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Original Article

PRINCIPAL COMPONENT ANALYSIS AND HPTLC FINGERPRINT OF *IN VITRO* AND FIELD GROWN ROOT EXTRACTS OF *WITHANIA COAGULANS*

PREETHI MP[1], SANGEETHA U[1], PRADEEPA D[1], VALIZADEH M [2], KALAISELVI SENTHIL[1,*]

[1]Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women University, Coimbatore 641043, Tamil Nadu, India, [2] Department of medicinal plants, College of Agriculture, High Educational complex of Saravan, I.R Iran.

Email: aubio.ptc.kalai@gmail.com

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ABSTRACT

Objective: The present study aims to standardize the HPTLC procedure for quantification of withanolides and to analyze phytochemical composition variance in *Withania* roots collected from different geographical areas.

Methods: Field roots of *Withania coagulans* and *Withania somnifera* were collected from different geographical locations and analyzed for its withanolide accumulation in comparison to *in vitro* roots using HPTLC. Standardization of HPTLC mobile phase, plant extraction solvent system, scanning wave length and other parameters for quantification of withanolide has been carried out. Also other phytoconstituents were quantified using phytochemical screening and the results were subjected to principle component analysis using XL STAT software.

Results: The HPTLC system was standardized and was found out that roots of *Withania coagulans*, AUF Wc 024 and AUF Wc 025 had maximum withanolide A accumulation (1.17mg/g). The extractive value was found to be high for AUF Wc 021 (392.4 mg/g). Toluene: Ethyl acetate: Formic acid (5:5:1) has been standardized as the best solvent system for HPTLC analysis of withanolides.

Conclusion: HPTLC analysis revealed that the banding patterns of *in vitro* adventitious roots of *Withania coagulans* were similar to the field grown roots. Moreover, Principle Component Analysis displayed wide variance in the phytocompounds accumulation and enormous deviation in the extractive value of collected root samples. It was found that the *in vitro* conditions are favorable for the accumulation of withanolides and are not diversely affected by external factors.

Keywords: *Withania coagulans*; *Withania somnifera*; HPTLC; Principle component analysis; withanolide.

INTRODUCTION

Since ancient times, plants have been an exemplary source of medicine and various Ayurvedic literatures mentions the use of plants as treatment for human ailments. Phytomedicines are dietary supplements in the form of powders, capsules, tablets, extract, fresh or dried plants and are usually taken to improve health conditions and for well being. These herbal phytomedicines are considered to be harmless and are increasingly consumed by people without proper prescription. The profiling of these traditionally used medicinal plants should be accomplished in order to analyze the quality and quantity of phytocompounds embodied in them. Pharmacological screening of natural products has led to discovery of a number of drugs, thus different civilizations have developed in their own indigenous system of medicines. It is the efficacy and safety of these herbal medicines that has turned the major pharmaceutical population towards medicinal plants research [1]. Plant produces several bioactive chemicals to protect itself but recent research demonstrates that these chemical constituents can also protect humans against various diseases. There are groups of bioactive chemicals called secondary metabolites in fruits, vegetables and herbs, and each of these compounds work in different ways [2]. Changes in the environmental factors produce a new and sometimes unexpected secondary metabolic profile resulting in their accumulation variance [3]. Hence the production of these secondary metabolites is usually higher in *in vitro* tissue culture compared to that of wild variety [4]. Approaches has been initiated in various active research programs either to isolate new lead compounds or to produce standardized extracts [5]. For this it is necessary to evaluate various qualitative and quantitative parameters, which may be helpful in setting standards for particular medicinal plant/parts of the plant. With the help of these standards one can easily identify and characterize single compounds, which may play a major role in maintaining quality and purity of that particular drug [6].

The genera *Withania* plays an extensive role in the indigenous medicine of South East Asia, e.g. in the Unani and Ayurvedic systems. The twenty-three known *Withania* species are widely distributed in the drier parts of tropical and subtropical zones [7].

Among them, only two (*Withania somnifera* (L.) Dunal and *Withania coagulans* Dunal) are economically significant and widely cultivated [8]. *Withania somnifera* and *Withania coagulans* are the most reputed medicinal plants of Ayurveda and has well-descript pharmacological activities such as physiological and metabolic restoration, anti-arthritic, anti-aging, cognitive function, improvement in geriatric states and recovery from neurodegenerative disorders [9, 10].

In vitro and *in vivo* molecular pharmacological investigations have elucidated associations of these properties of the herb with its specific secondary metabolites known as withanolides [11, 12, 13].

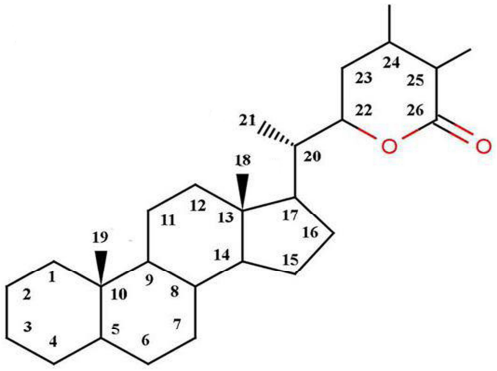


Fig. 1: Molecular structure of Withanolide A.

Withania coagulans is distributed in the east of the Mediterranean region and extends to South Asia. It shows the presence of esterases, lignan, alkaloids, free amino acids, fatty oils, essential oils in addition to withanolides [14]. By considering the demand of this herb, *in vitro* development and mass cultivation of the roots for the commercial purpose has been developed. *Withania coagulans* has come into focus of medicinal plant researchers in the recent years due to their diverse pharmacological activities. But until now very limited studies are available with respect to phytochemical analysis of this plant and their parts. The phytochemistry of *Withania* species has been studied extensively by several workers and various types of withanolides and others chemical constituents are isolated from this plant [15]. Although *Withania* have been extensively investigated yielding large number of steroidal structures, withanolide A is one of the most important one among them that contributes to the therapeutic potential of the plant [16] (Fig. 1). In the present study, a sensitive, simple and accurate High Performance Thin Layer Chromatographic (HPTLC) method has been established in order to analyze and quantify the withanolide accumulation variance among the root samples of *Withania coagulans* from different locations and its comparison with the *in vitro* cultivated adventitious roots.

MATERIALS AND METHODS

Chemicals and Equipments

HIMEDIA chemicals and Elix-3 water were used for the entire study. HPTLC was performed on precoated Silica gel aluminum $60F_{254}$ plates (E.MERCK, Germany) in a Semiautomatic CAMAG Linomat 5 device. Spectrophotometer and colorimeter was used in the quantitative phytochemical analysis to measure the absorbance of the samples..

Plant material

Dried root samples of 21 isolates of *Withania coagulans* and 2 *cultivars of Withania somnifera* roots from Iran were used for the quantitative assay. Apart from that, a Gujarat variety of *Withania somnifera* and *in vitro* root samples of both *W.coagulans* and *W. somnifera* were used for assays (Fig. 2).

Fig. 2: Field grown roots of *Withania coagulans* collected from various locations along with *in vitro* propagated roots used for phytochemical analysis and HPTLC finger print.

**(USB = AUF Wc; USB Ws = AUF Ws)*

Preparation of extracts from roots

The dried root samples were ground thoroughly using a mixer and the root powders were obtained. One gram of each root sample was extracted using 200ml of three different solvents namely Ethyl Acetate, Chloroform and Methanol.

Initially 1g of root was weighed and treated with 1ml of ammonia for 20mins. Followed by was sonicated for 20 min with 50 ml of the solvent and placed in a shaker for 2 hours at 104 rpm at 22^0C. At the end of 2hrs the extract was filtered using Whatmann no: 1 filter paper and the residue were again treated with 50 ml of the solvent. This step was repeated four times to obtain 200ml of the extract. For the final quantitative analysis all the 23 samples were extracted using Ethyl Acetate. These extracts were then concentrated by evaporation using a flash evaporator maintained at 45^0 C and 150rpm. After complete solvent evaporation, the residue was dissolved using HPLC grade methanol [17].

Method development for HPTLC

The High Performance Thin Layer Chromatography analysis was carried out on 20x10cm precoated silica gel aluminum plate $60F_{254}$ (E.MERCK, Germany). The sample extracts were applied to the plates as 6mm bands, under a stream of nitrogen, by means of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator fitted with a 100µl HPTLC Hamilton syringe. Linear ascending development to a distance of 8cm was carried out in 20x10cm twin trough chamber saturated for 30mins at room temperature (25ºC±2) with 20ml mobile phase (five different mobile phases were used). The banding patterns were visualized in 254nm, 366nm and white light and Densitometric scanning was performed

with Camag TLC scanner III in the reflectance –absorbance mode at 540 nm after spraying with either 10% Sulphuric acid or Anisaldehyde Sulphuric acid and analysed by Win CATS software (1.3.0 Camag) [18].

The developed chromatograms were then compared on the basis of the intensity of the spots obtained to finalize the best solvent for the extraction of the samples, best mobile phase for development of chromatograms and the best derivitization agent for visualizing spots. These results were then applied to prepare a final HPTLC fingerprint containing all the collected 24 root samples and also *in-vitro* samples of *Withania coagulans* and *Withania somnifera* were used. The final chromatogram was then observed to find the sample having maximum phytoconstituents by observing the spots. The plates were then scanned and the peaks were analyzed to estimate the amount of withanolide A in each of the samples.

Quantitative estimation of selected phytochemicals

Estimation of total Carbohydrates: The procedure of Hedge and Hofreiter (1962) [19] was followed for the estimation of total carbohydrates present in 1g of collected root samples.

Estimation of Proteins: The estimation procedure of Lowry *et al.,* (1951) [20] was followed for proteins present in 1g of root extract samples. The optical density read at 660nm gave the protein content of the samples.

Estimation of Flavonoids: The procedure by Cameron e*t al*. (1943) [21] was followed for estimation of flavonoids in the root samples.

Estimation of Steroids: A modified procedure of Wall *et al.*, (1952) [22] was followed for the estimation of steroids in1g of root, where the green color developed was observed at 640nm and the total Steroids present in1 g of the sample was calculated.

Estimation of Saponins: The amount of saponins present in 1g of roots was calculated by following the estimation procedure by Baccou *et al.*(1977) [23].

Estimation of physicochemical parameters

Various physicochemical parameters were studied in all the 23 root samples using standard procedures.

Determination of Total Ash Value: About 2 grams of air dried, powdered roots were accurately weighed and taken in a tarred silica crucible and incinerated in a muffle furnace by gradually increasing the temperature to 450^0 C to make it dull red hot and free from carbon. Cooled in a desiccator, weighed and the percentage of total ash was calculated [24].

Determination of moisture content: The moisture content (loss on drying) was determined by heating accurately 1g of the root powder in an oven at 105^0 C for 1 hour, cooled and weighed. The reduction in the weight was calculated and the percentage of weight loss indicates the amount of moisture in the sample [24].

Determination of extractive value: Extractive value was observed using ethyl acetate as a solvent in all the 23 root samples. The extracts were obtained from 1g of root samples, and were completely evaporated to dryness in water bath. The extractive was then weighed and the percentage of compound extracted was calculated [24].

Statistical analysis

The significance of the quantitative estimations were obtained by ANOVA using excel. They were further subjected to principal component analysis (PCA) using XL STAT software and cluster analysis using NT sys software.

RESULTS AND DISCUSSION

Quantitative analysis of phytochemicals

Quantitative estimation of proteins, carbohydrates, saponins, steroids and flavonoids in all 21 field roots and an *in vitro* root sample of *Withania coagulans,* and 2 field grown (Ws 025, Ws 157) and an *in vitro* root sample of *Withania somnifera* were carried out.

Table 1 explicates the quantitative analysis of the phytochemicals and is found that there is a major accumulation variance of phytochemicals among the samples. These variations among different plant samples of the same species are due to the influence of the geographical locations on the metabolic pathways.

From the analysis of AUF Wc 158 variety of *Withania coagulans,* it can be interpreted that this plant might have grown in a geographical condition that has suppressed the production of proteins, carbohydrates, saponins and flavonoids but has opposite effect on steroidal accumulation. It's also found that the protein content of all the samples was higher compared to the carbohydrate and most of the samples except a few had saponins content higher than that of protein.

Among the five phytoconstituents studied, the carbohydrate content was found to be the least in all the samples. The quantified values of the above phytoconstituents can be used as a major tool for obtaining a quality control profile for a drug. Secondary metabolism is not a static process. Rather, it changes in response to numerous factors [25]. The effect of modified external factors such as temperature and light intensity, and plant internal factors such as phenological phase and their possible interactions was found to influence bioactive secondary metabolite accumulations in plants [26].

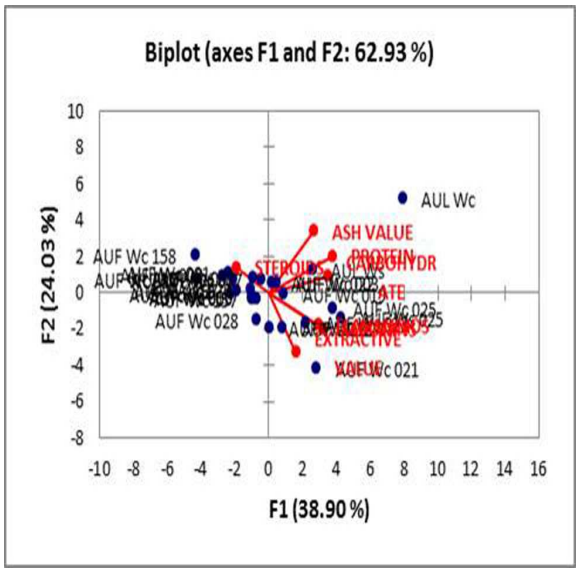


Fig. 3: Biplot graph revealing that neither steroids nor saponins are been affected in the *in vitro* samples of *Withania coagulans*.

Principal component analysis

The values of the phytochemical assays were subjected to principal component analysis using XL STAT software. The amount of carbohydrates in all the 25 samples were between 0.72 and 3.85mg/g with the deviation of 0.832 revealing that the geographical conditions has not much influence on the accumulation of carbohydrates whereas the amount of steroids in the plant samples were between 0.94 and 26.02 with a deviation of 4.821. The extractive values of the samples ranged from 20.400 to 402.000 with a deviation of 109.059 being maximum among all the parameters tested. The extractive value represents the amount of secondary metabolites in the plant which in turn contributes to the therapeutic potential of the plant. Hence the variation in extractive value merely means variation in the therapeutic potential of the plants obtained from different locations. The summary statistics of the 25 samples are displayed in Table 2.

The correlation among the different phytochemicals was also executed (Table 3). All the compounds had a positive correlation except steroids which are negatively correlated to rest of the phytochemicals indicating a decrease in its value along the increase

in the other phytochemical. This clearly indicates that the steroid metabolism is negatively regulated by the conditions that favour the other metabolic pathways. Thus paving the path to develop *in vitro* protocol development for the growth of plant with steroidal lactones as therapeutic compounds and preventing it from being negatively regulated by external factors and other influencing conditions.

Table 1: Quantitative analysis of phytochemicals in field grown and *in vitro* roots of *Withania coagulans* and *Withania somnifera* (expressed in mg/g dried root)

S.No	Sample	Extractive value	Protein	Carbohydrate	Saponins	Steroids	Flavanoids	Ash value
1.	AUF Wc 001	40.8	4.52±0.22	**0.72±0.22**	6.20±0.08	7.04±0.16	2.22±0.42	161.5
2.	AUF Wc 002	51.6	3.47±0.8	1.34±0.24	8.47±0.05	8.45±0.22	3.38±0.20	196.0
3.	AUF Wc 003	52.2	5.5±0.22	2.26±0.62	10.34±0.53	9.05±0.03	3.64±0.15	143.5
4.	AUF Wc 004	42	3.95±0.35	1.12±0.20	8.65±0.23	7.52±0.26	2.46±0.34	71.0
5.	AUF Wc 005	49.8	5.68±0.52	0.84±0.08	8.39±0.15	4.56±0.23	3.89±0.56	98.0
6.	AUF Wc 006	35.4	3.48±0.30	1.23±0.23	6.42±0.05	8.16±.014	2.86±0.10	136.3
7.	AUF Wc 007	45	6.58±0.59	1.72±0.32	7.52±0.22	8.64±0.24	3.69±0.11	128.3
8.	AUF Wc 008	28.2	4.23± 0.65	0.86±0.18	6.34±0.42	10.20±0.05	2.43±0.22	124.6
9.	AUF Wc 010	**20.4**	6.04±0.52	1.13±0.52	11.53±0.05	5.34±0.25	2.62±0.18	74.6
10.	AUF Wc 018	107.4	4.33±0.40	0.77±0.58	16.01±0.74	6.23±0.16	4.12±0.17	**69.5**
11.	AUF Wc 019	68.4	10.7±0.82	0.77±0.64	14.12±0.16	7.32±0.25	3.54±0.18	118.5
12.	AUF Wc 021	**392.4**	5.62±0.54	0.97±0.55	16.82±0.18	6.19±0.05	6.32±0.01	98.0
13.	AUF Wc 022	24.6	8.86±0.92	1.82±0	9.22±0.16	5.43±0.22	3.22±0.13	92.5
14.	AUF Wc 023	171.6	5.52±0.05	1.87±0.15	14.62±0.45	1.18±0.32	4.36±0.16	127.0
15.	AUF Wc 024	402	8.56±0.45	1.44±0.16	10.34±0.06	4.56±0.24	2.16±0.48	92.5
16.	AUF Wc 025	92.4	9.02±0.05	2.68±0	**17.12±0.83**	8.00±0.02	5.62±0.23	118.0
17.	AUF Wc 026	120.6	4.11±0.38	1.93±0.05	8.45±0.50	8.63±0.05	2.32±0.13	116.0
18.	AUF Wc 027	27.0	**2.08±0.67**	0.73±0.16	**4.45±0**	5.49±0.54	**0.87±0.10**	87.0
19.	AUF Wc 028	208.8	4.92±0.04	1.20±0.02	10.65±0.54	2.26±0.08	2.16±0.22	70.0
20.	AUF Wc 157	41.4	5.25±0.06	1.35±0.03	9.83±0.54	**0.94±0.05**	2.65±0.05	85.5
21.	AUF Wc 158	43.2	3.08±0.02	1.00±0.15	6.52±0.25	**26.02±0.02**	1.83±0.07	141.6
22.	AUL Wc	33.6	**22.06±0**	3.01±0.10	14.40±0	2.76±0	2.76±0.06	**490.0**
23.	AUF Ws 025	264	7.95±0.08	**3.85±0.02**	11.24±0.32	2.53±0.32	4.98±0.32	112.6
24.	AUF Ws 157	37.2	5.68±0	0.98±0.05	5.62±0.34	2.44±0	2.87±0.08	74.0
25.	AUL Ws	30.6	9.37±0.42	2.92±0.03	5.93±0.12	8.16±0.04	**6.42±0.20**	180

Data represents Mean± SE twice repeated, expressed in mg/g. P-value=0.683202; AUF – field root samples, AUL – *in vitro* root samples, Wc – *Withania coagulans*, Ws – *Withania somnifera*

Table 2: Summary statistics data of the field grown and *in vitro* roots of *Withania coagulans* and *Withania somnifera*

Variable	Observations	Minimum	Maximum	Mean	Std. deviation
Protein	25	2.080	22.060	6.422	3.926
Carbohydrate	25	0.720	3.850	1.540	0.832
Saponins	25	4.450	17.120	9.968	3.712
Steroids	25	0.940	26.020	6.684	4.821
Flavanoids	25	0.870	6.420	3.336	1.374
Extractive value	25	20.400	402.000	97.224	**109.059**
Ash value	25	69.500	490.000	128.260	82.710

Table 3: Correlation among the Phytochemicals observed in field grown and *in vitro* roots of *Withania coagulans* and *Withania somnifera*

Variables	Protein	Carbohydrate	Saponins	Steroids	Flavanoids	Extractive value	Ash value
Protein	1	0.557	0.420	-0.277	0.230	0.025	0.761
Carbohydrate	0.557	1	0.234	-0.178	0.481	0.130	0.445
Saponins	0.420	0.234	1	-0.281	0.515	0.454	0.124
Steroids	-0.277	-0.178	-0.281	1	-0.141	-0.238	0.015
Flavanoids	0.230	0.481	0.515	-0.141	1	0.297	0.025
Extractive value	0.025	0.130	0.454	-0.238	0.297	1	-0.198
Ash value	0.761	0.445	0.124	0.015	0.025	-0.198	1

To summarize the information of the data matrix briefly, factor analysis was applied using PCA as the method from extraction of factors. After orthogonal transformation, four components (PC) were obtained that explained the variance of the factors. Factor 1 and 2 with largest proportion of the variance (62.934 =38.901+24.033) were considered as the main principal components. The seven parameters were plotted against F1 and F2 and it was found that ash value, proteins and carbohydrates were positive for both F1 and F2. The biplot graph (Fig. 3) revealed that the variation in the *in vitro* samples of both *Withania coagulans* and *Withania somnifera* were contributed due to ash value, proteins and carbohydrates. *In vitro* condition has favorable factors for the development of both *Withania somnifera* and *Withania coagulans* and minimal detrimental influence caused on steroids and saponins accumulation under which major secondary metabolites are grouped.

Cluster analysis

For better understanding of the genetic diversity and relationship among the 25 samples a dendrogram was constructed using NT sys software. The graph obtained exhibited that among the 25 samples AUF Wc 018

and AUF Wc 021 were more closely related which was then nearly related to AUF Wc 025. The samples AUL Ws and AUF Wc 007 were distantly related to the samples AUF Wc 001 and AUF Wc 027. The *in vitro* samples of Withania coagulans was closely related to AUF Wc 019. Though the results of cluster analysis based on the phytochemical constitution revealed that the samples AUF Wc 018, AUF Wc 021 and AUF Wc 025 are closely related, their morphology remained different. There are considerable variations among the size, colour and texture of the root samples. The same morphological differences were observed among samples AUF Wc 001 and AUF Wc 027 which were clustered together in the dendrogram. The samples AUF Wc 006 and AUF Wc 008 were found to be of the same size, but clustered as different groups. This clearly reveals that the phytochemical characteristic of the plant is independent of the morphological characteristics though both vary with the geographical conditions.

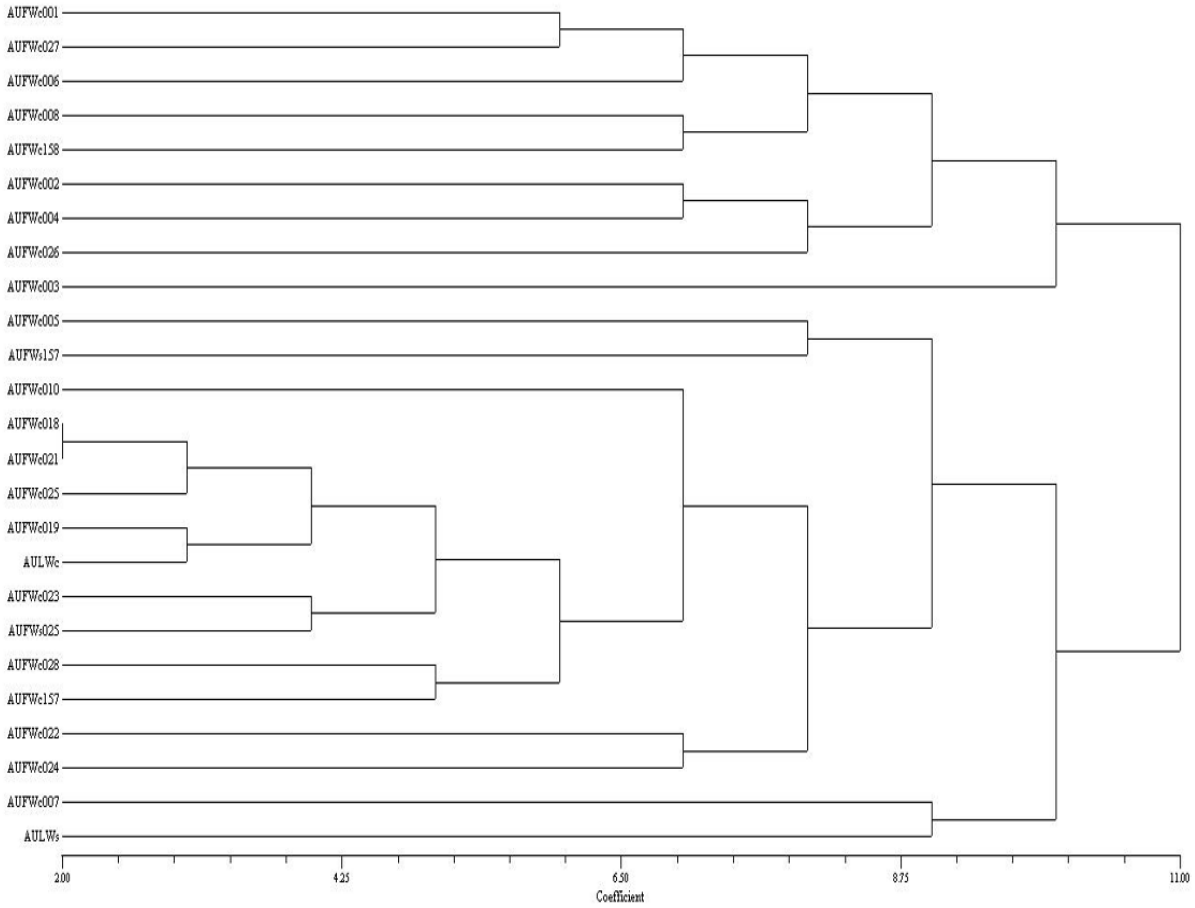


Fig. 4: Dendrogram showing the relationship among 25 varieties of *Withania coagulans* based on the five phytochemical characteristics.

Extraction of metabolites from root samples

For finding the best solvent for metabolite extraction, five root samples (AUF Wc 019, AUF Wc 008, AUL Wc, AUL Ws guj, AUL Ws) were chosen and extracted using three different solvents Ethyl acetate, Methanol and Chloroform. These samples were then subjected to HPTLC analysis. The visual interpretation of the developed HPTLC plates confirmed that out of the three solvents used for the extraction, Ethyl acetate extracted samples showed more number of spots and thus has maximum ability to extract metabolites and is considered as the best solvent over methanol and chloroform (Fig 5). There are no much studies on solvent system for extraction and most of the studies on Withania samples have employed methanol extracts [27] or methanol: water (4:1) [28].

HPTLC profiling of *in vitro* and field grown Withania root extracts using different mobile phases and derivitization agents

The root extracts were spotted on HPTLC plates and different combination of solvents were attempted to get a good separation and stable peak. The five different mobile phases applied for the study were Benzene: Ethyl acetate (2:1), Toluene: Ethyl acetate: Formic acid (5:5:1), Chloroform: Methanol (9:1), Ethyl acetate: Methanol: Water (7:2:1) and Toluene: Ethyl acetate (93:7). The chamber has been saturated with the mobile phase for 30 min at room temperature. The mobile phases resulted in disparate resolution of bands and among them, maximum number of compounds separated as distinct spots with better resolution was acquired in mobile phase Toluene: Ethyl acetate: Formic acid (5:5:1) (Fig 5). The same mobile phase had been applied by Sharma *et al*., (2007) and Patel *et al*., (2009) for estimating the amounts of Withanolide A and Withaferin A in different parts of *Withania somnifera*. Most of the studies on Withania plant are being done with the same mobile phase though no data has been employed on mobile phase standardization. Withanolide standard was visible as clear spots with different Rf values corresponding to each mobile phase used thus indicating the capability of the solvents in the mobile phase to dissolve and carry the withanolides (Table 4). The variation in the Rf values of the standard in different mobile phases is due to the effect of polarity of the solvents that determines its efficiency to carry Withanolide A. The plates were subjected to multi wavelength spectral detection scanning to identify the optimal wavelength for Withanolide A detection and it was determined as 234nm. The developed plates were scanned at this wave length using Camag TLC scanner III in the reflectance –absorbance mode. The literature search showed that to date the scanning for Withanolide is performed at 254nm [29] or at 530 nm in case of derivitized plate which are default Linomat HPTLC system settings [27].

Table 4: Rf values of withanolide A peaks in different mobile phases using *Withania* root samples (AUF 019, AUF 008, AUL Wc, AUF Ws guj, AUL Ws)

S.No	MOBILE PHASE	WITHANOLIDE A (Rf)
1.	Benzene: Ethyl acetate (2:1)	0.44
2.	Chloroform: Methanol (9:1)	0.68
3.	Ethyl acetate: Methanol: Water (7:2:1)	0.83
4.	Toluene: Ethyl acetate (93:7).	0.80
5.	Toluene: Ethyl acetate: Formic acid (5:5:1)	0.68

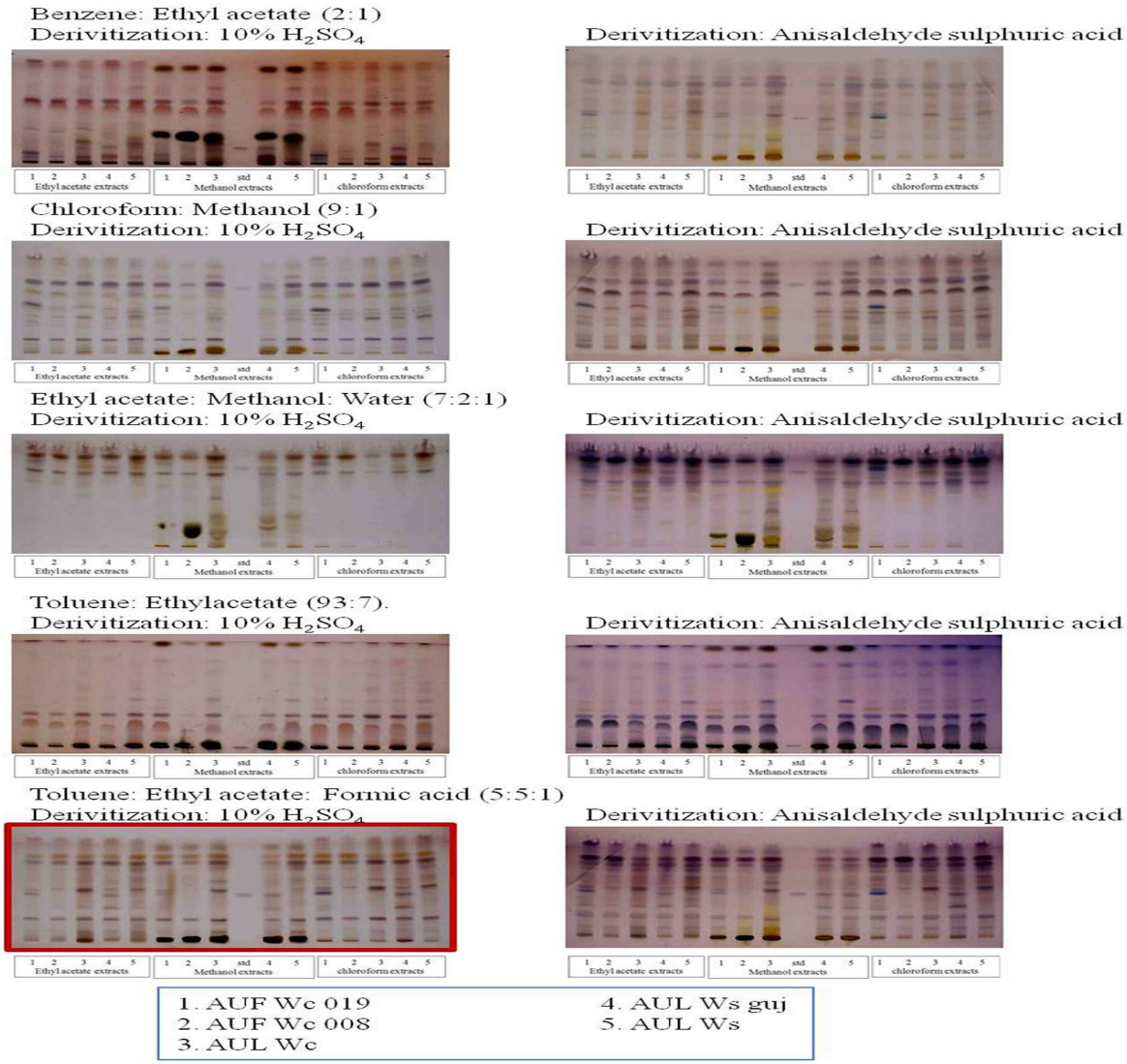


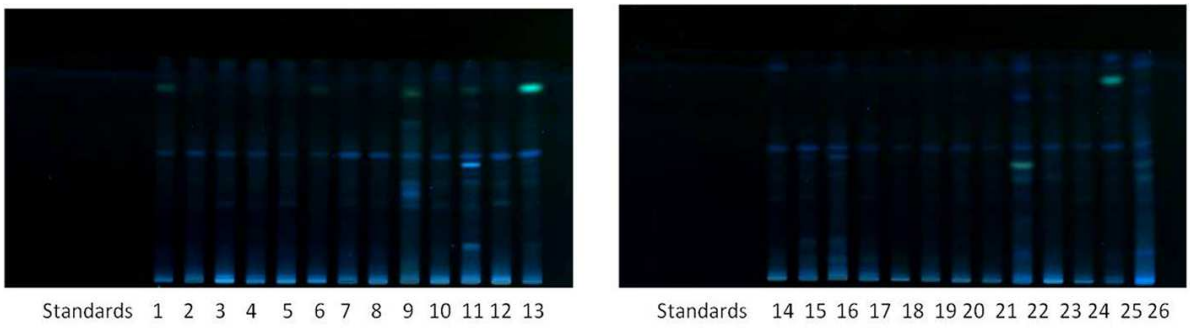
Fig. 5: Standardization of mobile phase and derivitization agent for HPTLC fingerprint.

The above solvents were chosen as per described in the monograph of *Withania somnifera.* (natural remedies) The developed plates were then derivitized separately using 10% H_2SO_4 and Anisaldehyde sulphuric acid. Though both the plates showed comparable results, the plates derivitized using the latter showed a little sharper visualization of spots compared to the plate's derivitized using 10% sulphuric acid. But taking into consideration the drawbacks of using Anisaldehyde sulphuric acid for derivitization like change in color of the plate and less interaction of the solution with the plate leading to difficulty in derivitization, 10% H_2SO_4 was proceeded for further analysis (Fig. 5).

HPTLC fingerprinting of *in vitro* and field grown Withania root extracts

HPTLC fingerprint of the Ethyl acetate extracts of *Withania coagulans* roots collected from different geographical locations of Iran and the *in-vitro* propagated root samples of *Withania coagulans* was performed using mobile phase Toluene: Ethyl acetate: Formic acid (5:5:1) to see the accumulation of various phytoconstituents. Three field grown root extracts and an *in vitro* root extract of *Withania somnifera* were also included in the study so as to have a comparison among the two species. Standard Withanolide A (0.1mg/ml) was spotted in varying volumes of 2µl, 6µl, 10µl, 12µl in order to quantitatively estimate the amount of Withanolide A present in all the root samples. The HPTLC analysis was carried out with all the 26 samples. The ethyl acetate extracts of the samples were applied to the plates as 6mm bands under a stream of nitrogen, at the rate of 150nl/s by means of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator fitted with a 100µl Hamilton HPTLC syringe. Linear ascending development to a distance of 8cm was carried out in 20x10cm twin trough chamber saturated for 30mins at room temperature (25ºC±2) with 20ml of mobile phase; the plates were dried in a current of air with the help of an air dryer. The banding patterns were visualized at 366nm respectively (Fig.6). Among all the samples analyzed the *in vitro* sample of *Withania coagulans* was

found to have large number of spots indicating a higher number of phytochemicals. All the field grown root samples gave nearly the same type of banding pattern with the sample AUF Wc 010 showing more number of spots followed by AUF Wc 019. The developed plates were then subjected to Densitometric scanning with Camag TLC scanner III using Savitsky-Golay7 filter in the reflectance –absorbance mode at 234nm at the speed of 200mm/s, the D2 and W lamp was chosen to scan the plates and the slit dimensions were set at 4.00 x 0.30 mm, Micro. All the tracks were scanned and the peaks were displayed. The Withanolide A peak was viewed as a separate spot with Rf values around 0.65-0.69 as obtained in the standardization of mobile phase. The peak analysis revealed increase in concentration of Withanolide along increase in volume.



1. AUF Wc 001	14. AUF Wc 023
2. AUF Wc 002	15. AUF Wc 024
3. AUF Wc 003	16. AUF Wc 025
4. AUF Wc 004	17. AUF Wc 026
5. AUF Wc 005	18. AUF Wc 027
6. AUF Wc 006	19. AUF Wc 028
7. AUF Wc 007	20. AUF Wc 157
8. AUF Wc 008	21. AUF Wc 158
9. AUF Wc 010	22. AUL Wc
10. AUF Wc 018	23. AUF Ws 025
11. AUF Wc 019	24. AUF Ws 157
12. AUF Wc 021	25. AUF Ws Guj
13. AUF Wc 022	26. AUL Ws

Fig. 6: Developed HPTLC fingerprint of field and *in vitro* propagated roots of *Withania coagulans* and *Withania somnifera* visualized at 366nm.

Table 5: Amount of withanolide A in field and *in vitro* propagated roots of *Withania coagulans* and *Withania somnifera*

S.No	SAMPLE	Rf	PEAK AREA	WITHANOLIDE A (mg/g)
1.	AUF Wc 001	0.68	2936.5	**0.27**
2.	AUF Wc 002	0.66	6301.7	0.59
3.	AUF Wc 003	0.66	8891.0	0.83
4.	AUF Wc 004	0.66	7805.5	0.73
5.	AUF Wc 005	0.66	4952.2	0.46
6.	AUF Wc 006	0.66	2977.3	0.28
7.	AUF Wc 007	0.68	7776.1	0.73
8.	AUF Wc 008	0.68	4308.5	0.40
9.	AUF Wc 010	0.67	6942.8	0.65
10.	AUF Wc 018	0.67	4940.0	0.46
11.	AUF Wc 019	0.67	9841.9	0.92
12.	AUF Wc 021	0.68	5957.7	0.56
13.	AUF Wc 022	0.69	9599.7	0.91
14.	AUF Wc 023	0.68	9118.0	1.12
15.	AUF Wc 024	0.68	9524.8	**1.17**
16.	AUF Wc 025	0.69	9504.4	**1.17**
17.	AUL Wc	0.69	1500.77	0.40
18.	AUF Wc 026	0.69	5026.6	0.62
19.	AUF Ws 025	0.66	10627.7	**1.30**
20.	AUF Ws 157	0.66	2200.9	**0.27**
21.	AUF Ws guj	0.65	2990.1	0.37
22.	AUL Ws	0.68	2220.68	0.58

The amount of Withanolide A in each of the samples was quantified comparing with the standards using peak area as an evaluation mode at multilevel calibration. A linear regression graph was obtained using CAMAG software. Fig.7 represents the linear regression graph of the standards and the peak analysis of all tracks at 234nm.

The amount of withanolide A varied considerably between the different root samples and is presented in Table 5. The Withanolide A concentration in root samples ranged from 0.27 mg/g (AUF Wc 001 and AUF Ws 157) to 1.30 mg/g (AUF Ws 025). The variations in the Withanolide A concentration can be attributed to

the growth condition which is influenced by the geographical location in which it is grown. The concentrations of various plant secondary products are strongly dependent on the growing conditions [30].

In addition to Withanolide A, four compounds with Rf 0.01, 0.40, 0.76 and 0.85 were found in all the samples including the *in vitro* samples. The *in vitro* samples of *Withania somnifera* and *Withania coagulans* were found to have a same pattern of banding except that three compounds with Rf 0.02, 1.07 and 1.18 present in *Withania coagulans* were absent in *Withania somnifera* indicating the presence of additional compounds in *Withania coagulans* than *Withania somnifera* which is an established medicinal plant.

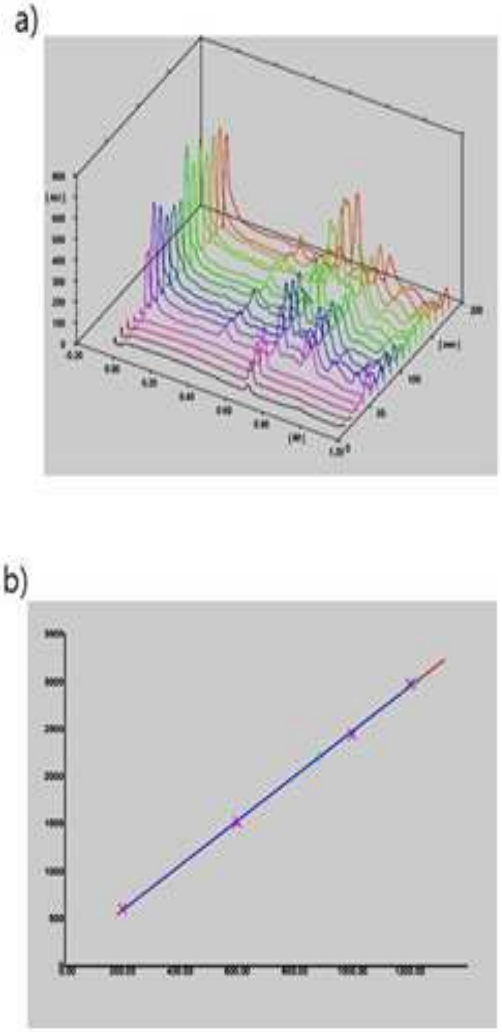


Fig 7: Quantification of withanolide A in different root samples of *W. coagulans* and *W. somnifera* a) 3D densitogram of *W. coagulans* and *W. somnifera* field grown and *in vitro* adventitious root samples along with withanolideA standard at 234nm. b) Calibration plot showing linearity for standard Withanolide A

SUMMARY AND CONCLUSION

The present study was carried out with an aim of standardizing the HPTLC procedure for Withanolide analysis and performing a final fingerprint of Withania root samples to find the best Withania coagulans root among the collected field roots from different geographical locations and also to analyse the variability in their phytochemical constituents. The estimation of physicochemical parameters showed that the sample AUF Wc 018 recorded a high ash content of 69.5mg/g. The extractive value was found to be high for AUF Wc 021 (392.4 mg/g) indicating that the respective root was found to accumulate more metabolic products.

The HPTLC profiling of the root samples using different solvents and mobile phases revealed that ethyl acetate had better capability of extraction of metabolites and the combination of Toluene, Ethyl acetate and Formic acid in the ratio 5:5:1 proved to be the best mobile phase for better separation of the metabolites. Derivitization with 10% Sulphuric acid was found to produce consistent results for visualization of bands under normal light. Among all the samples analyzed, the in vitro sample of *Withania coagulans* was found to have larger number of spots indicating an increased number of phytocompound accumulations. To conclude, as observed, there is a wide variation with the phytochemical contents of field grown roots collected from different locations, and hence its quantification would be a useful tool for selecting the best source of the phyto-constituents for drug development and also prevent contamination with other plant samples.

Until date not much study has been carried out with such large number of morphologically different root samples of *Withania coagulans*. This is the first report presenting such an elaborate study. Further the standard HPTLC procedures optimized would give better resolution of spots and would help in the screening of Withanolides and other phytochemicals consistently. Also the respective seeds of AUF Ws 025, AUF Wc 024 and AUF Wc 025 is been germinated *in vitro* in our plant tissue culture laboratory, and the accumulation of the withanolide A from the *in vitro* cultured roots of these samples will be analyzed to see the effect of *in vitro* conditions on the plant in comparison with the field grown variety in a clear perspective.

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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