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Quality Control of *Withania somnifera* and its Marketed Formulations by Validation through High Performance Thin Layer Chromatography

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Abstract | **Review Article**

Withania somnifera is the most valuable herbaceous plant, also known as Ashwagandha used in the traditional systems of Indian medicine having many therapeutic effects. It is obtained from the dried roots and stems of *Withania somnifera* belonging to family Solanaceae. The active chemical constituents of *Withania somnifera* are withaferin A, withanolide A, withasomniferin-A, isopelletierine, withasomidienone, tropine, withanone, cuscohygrine, anaferine, hygrine, anahygrine, somniferine, mesoanaferine, etc. It is used as a liver tonic, aphrodisiac, in asthma, emaciation, in bronchitis and ulcers. It has life prolonging, rejuvenating effect & also used for the treatment of insomnia, anxiety, skin disease, nervous exhaustion, impotency, enhancing memory and insulin secretion etc. Marketed formulations of Ashwagandha are Ashwagandharista, Himalaya Ashwagandha, Stresswin, Stresscom, Inlife Ashwagandha capsules**,** Himalaya massage oil**,** Ancient Apothecary**,** KSM 66 Ashwagandha**,** Vigomax**,** Baidyanath Ashwagandha Amrita 450 ml, Vital plus, Amrutha kasthuri and Brento etc. To maintain the quality of marketed formulations validation should be done. Different validation parameters such as LOQ, LOD, range & linearity, accuracy, ruggedness and specificity have been studied. Ashwagandha is in demand as a good health promoter is expanding in global market. Many efforts were undertaken to develop a better *Withania somnifera* variety with a specific chemotype. HPTLC (High performance thin layer chromatography) is most important in evaluating the quantity and quality of herbal drug. This technique is modern & effective form of TLC which is used for qualitative and quantitative analytical determination of analytes. It is the simple, rapid, precise, specific, robust and accurate technique. The effect of different extraction methods on marker compound Withaferin-A & other than Withaferin-A, effect of different climatic zones on phytochemical profile of *Withania somnifera*, different mobile phase used and quantification & validation of withaferin-A by using HPTLC have also been studied.

Keywords: Ashwagandha, Withania somnifera, marketed formulations, HPTLC, Validation, Withaferin A, Withanolide A.

1. INTRODUCTION

In the present era, mind of people has been changed from synthetic to herbal medicines. With increasing the need of herbal products it is necessary to maintain the quality of them for betterment of human being (Sharma *et al*., 2008). Various quality control tools are there which are used to ensure the quality of herbal drugs. Both quantitative & qualitative parameters are required for the quality assurance of them. Techniques such as IR, UV which are commonly used for qualitative determinations whereas HPLC(high pressure liquid chromatography), HPTLC (high performance thin layer chromatography), SFC (supercritical fluid chromatography), GC-MS (gas chromatography-mass spectroscopy), ICP-MS (inductively coupled plasma-mass spectroscopy), thermal analysis, GC-MS (gas chromatography-mass spectroscopy) are used to quantification of herbal medicines for the purpose of quality control (Balekundri *et al*., 2020).

Withania somnifera also known as Ashwagandha is most valuable herbaceous plant in the traditional systems of Indian medicine having many therapeutic effects (Gupta *et al*., 2007). It is also called "Indian Winter cherry" or "Indian Ginseng" (Singh *et al*., 2011). It is a desert plant which grew up in dried and rain-forest regions (Meher *et al*., 2016). The active chemical constituents of *Withania somnifera* are withaferin A, withanolide A, ashwagandhine,

withasomniferin-A, isopelletierine, withasomidienone, tropine, withanone, cuscohygrine, β-sitosterol D glucoside, gallic acid, anaferine, hygrine, anahygrine, somniferine, rutin, mesoanaferine, etc (Saleem *et al*., 2020). It has life prolonging, rejuvenating effect & also used for the treatment of insomnia, anxiety, convulsion, skin disease, inflammatory conditions, nervous exhaustion, impotency, enhancing memory or cognitive and enhance insulin secretion and has been shown to be a successful treatment for cancer cells (Krutika *et al*., 2016). Some marketed formulations of *Withania somnifera* are Ashwagandharista, Himalaya ashwagandha, Stresswin, Stresscom, MuscleBlaze Ashwagandha 1000mg Tablet, Inlife Ashwagandha Capsules, Himalaya massage oil, Ancient Apothecary, KSM 66 Ashwagandha, Vigomax, Baidyanath Ashwagandha Amrita 450 ml, Vital plus, Amrutha kasthuri and Brento etc. To know the quality of marketed formulations, validation is done. And in this review different extraction methods, solvents used for exytraction, different validation methods and effect of different climatic zones on phytochemical profile on are discussed.

HPTLC is a prominent analytical method for quantification and fingerprinting of marker compounds in herbal medicines because of its simplicity, accuracy, sensitivity and applicability for high throughput screening of herbal medicine. The mobile phase of HPTLC has a high speed capillary flow range (Modi *et al*., 2016). This technique is superior to more analytical techniques in case of cost and time for analysis (Sonia *et al*., 2017).

Validation is the technique used for confirming that the scientific technique hired for a particular test is appropriate for its intended use. It is an aspect of quality assurance since it entails a systematic examination of process, facilities and system to see if they execute their intended functions consistently and effectively. For compound evaluation, there are eight parameters which are Robustness, LOD, LOQ, Range & Linearity, Accuracy, Ruggedness, Specificity and Precision (Ahir *et al*., 2014).

Robustness is the method which is used to estimate of its volume persist unaltered at little yet intentional convert in technical variables and lay out a sign of its reliability throughout common use (Patil *et al.,* 2017).

Limit of detection defined as the lowest concentration of a specimen in sample which can be identified, still not definitely quantified.

Limit of quantification explained as the smallest concentration of an analyte in a sample with acceptable precision and accuracy can be quantified (Rashmin *et al.,* 2012).

Linearity is defined as its ability to obtain test results which are shortly, or beyond means of well-stated mathematical variation, proportional to the mass of specimen in samples in a given value.

Accuracy of a systematic technique is the extent to which true values agree after providing test results by the method. For estimation of accuracy true value can be obtained in different ways. It expresses the correctness of the method (Patil *et al.,* 2017).

Ruggedness define the degree of reproducibility of test outcome prevail by analyzing the same sample under different test conditions.

Specificity defines the ability of the method to compute accurately and specifically the substance of interest in the sample as impurities (Patil *et al.,* 2017).

Precision refers to the accordance between the single test products when a technique is appeal frequently to the same sample. It is usually indicated as relative standard deviation (Rashmin *et al.,* 2012).

The major component present in *Withania somnifera is* withaferin-A and minor components are withanolide A and 12 deoxy withastramonolide. This extraction methods and extracting solvents plays an important role in extracting the analytes from the drug.

2. Effect of different extraction methods on marker compound Withaferin-A & other than Withaferin-A

The most commonly used conventional and novel extraction methods for extracting the withaferin-A from *Withania somnifera* with percentage yield is mentioned in table 1and other than withaferin-An are mentioned in table 2. Soxhaltion is based on cell permeation and the extracting solvent is used to solubilize the active ingredients. In maceration technique, coarsely powdered drugs placed in a container & the menstruum is used for the extraction. These traditional procedures are solvent and time consuming, as well as thermally hazardous. Microwave assisted extraction has been developed and refined for quick extraction for withanolides in response to the increasing demand for more environmental friendly approaches. MAE involves using microwave energy to heat the solvent in contact with the sample. Different extraction techniques & different solvents show variations in extraction yield due to the polarity of the solvents. By changing the solvent methanol to water in maceration technique, gives high percentage yield of withaferin-A but this technique is very time consuming.

Table-1: Effect of different extraction methods on marker compound Withaferin-A (Jyothi *et al.*, 2010 and Jain *et al.*, 2010)

Extraction methods	Extracting solvent	Solvent used (ml/g)	Extraction time	%age Yield of withaferin-A
Soxhlation	Methanol	50	14h	0.16
Maceration	Water	50	10h	4.80
Microwave Assisted Extraction	Methanol	20	2min	0.69

Table-2: Effect of different extraction methods on marker compound other than Withaferin-A (Dhanani *et al.*, 2017)

Extraction method	Solvent used for extraction	%age Yield	Withanolide A (µg/mg)	12 deoxy withastramonolide (µg/mg)
Soxhlet	Water	9.51	1.14	0.36
	Ethanol	9.08	3.57	1.22
	Water:Ethanol	9.43	1.25	0.40
Microwave assisted extraction	Water	11.18	0.59	0.20
	Ethanol	10.01	4.35	1.39
	Water:Ethanol	11.39	1.09	0.28
Ultrasonic extraction method	Water	9.90	0.91	0.26
	Ethanol	2.85	6.04	2.04
	Water:Ethanol	9.74	1.21	0.41

3. Effect of different climatic zones on phytochemical profile of *withania somnifera*

Different agro-climatic conditions have a significant impact on phytochemical composition in plants. It has been shown that plant physical and chemical behaviour varies greatly depending on the climatic conditions, resulting in differential variance in the active ingredients present in that particular plant mentioned in table 3. Maximum amount of withaferin-A 1.19% was found in desert region & withanolide-A content 3.22% also from desert region. It was observed that when the samples collected from the desert region it has a good phytochemical profile than the other samples collected from the different regions.

Table-3: Effect of different climatic zones on phytochemical profile of *Withania somnifera* (Kherde *et al.*, 2020)

Quantification by HPTLC	Coastal Region	Desert Region	Plateau Region	Plains Region
Withaferine-A	0.82%	1.19%	0.49%	0.42%
Withanoloide-A	2.40%	3.22%	0.63%	1.41%

4. Effect of different solvents of increasing polarity on the extraction of total withanolides (wda, wa, 12wd) from *withania somnifera*

Different solvents according to their polarity are used. Hexane, chloroform, ethyl acetate and methanol are used to demonstrate the extraction effectiveness of aqueous alcoholic solvents with percentage yield are mentioned in table 4. Highest concentration of three withanolides was yielded by organic solvents chloroform and ethyl acetate. Upscaling process development in the preparation of enriched extracts from Withania somnifera, bio-prospecting investigations, crop improvement and quality control could all benefit from an optimised extraction process. Maximum yield was obtained from methanol and minimum from hexane. It was concluded that higher composition of polar solvent gives better extraction yield.

Table-4: Different solvents of increasing polarity on the extraction of total withanolides (Kumar *et al.*, 2010)

Solvent	%age yield of extract	%age yield of Withanolide- A	%age yield of withaferin-A	%age yield of Withanolide- D	Withanolide, % in extract
Hexane	1.60	0.7184	NQ*	0.0060	0.7244
$CHCl_3$	2.90	0.9268	0.2705	0.1615	1.3588
EtOAc	6.00	1.0182	0.1676	0.1468	1.3326
MeOH	12.47	0.1762	0.0344	0.0305	0.2410

*Not quantifiable

The mobile phase for quantification of withanolides is chosen depending on the adsorbent material employed as the stationary phase, as well as the physical and chemical properties of the analyte. List of different mobile phases at specific composition for the separation of withanolides are mentioned in table 5.

Table-5: List of different mobile phase used for quantification of different withanolides by HPTLC (Srivastava *et al*., 2008, Sharma *et al*., 2007, Devkar *et al*., 2012, Sangwan *et al*., 2004)

Sr. no.	Mobile phase	Withanolides
1	Dichloromethane-methanol-acetone-diethyl ether(15:1:1:1v/v)	Withanolide A, withaferin-A & 1,2 deoxy-withastraminolide
2	Ethyl acetate-toluene-formic acid(5:5:1)v/v	Withanolide A & withaferin A
3	Dichloromethane-toluene-methanol-diethyl ether-acetone(7.5:7.5:3:1:1)	1,2 deoxy-withastraminolide, withanolide A, withanolide B & withaferin-A
4	Chloroform –benzene- methanol–ethyl acetate(74:24::4)	Withanolide A, withaferine A & Withanone

The amount of the marker compound withaferin-A was varying with different plant parts of *Withania somnifera.* The R_f values, LOD and LOQ were different in different plant parts of *Withania somnifera.* Different mobile phases has been used for the quantification of marker compound withaferin-A. Validation and quantification of withaferin-A from different plant parts of *Withania somnifera* and its marketed formulations by HPTLC are mentioned in table 6 and 7.

Table-6: Validation and quantification of withaferin-a from different plant parts of *W. somnifera* by HPTLC (Devkar *et al*., 2012, Sharma *et al*., 2007, Srivastava *et al*., 2008, Mirjali *et al*., 2009, Nayak *et al*., 2009, Tomar *et al*., 2019)

Plant part used	Mobile phase	%age yield of Withaferin-A	LOD (ng)	LOQ (ng)	Average recovery (%)	Precision (%) RSD	Absorption reflection mode(nm)	R_f values
Root	Dichloromethane–toluene–methanol–acetone–diethyl ether (7.5:7.5:3:1:1 v/v)	7.46	120	350	98	1.16	235	0.58
Whole plant	Toluene: ethyl acetate:formic acid (5:5:1)	1.46	120	800	96.3	1.28	530	0.33
Plant tissues	dichloromethane–methanol–acetone–diethyl ether (15 + 1 + 1 + 1, v/v/v/v)	2.19	19.62	65.39	98.44	0.18	230	0.61 ± 0.04
Aerial part & Root	Ethyl acetate/toluene/formic acid/2-propanol (7:2:0.5:0.5)	2.2	18.28	60.31	98	0.99	215	0.29
Root	Toluene–ethyl acetate–formic acid 5:5:1	0.0376	100	800	101	0.81	200	0.57
Root, stem, leaves	Toluene, ethyl acetate and acetic acid (60:40:4)	0.99	200	666	203± 1.4	0.69	221	0.10

Table-7: Validation and quantification of withaferin-a from marketed formulations of *W. somnifera* by HPTLC (Tatke *et al*., 2010, Bhondave *et al*., 2014, Mistry *et al*.,2015, Trivedi *et al*.,2009)

Marketed formulations	Mobile phase-	%age yield of Withaferin-A	LOD (ng)	LOQ (ng)	Average recovery (%)	Precision (%)	Absorption reflection mode	R_f values
Churna	Methanol: Chloroform 2:8	0.458	100	300	101.98±0.11	0.006	207nm	0.59
Arishta	Methanol: Chloroform 2:8	0.137	100	300	103.37±0.16	0.057	207nm	0.59
Capsule	Methanol: Chloroform 2:8	0.437	100	300	102.44±0.12	0.059	207nm	0.59
Vati	Methanol: Chloroform 2:8	0.698	100	300	103.21±0.28	0.046	207nm	0.59
Ashvagandharishta	toluene: ethyl acetate: formic acid: methanol(6:3:0.1:0.6, v/v/v/v)	81.14 / ±1.37ng /band	12.0487	36.5114	101.42 / ±1.68	0.80 - 0.89	474nm	0.37±0.02
Union Total capsule	chloroform: methanol: toluene: formic acid (6.5: 0.5: 3: 0.25 v/v/v/v)	0.722	23.818	72.175	100.06 % to 100.46 %	0.744 – 1.507	530	0.22
Brento Tablet	Chloroform- Ethyl acetate- Toluene- glacial acetic acid (2:5:5:1) v/v/v/v)	0.478	0.037	0.112	99.14 ± 0.465	0.435	223	0.27 ± 0.02

Different plant parts of *Withania somnifera* have been used for the quantification and validation of Withanolide A, 1,2 Deoxy-withastranolide, Withanolide B, 12-Deoxywithastramonolide are mentioned in table 8.

Table-8: Validation & quantification of withanolides (other than withaferin-a) by HPTLC using different plant parts of *W. somnifera* (Devkar *et al*., 2012, Srivastava *et al*., 2008)

Compound	Plant part used	Mobile phase-	Amount present	LOD (ng)	LOQ (ng)	Average recovery (%)	Precision (%) RSD-	absorption reflection mode at	R_f values
Withanolide A	Root	Diethyl ether: acetone: methanol: toluene: dichloromethane (1:1:3:7.5:7.5 v/v/v/v/v	2.78	60	250	98	1.75	235	0.68
1,2 Deoxy-withastranolide			1.12	80	300	99.5	1.14	235	0.61
Withanolide B			2.19	80	300	99	1.60	235	0.79
12-Deoxywithastramonolide	Plant tissues	Acetone: methanol–dichloromethane diethyl ether 1:1:15:1	0.094	13.66	45.55	98.00–101.25	0.18–0.74	230	0.72 ± 0.03
withanolide-A	Plant tissues	Acetone: methanol–dichloromethane diethyl ether 1:1:15:1	23.13	0.109	72.76	95.83–98.95	0.18–0.85	675	0.86 ± 0.03

Marketed formulations like vati, capsule, churna and ashwagandharistha has been quantified and validated by HPTLC for the determination of β-sitosterol D glucoside, gallic acid and Rutin are mentioned in table 9. All developed HPTLC methods were validated in terms of LOD, LOQ, precision, accuracy and specificity. For the quality determination and standardisation of polyherbal preparations, it is critical to combine modern scientific knowledge with sensitive analytical techniques.

Table-9: Validation & quantification of withanolides (other than withaferin-a) by HPTLC using marketed formulations of *W. somnifera* (Tatke *et al*., 2010, Bhondave *et al*., 2014, Tiwari *et al*., 2012)

Compound	Marketed Formulations	Mobile phase-	Amount present	LOD (ng)	LOQ (ng)	Average recovery (%)	Precision (%) RSD-	absorption reflection mode at	R_f values
β-sitosterol D glucoside	Capsule	methanol: chloroform 2:8 v/v	0.293	10	30	97.13 ±0.09	0.007	207nm)	0.21
β-sitosterol D glucoside	Aristha	methanol: chloroform 2:8 v/v	0.250	10	30	99.00±0.09	0.069	207nm)	0.21
β-sitosterol D glucoside	Vati	methanol: chloroform 2:8 v/v	0.278	10	30	98.67 ±0.10	0.067	207nm)	0.21
β-sitosterol D glucoside	Churna	methanol: chloroform 2:8 v/v	0.234	10	30	98.06±0.22	0.057	207nm	0.21
Gallic acid	Ashvagandharishta	toulene:: formic acid: methanol: ethyl acetate (6:0.4:0.2: 3)	0.064	17.7444	53.7711	98.77 - 101.96	0.64 - 0.78	474	0.26
Rutin		toulene:: formic acid: methanol: ethyl acetate (6:0.4:0.2: 3)	0.00464	120	400	99.86	0.76	280	0.59

CONCLUSION

Quality control is a very important aspect for the safety and efficacy of herbal formulations. And to maintain the quality of marketed formulations validation plays an important role. For this purpose validation parameters were studied and discussed. Different validation methods were used to standardize different kind of formulations containing Ashwagandha by HPTLC.

HPTLC methods can be used to determine batch to batch variations and routine analysis by he rbal manufacturers of its formulations. Due to potential behaviour of *W. somnifera* in many diseases, it has been used from so many years but more clinical trials are still required for better therapeutic efficacy. So there is a need for more research work to maintain the quality of herbal formulations for quantification as well as qualification of withaferin-A, withanolide-A, B and D in crude drug as well as in marketed formulations.

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