TLC (Thin Layer Chromatography) to construct chemical fingerprints of Ashwagandha (*W. Somnifera)* – A Rejuvenating Herb

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Abstract

Withania somnifera has been used in Ayurvedic medicine for the treatment of depression and inflammation and as an aphrodisiac. It contains many phytochemicals such as Withaferin A, Withanine, Withanolides and Anahygrine. Withaferin 'A' being the most active compound was estimated in herbal extracts and polyherbal formulation using TLC in number of studies from Withania somnifera. Dried leaves and roots from each of the 30 genotypes were extracted for phytochemicals using methanolic extracts. Chromatograms were developed using solvent system consisting of chloroform: methanol (90:10) for methanolic extracts and a mobile phase consisting of toluene: ethyl acetate: formic acid (75:18:7) on precoated TLC aluminum plates $Si_{60}F_{254}$ for methanolic leaf extracts from each of the 30 genotypes. Based on the TLC profile of phytochemicals obtained from leaves and roots, all the 30 genotypes of ashwagandha were grouped together in five major clusters irrespective of their geographical conditions.

The genotypes 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 showing relation among them. The TLC 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.

Keywords: *Ashwangandha*, Chemotyping, TLC, chromatogram, Dendrogram, Cluster Tree Analysis.

Introduction

Chemical profile is mostly used to describe the chemical pattern for a certain plant material based on the chemical components found in its fractions or extracts. Most regulatory guidelines and pharmacopoeias advise macroscopic and microscopic examination as well as chemical contents (profile) of botanical products for quality control and standardization. Macroscopic identification/ evaluation involves contrasting a sample with a reference material that serves as a standard in order to identify traits such as shape, size, color, texture, surface characteristics, fraction properties, odor, taste and other organoleptic qualities.

The initial step in microscopic analysis is a comparative microscopic study of broken and powdered raw botanical materials. These simple procedures like macroscopic and microscopic evaluations are widely used methods for identification of a particular drug plant. However, such methods are inapplicable in case of crude drug powders and herbal drug extracts as they do not bear the characteristics morphological and anatomical features of the parent plant. Although complementary/supplementary methods like organoleptic evaluation, simple chemical tests and UV spectral studies are helpful to some extent, none of these is completely enough to identify a particular chemotype. In such situation, depiction of molecular or biochemical profile of the material using chemical fingerprinting technique is of great importance.

In the Ayurvedic system of Indian medicine, Ashwagandha also known *as Withania somnifera (L.) Dunal*, occupies a unique place. The central Indian States of Madhya Pradesh, Rajasthan and portions of Punjab, Himachal Pradesh and Uttar Pradesh are the primary growing regions. There are 23 species, two of which, *W. somnifera (L.) Dunal* and *W. coagulans (L.) Dunal*, are thought to have therapeutic properties. India has 4000 hectares dedicated to its cultivation.[17,25] Seeds can be sown directly in the fields and seedlings can be raised and transplanted in addition to direct seeding. It thrives on sandy loam or light red soils with a pH range of 7.5 to 8 and needs an annual rainfall of 660-750 mm. It is known as Indian ginseng due to the similarity between the roots of Ashwagandha and ginseng roots.[4,23]

It contains very high concentration of alkaloids and other secondary metabolites such as flavonoids and steroidal lactones. The steroidal lactones have been collectively given the name withanolides. These withanolides produce mild ionotropic and chronotropic effects on cardiovascular system. The ingredients have pharmacological properties with curative effects against a number of problems such as diabetes[6,13,15], gastro intestinal and reproductive system disorders[12,16,21], anti-ageing agent, antitumor and as general health/memory tonic[8] etc. Recently it has found its potential use in cancer chemotherapy.[7] Different kinds of biological activities have been ascribed to some of the purified chemical constituents of Ashwagandha and to the raw material extracted from its leaves, roots, bark and fruits.[14,29]

Withania somnifera being a plant of medicinal importance especially in India and South Africa has been investigated for pharmacological properties of crude extracts of its roots and leaves. The first scientific investigation regarding the medicinal aspect of plant started date back to 1911 when work on the chemical constituents of *W. somnifera* collected from South Africa was carried out and withanol ($C_{25}H_{34}O_5$) was reported from the roots extract and two other compounds somnirol ($C_{32}H_{46}O_7$) and somintol ($C_{33}H_{46}O_7$) were reported from leaf. Chemical profiling establishes a specific chemical pattern for a plant material and its fractions or extracts. Thin layer chromatography can be used to profile the phytochemical components of plants or their products.

The choice of the appropriate plant chemotype is crucial for effectiveness.[20] 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. Thin layer chromatography (TLC) is frequently employed as useful instrument for the qualitative identification of minute amounts of contaminants. In addition to these procedures including spectrophotometric analysis, volumetric analysis, gravimetric calculations, gas chromatography, column chromatography are widely employed to examine chemical profiles.[24]

Material and Methods

Plant Material: Thirty genotypes of *W. somnifera* (L.) Dunal, seeds were obtained from the Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana's Medicinal Aromatic and Under Utilized Plant (MA&UUP) Section, Department of Plant Breeding (Table 1). During the Kharif season, seeds were sown in the fields of the MA&UUP Section of the Department of Plant Breeding and in the screen house of the Department of Biotechnology. The genotypes were grown in an enhanced design using all agronomic practices and packages.

Reagents and Chemicals: All the chemicals and reagents used in the current study for thin layer chromatography were of high purity analytical grade and were purchased from S.D. Fine Chemicals Ltd., India, Bangalore Genei Pvt. Ltd., India and Genetix, India. Standard substances Withaferin 'A' and Withanolide 'A' were purchased from Life Technologies, India.

Sample preparation: Thirty genotypes leaves and roots were separately dried for 24 hours at 70°C (until the weight remained consistent) (Fig. 1). Using a pestle and mortar, dried samples were crushed to a fine powder. Ten gram of powdered sample was extracted with 200 ml of 100% methanol for four hours on a boiling water bath. The extracts were collected by filtration using Whatmann filter paper no. 1 and the filterate was concentrated to 1/3rd of its original volume (Fig. 2).

Preparation of TLC Plates: The glass plates (20 x 10 cm) were cleaned and rinsed with acetone before being coated with silica gel G_{254} that had been made by dissolving 5g in 20 ml of distilled water. The coating was between 0.2 and 0.3 mm thick. The plates were dried at room temperature before being activated in an oven at 100°C for 30 minutes and cooled to room temperature. Glass plates that were already coated with silica gel made the procedure easier to manage faster.

Development of Thin layer chromatogram: With the use of a micropipette, methanolic extracts (1µl) made from leaf and root samples were individually put 1cm above the edge of the plates together with the standard reference. Various solvent solutions were used for fractionation (Tables 2 and 3). The solvents utilized can be either a single solvent system or a combination of two, three, or even more solvents.

To separate the various components, absorbent is used as the stationary phase while polarity gradient, extracted plant extracts, which can be either crude extracts or organic solvent extracts, are used as the mobile phase. The solvent systems that resulted in best resolution of various components from leaf and root extracts, were LS-8 and RS-1 respectively.

Visualization and Documentation: The TLC plates were air dried before being evenly coated with a 5% methanolic solution of concentrated sulfuric acid and baked in a hot air oven for 10 minutes at 100°C. The chromatogram that resulted from development, was instantly captured on film.

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

S.N.	Genotypes	Source
1.	WS-124,WS-201,WS-202,WS-204,WS-205,WS-206,WS-210,WS-213,WS-218,WS-220,WS-223,WS-224,WS-226	Udaipur, Rajasthan
2.	WS-90-100,WS-90-103,WS-90-104,WS-90-105,WS-90-117,WS-90-125,WS-90-126,WS-90-129,WS-134(C),WS-90-135,WS-90-136,WS-20 (C)	Mandsaur (M.P.)
3.	Adinath	Neemuch (M.P.)
4.	Local	Hisar, Research Farm Area
5.	HWS-04 -1,HWS-04 -2,HWS-04 -3	Haryana

Fig. 1: Samples collected and stored for leaf and root from different genotypes and methanolic extracts of thirty genotypes prepared for TLC using a) leaves and b) roots

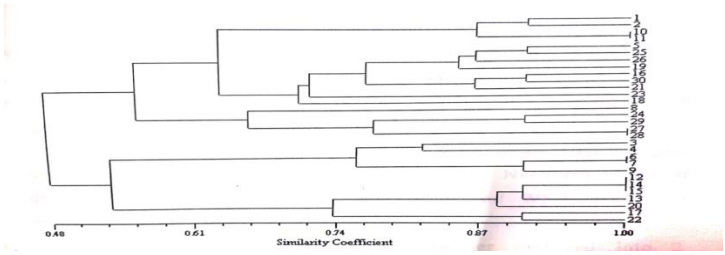

Fig. 2: Dendrogram of leaf samples created using UPGMA analysis from 30 genotypes

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

Solvent system code	Solvent system	Ratio
LS-1	EtoAc : MeoH	5:5
LS-2	Butanol : H_2O : AcoH	7:3:4
LS-3	EtoAC : H_2O : MeoH	40:54:64
LS-4	Hexane : EtoAc	7:3
LS-5	$CHCl_3$: MeoH	9:1
LS-6	$CHCl_3$: EtoAc	1:1
LS-7	CH_2Cl_2 : MeoH	1:1
LS-8	Toluene : EtoAc : HCOOH	75:18:7
LS-9	Hexane : Butanol	1:3
LS-10	$CHCl_3$: EtoAc : MeoH : Benzene	70:4:8:24

Table 3
Solvent systems used for TLC analysis of root extracts in *W. somnifera* (L.) Dunal

Solvent system code	Solvent system	Ratio
RS-I	$CHCl_3$: MeoH	9:1
RS-II	Acetonitrile : H_2O	4:6
RS-III	nHexane : EtoAc	7:3
RS-IV	Toluene : EtoAc	7:3
RS-V	Toluene : EtoAc : HCOOH	60:15:5
RS-VI	Toluene : EtoAc	5:5
RS-VII	$CHCl_3$; MeoH	1:1
RS-VIII	$CHCl_3$: MeoH	7:3
RS-IX	EtoAc : MeoH : H2O	40:6.4:5.4
RS-X	Benzene	(100%)
RS-XI	EtoAc	(100%)

The distance that each spot moved on the chromatogram relative to the distance that the developing solvent went, was used to calculate its Rf value.

$$Rf = \frac{Distance\ travelled\ by\ sample}{Distance\ travelled\ by\ solvent}$$

Results and Discussion

Thin layer chromatography: TLC offers a number of advantages out of the box such as simplicity, high sample throughput, speed and fixed and working capital economy. In investigations pertaining to the chemical profile of a certain chemotype as well as in current herbal pharmacopoeias as a medication identification tool,

conventional TLC has a desired position. To establish TLC as an accurate and reliable tool for studying chemical fingerprints and ensuring the efficacy of herbal medicines, careful consideration of the chromatographic parameters is required. This results in well formed spots with enough separation and consistent Rf values. By including a marker or common compounds, its credibility was boosted.

The chemical fingerprint or chemo profile of 30 genotypes of Ashwagandha (*W. somnifera* L.) was studied using thin layer chromatography. To identify certain plant species, a variety of straightforward techniques include botanical description, comparisons with herbaria, observation with the unaided eye and microscopic investigation which are frequently utilized. These techniques, however, cannot be used with herbal drug extracts, drug powders, or crude drugs since they lack the parent plant's distinctive morphological and anatomical characteristics. While procedures like organoleptic evaluation, chemical testing and UV spectral investigations are additional, none of them are comprehensive enough to describe chemical profile.

Due to its adaptability, speed, simplicity and affordability, thin layer chromatographic fingerprinting is preferred in such circumstances.[19,27,28] TLC fingerprinting of medicinal plants has been tried by a number of researchers. In the current work, phytochemicals were isolated from the leaves and roots of mature *Withania somnifera* plants of different genotypes and were evaluated by TLC. The solvent system LS-8 with a composition of toluene: ethyl acetate: formic acid in a ratio of 75: 18:7 was found to be the best at producing round, legible and well-resolved spots when using methanolic leaf extract in all 30 genotypes of Ashwagandha out of the ten different solvent combinations that were tried (Tables 2 and 3). Solvent system RS-1, which has chloroform: methanol in ratio of 90:10, was able to resolve methanolic root extracts.

Thin layer chromatography has been shown to be a useful tool for studies of alkaloids and chemical profiles in plants of medicinal importance.[1,2] TLC fingerprints were developed in ten significant crude drugs obtained from various plant parts including roots (*Plumbago indica*, *Rauvolfia serpentine*), shoots (*Centella asiatica*, *Andrographis paniculata*) and tubers (*Holostemma adakodein*).

TLC fingerprints using leaf extracts in Ashwagandha (*W. somnifera*): A nine-spotted thin layer chromatogram was obtained using solvent system LS-8 having toluene: ethyl acetate and formic acid in a ratio of 75: 18: 7 differentiating the genotypes of Ashwagandha. A maximum of eight spots were observed with Rf value (0.07, 0.12, 0.17, 0.58, 0.64, 0.73, 0.80 and 0.98) in genotype WS-223 and WS-224 from Udaipur, (Rajasthan) followed by 7 spots in genotypes WS-90-126 from Mandsaur district, M.P. with Rf value (0.12, 0.14, 0.17, 0.64, 0.73, 0.80 and 0.98) and WS-90-129 (M.P.) with Rf value (0.07, 0.12, 0.14, 0.64, 0.73, 0.80 and 0.98). 3 spots were resolved in three genotypes WS-205 (Rf value 0.07, 0.58, 0.64) from Udaipur, Rajasthan, WS-90-100 (Rf value 0.07, 0.64, 0.73) from Mandsaur (M.P.) and HWS-04-2 (Rf value 0.12, 0.64, 0.80) from Haryana in the chromatogram.

The spot six corresponding to Rf value of 0.64 was found to be present in 29 genotypes out of a total of 30 except WS-202 (Raj.) followed by spot one (Rf value 0.07) as observed in 24 genotypes except these six genotypes, WS-90-104, WS-90-105, WS-90-126, WS-90-136 from Mandsaur district (M.P.) and Local, HWS-04-2 from Haryana. Similarly, twenty genotypes revealed the presence of spot seven with Rf value (0.73) including 9 genotypes from Udaipur (Raj.), 10 genotypes from Mandsaur dist. (M.P.) and one genotype (Local) from Hisar, Haryana. Eighteen genotypes showed the presence of spot five (Rf value 0.58) which comprise of 10 genotypes from Udaipur (Raj.) four genotypes from Mandsaur (M.P.) and one genotype from Hisar (Haryana). Spot nine with a Rf value (0.98) was resolved in 17 genotypes of Udaipur (Raj.), 8 genotypes from Mandsaur (M.P.) and 2 genotypes from Haryana.

Spot 2nd with Rf value (0.12) was resolved in 14 genotypes of Udaipur (Raj.), 9 genotypes from Mandsaur (M.P.) and 2 genotypes from Haryana. Spot eight with Rf value (0.80) was observed in eleven genotypes, 3 from Haryana, 6 genotypes from M.P. A minimum of 2 spots were resolved in genotype WS-202 (Rf value 0.07, 0.58) from Udaipur (Raj.) and genotype WS-90-103 (Rf value 0.07-0.64) from Madhya Pradesh and 2 from Udaipur, (Raj.) Three genotypes one WS-90-126 from Mandsaur (M.P.) and two WS-223 and WS-224 from Udaipur, (Rajasthan) showed the presence of spot 4 (with Rf value 0.17). A minimum of 2 genotypes WS-90-126 and WS-90-129 both from Mandsaur dist. (M.P.) showed the presence of spot 3 (with Rf value 0.14) as shown in table 4.

TLC fingerprint using root extracts: The chromatogram obtained after running the samples in solvent system RS-1 having chloroform: methanol in a ratio of 90:10 revealed a maximum of 5 spots with Rf value 0.16, 0.29, 0.40, 0.58 and 0.93 respectively. A spot with Rf value 0.40 was observed in all genotypes taken in present investigation except local genotype from Hisar, Haryana which lacked this component. A total of eighteen genotypes showed the presence of fifth spot with Rf value 0.93 except WS-201, WS-206, WS-220, WS-223, WS-224, WS-226, WS-90, 100, WS-90-103, WS-90-105, WS-20 (C) and 2 genotypes from Haryana i.e. local and HWS -04-3. This is further followed by 23 genotypes which showed the particular spot of Rf value (0.29) except genotypes WS-124, WS-201, WS-202, WS-204, WS-205, WS-206, WS-210 all procured from Udaipur (Rajasthan). As compared to above, a maximum of eight genotypes showed the presence of spot I with Rf value 0.16 which includes WS-223, WS-224, WS-226 from Udaipur (Raj.) and WS-90-103, WS-90-104, WS-90-117, WS-90-129, WS-134 (C) taken from Mandsaur district (M.P). This particular spot was not

observed in other genotypes except those mentioned. 6 genotypes showed the presence of 4th spot with Rf value 0.58 as observed in five genotypes from Udaipur which includes WS-202, WS-204, WS-205, WS-206, WS-210 and one genotype WS-90-135, from Mandsaur distt. (M. P.). Maximum of 4 spots were resolved in four genotypes, WS-90-104 with Rf value (0.16, 0.29, 0.40 and 0.93), genotype WS-90-117 (Rf value of 0.16, 0.29, 0.40 and 0.93) genotype WS-90-129 (Rf value (0.16, 0.29, 0.40 and 0.93) and WS-90-135 with Rf value 0.29, 0.40, 0.58 and 0.93 all belonging to Mandsaur districts (M.P.).

This is followed by 3 spots in 17 genotypes with maximum frequency as observed including nine genotypes from Udaipur (Rajasthan) WS-202 (Rf value 0.40, 0.58, 0.93), WS-204 (Rf value 0.40, 0.58, 0.93), WS-205 (Rf value 0.40, 0.58 and 0.93), WS-210 (Rf value 0.40, 0.58, 0.93) WS-213 (Rf value 0.29, 0.40 and 0.93), WS-218 (Rf value 0.29, 0.40 and 0.93) and genotype WS-223, WS-224, WS-226 (Rf value 0.16, 0.29, 0.40), each along with 6 genotypes from Mandsaur district (M.P.) i.e. genotypes WS-90-125, WS-90-126, WS-134 (C) WS-90-136 and Adinath with a Rf value of 0.29, 0.40, 0.93) each and genotype WS-90-103 (Rf value 0.16, 0.29, 0.40) and also two genotypes from Haryana HWS-04-1 and HWS-04-2 with a Rf value of 0.29, 0.40, 0.93) each.

Out of 30 genotypes, only 2 spots were observed in seven genotypes from Udaipur (Rajasthan) which include WS-124 (RF value 0.40, 0.93), WS-206 (Rf value 0.40 and 0.58) and WS-220 (Rf value 0.29 and 0.40), 3 genotypes from Mandsaur (M.P.) viz. WS-90-100 (Rf value 0.29 and 0.40) WS-90-105 (Rf value 0.29 and 0.40) and one genotype from Haryana HWS-04-3 (Rf value 0.29 and 0.40).A minimum of one spot was resolved in the fingerprints of only two genotypes one from Udaipur (Rajasthan),WS-201 with Rf value 0.40 while another genotype local from Hisar (Haryana) with a single spot of Rf value of 0.29 as shown in table 5.

Relationship among different genotypes using TLC chromatogram from leaves and roots in Ashwagandha: The simqual subprogram of the NTSYS-pC software was used to compute similarity matrices, taking into account the presence of a specific spot with a specific Rf value as 1 and its absence as '0' in a particular genotype similar to RAPD fingerprints data used to construct dendrogram, in order to study relationships among various genotypes of aswhagandha based on chemical fingerprints obtained. Only the finest, round, readable and ultimately resolved spots were taken into consideration; the faint spots were excluded. The results from the similarity matrices varied from 0.11 to 1.0, indicating substantial genotype variability consistent with DNA level observations. Regardless of the local climatic circumstances, a maximum of 100% similarity was found between certain genotypes from the same place as well as between genotypes from various locations like Madhya Pradesh, Haryana and Rajasthan.

It is not surprising that all of these genotypes were found in Udaipur, Rajasthan including WS-124 and WS-204, between WS-124 and WS-210, between WS-201 and WS-206, between WS-201 and WS-213, between WS-201 and WS-218, between WS-206 and WS-218 and between WS-223 and WS-224.

Table 4
R_f values of TLC spots from leaf extracts of *W. somnifera* (L.) Dunal genotypes

S.N.	Genotypes	R_f values	S.N.	Genotypes	R_f values
1	WS-124	0.07, 0.58, 0.64, 0.73, 0.98	16	WS-90-104	0.12, 0.58, 0.64, 0.73
2	WS-201	0.07, 0.58, 0.64, 0.73	17	WS-90-105	0.12, 0.58, 0.64, 0.73
3	WS-202	0.07, 0.58	18	WS-90-117	0.07, 0.12, 0.58, 0.64, 0.80, 0.98
4	WS-204	0.07, 0.58, 0.64, 0.73, 0.98	19	WS-90-125	0.07, 0.12, 0.64, 0.73, 0.98
5	WS-205	0.07, 0.58, 0.64	20	WS-90-126	0.12, 0.14, 0.17, 0.64, 0.73, 0.80, 0.98
6	WS-206	0.07, 0.58, 0.64, 0.73	21	WS-90-129	0.07, 0.12, 0.14, 0.64, 0.73, 0.80, 0.98
7	WS-210	0.07, 0.58, 0.64, 0.73, 0.98	22	WS-134 (C)	0.07, 0.12, 0.64, 0.98
8	WS-213	0.07, 0.58, 0.64, 0.73	23	WS-90-135	0.07, 0.64, 0.73, 0.80, 0.98
9	WS-218	0.07, 0.58, 0.64, 0.73	24	WS-90-136	0.12, 0.64, 0.73, 0.80, 0.98
10	WS-220	0.07, 0.58, 0.64, 0.73	25	WS-20 (C)	0.07, 0.12, 0.64, 0.73, 0.80, 0.98
11	WS-223	0.07, 0.12, 0.17, 0.58, 0.64, 0.73, 0.80, 0.98	26	Adinath	0.07, 0.58, 0.64, 0.73
12	WS-224	0.07, 0.12, 0.17, 0.58, 0.64, 0.73, 0.80, 0.98	27	Local	0.12, 0.58, 0.64, 0.73
13	WS-226	0.07, 0.12, 0.58, 0.64, 0.98	28	HWS-04-1	0.07, 0.64. 0.80. 0.98
14	WS-90-100	0.07, 0.64, 0.73	29	HWS-04-2	0.12, 0.64, 0.80
15	WS-90-103	0.07, 0.64	30	HWS-04-3	0.07, 0.64, 0.80, 0.98

Table 5
R_f values of TLC spots from root extracts of *W. somnifera* (L.) Dunal genotypes

S.N.	Genotypes	R_f values	S.N.	Genotypes	R_f values
1	WS-124	0.40, 0.93	16	WS-90-104	0.16, 0.29, 0.40, 0.93
2	WS-201	0.40	17	WS-90-105	0.29, 0.40
3	WS-202	0.40, 0.58, 0.93	18	WS-90-117	0.16, 0.29, 0.40, 0.93
4	WS-204	0.40, 0.58, 0.93	19	WS-90-125	0.29, 0.40, 0.93
5	WS-205	0.40, 0.58, 0.93	20	WS-90-126	0.29, 0.40, 0.93
6	WS-206	0.40, 0.58	21	WS-90-129	0.16, 0.29, 0.40, 0.93
7	WS-210	0.40, 0.58, 0.93	22	WS-134 (C)	0.16, 0.40, 0.93
8	WS-213	0.29, 0.40, 0.93	23	WS-90-135	0.29, 0.40, 0.58, 0.93
9	WS-218	0.29, 0.40, 0.93	24	WS-90-136	0.29, 0.40, 0.93
10	WS-220	0.29, 0.40	25	WS-20 (C)	0.29, 0.40
11	WS-223	0.16, 0.29, 0.40	26	Adinath	0.29, 0.40, 0.93
12	WS-224	0.16, 0.29, 0.40	27	Local	0.29
13	WS-226	0.16, 0.29, 0.40	28	HWS-04-1	0.29, 0.40, 0.93
14	WS-90-100	0.29, 0.40	29	HWS-04-2	0.29, 0.40, 0.93
15	WS-90-103	0.16, 0.29, 0.40	30	HWS-04-3	0.29, 0.40

Although Adinath is from a different Madhya Pradesh tehsil (Neemuch), Adinath is still related to the genotypes of Udaipur (Rajasthan) and Mandsaur district (M.P.) which includes WS-218 and Adinath, WS-213 and Adinath, while WS-90-104 and WS-90-105 are both from Mandsaur (M.P.). There was at least 1% resemblance between the genotypes WS-205 from Udaipur, Rajasthan and WS-90-126 from Mandsaur, Madhya Pradesh. A value of 0.647 was discovered for the average similarity index.

Cluster tree analysis: A dendrogram was constructed taking Rf value as basis to construct similarity matrices table in both leaf and root samples of Ashwagandha. All the 30 genotypes of Ashwagandha were grouped together in a major cluster at a similarity coefficient of 0.48. This major cluster further bifurcates into two major clusters, cluster I and cluster II at a similarity coefficient of 0.53 and 0.55 respectively.

The cluster at a similarity coefficient of 0.53 was further bifurcated into two clear and distinct sub clusters: one at a similarity coefficient of 0.74 and sub cluster II at a coefficient of 0.76 separating five genotypes of Udaipur (Rajasthan), WS-202, WS-204, WS-206, WS-210 and WS-218 from that of WS-224, WS-226 clustered together from five genotypes of Mandsaur (M.P.), WS-90-100, WS-90-103, WS-90-126, WS-90-105, WS-134(C).

Sub cluster one (I) was further diverged into two groups at a similarity coefficient of 0.88 and 0.89 separating genotypes WS-90-105 and WS-134(C) from Mandsaur (M.P.) from that of WS-224, WS-226 from Udaipur (Raj.) along with genotype WS-90-100, WS90-103, WS-90-126 from Mandsaur (M.P.) at a coefficient of 0.89. The group II of sub cluster 1 of cluster 1 further out grouped the genotypes WS-90-126 (MP) at a coefficient of 0.88 and got further delineated into 2 subgroups I and 2 at a coefficient of 0.89. Subgroup I of group II included genotypes WS-226 only while the subgroup II included genotypes WS-224 from Udaipur (Raj.) clustered together with genotype WS-90-100 and WS-90-103 altogether from Mandsaur dist. (M.P.).

The sub cluster II of cluster I is further bifurcated into two major subgroups, subgroups 3 at a coefficient of 0.81 and subgroup 4 at a coefficient of 0.90. Subgroup 3 of sub-cluster II comprised of two genotypes WS-202 and WS-204 from Udaipur (Raj). Subgroup 4 comprised of genotypes WS-206, WS-210 and WS-218 from same location. Thus the genotypes of same location (Udaipur, Raj.) were grouped apart from each other. The cluster II at a similarity coefficient 0.55 was subdivided into sub cluster III and sub cluster IV at a coefficient of 0.63 and 0.66 respectively, separating the 5 genotypes WS-213, WS-90-136, Local, HWS-04-I from that of WS-124, WS-20I, WS-220, WS-223, WS-205, WS-20(C), Adinath, WS-90-125, WS-90-104, WS-90-129, WS-90-135 and WS-90-117 which were grouped together irrespective of their origin/or geographical location.

The sub cluster III at a similarity coefficient of 0.63 is further delineated into two distinct groups, group 3 and group 4 at a coefficient of 0.87 and 0.69 respectively. The group 3 at a coefficient of 0.87 out grouped the two genotypes from Udaipur (Rajasthan) WS-220 and WS-223 from that of WS-124, WS-201 at a similarity coefficient of 0.90 in spite of being from same location Udaipur (Rajasthan).Sub cluster III at a coefficient of 0.69 out grouped the genotype WS-90-117 from Mandsaur (M.P.) from that of genotypes from both Udaipur (Rajasthan) and Mandsaur (M.P.) which are clustered together including WS-205, WS-20 (C), Adinath, WS-90-125, WS-90-104, WS-90-129, WS-90-135 and one genotype HWS-04-3 from Haryana. It again out grouped the genotype WS-90-135 at a similarity coefficient of 0.71 from the above cluster and got further divided to separate genotypes WS-124 and WS-201 together at a coefficient of

0.90, while out grouping genotype WS-220, WS-223 from this cluster at a coefficient of 0.87.

Group 4 of sub cluster III of cluster II out grouped the genotype WS-90-117 (M.P.) at a coefficient of 0.69 while again out grouping the genotype WS-90-135 at a coefficient of 0.71. This group is further delineated at a coefficient of 0.76 into 2 distinct subgroups, out grouping genotype WS-90-129 from that of WS-90-104 and HWS-04-3. Genotype WS-90-125 was again out grouped at a coefficient of 0.85. This subgroup again delineated the genotype (Adinath) at a coefficient of 0.87 while clustering together genotype WS-205 from Udaipur (Rajasthan) and genotype WS-20 (C) from Mandsaur district (M.P.) at a coefficient of 0.90. Subcluster 4 of cluster II at a coefficient of 0.66 out grouped the genotype WS-213 from WS-90-136, from Mandsaur (M.P.) and genotype HWS-04-1, HWS-04-2 and local from Haryana.

Thus, showing high variations in chemical composition, this sub cluster is further diversified into sub sub-group I and II at a coefficient of 0.77 separating genotype Local and HWS-04-1 of Haryana from that of WS-90-136 and HWS-04-2 from two different locations one being from Mandsaur (M.P.) and other from Haryana as shown in fig. 2.

Similarity among different genotypes of Ashwagandha using chromatograms obtained from root extracts: The cluster tree analysis grouped the genotypes into one major cluster at a similarity coefficient of 0.51. This major cluster was further diverged into 2 major clusters, cluster I and cluster II at a similarity coefficient of 0.67 and 0.68 respectively. The cluster I of major cluster at a similarity coefficient of 0.67 was further separated the 30 genotypes into two sub clusters, sub cluster I, II. The sub cluster I at a coefficient of 0.76 further out grouped the genotype WS-90-135 from that of five genotypes WS-202, WS-204, WS-205, WS-206, WS-210 all grouped together and belonging to same location Udaipur, Rajasthan, while genotype WS-206 was further put apart from this cluster at a coefficient of 0.79 even being from same location as shown in fig. 3.

The sub cluster II at a coefficient of 0.79 again bifurcated two genotypes WS-124 and WS-201 from Udaipur (Rajasthan) separating them from other genotypes as already described in sub cluster I, which included genotypes WS-202, WS-204, WS-205, WS-206, WS-210 and genotype WS-90-135, all together grouped into one cluster in spite of being from different locations. Cluster II (a bifurcation of major cluster) was further divided into two sub clusters, sub cluster 3 at a coefficient of 0.76 and sub cluster 4 at a coefficient of 0.79. The first sub cluster 3 at coefficient of 0.76 out grouped the genotypes WS-223, WS-224, WS-226 and WS-90-103 from two different locations from that of a cluster of genotypes comprising WS-220, WS-223, WS-224, WS-226, WS-90-103, WS-90-105, WS-90-100, WS-20 (C) and HWS-04-3 and local, a total of 10 genotypes, from different location at a coefficient of 0.76. It got further delineated at a similarity coefficient of 0.79 into two different sub clusters separating genotypes WS-90-105 and Local (Haryana) from that of genotypes WS-220, HWS-04-3, WS-90-100 and WS-20 (C) all from different places. The sub cluster 4 of cluster II was further divided into sub sub-clusters at a similarity coefficient of 0.79, separating genotypes WS-213, WS-218, WS-90-125, WS-90-126, WS-90-136 Adinath clustered together with HWS-04-I and HWS-04-2 (2 genotypes from Haryana) all together from that of 3 genotypes which were WS-90-104, WS-90-129 and WS-90-117, all three being from Mandsaur district (M.P.).

Presence of standard reference compound in 30 genotypes of Ashwagandha: The methanolic root and leaf extracts prepared were spotted along with standard reference compounds namely Withaferin 'A' and Withanolide 'A'. The chromatogram obtained using solvent system RS-1 having chloroform: methanol in the ratio of 90:10 and plates when sprayed with 5% methanolic fraction of sulphuric acid revealed the presence of Withanolide 'A' as a spot with an Rf value of 0.40. A total of twenty nine genotypes out of 30 (96%) revealed the presence of Withanolide 'A' as a major component of root extracts prepared from each of the 30 genotypes except one genotype i.e. local from Hisar (Haryana).

Withaferin 'A' which is a major withanolide in leaves, was not observed in any of the genotypes as shown by absence of a spot with a Rf value of (0.07) (Fig. 4). The methanolic leaf extracts spotted along with the reference standard compound revealed the presence of Withaferin 'A' in twenty four genotypes out of 30 genotypes undertaken in present investigation with a Rf value of 0.07. However, all the leaf extract samples lacked the presence of Withanolide 'A' in them, indicating Withaferin 'A' to be a major Withanolide of leaves and Withanolide 'A' as a major constituent of roots. Out of 9 spotted chromatogram obtained using leaf extracts spot with a Rf value 0.07 corresponded to the standard reference compound spotted along with other genotypes revealing the presence of 'Withaferin A' in 24 genotypes out of 30 (86%).

A high variability in alkaloid profile was obtained using thin layer chromatography showing the presence of hyoscyanine and scopolamine from hairy root cultivars of *Datura stramonium* and *Hyosyamus niger* belonging to *Solanaceae* family. Scopolamine was found nearly pure in alkloid fraction of transformed root strains of *P. stramonium*. Alkaloid reagents showed only traces of other unidentified constituents on TLC plates.[3,5]

Fresh dried and powdered samples of leaf stem and root of *Acalypha indica* (Indian-nettle) were subjected to TLC analysis after extraction using different solvents such as hexane, chloroform, acetone ethanol.[9,10] TLC analysis separated the active compounds on plates. Leaf, root and stem extracts gave distinct spots respectively at Rf value of 0.371± 0.0009.

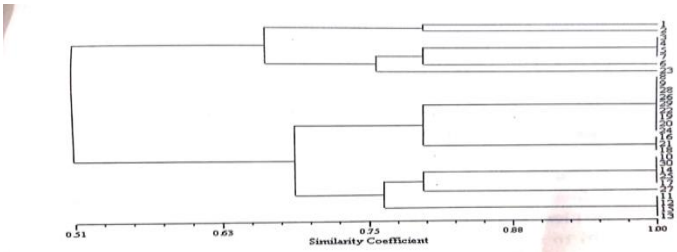

Fig. 3: Dendrogram of root samples created using UPGMA analysis from 30 genotypes

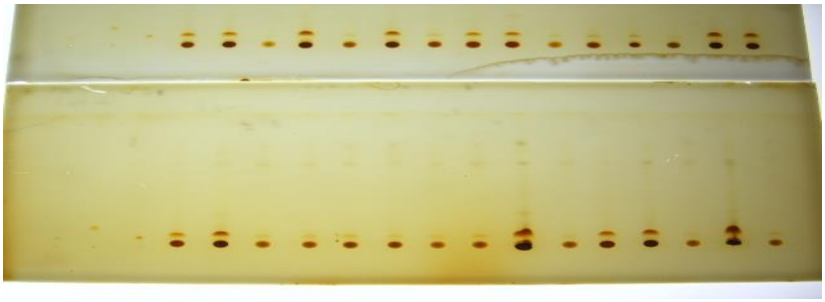

Fig. 4: TLC plate showing presence of standards in root samples in 30 genotypes (15 above, 15 samples below)

Phytochemicals screening showed presence of maximum bioactive compounds tested while ethanolic extracts revealed different Rf values showing that plants are rich source of secondary metabolites, alkaloid, coumarins. Kowalska and Sajewicz[9] gave comprehensive view of screening of botanicals using TLC as a modern analytical technique. The review plotted described almost all sorts of psychoactive plants, herbal medicines, cosmetic products and chemotaxonomic determination of biologically active components.

Conclusion

There is burgeoning need for the promotion of medicinal plants in India because they are re-emerging as a health tonic due to the mounting costs of prescription drugs in the maintenance of personal health, secondly, these are providing livelihood to a significant number of people in the rural as well as in urban communities. Moreover, due to over exploitation of medicinal herbs from the natural resources, some of the species are threatened of being extinct from ecosystem. Last but not the least in the international market, the opportunities are emerging day by day for trade of medicinal herbs obtained from medicinal plants to fetch foreign exchange for country. Thin layer chromatography was opted to study chemical fingerprint or chemo profile in 30 genotypes of Ashwagandha. The leaf samples of each 30 genotypes gave round, clear, legible spots using solvent system LS-8 having toluene; ethyl acetate and formic acid in a ratio of (75:18:7) while in contrary, root samples were better resolved in solvent system RS-I having $CHCl_3$: MeOH in a ratio of (90:10).

Similarity matrix computed using TLC data of 30 genotypes ranged from 0.11 to 1.0 in both leaf and root samples. The standard reference compound spotted along with the methanolic root and leaf extracts clearly demarcated the leaves and roots constituents. A total of twenty nine genotypes out of 30 (96%) revealed the presence of Withanolide 'A' (Rf value, 0.40) in root extract prepared from each of the 30 genotypes except one genotypes. As compared to the roots, the leaf extracts showed the presence of Withaferin 'A' (Rf value 0.07) in 26 genotypes out of 30 (86%), except genotypes WS-90-104, WS-90-105, WS-90-126, WS-90-136, Local and HWS-04-02. All the roots samples lacked withaferin 'A' while all leaf samples lacked Withanolide 'A' which was elucidated earlier in many reports. The results revealed that chemical markers in fingerprinting genotypes can successfully determine the genetic relationships and can estimate genetic diversity among various genotypes.

Chromatography and chromatograms disclose elaborate key phytoconstituents in qualitative and quantitative identification and screening, analogous to finger printing and fingerprints. Researchers can examine metabolomes to expand their research options and can conduct extensive studies of various metabolites. The main restriction is the amount and variance in various metabolites according to conditions, environmental variables and other changes. The TLC method, which produces a chromatograph to separate non volatile substances from distinct plant components, is the first step to identify and it is the fundamental approach.

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