HPTLC-A simple and sensitive method to characterize thirty chemotypes and total alkaloid content in Ashwagandha (*W. Somnifera)*

Khatak S.* and Kumar R.
Department of Biotechnology, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra-136119, Haryana, INDIA
*sunitakhatak2019@gmail.com

Abstract

Withania somnifera is not just a name of a herb, it is a multipurpose medicine ranging from a curative agent for cardiovascular disease, cancer, reproductive health care etc. Dried roots and leaves from 30 genotypes collected from nearby States of Madhya Pradesh, Haryana and Rajasthan were extracted using HPTLC for generating chromatograms. Chromatograms were developed using solvent system consisting of chloroform: methanol (90:10) for methanolic extracts while a mobile phase consisted of toluene: ethyl acetate: formic acid (75:18:7) on precoated TLC aluminum plates Si60F$_{254}$ for all the thirty genotypes. The HPTLC profile of leaves phytochemicals resulted in grouping of various genotypes in five clusters and four clusters using roots.

The genotypes collected did not reveal distinct cluster based on their collection sites, instead were interspersed with each other similar to earlier reports and our previous investigation using TLC. The present research revealed presence of different components in different genotypes. Still the steroidal lactones withaferin-A and withanolide- A reported by earlier researchers were found in maximum genotypes irrespective of their locations.

Keywords: Ashwagandha, Chemotyping, HPTLC, Chromatogram, Fingerprints.

Introduction

A 4-5 feet tall shrub having common occurrence in Madhya Pradesh, Rajasthan and portions of Punjab, Himachal Pradesh and Uttar Pradesh named Ashwagandha, also known *as Withania somnifera (L.) Dunal*, is a multipurpose drug resource. It belongs to popular family of *Solanaceae* and easily available as wild species being ubiquitous and growing in the drier regions of subtropical India[30,34,41]. There are 23 species *W. somnifera (L.) Dunal* and *W. coagulans (L.) Dunal*, having immense potential in medical sector owing to steroidal lactones as bioresource found in its roots and leaves[7]. India has 4000 hectares dedicated to its cultivation[29,35] and most of the metabolites phytochemicals have already been discovered.

Sominfera means primarily a sleep inducer. It increases vitality and longevity, its sedative properties and other rejuvenating potential makes it comparable to Chinese ginseng plant[9]. The plant is used as single entity or as phyto-complex with other medicinal herbs in number of medicinal formulations for cure of anxiety, cardiovascular disease, anti inflammatory agent, thyroprotective, hypoglycemic and in common cold and chills. The steroidal lactones named withanolides produce mild chronotropic and ionotropic effects on cardiovascular system[9,35]. The ingredients have pharmacological properties[21,22,30] gastro intestinal reproductive system disorders[27,37], anti-ageing agent, antitumor and as general health/memory tonic[18] etc. Recently it has found its potential use in cancer chemotherapy[16].

Decoction of roots and leaves is used for toning of uterus after birth or miscarriages. Root bark is used against inflammation, anxiety and swelling tumors[10,23,44]. Roots have 18 fatty acids, beta sito-sterols, polyphenols and phyto-sterols. Number of withanolides namely withanine, withananine, pseudowithanine, somnine, somniferine, somniferinine have been detected. Leaves have withanolides as withaferin-A as reported in present research. The choice of the appropriate plant chemotype is crucial for effectiveness. Even when a plant species has a large number of chemotypes, choosing the proper one to attribute clinical effects is exceedingly challenging.

The environment, genotype, timing of plant material collection etc. can all affect a crop's chemical composition. So consequently, technologies that utilize advancement in existing techniques like HPTLC allow complete sampling of the genome with greater power of discrimination and are becoming the method of choice[15,24,31].

Review of Literature

Dhawan et al[9] reported on role of withanolides of Ashwagandha against corona virus disease agent, SARS cov-2 causing a swipe of almost 87 million people during 2019-2020. The aftermaths still exist in people who got vaccinated having severe heart attacks and other side effects. An alternative is needed to develop for combating effects of SARS infection in humans. Withaferin-A in leaves have been found effective as antitumourigenic in nature. It binds to SARS cov-2 S protein and alters its access into host cells. Withanone A and withanoside –V cause interference to functional activity of main protease SARS –COV-2 and withanolide-A control cytokine secretion during infection and alleviate cytokine storm in lungs. It also has anti inflammatory immunomodulatory aspects.

The report influenced on two most exploited withanolides responsible for most of the biological activity, Withaferin A and withanolide –D are in addition to withanosides, alkaloid, steroids and sitoindosides. Withaferin-A has free radical scavenging properties, while withanolide –D increases radio sensitivity of human cancer cells via inhibiting DNA damage to non homologous end joining repair pathways. They reported withanolide –V active against SARS Cov-2 virus and withanolide-A to inhibit acetyl cholinesterase in body. The plant has been found to treat Alzheimer too.

Mikulska et al[23] reported the plant benefits to human health care. The plant is boon in adaptogenic effects, antimicrobial agent, cardio protecting, anti diabetic and as a valuable remedy to cure many ailments[4,13]. High performance thin layer chromatography (HPTLC) is frequently employed as useful instrument for the qualitative identification of minute amounts of contaminants and preferred over TLC which has been the most and 1st choice of pharmacist and ethno pharmacologists to study secondary metabolites in number of plants. Inspite of simplicity and convenience, high sample throughput speed TLC is associated with some of the major drawbacks. The crude drug is extracted with methanol and silica gel plated TLC plates are developed using a selected solvent system. Methanol yields an extract of almost all the phytoconstituents like salts, sugars, amino acids, alkaloids, glycosides, steroids, saponins, oil waxes etc. Such a heterogenous extract usually yields a poor TLC due to overcrowding, asymmetry and poor resolution of spots. Lack of reproducibility of retention factor (R_f) values is yet another factor that should be taken into consideration. Documentation of fingerprint is an essential factor to be under taken for quality control (QC) systems.

Thus, a refinement of whole techniques is a pre-requisite to make fingerprint more unique, reproducible, authentic and adaptable to modern standards to study finger print.

Material and Methods

The leaf samples prepared were marked as L1 TO L30 and roots samples as R1 to R30 for ease in writing and depicting in text to study chemical fingerprinting.

Table 1
A brief description of *W. somnifera* genotypes used in present investigation

Genotypes	Source	Designated Samples(Leaves)	Designated Sample(Roots)
1.WS-124, 2.WS-201, 3.WS-202, 4.WS-204, 5.WS-205, 6.WS-206, 7.WS-210, 8.WS-213, 9.WS-218, 10.WS-220, 11.WS-223, 12.WS-224, 13.WS-226	Udaipur, Rajasthan	L1-L13	R1-13
14.WS-90-100, 15.WS-90-103 16.WS-90-104, 17.WS-90-105 18.WS-90-117, 19.WS-90-125 20.WS-90-126, 21.WS-90-129 22.WS-134(C), 23.WS-90-135, 24.WS-90-136, 25.WS-20 (C)	Mandsaur (M.P.)	L14-L25	R14-R25
26.Adinath	Neemuch (M.P.)	L26	R26
27.Local	Hisar, Research Farm Area	L27	R27
28.HWS-04 -1, 29.HWS-04 -2 30.HWS-04 -3	Haryana	L28-L30	R28-30

All the chemicals and reagents used in the current study for High performance thin layer chromatography were of high purity analytical grade and were purchased from S.D. Fine Chemicals Ltd., India. Standard substances, withaferin 'A' and withanolide 'A,' were purchased from Life Technologies, India.

High Performance Thin Layer Chromatography (HPTLC): Thin layer chromatography has an automated variant called HPTLC. The HPTLC analysis procedure was as follows:

Sample Preparation: 100 mg of powdered leaf/root material was combined with 1 ml of ammonia (25-27% in water) in a glass tube. Shake well, then add 10 ml of methanol and stir on a vortex. This mixture was heated to boiling in a water bath for one hour. Filter through Whatmann paper no. 1. The filtrate was collected, dried with an oven and then reconstituted with 1 ml of methanol. 1 ml of 100% methanol was used to dissolve 1 milligram of withaferin A and withanolide A in order to prepare the standard sample.

Plate Preparation: Aluminum plates precoated with Si60 F_{254} for HPTLC LiChrosphere analysis were employed.

Development of chromatogram: On HPTLC plates that were 0.5 cm apart and 1.5 cm from the lower border of the plate, samples (1µl) or methanolic extracts of each sample were applied. The plates were developed in a 25cm twin trough chamber with 100 cc of developing solvent per trough using the HPTLC grade solvents LS-8 and RS-1 (Table 2).

Following the development of the chromatogram, the plates were dried with cold air for five minutes at room temperature. The developing distance was maintained at 0.7 cm from the lower edge of the plate.

Detection: In the CAMAG HPTLC model, detection and quantification were carried out using densitometry at 254nm wavelength as shown in fig. 1.

Alkaloid quantification: Total alkaloids from roots and leaves were extracted using method of Harbourne[13] with suitable modifications. The leaves and roots were collected from fully mature plants and dried in an oven at 70ºC for 24 hours (till the weight was constant). Dried samples were ground to a fine powder with the help of pestle and mortar. Ten gram of each sample was extracted with 50 ml of methanol for 4 hours on a boiling water bath. The extract was collected after removal of the residue by centrifugation. About 10 ml of the extract was taken in a beaker and evaporated to dryness on a water bath. The residue obtained was dissolved in 1 ml of 10% HCl and shaken with 10 ml $CHCl_3$.

The organic layer was discarded and aqueous layer was made slightly basic with 5% sodium carbonate solution. It was partitioned with 20 ml ethyl acetate; the organic layer was collected and was dried by shaking with 1-2 gm anhydrous sodium sulphate. The material was dried in an oven at 100ºC for 10-15 minutes, cooled in desiccators and weighed.

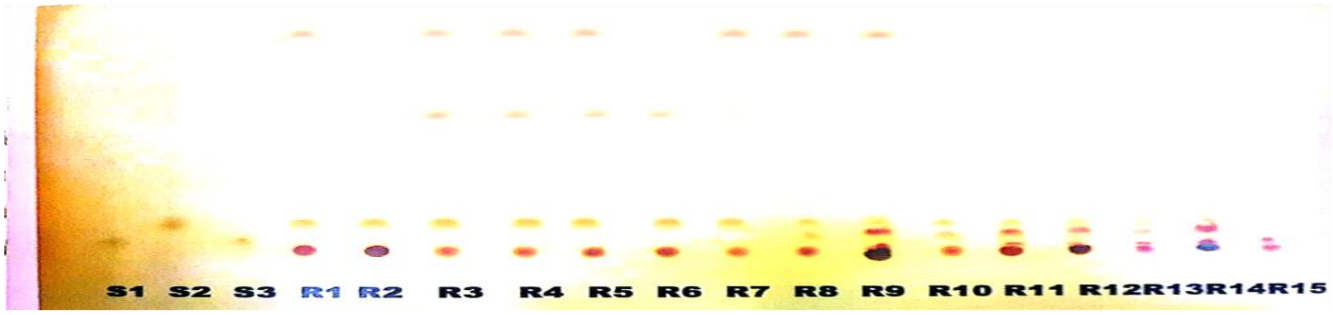
Results and Discussion

High Performance thin Layer Chromatography: A rapid, improved simple and precise high performance thin layer chromatography method has been developed to determine the distribution of phytochemicals in 30 genotypes of Ashwagandha procured from different States of India viz. Haryana, Rajasthan and Madhya Pradesh having different climatic conditions. Chemical fingerprints were obtained in all 30 genotypes to determine the presence of chemotypes and the ones containing medicinally important withanolides. Dried leaves and root samples from each of the 30 genotypes were collected and extracts were prepared as described.

Table 2
Solvent systems used for HPTLC analysis of leaf and root extracts in *W. somnifera* (L.) Dunal

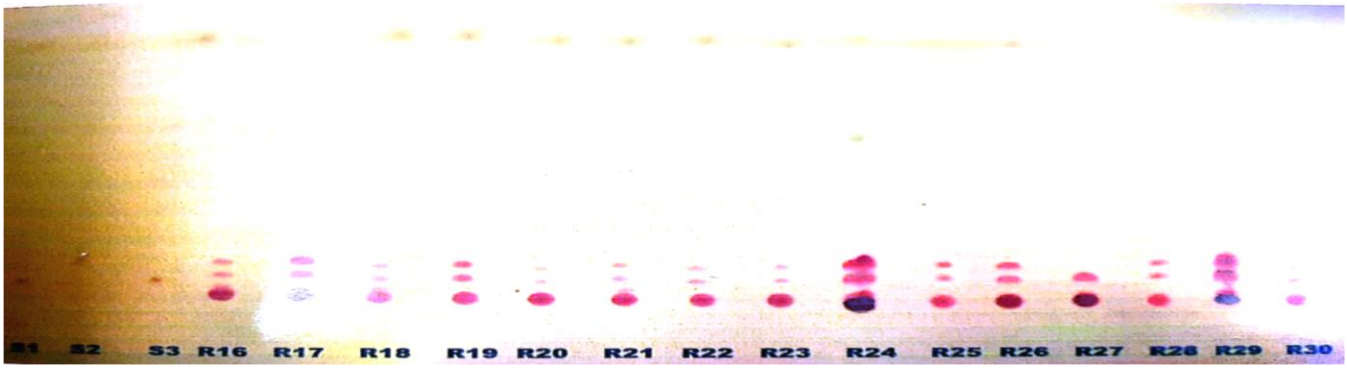
Solvent system code	Solvent system	Ratio
LS-1	Toluene :EtoAc : HCOOH	75:18:7
RS-1	$CHCl_3$:MeoH	9:1

Fig. 1: CAMAG, HPTLC model at Buffalo Research Centre, Hisar, Haryana.



R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04
0.24	0.31	0.26	0.18	0.34	0.18	0.28	0.21	22	0.22	0.24	0.16	0.16	0.44	0.25
0.26	0.47	0.32	0.33	0.48	0.29	0.34	0.29	0.35	0.36	0.29	0.25	0.24	53	0.31
0.31	0.49	38	42	0.62	0.34	0.5	0.35	41	0.46	0.51	0.29	0.29	55	54
0.47	0.51	44	48	0.71	0.48	0.66	0.52	45	0.49	0.63	0.4	0.45	62	58
0.57	0.54	46	0.6	0.82	54	0.74	0.63	48	59	0.77	0.5	0.52	66	66
0.67	0.58	54	0.69	1.06	55	0.85	0.66	51	75	1.03	0.54	0.56	69	68
0.77	0.65	59	0.8		63	1.08	0.68	55	78		0.64	0.61	78	73
1.05	0.68	61	1.06		73		77	0.7	85		77	0.62	82	0.8
	1.06	66			1.07		87	0.81	1.09		1.02	0.64	1.04	1.04
		68					1.08	0.9				0.77		
		79						1.09				1.02		
		1.06												

Fig. 2a): Depicting first 15(R1-R15) genotypes fingerprint spotted on plates using methanolic root extracts, with standards S1, S2, S3.

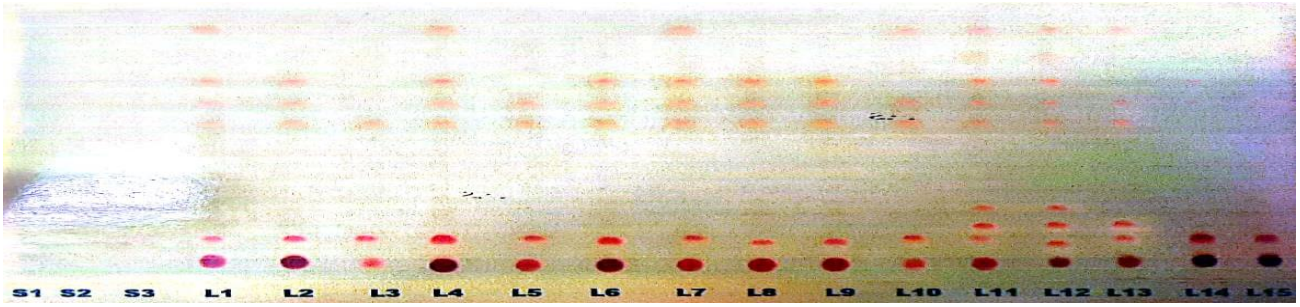


R16	R17	R18	R19	R20	R21	R22	R23	R24	R25	R26	R27	R28	R29	R30
0.04	0.04	0.04	0.04	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.03	0.03	0.03	0.04
0.18	0.29	0.34	0.36	0.24	0.19	0.18	0.29	0.12	0.3	0.3	0.2	0.31	0.32	0.33
0.26	0.33	0.5	0.47	0.32	0.3	0.3	0.33	0.19	0.52	0.51	0.3	0.61	0.53	0.44
28	38	57	0.54	0.37	0.41	37	0.36	0.3	0.6	0.55	0.49	0.63	0.58	0.56
32	39	63	0.58	45	0.63	0.4	0.64	0.49	0.64	0.63	55	0.66	0.65	
48	0.5	74	0.67	58	71	43	0.71	0.52	0.72	0.72	61	0.7	0.76	
57	0.55	86	0.7	69	82	0.5	0.82	0.62	0.82	0.82	67	0.72	0.86	
0.69	64	1.06	79	0.73	1	0.53	1	0.65	1.01	1.01	73	0.83	1.02	
81	72		89	75		0.62		0.71		1.01	82	1.02		
N/A	83		1.07	1		0.62		0.82			1.02			
1.05	1.05			1.07		0.65		1						
						0.72		1.11						
						82.89								
						10.07								

Fig. 2b): Depicting last15(R16-R30) genotypes fingerprint spotted onplates using methanolic rot extracts, with standards S1, S2, S3.

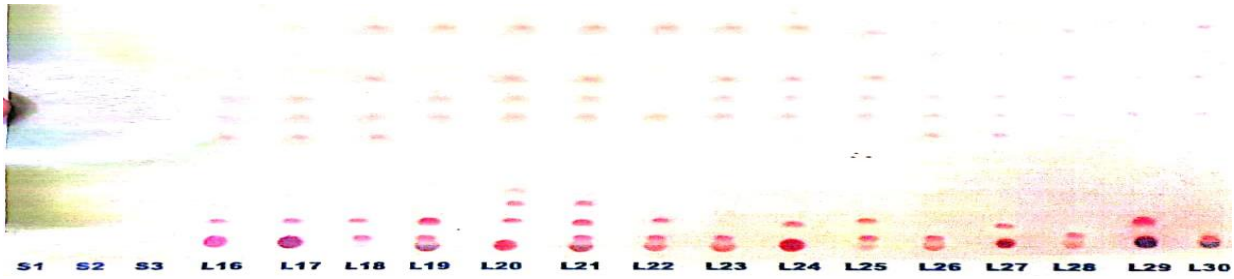
Separation of phytochemical constituents was achieved by employing a mobile phase consisting of chloroform, methanol (90:10) for methanolic root extracts, while a mobile phase consisting of toluene: ethyl acetate: formic acid of (75:18:7) was used on precoated HPTLC aluminium plates Si 60F 254 from each of the 30 genotypes. The derivatized plates were detected by keeping the plates in a chamber equipped with UV system at 254 nm. Similar solvent system has earlier been reported to analyze withaferin-A from herbal extract and polyherbal formulation of *W. somnifera* by Mahadevan et al[21] using HPTLC method. Solvent system employed was toluene: ethyl acetate: formic acid in the ratio of 50:15:5.

The HPTLC profile obtained using methanolic root extracts along with standard reference compound revealed the presence of withanolide A in all the 30 genotypes with an Rt (Retention time) of 0.33 as shown in table 3 and fig. 2a (R1-R15) and 2b (R16-R30). According to Pradhan and Sharma[32], medicinal herbs are preferred over allopathy because they have fewer side effects and are more cost-effective. HPTLC will be used as a very accurate quantification tool to investigate phytochemicals and components in many plants that have been referenced in ancient literature.



S.N.	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15
1.	0.04	0.04	0.04												…..
2.	0.13	0.13	0.56	0.13	0.11	0.13	0.11	0.12	0.12	0.11	0.11	0.11	1.16	0.13	…….
3.	0.46	0.66	1.09									1.08			……..
4.	0.69	0.8													…….
5.	0.8	1.07													……
6.	1.09														……..

Fig. 3a): Genotypes 1 to 15(L1-L16) according to Table-1 spotted on plate for fingerprint analysis using methanolic leaf extracts and spotted phytochemicals retention factor below.



S.N.	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L27	L28	L29	L30
1.															
2.	0.11	0.12	0.12	0.13	0.13	0.14	0.15	0.16	0.15	0.17	0.18	0.19	0.05	0.2	0.07
3.	0.18	0.2	0.18	0.19	0.2	0.21	0.21	0.22	0.26	0.24	0.26	0.28	0.2	0.31	0.21
4.	0.52	0.35	0.63	0.77	0.55	0.55	0.8		0.41	0.53	0.57	0.43	0.26	0.45	0.28
5.	0.74	0.47	0.76		0.77	0.63			0.57	0.59		0.61	0.42		0.52
6.	0.85	0.52			0.88	0.78			0.81	0.82		0.84	0.85		0.62
7.		0.75				1.07			0.92			0.94	0.95		0.66
8.		0.85											1.01		0.72
9.		1.13													0.89
10.															1.05

Fig. 3b): Genotypes 16 to 30(L16-L30) according to table 1 spotted on plate for fingerprint analysis and spotted phytochemicals retention factor.

On the basis of HPTLC profile of root alkaloids, all the 30 genotypes of Ashwagandha were grouped together in four different clusters irrespective of their geographical location (Table 3). First cluster contains one peak in accordance to standard compound withanolides- A at Rt 0.33 and clustered 10 genotypes from Udaipur, Rajasthan all together which includes: WS-124, WS-20I, WS-202, WS-204, WS-205, WS-205, WS-206, WS-210, WS-213, WS-218, WS-220, along with genotypes WS-90-103, WS-90-100, WS-20 (C) from Mandsaur district, Madhya Pradesh and also 2 more genotypes local and HWS-01-I from Haryana. A total of 15 genotypes showed similar pattern for rt 0.33 in different genotypes showing presence of standard reference compound withanolide-A. Second cluster contains only 4 genotypes on the basis of their chemical profile including genotypes WS-223, WS-224, WS-226 from Udaipur, Rajasthan while genotypes WS-90-104 from Mandsaur district, Madhya Pradesh had a Rt of (0.29, 0.64).Third cluster revealed 3 peaks of Rt 0.33, 0.72, 0.83 approximately and clustered genotypes WS-90-105, WS-90-117, WS-90-125, WS-90-126, WS-90-129, Adinath from Mandsaur district, Madhya Pradesh along with 2 genotypes from Haryana which include HWS-04-2 and HWS-04-3. A fourth cluster was observed with many peaks which includes genotypes WS134(C), WS-90-135, WS-90-136 from Mandsaur district, Madhya Pradesh with Rt of approximately 0.30, 0.63, 0.72, 0.89, 1.00. 0.82. The study revealed that HPTLC profiles were in quite accordance with the results reported by earlier researchers[3,12,19,20,24,26,31,32,38,42].

HPTLC profiles for leaf extracts resulted in grouping of various genotypes into five clusters [Table 4 and fig. 3a (L1-L16) and L16-L30)]. Cluster 1 comprised of genotypes WS-204, WS-205, WS-206, WS-210, WS-213, WS-218, WS-220, WS-223, WS-224 from Udaipur Rajasthan alongwith genotypes WS-90-100, WS-90-103, WS-90-104 from Mandsaur district (Madhya Pradesh) with a Rt (Retention time) of approximately (0.11-0.14). Cluster 2 includes only 4 genotypes all from Mandsaur (M.P.) i.e. WS-90-105, WS-90-117, WS-90-125, WS-90-126, with 2 peaks of Rt (retention time 0.12, 0.77). Cluster 3 contains 6 genotypes with Rt 0.15 and one peak which includes WS-134 (C) WS-90-135, WS-20 (C), Adinath, from Mandsaur and Neemuch (M.P.) and 2 genotypes HWS-04-1 and HWS-04-3 from Haryana. Cluster 4 comprised of 6 genotypes with 3 peaks at Rt of (0.20, 0.31, 0.77) which includes genotypes WS-90-136, Local. HWS-04-2 and 3 genotypes from Udaipur, Rajasthan, Cluster 5 includes only 2 genotypes WS-124 and WS-201, which show peaks at Rt of 0.13. 0.46, 0.80.

The major-chemical components of dried plant materials or chemical fingerprints have been studied using HPTLC in large number of medicinal plants viz. *Andrographis paniculata, Adhatodavasica, Gymnemasylvestre, Curcuma longa, Asparagus racemosus, Amaryllis belladonna, Papaver somniferum L. and Enicostemma littoral*[17]. Srivastava et al[39] determined the chemical fingerprint of *Andrographis paniculata* (*Acanthaceae*) using high performance thin layer chromatography.

Major alkaloids andrographolide and neoandrographolide were absent in the hexane extracts but were present in greater amounts in the methanol extracts as compared to other extracts[38].

Table 3
Ashwagandha genotypes differentiated on the basis of alkaloid profile from roots using HPTLC

1.	Cluster-I	15/30, (0.33 Rt) WS-124, WS-20I, WS-202, WS-204, WS-205, WS-206, WS-210, WS-213, WS-218, WS-220, WS-90-103, WS-90-100, WS-20 (C), HWS-04-I, Local
2.	Cluster IInd	4/30, (0.29, 0.64 Rt) WS-223, WS-224, WS-226, WS-90-104,
3.	Cluster IIIrd	8/30, (0.33, 0.72, 0.83 Rt) WS-90-105, WS-90-117, WS-90-125, WS-90-126, WS-90-129, Adinath, HWS-04-02, HWS-04-03
4.	Cluster IV	3/30 at Rt ≃ 0.30, 0.63, 0.72, 0.82, 0.89, 1.00 WS-134 (c), WS-90-135, WS-90-136

Table 4
Genotypes of Ashwagandha clustered on the basis of alkaloid profile from leaves using HPTLC

1	Cluster-I	(12/30)(Rt -0.11-0.14) WS-204, WS-205, WS-206, WS-210, WS-213, WS-218, WS-220, WS-223, WS-224, WS-90-100, WS-90-103, WS-90-104
2	Cluster-2	(4/30),Rt (retention time 0.12, 0.77). WS-90-105, WS-90-117, WS-90-125, WS-90-126
3	Cluster-3	(6/30)-WS-134 (C), WS-90-135, WS-20 (C), Adinath, HWS-04-I, HWS-04-3,
4	Cluster-4	(6/30), Rt of (0.20, 0.31, 0.77) -WS-90-136, Local, HWS-04-2, WS-202, WS-226, WS-90-129
5	Cluster-5	(2/30), (Rt of 0.13. 0.46, 0.80) -WS-124, WS-20I

Withania somnifera contains many phytochemicals such as withaferin -A, withanine, anahygrine etc. Withaferin 'A', being the most active compound was estimated in herbal extracts and polyherbal formulation using HPTLC[26] from *Withania somnifera*. Similar studies were carried out by Bishnupria et al[8] who reported that chemical composition of *W. somnifera* particularly alkaloids revealed a reduction in number and quantity in leaves due to air pollution. Upadhyay et al[43] reported HPTLC fingerprint profile of an aqueous extract of *E. littorale*. They performed successive extraction of aqueous extract using petroleum, ether, toluene, chloroform, ethyl acetate and n-butanol.

Phytochemical testing revealed that n-butanol fraction contained mainly terpenoids whereas chloroform and ethyl acetate fractions contained different chemical groups including flavonoids, alkaloids, phenols and tannins. Similar to our study Pathania et al[30] achieved the separation of curcuminoids from *Curcuma longa* by employing a mobile phase consisting of chloroform and methanol (98:2) on precoated HPTLC Lichrosphere aluminum plates Si60F254. Mahadevan et al[21] opted similar conditions for estimation of withaferin-A from herbal and polyherbal extracts from *W. somnifera* using precoated silica gel G. (aluminium backed) plates as stationary phase while toluene: ethylacetate; formic acid (50:15:5) as mobile phase.

As compared to this, Simonovska and Vovk[35] developed the chromatograms for estimation of potato glycoalkaloids using chloroform-methanol 2% aqueous NH_4OH (70:30:5) mixture as mobile phase from methanolic extracts in different parts of potato plant. Based on the HPTLC profile of phytochemicals obtained from leaves, all the 30 genotypes of ashwagandha were grouped together in five major clusters irrespective of their geographical conditions. The genotypes here also did not reveal a clear cut grouping or clustering of genotypes of particular locations but were found to be interspersed with each other.

The first cluster revealed 15 genotypes from different locations namely, Udaipur (Raj.), Mandsaur (M.P.) and that of Haryana. The HPTLC profile of root phytochemicals resulted in grouping of various genotypes in five clusters and here also the genotypes did not form distinct location specific clusters but were interspersed with each other similar to earlier reports.

Adin et al[2] used HPTLC to quantitatively analyze and to verify the presence of Baicalin in extracts of several *Oroxylum indicum* plant sections. Acetone, ethyl acetate, water and formic acid were utilized as the mobile phase at 318nm wavelength. Baicalin is an active component of *O. indicum*, the primary component of the flavocoxide authorized medical food, which is categorized as RAS by the USFDA. Lingfa et al[20] used HPTLC fingerprinting at dual wavelength of UV (254/366nm) and methanol as an organic solvent to clarify the phytochemical profile of *Withania somnifera* plant leaf and roots during various/different seedling, vegetative and reproductive stages. At various wavelengths and developmental phases, varied bands were seen.

Using HPTLC at the seedling stage, methanolic leaf extracts showed five peaks at 450 nm with R_f values ranging from 0.42 to 0.90. In contrast, nine R_F value peaks in the range of 0.10 to 0.89 were found during the negative stage. Six peaks in the reproductive stage with R_f values ranging from 0.30 to 0.92 were seen. Compared to leaves, roots showed six peaks with Rf values ranging from 0.00 to 0.82 during the seedling stage.The vegetative stage yielded 10 peaks with R_f values ranging from 0.00 to 0.96 while reproductive phases produced maxima that ranged from 0.01 to 0.91 overall. When compared to the vegetative stage (1.65%), the peak area in the reproductive stages was larger (3.33%).

Khalid and coworkers[19] screened phytochemicals and phytoconstituents from leaf tissue from *Gmelina arborea* Roxb.L. using HPTLC**.** Methanol was used as the best organic solvent, coupled with hydroalcoholic and aqueous extracts. Additionally, the presence of *G. arborea* leaf tissues was examined in hexane, ethyl acetate and methanolic extracts and HPTLC fingerprints were created. A considerable amount of flavonoid content (8.843-0.0185 to 11.242-0.021 mg QE/g) and phenolic content (19.395-0.01 to 20.202-0.0058 mg GAE/g) were found in the leaf portion, a plant component chosen from the genus *G. arborea*. The hydroalcoholic contents demonstrated strong antioxidant activity and a unique chromatogram at 2 separate wavelengths of 254 and 366 nm depending on dissolution. The greatest number of bands were seen in ethyl acetate extracts in contrast 10 at 254 nm and 12 at 366 nm, respectively.

For scavenging (antioxidant activity) of 90 mg practically, hydroalcoholic extracts were the best extracts. The outcomes might be a direct result of the greater phenol and flavonoid content and they hold the promise of lowering stress and scavenging ROS in the future[19]. Pradhan and Sharma[32] have also investigated the possible use of HPTLC to create fingerprints or chromatograms using various *Inula grandiflora* plant sections. Abdelwahed et al[1] reported analytical tool and techniques to elucidate different chemical constituents of *Withania* quantitatively or qualitatively way. Chromatographic methods were used to analyze chemical constituents which mainly include HPLC, UPLC with UV detection, mass spectroscopy, gas chromatography, TLC, HPTLC, UV visible spectroscopy, FTIR and NMR.

Total alkaloid content: The alkaloid content in leaves ranged from 0.65% to 2.68% while in roots it was observed to range from 0.31% to 3.87% (Table 5). The highest percentage of alkaloid content was observed in genotype HWS-04-2 (2.68%) from Haryana while only 0.645% was observed in genotype Adinath from Neemuch tehsil of Mandsaur district, Madhya Pradesh. In roots, highest percentage of 3.87% was observed in genotype WS-226

from Udaipur (Rajasthan), while lowest 0.31% in genotype Adinath from Neemuch tehsil of Mandsaur district (M.P.) was observed. Alkaloids content is known to be influenced by a number of factors including genotype, environmental conditions, date of sowing and developmental stage of the plant.

Sreerekha et al[38] reported 0.2413% withanolides in roots of Ashwagandha up to 105 days after sowing which further goes down significantly to 0.1997%. Barath Kumar et al[5] reported a total alkaloid content of (1.302%) primarily from roots of Ashwangandha (*W. somnifera* L. Dunal) and further reported an increase in alkaloid content by application of chloroethyl triammonium chloride (CCC) (Chlormequat) at the rate of 2000 ppm after transplanting. Baraiya et al[4] studied the initiation of alkaloid synthesis, its partitioning and accumulation pattern in different plant parts of Ashwangandha (*W. somnifera* L. Dunal) from sowing to physical maturity and revealed higher concentration of alkaloids assimilation (14.52%) in leaves (source) which was transported to roots via stem so that the alkaloid per cent in roots increased with advancement of age of the crop up to 105 days (71.75%), which was further reported to be (122.19%) after first fruit maturity. A reduction in total alkaloid content was reported, when crop reached 135 days age. Sreerekha et al[38] reported withanolide content in different plant parts (leaf, berry and root) of wild and cultivated (WS-100) Asgandh (*W. somnifera*). Highest amount of withanolide was reported in berries (2.95-3.35%) and roots (0.23-1.11%), while in leaves withanolide content ranged from 0.16-2.07%. The total withanolide content in all plant parts put together was found to be 4.95%.

Conclusion

An advanced version of TLC (HPTLC) was used to study alkaloid profile/chemoprofile in 30 genotypes of Ashwagandha. On the basis of different Rt (Retention time) and peak areas, all the 30 genotypes of Ashwagandha were grouped in 4 different clusters using leaf extracts while root extracts formed 5 clusters in all. In conclusion, a fairly high level of polymorphism was observed among various genotypes of *W. somnifera* included in the present study using different types of markers.

Table 5
Total alkaloid content (%) in leaves and roots of *W. somnifera*(L.) Dunal genotypes

S.N.	Genotypes	Alkaloid content (%) on dry wt. basis	
		Leaves(%)	Roots(%)
1	WS-124	2.13	0.35
2	WS-201	2.07	0.37
3	WS-202	0.53	0.48
4	WS-204	2.17	0.65
5	WS-205	0.98	0.52
6	WS-206	1.64	0.73
7	WS-210	1.07	1.54
8	WS-213	1.36	1.41
9	WS-218	1.47	0.61
10	WS-220	0.67	0.85
11	WS-223	1.35	3.30
12	WS-224	1.27	1.92
13	WS-226	0.88	3.87
14	WS-90-100	2.19	2.21
15	WS-90-103	2.02	0.51
16	WS-90-104	1.11	3.17
17	WS-90-105	2.0	0.45
18	WS-90-117	0.79	1.08
19	WS-90-125	0.93	0.58
20	WS-90-126	1.12	1.37
21	WS-90-129	1.14	0.46
22	WS-134(C)	0.22	1.96
23	WS-90-135	0.82	3.36
24	WS-90-136	1.78	0.40
25	WS-20 (C)	0.88	1.05
26	Adinath	0.65	0.31
27	Local	2.46	2.83
28	HWS-04 -1	0.77	3.53
29	HWS-04 -2	2.68	0.38
30	HWS-04 -3	0.84	0.38

The results revealed that chemical markers in fingerprinting genotypes could successfully determine the genetic relationships and estimate genetic diversity among various genotypes. The content of alkaloid in leaves ranged from 0.65% to 2.68% being highest (2.68%) in HWS-04-2 genotype from Haryana, while 0.645% was observed in genotype Adinath from Neemuch tehsil of Mandsaur district (MP). The roots revealed a maximum of 3.87% in genotype WS-226 from Udaipur (Raj.) while a minimum of 0.31% was observed in genotype Adinath from Neemuch tehsil of Mandsaur district (M.P.). The standard reference compound spotted along with the methanolic root and leaf extracts clearly demarcated the leaves and roots constituents.

All the roots samples lacked withaferin 'A' while all leaf samples lacked withanolide 'A' which is elucidated earlier in much reports.The main restriction is the amount and variance in various metabolites according to conditions, environmental variables and other changes. The HPTLC method, which produces a chromatograph to separate non volatile substances from distinct plant components, is the first step to identify as the fundamental approach.

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