, which is cultivated in India for the medicinal purposes.[3]



Structure of Withaniferin A

The roots of the plant are categorized as rasayanas, which are reputed to promote health and longevity by augmenting defence against disease, arresting the ageing process, revitalizing the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors and by creating a sense of mental well being. The roots and leaves of Ashwagandha contain various alkaloids, viz., withanolides[5], withaferins[4] and withanosides.[6]

The withanolides are steroidal compounds and bear resemblance, both in action and appearance to the active ginsenosides of Asian ginseng. Studies show that the plant has been used as an antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent, antitumor, astringent and more recently to treat ulcers, bacterial infection, venom toxins and senile dementia. The biological activities of withanolides, especially of the dominant withanolide A and withaferin A, have been studied extensively and, more recently, have been shown to have anti-cancerous activity.[7, 8]

In this paper, we discussed about the purification of the secondary metabolite from the *in vivo* root extract of *W. somnifera* by simple techniques for the effective elution of a single compound withaferin A.

Figure 1: *Withania Somnifera* (Ashawgandha)

Common names – Ashawgandha, Indian Ginseng & Indian Winter cherry

Family – Solanaceae

Biological Source – Leaves, Root of *Withania Somnifera*

It grows in dry parts in sub-tropical regions. Rajasthan, Punjab, Haryana, Uttar Pradesh, Gujarat, Maharashtra & Madhya Pradesh.

Active constituents are

At present, 12 alkaloids, 35 withanolides, & several sitoindosides from this plant have been isolated & studied.
Mainly contains Withanolides & Withaferin Withanolides I, II, & III With none, Withasomnine & Withanine

Medicinal Use: Anti-inflammatory**,** Analgesic**,** Anti-stress, Osteoarthritis**,** Immunomodulatory **&** Anxiety & Cancer

MATERIALS & METHODS:

(1) Plant material and Chemicals:

Roots and Leaves of *W. somnifera* were the forest of aravali Bharathari District Rajasthan. Materials were air dried at room temperature and powdered mechanically. The powdered of roots and leaves were used as the plant material for all analysis. Chemicals, instruments, glassware used in the process were listed in table 1 and 2.

(2) Isolation and Extraction:

Isolation was carried out by using newer and conventional method. 200 gms ashwagandha punchang (root and leave) powder in to 2 Litre Round bottom flask (R.B.F.).then 750 ml petroleum ether was added to deffate the material. It was Refluxed at 60 ^{o}C for 90 min. after 90 min materials was filtrate and added further 800 ml of 75 % Methanol and reflux continue for four hours at 60^{o} C. After that, flask was allowed to stand for cool and filtered all solutions.

About 750 ml methanolic extract was collected, and transferred it into separation funnel.Now, extract with water (100 ml) followed by dichloromethane (3×200ml) and collect the lower layer separate out in to 1 liter beaker(total 600ml solution). Now about 8 to 10gms of activated charcoal were added into beaker and place beaker on water bath for 10 min.

Then filter well by using whattmann filter paper. Yellowish color solution obtains after filtration, which allows evaporating on water bath in evaporating dish. Allowed to Complete dry it on water bath and then cool the evaporating dish for 10 min at room temperature. Blackish color semi solid material was obtained at bottom of the dish which was gummy in nature. Then it was washed out with 70-80ml n-hexane and shakes well the dish, allow to stand for 5 min, then decant the n-hexane in to other dish (because decanted hexane may contain powder.) Again add 70ml n-hexane in to dish which containing blackish material. Material was crushed by using mortar pastel to make dry powder. Yellowish-green powder obtains which was free flowing in nature.

Obtained yellowish free flowing powder was subjected to thin layer chromatography (TLC) for Identification, and was subjected to High performance liquid chromatography (HPLC).\ for purification.

Table 1: List of Chemicals Used In Isolation Process

S. N.	Name of Chemicals	Grade of chemicals
1	Petroleum ether	GR grade, Merck
2	Methanol	For synthesis, Merck
3	Dichloromethane	GR grade, Merck
4	n-hexane	GR grade, Merck
5	De-mineralized(DM) water	Extra purified
6	Ethyl acetate	GR grade, Merck
7	Acetone	GR grade, Merck
8	Diethyl ether	GR grade, Merck
9	Hydrochloric acid (HCl)	AR grade, Merck
10	Sulphuric Acid (H2SO4)	AR grade, Merck

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Table 2: List of Chemicals Used In TLC and HPLC analysis

S. N.	Name of Chemicals	Grade of chemicals
1	Chloroform	HPLC grade, Merck
2	Toluene	AR grade, Merck
3	Methanol	HPLC grade, Merck
4	Acetonitrile	HPLC grade, Merck
5	Formic acid	AR grade, Merck
6	Glacial acetic acid	AR grade, Merck
7	Acetone	HPLC grade, Merck
8	Ethyl acetate	AR grade, Merck
9	Water for HPLC	HPLC grade, Merck

(3) Thin Layer chromatography: [9, 10, 11]

Thin-layer chromatography was Carry out on a precoated silica gel 60F254 plate using *withaferin A* as a reference standard**.** **Mobile Phase:** Chloroform: methanol (9.0: 1.0), **Test solution:** To 3 g of the substance being examined, add 25 ml of *methanol*, heat on a water bath for 10-15 minutes, cool and filter, **Standard solution:** Dissolve 10 mg of *withaferin A* in 10 ml of methanol, **Procedure:** Apply 10 µl each of the test and standard solutions on a TLC plate as bands of 10 mm. Develop the plate to a distance of 8 cm from the line of application. Dry the plate in air and examine under 254 nm. Spray the plate with a solution of *anisaldehyde sulphuric acid reagent* (figure 1) Heat the plate at 110 Celsius for about 5 minutes till the bands are clearly visible. Results are shown in table 3

(4) High performance liquid chromatography: [12, 13, 14, 15]

High performance liquid chromatography was performed during different trials for isolation methods. HPLC analysis was performed on a Shimadzu LC-20AD pump system equipped with a Shimadzu SPD-20AT UV- Visible detector with the detection wavelength set at 230 nm and 20µL Rheodyne injector loop. A column was a reversed-phase (Luna C18 4.6 mm x 260 mm – particle size 5µ) eluted at a rate of 1.0 mL/min with a solvent system {acetonitrile: 1% Glacial Acetic acid – 6:4 (V/V)}. Sample was prepared in the HPLC grade methanol. Results are shown in table 4

RESULTS AND DISCUSSION:

Withaferin-A was successfully isolated from the plant material of *withania somnifera*. Isolated withaferin-A was found to be above 90 % pure by HPLC analysis. Isolated withaferin-A show single spot at same Rf value corresponding to Standard withaferin-A. Thin layer chromatography and High performance liquid chromatography was performed for identification of final compound.

Silica coated TLC is convenient and suitable for the analysis of withanolides including withaferin A. It is often used to monitor fractions or finally purified withanolides. Chloroform - methanol (95:5) is frequently used solvent system for aglycones and chloroform - methanol (90:10) for glycosides.

The obtained purified compound is expected to be withaferin A, so the mobile phase for TLC of sample and standards were changed as chloroform: methanol in the ratio 9:1 with same spraying reagent. The single compounds were analyzed in TLC with standards Withaferin A. The standard withaferin-A showed the same Rf value as 0.65. From this result it can be stated that the purified compound is withaferin A.

Results are shown in table 3, and TLC chromatogram shown in below figure 1.

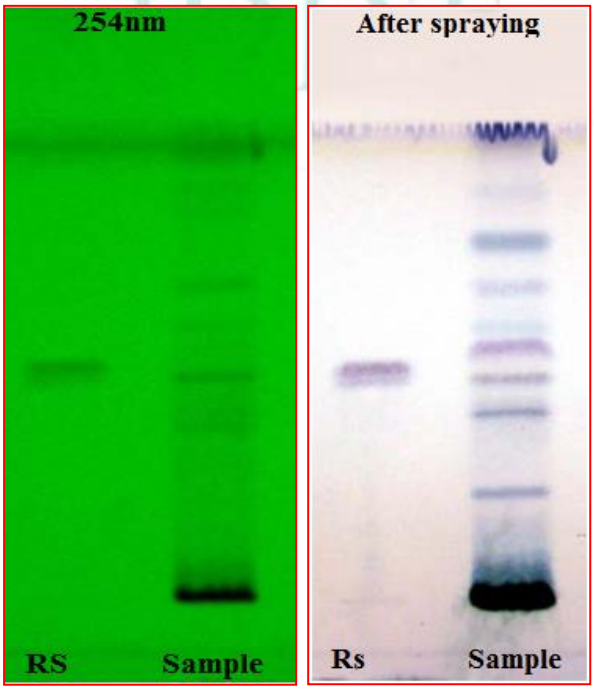

Figure 4: Thin-Layer Chromatogram of Ashwagandha *RS*: *Withaferin A*, T: Test solution (Detection by *anisaldehyde sulphuric acid*)

Table 3: Results of TLC study in Rf value

TLC Figure Nnumber		RF Value		
		Detection at		
		365nm	254nm	After spraying
Figure 1	RS	-	0.65	0.65
	T	-	0.28, 0.45, 0.55, 0.66,0.72	0.28, 0.45, 0.55,0.66, 0.72, 0.78, 0.84
Figure 2	RS	0.65	0.65	0.65
	T	0.65	0.28, 0.45, 0.54, 0.58, 0.65,0.76, 0.81, 0.82, 0.84	0.28, 0.45, 0.58, 0.65,0.76

The purified compound was further confirmed by High Pressure Liquid Chromatography analysis with standard Withaferin A as reference compound. Both standard and purified compound obtain peak at same retention time (3.957 min) as shown in figure 2 and 3 respectively. Results of HPLC analysis are shown in below table 4

Table 2: Results of HPLC study

Sample Name	Fig. No.	Retention Time	Peak Area	Wt/ml (Mg/ml)	Conc. (ppm)	% Purity
Withaferin A	3.2	8132.155	3.957	1mg/10ml	100ppm	97.00%
Test	3.3	7753.429	3.957	1mg/10ml	100ppm	92.48%

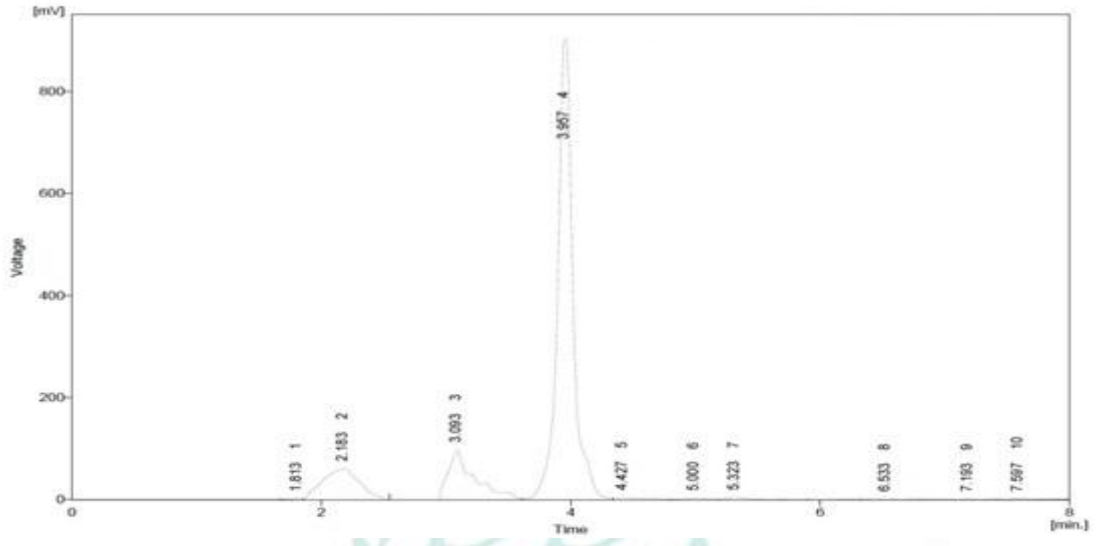

Figure 2: HPLC chromatogram of Sample Standard Withaferin A (97%)

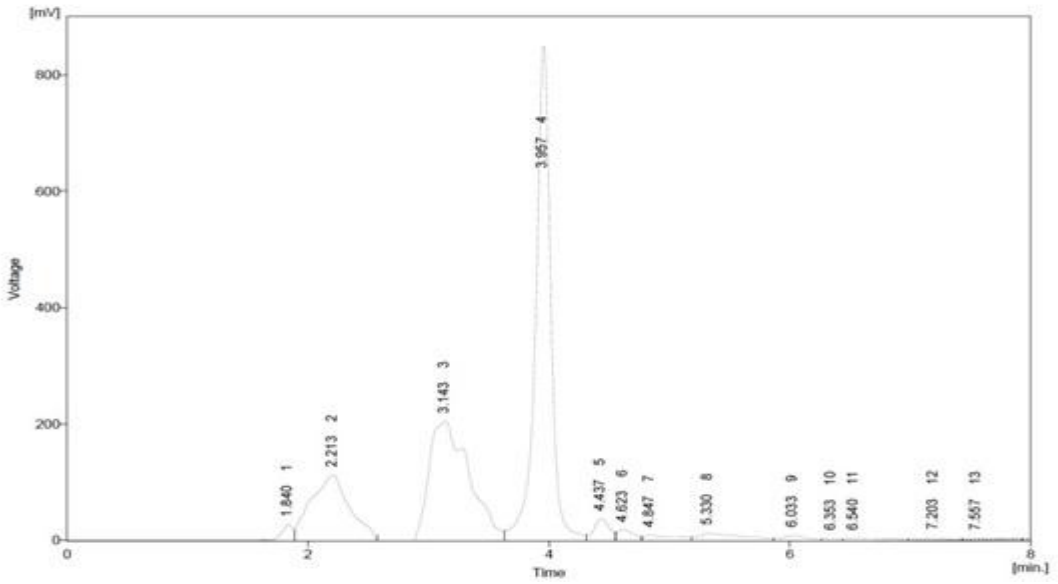

Figure 3: HPLC chromatogram of test withaferin A

CONCLUSION:

From the present study, it can be concluded that the *Withania somnifera* that contain Withaferin A compound were eluted using simple techniques with less cost effect and they are quantified with the HPLC techniques. So, the obtained Withaferin A compound will be used as the marker for analyzing the unknown compounds. 500 micro gram withaferin A compound obtains approximately from the 200 grams of the dried roots of *Withania somnifera*.

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