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Phytochemical Evaluation of Withanolide-A in Ashwagandha Roots from Different Climatic Regions of India

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Abstract

Ashwagandha [*Withania somnifera* (L.) Dunal] is a highly valued medicinal plant in Ayurveda, used either singly or in combination with other herbs. Plants growing under different agro-climatic condition often show qualitative and quantitative variations in their phytoconstituents. In the GC-MS analysis, 24 bioactive phytochemical compounds were identified in roots and leaves of *Withania somnifera*. Present investigation deals with comparison of Ashwagandha plants (WsL, WsK, WsN and WsM) obtained from various regions (Lucknow, Karnataka, Nimuch and Mumbai) of India, for their withanolide-A contents using HPTLC. Methanolic root extracts of WsN showed maximum withanolide-A content (1.3%), WsK being close behind at 1.2% followed by WsM (0.7%); WsL showed minimum withanolide-A content at 0.4% only.

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Introduction

Withania somnifera (L.) Dunal, commonly known as 'Ashwagandha' belongs to family-Solanaceae. Ashwagandha sometimes referred to as Indian ginseng, is a valued herb in Ayurveda and has been cultivated for centuries in India. Ayurvedic Pharmacopoeia of India cites it as a strengthening tonic, aphrodisiac, and for the treatment of arthritis. It's the primary component of numerous traditional Ayurvedic tonics and anti aging compounds (Ayurvedic Pharmacopoeia of India, 1985; Tripathi et al., 1996).

The primary constituents of interest in Ashwagandha are alkaloids and steroidal lactones the latter of which are known as withanolides. Alkaloids constitute about 0.3-4.3% of total constituents. Several withanolides are identified in Ashwagandha including withanolides A-Y (Tripathi et al., 1996).There are as many as 40 similar structures synonymously identified in the literature as withaferin-A (Kapoor, 1990.). Withanolide-A is also the major compound of *Withania somnifera* and is a biologically active steroidal lactone. The present research work is aimed to describe the phytochemical variations among different climatic regions.

At least five different morpho, chemo and geographic forms have been identified in this species, though names have not been assigned to them. The five primary chemotypes originate from different areas and are categorized as Form I to Form V. Form I is cultivated in Madhya Pradesh and is the primary source of commercial material in India. Form II originates from sandy deserts of Rajasthan, Form III grows in Chandigarh and other mountainous area of Punjab, Form IV grows near Delhi and Form V grows near Delhi and Ahmadabad.

Various agro climatic conditions also influence the phytoconstituents, hence an effort was made in the present investigation to evaluate Ashwagandha root samples from four different regions of India viz. Lucknow (WsL), Karnataka (WsK), Mumbai (WsM), and Nimuch (WsN) for their withanolide-A contents.

Materials and methods

Withania somnifera plants were collected in winter season from different regions of India viz. Lucknow, Karnataka, Nimuch and Mumbai. The authenticity of the plant materials was conformed at the Department of Botany, Institute of Science, Mumbai.

Extraction of the plant materials

The roots were cleaned, air dried and crushed into coarse powder using mortar and pestle. 10gm of powdered roots were extracted with 250ml of methanol for 36hrs using Soxhlet apparatus. The extract was evaporated on water bath to 25ml (Trease and Evans, 1989).

Preliminary phytochemical analysis

Roots extracts were analyzed for alkaloids, flavonoids, tannins, saponins, anthroquinones, sterols, triterpenes and glycosides by HPTLC method.

HPTLC analysis for withanolide-A

Samples were analyzed for withanolide-A by HPTLC technique and UV spectral analysis using WINCAT software (CAMAG, Switzerland) supplied by Anchrom Pvt. Ltd., Mumbai. Precoated TLC plates (Silicagel 60 F_{254}) of 0.2mm thickness and 20 × 20cm size were purchased from Merck (KGaA, Germany) and standard withanolide-A was purchased from Natural Remedies (Bangalore). The stock solution of standard withanolide-A was prepared in methanol (1 mg/ml). 5µl sample extracts were loaded as an 8mm streak on HPTLC at 10mm distance between two streaks using a Linomat IV, an automatic spotter. The solvent system used was Chloroform: Methanol (9:1) (Mukherjee, 2002).

After drying, plates were scanned and chromatograms were recorded. Spectra, R_f values, AUC and λ max of withanolide-A of all the samples were recorded. Plates were derivatized using Anisaldehyde sulphuric acid, observed under 254nm, and 366nm as well as under white light.

Calibration studies

Linearity for withanolide-A was evaluated in the range of3.0-7.0.µg mL^{-1},peak area versus concentration was subjected to least square linear regression analysis and the slope, intercept and correlation coefficient for calibration were determined. Limit of detection (LOD) and quantification (LOQ) were determined from the calibration curve using expression 3σ/s and 10σ/s where σ is standard deviation and s is the slope of calibration curve. Accuracy of method was evaluated through the percentage recoveries of known amounts of withanolide-A.

GC-MS Analysis

The GC-MS analysis of methanolic root extracts of *Withania somnifera* collected from different climatic region of India was performed using an Agilent Technologies GC-System HP5 coloumngas chromatography equipped with a Elite-5 capillary column (5% Diphenyl 95% dimethyl poly siloxane) (30nm × 0.25mm ID × 0.25µmdf) and mass detector tub mass gold of the company which was operated in EI mode. Helium was the carriers gas at a flow rate of 1 ml/min. the injector was operated at 200^0C and the oven temperature was programmed as follows: 60^0C for 15min, then gradually increased to 270°C at 3 min. the identification of components to was based on comparison of their mass spectra with those of Wiley and NIST Libraries and those described by Adams as well as on comparison of their retention indices Senthis with literature.

Results and discussion

Preliminary phytochemical analysis using chemical tests revealed presence of alkaloids, flavonoids, tannins, saponins, sterols, triterpenes and glycosides. Anthraquinones could not be detected either in leaf or root extracts (Table 1).

Table 1. Preliminary phytochemical analysis of methanolic root extracts of *Withania somnifera* from selected regions.

Sr. No.	Phytochemicals	Roots
1.	Alkaloids	+
2.	Flavonoids	+
3.	Tannins	+
4.	Saponins	+
5.	Anthraquinones	-
6.	Sterols	+
7.	Triterpenes	+
8.	Glycosides	+

Phytochemical fingerprinting of root extracts by HPTLC

Presence of secondary metabolites detected by preliminary method was confirmed by HPTLC analysis. The results are depicted in (Table 2). The correlation coefficient of withanolide-A was found to be 0.997. The peak area (y) is proportional to the concentration of withaferin-A (x) following

the regression equation y=40.742+1.949x. (Fig. 1) Experimentally derived LOD and LOQ was 300 and 700 ng/mL^{-1}.

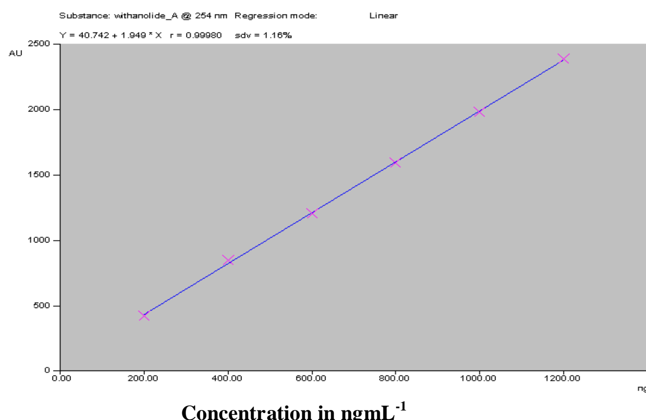

Fig. 1: Calibration curve of sample and standard withanolide-A.

Table 2. Phytochemical fingerprinting of methanolic root extracts of *Withania somnifera* by HPTLC.

Sr. No.	Phytochemicals	Roots
1.	Alkaloids	+
2.	Flavonoids	-
3.	Tannins	-
4.	Saponins	+
5.	Anthraquinones	-
6.	Sterols	+
7.	Triterpenes	+
8.	Glycosides	+

Stability: Stability of withanolide-A solution was evaluated to verify whether spontaneous degradation occurred within 2 days. The results were calculated as the percentage content of the standards at 24 and 48 hours. Standard showed less than 5% degradation at investigated temperature (Table 3).

Table 3. Stability of standard withanolide-A (%).

Phytochemical	Temperature	24hrs	48hrs
Withanolide-A	4°C	96.9904	92.448

Table 4. Results of recovery studies of withanolide-A

Concentration	Recovery (%)
0.2µg	60.7
0.3µg	66
0.4µg	63

Precision studies showed RSD less than 1% indicating sufficient precision. Recovery studies (60.7% to 66%) indicated that the method was reliable for the quantification of withanolide-A (Table 4) in the test samples. Results of method validation are in keeping with those of (Jirge et al., 2011; Silvia, 2010; Rothenbuhler, 2010).

The HPTLC analysis showed the presence of withanolide-A was confirmed by comparing with the standard peak of withanolide-A (Fig. 1). Maximum R_f of Withanolide-A was 0.65 for all the test sample which was very close to 0.64 of standard withanolide-A (Fig. 2). Lamda of test sample and standard for withanolide-A is shown in (Fig. 3). Withanolide-A content ranged from 0.4 to 1.3%. Maximum quantity was detected in WSN (1.3%) followed by WSK (1.2%) WSM (0.7%) and WSL (0.4%) (Table 5).

Table 5. Withanolide-A content of Ashwagandha collected from different regions of India.

Sr. No.	Sample	Withanolide-A (%)
1	WsL	0.4
2	WsK	1.2
3	WsN	1.3
4	WsM	0.7

GC-MS analysis

Using GC-MS, the linear retention indices for all the compounds were determined by coinjection of the sample. The individual constituents were identified by their identical retention indices, referring to known compounds from literature (Adam, 1995) and also by comparing their mass spectra with either the known compounds or with Wiley mass spectral database. More number of compounds was detected in leaves than in roots.

Identification of phytochemicals from leaf extract

Maximum number of chemicals (12) could be detected in the leaf collected from Nimuch region. Karnataka and Lucknow samples gave four and three peaks respectively while leaves collected from Mumbai region gave only two peaks. DL-Proline, 5-oxo, methyl ester was a common compound detected in leaves of WSM and WSK region where as 1- Phenylalanine, methyl ester was unique to WSM and Quinic acid, n-Hexadecanoic acid and 9,12,15-Octadecatrienoic acid to WSK. Similarly, 9H-Pyrido [3,4-b] indole was a common compound in leaf samples of Nimuch and Lucknow. All the other compounds (11-in WSN and 2-in WSL) were unique to that particular region (Table 6).

Identification of phytochemicals from root extract

Based on peak area, molecular weight a total of 15 compounds were identified from root extract *Withania somnifera* from four different regions. Maximum numbers of peaks were detained (6) for Nimuch area followed by Lucknow (4) and minimum being Mumbai and Karnataka (3 each). None of the constituents identified from the roots were common to any of the selected region (Table 7).

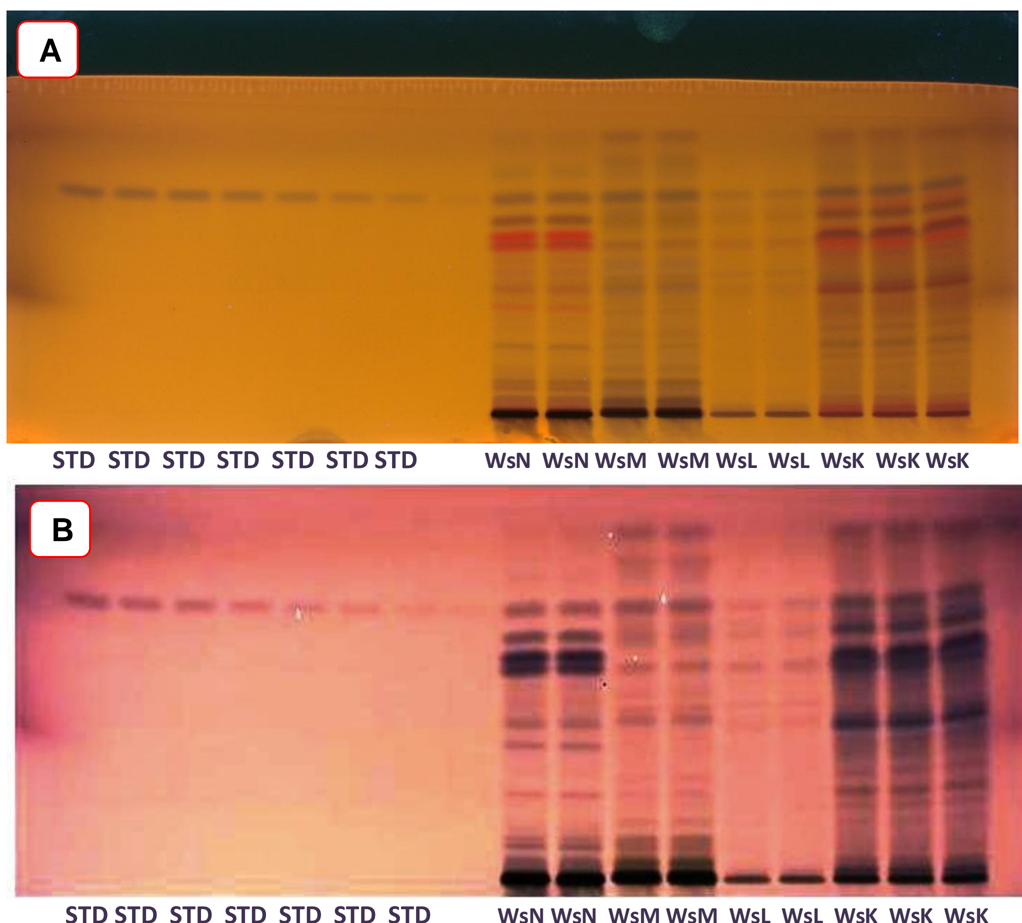

Fig. 2: HPTLC profile of Ashwagandha test sample – Withanolide-A content in *Withania somnifera*. (A) After derivatization - image of fluoresces at 366nm. (B) Image of white light.

Table 6. GC-MS detection of other phytochemicals from methanolic leaf extracts of *Withania somnifera* from selected regions.

Sample	Details of compounds detected		
	Name	**Retention time (min.)**	**Molecular formula**
WsM	DL-Proline,5- oxo, methyl ester	7.4	$C_6H_9NO_3$
	1-Phenylalanine, methyl ester	8.1	$C_{10}H_{13}NO_2$
WsK	DL-Proline,5-oxo, methylester	7.4	$C_6H_9NO_3$
	Quinic acid	10.4	$C_7H_{12}O_6$
	n-Hexadecanoic acid	14.1	$C_{16}H_{32}O_2$
	9,12,15-Octadecatrienoic acid	15.8	$C_{18}H_{30}O_2$
WsN	Pyrrolidine, 1-[1-(phenylmethyl)butyl]	3.5	$C_{15}H_{23}N$
	D-Alanine,N-Propargyloxycarbonyl-, isohexyl ester	3.5	$C_{13}H_{21}NO_4$
	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	4.4	$C_6H_8O_4$
	2-Furancarboxaldehyde,5-(hydroxymethyl)-	5.4	$C_6H_6O_3$
	Octanamide.N-(2-mercaptoethyl)-	6.1	$C_{10}H_{21}NOS$
	1,3-Diazacyclooctane-2-thione	6.1	$C_6H_{12}N_2S$
	Geranyl isovalerate	8.5	$C_{15}H_{26}O_2$
	Guanosine	8.5	$C_{10}H_{13}N_5O_5$
	Ppropiolic acid, 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl)-	12.3	$C_{13}H_{20}O_3$
	2-Pentyne-1,4-diol,4-methyl-1-(2-thienyl)-	12.3	$C_{10}H_{12}O_2S$
	9H-Pyrido[3,4-b] indole	14.4	$C_{11}H_8N_2$
	1-Naphthalenecarbonitrile,8-amino-	14.4	$C_{11}H_8N_2$
WsL	13.Oxadispiro [5.0.5.1] tridecan-1-one	10.4	$C_{12}H_{18}O_2$
	2H-Cyclodeca[b]pyran, 3,4,5,6,7,8,9,10,11,12-decahydro-4-methyl-	10.4	$C_{14}H_{24}O$
	9H-Pyrido[3,4-b] indole	14.4	$C_{11}H_8N_2$

Table 7. GC-MS detection of other phytochemicals from methanolic root extracts of *Withania somnifera* from selected regions.

Sample	Details of compounds detected		
	Name	**Retention time (min.)**	**Molecular formula**
WSM	Hexadecanoic acid, methyl ester	13.8	$C_{17}H_{34}O_2$
	Oxiraneoctanoicacid , 3-octyl-, cis-	17.9	$C_{18}H_{34}O_3$
	Linoleic acid ethyl ester	17.9	$C_{20}H_{36}O_2$
WSK	Diethyl Phthalate	10.3	$C_{12}H_{14}O_4$
	Estra-1,3,5(10)-trien-17 β-ol	14.1	$C_{18}H_{24}O$
	N-2,4-Dnp-L-arginine	14.1	$C_{12}H_{16}N_6O_6$
	9,12-Octadecadienoic acid	15.8	$C_{18}H_{32}O_2$
WSN	α-D-Glucopyranoside, O- α-D-glucopyranosyl-(1.fwdarw.3)- β-D-fructofuranosyl	8.5	$C_{18}H_{32}O_{16}$
	Guanosine	8.5	$C_{10}H_{13}N_5O_5$
	Pentanoic acid,2-propyl-,8-methyl-8-azabicyclo[3.2.1]octyl-3-yl ester, endo-	10.1	$C_{16}H_{29}NO_2$
	3,4-Dichloroatropine	10.1	$C_{17}H_{21}C_{12}NO_3$
	α-Pyrrolidone,5-[3-hydroxy-1-hexyl]	10.4	$C_{10}H_{19}NO_2$
	2-Cyclohexylpiperidine	10.4	$C_{11}H_{21}N$
WSL	8-Azabicyclo[3.2.1]octan-3-ol,8-methyl-, endo-	5.2	$C_8H_{15}NO$
	Ergost-5-en-3-ol,(3β)-	21.2	$C_{28}H_{48}O$
	Campesterol	21.2	$C_{28}H_{48}O$
	Stigmasterol	23.3	$C_{29}H_{48}O$

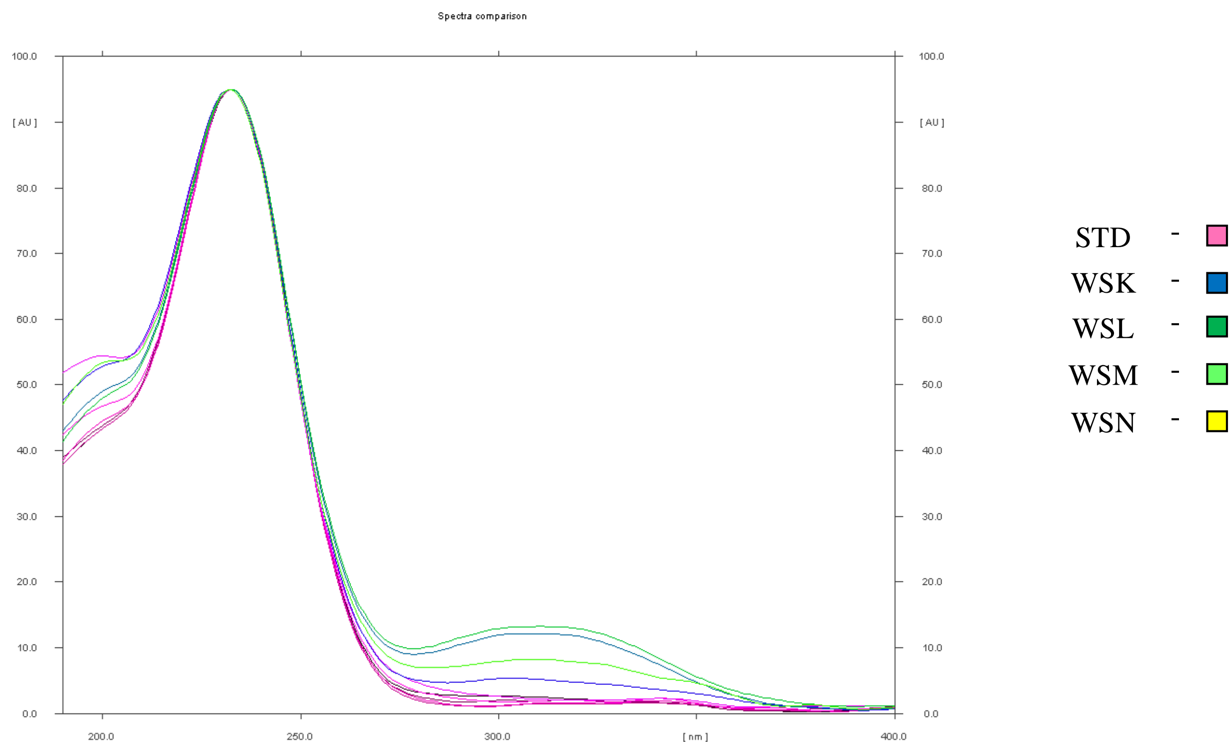

Fig. 3: Lamdamax of Ashwagandha test samples corresponding to Standard withanolide-A.

The formation of secondary metabolites is known to be highly influenced by varied climatic condition prevalent in different geographical regions. Hence, we have tested the metabolites present in crude extract of roots of *Withania somnifera* taken from several regions in India. Plants cultivated in four different states of India *viz.*, Madhya Pradesh (Nimuch), Uttar Pradesh (Lucknow), Maharashtra (Mumbai) and Karnataka (Kalasapur) were selected for the study. Our earlier data showed that the plants collected in the same season and identical age of the plants showed differing content of withaferin-A (Shah and Khan, 2012). The regional differences in

levels of the secondary metabolites are perhaps due to prevalent climatic conditions. Our data suggest that it is important to select and obtain the plant samples, for research or manufacturing after screening from various regions.

Withania somnifera is chemically very complex and more than 80 compounds are known from it (Van Wyk et al., 2000). Different parts of the plant contain a number of chemical compounds like alkaloids, steroidal compounds, steroidal lactones, saponins, glycosides, tannins etc. (Costa et al., 1999; Rajeshwar and Lalitha, 2013). This necessitates phytochemical profiling of the plant. The plants from selected regions were fingerprinted for their phytochemicals by spot tests, HPTLC and GC-MS method.

Preliminary phytochemical analysis of methanolic leaf and root extracts revealed the presence of alkaloids, saponins, sterols, triterpenes and glycosides. Anthraquinones could not be detected in any of the samples. HPTLC fingerprinting did not resolve flavonoids, tannins and anthraquinones. *Withania somnifera* has been shown to display an appreciable spectrum of phytochemical variability (Kumar et al., 2007). Therefore, a large number of researchers have profiled various parts of this medicinal plant for its phytochemicals. Visweswari et al. (2013) reported presence of alkaloids, flavonoids, steroids, saponins, phenols and glycosides from the methanolic extracts of dried roots of this plant and reported absence of terpenoids and tannins. Work of Saidulu et al. (2014) also revealed presence of flavonoids, alkaloids, glycosides, sterols, phenols, terpenoids, saponins, tannins and cardiac glycosides in methanolic extracts of roots, leaves and stems of Ashwagandha. Flavonoids were shown to be absent in ethanolic extracts of leaves and stems of *Withania* by Pralhad and Mishra (2014) though it could be detected in root extracts. Santhi and Swaminathan (2011) also reported glycosides, alkaloids, phytosterols, phenolics and flavonoids in Ashwagandha leaves extracted with water, ethanol and acetone. Presence of these secondary metabolites was also illustrated by Singh et al. (2010) in ethanolic root extracts.

GC-MS analysis of methanolic leaf and root extracts was performed, the identification of compounds was made on the basis of comparison of their mass spectra with those of Wiley and NIST Libraries and those described by Adam (1995) as well as on comparison of their retention indices (Vanden and Kratz, 1963) with literature. A total of 12 compounds could be detected in leaves and 15 in roots. Maximum number of phytochemicals could be detected in the leaf (12) and root (06) samples obtained from Nimuch region. Except for DL-Proline, 5-oxo, methyl ester which was common to WSM and WSK leaves and 9H-Pyrido [3,4-b] indole common to WSN and WSL leaves, each sample had its own unique profile. Roots from four selected regions did not show any similarity in their phytoconstituents. Twenty one constituents were analyzed in alcoholic root extracts of *Withania somnifera* (Senthil Kumar et al., 2011). One of the constituents being n-Hexadecanoicacid which is reported in leaf extract and its methyl ester in root extracts of our study.

The constituents of *Withania somnifera* roots are the steroidal alkaloids and steroidal lactones. They belong to a class of constituents called withanolides (Elsakka et al., 1990; Mishra et al., 2000) with the main active chemical constituent withaferin-A, a phytosterol (Lavie et al., 1965). Much of the pharmacological activity of *Withania somnifera* has been attributed to two main withanolides *viz.,* withaferin-A and withanolide-D (Sharma et al., 2011). The leaves of Indian chemotype are reported to contain 12 withanolides. The leaves of the plant from different habitat contain different Withanolides- a group of C28 steroids (Uddin et al., 2012).

Conclusions

The present study can be used in future for economical formulations of the active chemical ingredients in natural drugs against a variety of inflammatory diseases. No significant correlation could be drawn between edaphic factors and Withanolide contents indicating that phytochemical variations are by and large gene related. Probably more than one chemotypes of this plant of medicinal value exists in India. Further studies probably at the gene expression level will be necessary to ascertain the causes of phytochemical diversity, variations in the quantity of phytochemicals and difference in the medicinal potential of plants from one region to another.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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