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Comparison of analytical parameter of genetically transformed hairy roots of *withania somnifera* with normal roots

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ABSTRACT

Withania somnifera, known as Ashwagandha, widely considered as Indian Ginseng, is a plant of repute in Indian system of traditional medicine. The present study was aimed to compare high performance thin layer chromatography(HPTLC) profile of methanol extract of hairy roots of *Withania somnifera* with normal roots extracts purchased from different places. The normal roots extracts were obtained from different places like Tulsi Amrit, Indore; Natural Remedies, Bangalore; Ansar Industries, Surat; Amsar Private Limited, Indore; Prashant Pharmaceuticals, Rajpipla. Transgenic hairy roots were induced in *W. somnifera* by infecting leaf explants with two wild type strain of *Agrobacterium rhizogenes* ATCC 15834 and MTCC 4364 using MS media. Chromatographic method was used for separation of withanolide-D from extracts of roots. The *W. somnifera* hairy roots extract and normal root extracts purchased from different places and standard Withanolide-D sample were used in HPTLC using Solvent system: Toluene: Ethyl acetate: Formic acid (5:5:1) [V]. HPTLC profiling of extracts confirm about presence of Withanolide-D.HPTLC fingureprint profile scanned at 530nm for methanolic extract revealed with Rf value in range of 0.51 to 0.55. The hairy root extract of *Withania somnifera* showed almost similar rf (0.53) value and similar peak area (5934.27) when compared with the standard Withanolide-D. The HPTLC method for routine quality control and comparison of present species can be carried out by using method for extracts of plant which serve in qualitative, quantitative and was appropriate for standardization of extracts.

Keywords: *Withania somnifera*, Withanolide-D Authetification, Fingureprint, HPTLC profile, Standardization.

INTRODUCTION

India has one of the oldest, richest and most diverse cultural traditions associated with the use of medicinal plants. This knowledge is accessible from thousands of medical texts and manuscripts. The substances having medical value have been

extensively used for treating various disease conditions. Herbs being easily available to human beings have been explored to the maximum for their medicinal properties. Products of primary metabolism such as amino acids, carbohydrates and proteins are vital for the maintenance of life processes, while others like alkaloids, phenolics, steroids, terpenoids are products of secondary metabolism and have toxicological, pharmacological and ecological importance. [1]. Many medicinal plants, traditionally used for thousands of years, are present in a group of herbal preparations of the Indian traditional health care system (Ayurveda) and proposed for their interesting multilevel activities. Amongst the medicinal plants used in Ayurvedic preparations for their therapeutic action, some have been thoroughly investigated and some need to be explored. [2] The goals of using plants as sources of therapeutic agents are to isolate bioactive compounds for direct use as drugs, to produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity and/or lower toxicity, to use agents as pharmacological tools, to use the whole plant or part of it as a herbal remedy [1].

Ashwagandha [*Withania somnifera* L. Dunal Solanaceae)] is an important medicinal plant, widely used as a home remedy for several diseases in India as well as other parts of the world. Historically, the plant has been used as an aphrodisiac, liver tonic, anti-inflammatory agent, astringent, and more recently to treat bronchitis, asthma, ulcers, emaciation, insomnia, and senile dementia. Clinical trials and animal research support the use of ashwaganda for anxiety, cognitive and neurological disorders, inflammation, and Parkinson's disease. Ashwaganda's chemopreventive properties make it a potentially useful adjunct for patients undergoing radiation and chemotherapy.It is described as an herbal tonic and health food in Vedas and considered as 'Indian Ginseng' in traditional Indian system of medicine. In fact, it is mentioned as an official drug in the Indian Pharmacopoeia. [3] Standardization of plant materials is the need of the day. HPTLC offers better resolution and estimation of active constituents can be done with reasonable accuracy in a shorter time [4]. Withaferin A and withanolide D are the two main constituents that contribute to most of biological activity of *Withania somnifera* In the present study, we established analytical activity of ashwagandha roots extract. Although lot of work has been carried out on HPTLC of *Withania somnifera*, there is still no information on comparison of analytical activity of normal and hairy root extract. The study confirms that methanolic extract of hairy root possess more percentage of withanolide D than normal root.

MATERIAL AND METHODS

Collection and Authentication of Drug Material

Plant material was collected from medicinal plant garden of Poona College of Pharmacy, Bharati Vidyapeeth University, Pune. It was authenticated by Agharkar Research Institute, Pune (Authentication No. 1946-2006).

Initiation of Hairy Roots of *Withania Somnifera*

The hairy root culture were initiated from *W. somnifera* leaf explants which was infected by *A. rhizogenes* (ATCC15834).The standard protocol was followed for establishment of genetically transformed hairy root cultures from *W. somnifera* leaf explants.

Collection of the Drug Extract

Ashwagandha root extracts were provided by the following Indian companies: Alchemy Chemicals, Ujjain; Amsar Pvt. Ltd., Indore; Ansar industries, Surat; Natural Remedies, Banglore; and Tulsi Amrit, Indore; Prashant Pharmaceuticals, Rajpipla; Green Pharmacy, Pune. All these extracts were procured from a standard supplier in India and were stored in suitable conditions.

Solvents and Chemicals

Methanol, Chloroform, Toluene, Ethyl acetate, Formic acid, Vanillin, Boric acid, Conc. H_2SO_4 were used. All chemicals were purchased from Himedia laboratories, Mumbai.

Sample Preparation

The weighed amount of root extracts (20mg) were dissolved with 10 ml of methanol at room temperature.

Standard Preparation

The solution of standard withanolide-D sample was prepared in distilled water same as sample .(Standard was Collected from Bharati Vidyapeeth Campus,Katraj branch)

Extraction of Hairy Roots

Six months old hairy roots, were selected for extraction. About 50 mg of dried hairy roots were taken for analysis. The tissues were ground in a precooled mortar and pestle, and extracted overnight in methanol (5 times w/v) on a rotary shaker at 26°C and 100 rpm. The procedure was repeated three times and the methanolic extracts were pooled together. The extracts were filtered through 0.22µ filter. The resultant solution was extracted with 3 volumes of chloroform. Chloroform layer was separated from other layers through a separating funnel. The chloroform extract was dried in Eppendorf tubes. The residue was redissolved in 1 ml methanol, filtered through a nylon filter and this filtrate was used for further analysis. The extract obtained from different hairy root cultures were subjected to qualitative and quantitative estimation of withanolides [6].

ANALYSIS OF WITHANOLIDE D

Tests for identification of alkaloids

The alcoholic extracts were evaporated separately. To residue, dilute HCl was added. It was Shaken well and filtered. With filtrate, following tests were performed according to the procedures of [7]:

- Dragendorff's tests: To 2-3ml filtrate, few drops of Dragendorff's reagent was added.
- Mayer's test: To 2-3ml filtrate, few drops of Mayer's reagent was added.
- Hager's test: To 2-3ml filtrate, few drops of Hager's reagent was added.
- Wagner's test: To 2-3ml filtrate, few drops of Wagner's reagent was added.

Qualitative Estimation by Tlc

The *W. somnifera* hairy roots extract and normal root extracts purchased from different places were tested for the presence of Withanolides by HPTLC [5].

Solvent system: Toluene: Ethyl acetate: Formic acid (5:5:1)

Detecting agent: Vanillin: Boric acid: Conc.H_2SO_4: Methanol (0.5gm: 10gm: 20ml: 1000ml)

METHOD

Silica gel G plates was prepared and activated. The spots of the test samples (extract obtained from hairy root culture and normal root extract of *W. somnifera*) were applied on activated plates with the help of fine capillary tubes and placed in air tight chromatography chamber. The chromatography chamber was previously saturated with the solvent system. The solvent was allowed to run up to $3/4^{th}$ height of the TLC plate. Then the plate taken out air-dried and sprayed with the detecting agent. The plate was then heated at 110^{o}c for 2mins and evaluated in visible light.

Qualitative estimation by HPTLC

The *W. somnifera* hairy roots and normal root extracts purchased from different places were tested for the presence of withanolide D by HPTLC [5].

Hptlc Instrumentation

A Camag HPTLC system equipped with an automatic TLC sampler (ATS_4), TLC scanner 3 and integrated software win-CATS version 1.2.3 was used for analysis. HPTLC was performed on a pre-coated silica gel HPTLC plate (10cm by 10cm) of 100µm layer thickness for detection of withanolide-D in *W. somnifera* hairy root culture. Various spot from hairy root extract of *Withania somnifera* and normal root extract of *W. somnifera* obtained from different places were applied on the plate with 6mm as their bandwidth, with constant application and with band space 6mm.

Detection and estimation of withanolide D

The linear ascending development was carried out in a Camag with trough chamber (20cm X 20cm), which was pre-saturated with 20ml mobile phase at room temperature. The length of the chromatogram run was up to 80mm.Subsequent to the development, the TLC plate was dried in a current of air. The dried plate was dipped into freshly prepared reagent, followed by heating at 110^{0}C for 2min. Quantitative evaluation of the plate was performed in the absorption mode at 530nm.The source of radiation utilized was a tungsten lamp.

Solvent system: Toluene: Ethyl Acetate: Formic acid (5:5:1)

Detecting agent: Vanillin: Boric acid: Conc.H_2SO_4: Methanol
(0.5gm: 10gm: 20ml: 1000ml).

Calibration Curve for Withanolides in HPTLC

200, 400, 600, 800, 1000, 1200, 1400 and 10000 ng/spot of 10 µl of each of the standard solutions of withanolide was applied on a HPTLC plate. The plate was developed in a solvent system Toluene: Ethyl acetate: Formic acid (5:5:1) at 25 ± 2 ºC temperature and 40% relative humidity up to a distance of 8 cm. After development, the plate was dried in air and scanned at 230nm.The peak areas were recorded. Calibration curves were prepared by plotting peak area vs. concentration.

RESULTS

Results obtained in the present study relieved that the hairy root extracts posses potential analytical activity when compared with the normal root extract from all manufacturers.They were tested by the HPTLC overlay method. The hairy root extract of *Withania somnifera* showed almost similar rf value (0.53) when compared with the standard withanilide-D.

RESULTS

HPTLC results

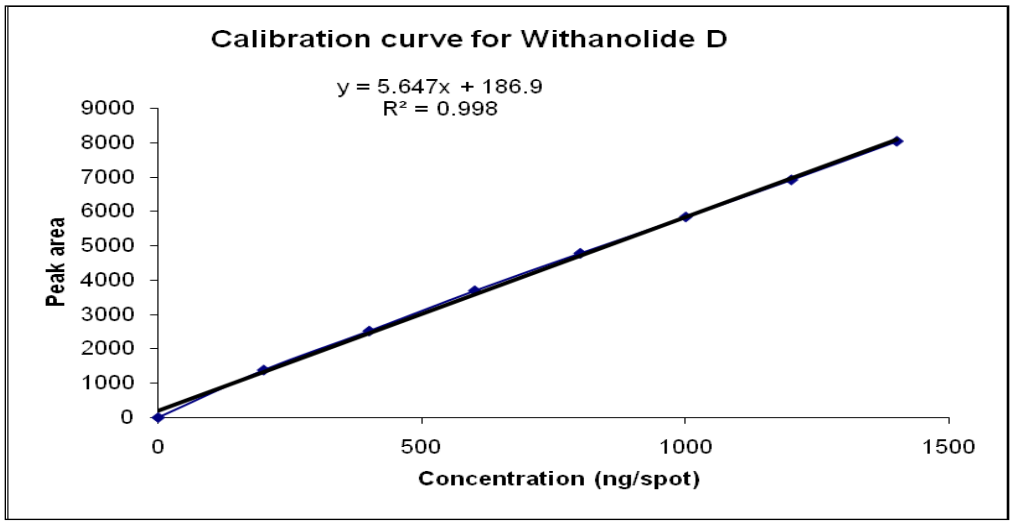
Calibration curve of withanolide-D

The concentration of withanolide in methanolic extract of hairy roots of *Withania somnifera* was calculated by the regression equation (Y= 5.647+186.9, r^2 =0.998). For determination of calibration curve different concentration taken were given in Table No.1 and calibration curve of withanolide-D was shown in Table No.2

Table No.1 Determination of Calibration curve of Withanolide D

Sr.No.	Concentration(ng/spot)	Peak area
1.	200	1380
2.	400	2515
3.	600	3686
4.	800	4774
5.	1000	5830
6.	1200	6908
7.	1400	8030
8.	10000	40234

Table No.2 Calibration curve of Withanolide D



DETERMINATION OF FINGER PRINTING PROFILE OF NORMAL ROOTS AND HAIRY ROOTS IN *W. SOMNIFERA* BY HPTLC

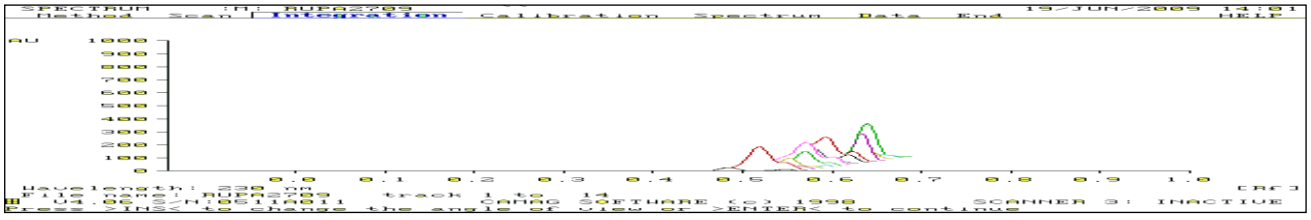
The normal root extract of *W.somnifera* obtained from Tulsi amrit, Indore; Natural Remedies, Bangalore; Ansar industries, Surat; Amsar Private Limited, Indore; Prashant Pharmaceutical, Rajpipla and hairy root of *W. somnifera* grown in grown in Poona College of Pharmacy were tested for the presence of withanolides by HPTLC. The data obtained from chromatographic method of hairy roots extract and normal root extracts from different places of *Withania somnifera* were shown in Table no.3. HPTLC overlay of comparative finger printing of normal roots and hairy roots of *W.somnifera* is given in Table no.4. This showed the chemical constituents produced by hairy roots of *W.somnifera* are same as in natural *Withania* roots.

Table No. 3. Fingure print profile of Withanolides content in normal roots and hairy roots of *W. somnifera:*

Sr. No.	Sample	Rf	Peak area	Withanolide content(%w/w)
1.	TAI	0.51	526.31	0.12
2.	NRB	0.53	475.35	0.10
3.	AIS	0.55	512.49	0.11
4.	APLI	0.53	521.44	0.11
5.	PPR	0.51	506.91	0.11
6.	HR	0.54	5628.34	0.18
7.	Standard	0.53	5934.27	0.23

Samples used in table are extract obtained from different manufacturer:
TAI- Tulsi amrit, Indore (NR), NRB- Natural Remedies, Bangalore (NR), AIS- Ansar industries, Surat (NR), APLI- Amsar private limited, Indore (NR), PPR- Prashant Pharmaceuticals, Rajpipla (NR), HR-Hairy roots, NR:-Normal roots
.

Table No. 4. Comparative finger printing profile of normal roots and hairy roots in *W. somnifera*.



DISCUSSION

Phytochemicals are chemical compounds synthesized during the various metabolic processes. Various phytochemicals possess a variety of pharmacological activities. These chemicals are often called secondary metabolites. *W. somnifera* contain Withanolide-D as secondary metabolite. Withanolide-D content in different roots (normal and genetically modified roots) extracts of *W. somnifera* was confirmed by HPTLC (Rf 0.53). Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The present study is first to report the comparative HPTLC fingerprint profile of extracts of *W.somnifera* roots showing active component Withanolide-D at 230nm with solvent system solvent system Toluene: Ethyl acetate: Formic acid (5:5:1). In present study, Peak area of HPTLC overlay was determined to compare analytical activity of untransformed normal roots obtained from different manufacturers of *W.somnifera* with that of transformed hairy root extract. The HPTLC results observed in hairy root extract was found to be having rf value with standard peak area than that of normal root extracts. *W.somnifera* consist several withanolides, but we compare with standard of withanolide-

D.The results of present investigation clearly indicate that the analytical activity vary according to the manufacturers of the normal root extract with hairy root extract. Thus, the study ascertains the value of genetically modified plant of *W.somnifera* , which could be of considerable interest to the development of new drugs. This densitometric HPTLC fingerprint profile may be used as marker for quality evaluation and standardization of the drug. Thus, HPTLC fingerprint profile along with their Rf values were recorded, which would serve as a reference standard for the scientist engaged in research on the medicinal properties of plant.

CONCLUSION

HPTLC fingerprint analysis not only gives the idea for the authentication of the plant extracts and its constituents but also provides the parameters for quality of herbal formulations. In HPTLC technique, as the sample is applied as a rectangular band it provides more resolution and better separation of spots as compared to the TLC technique because of the shape of the area in which the compounds are present on the plate. The chromatographic fingerprint, therefore is suitable for monitoring the identity and purity profile of a plant extract. HPTLC fingerprint analysis can be used as a diagnostic tool for the correct identification of the plant. In conclusion, the results obtained from qualitative evaluation of HPTLC fingerprint images will be helpful in the identification and quality control of the drug and ensure therapeutic efficacy. The normal roots extract of *W. somnifera* obtained from Tulsi Amrit, Indore; Natural Remedies, Bangalore; Ansar Industries, Surat; Amsar Private Limited, Indore; Prashant Pharmaceuticals, Rajpipla and hairy roots extract of *W. somnifera* were tested for the presence of withanolides by HPTLC (Rf 0.53). It can be finally concluded that HPTLC fingerprint analysis of hairy roots extract of *W. somnifera* can be used as a diagnostic tool for the correct identification of the plant and it is useful as a phytochemical marker and also a good estimator of genetic variability in plant population.

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