QUALITATIVE AND QUANTITATIVE DETERMINATION OF WITHAFERIN-A FROM WITHANIASOMNIFERA BY ANALYTICAL METHOD HPTLC

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ABSTRACT

Withaferin-A, an exceptionally oxygenated steroidal lactone found in the medicinal plant Withaniasomnifera and this plant belong to the Solanaceae family, is a major component of the withanolide group. Withaferin A have diverse functions including cytotoxic activity against cancer cell.The current study design to aim determination of the qualitatively and quantitatively presence of withaferin A from root powder extract of Withaniasomnifera. Withaniasomnifera root powered was extracted by using soxhlet extraction apparatus and obtained thick pest (extract) by using rota vapour. The extract was analysed by high performance thin layer chromatography (HPTLC). As a solvent methanol was used. Total three tracks were used to run the sample (extract of root power of Withaniasomnifera).Anti tumor compound withaferin A was obtained in the form of band qualitatively and area quantitavely.It is evident from the performed experiments that withaferin A is a major constituent of extract of root power of Withaniasomnifera.

Keywords: Withaniasomnifera, HPTLC, Withaferin-A, Qualitative determination, Quantitative determination, Soxhlet extraction.

INTRODUCTION-

Withaferin-A, an oxygenated steroidal lactone found in Withaniasomnifera (Solanaceae family), is a principal component of the withanolide group of herbal products [1]. Vigrous and diverse actions such as cytotoxicity, anti-stress, central nervous system, immunomodulatory activity, and cardioactivity are well-known for withanolides[2]. Withaferin-A's anti-tumor cytotoxic action has received a considerable interest since its discovery in the 1960s [3][4]. In Ayurvedic medicine, the roots of Withaniasomnifera are used for hypnotic, nervous sedative, aphrodisiac tonic, and astringent reasons [5]. Since the herbal products have gained recognition in international market, it is imperative to standardize them using their isolated constituents—ideally, the bioactive components. In the international market, standarization based on the maximum number of biomarkers is currently widely accepted. Given the significant role that the herb plays in the traditional Indian medicine and their very widespread reputation as health-promoting agents, it was decided to use standards to carry out the phytochemical profile of their constituents. In the present investigations phytochemical profiling of Withaniasomnifera was carried out to determine the component withaferin-A.

Numerous HPLC techniques are available for the simultaneous detection and quantitative measurement of two or three withanolides[6]. The primary drawback of the acetylation procedure is that it frequently obstructs the analysis of withaferin-A. It is essential to perform the acetylation

procedure prior to the analysis and profiling of withaferin-A in order to minimize this drawback. To our knowledge, there hasn't been any publication on the simultaneous measurement of withaferin-A using HPTLC. This study outlines the qualitative and quantitative approach for using HPTLC to determine the level of withaferin-A.

Plants have long been used as a common source of medicine, and several Ayurvedic texts attest to the usage of medicinal plants to cure human illnesses. Phytomedicines are nutritional extension in the form of powders, tablets, extract, capsules, fresh or dried plants and are usually taken to enhance health conditions and for good health. These botanical medicines are considered to be safe and has been continuously taken by people without proper recommendation. The profiling of these conventionally used medicinal plants must be carry out in order to analyse the quantity and quality of bioactive fraction incorporated in them. Numerous pharmaceuticals have been found via pharmacological analysis of herbal plants, and as a result, several civilizations have developed their own unique medical systems. Lack of side effects and effectiveness ofthese botanical medicines attracted the major pharmaceutical population towards drug yielding plants research [7].A number of bioactive compounds are produced by plants to defend themselves, but recent studies have shown that these bioactive substances can also safeguard humans against numerous illnesses. Plants including fruits, vegetables, and herbs generate a group of bioactive substances known as secondary metabolites, each of which functions differently [8]. In several research programmes strategies have been triggered off either to identify new lead substances or to develop standardized extracts [9]. For this it is imperative to assess countless qualitative and quantitative parameters, which may be beneficial in setting standards for specific medicinal plant/parts of the plant. This makes it simple to recognize and describe individual molecules, which may be essential for preserving the integrity and purity of the bioactive fraction [10].

Indian Pharmacopoeia contain the information about Withaferin-A, it means that Withaferin A is an official drug [11]. Withferin A is usually used as an abortion inducing drug (aborticide), amebicide, bacteriostatic, anti-stress, antiphlogistic, anti-arthritic, anti-oxidant, anti-tumoral (anti-cancerous), and contraceptive [12] [13] [14]. The plant Wathaniasomniferais extensively distributed geographically from Palestine to North India covering Pakistan, Israel, Baluchistan, Jordan, Sudan, Iran, Egypt, and Afghanistan; from Southern Mediterranean region to the Canary Islands and to South and East Africa. In India, the plant grows wild mostly in the northwest, which includes Punjab, Jammu, and Himachal Pradesh[14]. Therapeutic properties of Withaniasomnifera is credited to the potentialities of presence several alkaloids and steroidal lactone in the roots and leaves [14] [15]. There were many reports that demonstrates antitumor effects, immunomodulatory properties and antifungal activity of Withaniasomnifera[16] [17] [18] [19]. Withanolides are the major phytochemical constituent reported and tested from this medicinal plant. Withanolides are steroidal substances with an ergosterol skeleton that undergo oxidation to produce d-lactone from C-22 and C-26 [20]. One of the most significant withanolides with several preventative qualities is withaferin-A [12].

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EXPERIMENT

Reagents and solution

ELECTROCRAFTS (FBD), Faridabad, Haryana, India was the supplier of the bioactive reference standards containing Withaferin-A. HPLC grade or analytical grade chemicals and solvents procured from E-Merck and other prominent companies were used in the current investigation.

Plant material

The dried root sample of Withaniasomnifera, often known as Ashwagandha, was acquired from the Government Ayurvedic College and Hospital, Patna, Bihar, which is also known as Herbarium. Dried ashwagandha root sample was grounded thoroughly with the help of mechanical grinder (Hanil Co. Seoul, South Korea) and passed through mesh size 120 mm. The root powder obtained was stored in air tight container at 4°C, for further analysis.

Sample Preparation

Using a Soxhlet extraction device, 50 g of powdered ashwagandha root sample was extracted for 5–10 hours at 60°C using 500 ml of 80% ethanol. The best extraction duration for mass yield was determined to be seven hours. The resulting extract was filtered through Whatmann No. 1 filter paper, and the residue was concentrated until it was dry. The alcoholic content was then evaporated using Rota vapour at a temperature of 50°C. The lyophilized extract was then stored at 4°C for additional research.

Withaniasomnifera Withaferin-A HPTLC Analysis

Stationary phase HPTLC Silica gel 60 F_{254}(Merck) was used for HPTLC, Plate format 100 x 100 mm at application Position Y: 8.0 mm, length: 8.0 mm, width: 0.0 mm with track first position X: 15.0 mm, distance: 11.4 mm at solvent front position 70 mm.

For the qualitative determination Linomat 5 (S/N:241506), TLC Scanner 4 (S/N:241072) and TLC Visualizer 2 (S/N:241537) were used. The long-distance travel by developing solvent was 70 mm. Methanol was used as a solvent with speed 150 nl/s and volume per dose was 0.20 µl. The development chamber tank used was TTC 10×10. The development chamber saturation time was 20 mins., front volume through 5ml and rear volume through 10ml with 5 mins. drying at room temperature. After development, the plates were taken out of the chamber, allowed to dry for five minutes, and then the spots were seen under a UV lamp. The TLC plate was visualized by TLC Visualizer 2 at 227 nm and 366 nm in the form of bands. For the Scanning of the developed plate single λ TLC Scanner 4 was used. Bands were optimized for resolution. Bands were scanned with the speed of 20 mm/s with resolution 100 µm/step and Slit 5 x 0.2 mm. The use of deuterium and tungsten lights for the scanning of bands at wavelength 227nm. For the sample analysis per dose volume was 0.02µl.

Three tracks were used to determine the qualitative presence of Withaferin-A in the methanol-extracted root powder of Withaniasomnifera:Track 1, track 2 and track 3. 1.0 µl of quantity was loaded to the all three tracks. Track 1 was loaded with sample AW01, track 2 was loaded with reference withaferin-A (standard) and track 3 was loaded with sample AW01(Diluted) as shown in Table 1.

Table1. Detail of sample with track and loaded volume

TRACKS	DESCRIPTION	VOLUME (µl)
01	Sample AW01	1.0
02	Withaferin-A	1.0
03	Sample AW01 (diluted)	1.0

Withaniasomnifera stationary phase root powder extract was subjected to an HPTLC fingerprint analysis using 60 F227 (Merck) silica gel plates. The study was conducted using two tracks, identified as track 1 and track 2 for quantitative determination. Toluene: ethyl acetate (8:2) was the solvent employed for the chamber saturation. Derivatization took place for three minutes at 100ºC. A 10% methanolicsulfuric acid derivatizing agent was employed. Following derivatization, the plate was scanned at 227 nm with a single wavelength speed of 20 mm/s using scanner 4. The software used for the HPTLC fingerprint analysis Server DESKTOP-60R1I2G was version 2.5.18053.1.

Plotting peak area versus the quantity of withaferin-A applied allowed for the preparation of seven points calibration plot, which were used for calibration and linear range assessment. Various amounts (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5µl) of the 1 mg/ml withaferin-A stock solution were applied to a plate. Table 2 provides a track assignment for every amount.

Table2: Track assignment of reference standard withaferin - A

Track	Description	Volume (ul)
1	Withaferin-A standard	0.5
2	Withaferin-A standard	1
3	Withaferin-A standard	1.5
4	Withaferin-A standard	2
5	Withaferin-A standard	2.5
6	Withaferin-A standard	3
7	Withaferin-A standard	3.5

RESULT& DISSCUSION

Using HPTLC fingerprint profiling, the anticancer component Withaferin-A was detected in the powdered root of Withaniasomnifera. The peak purity was tested for Withaferin-A by comparing the spectra obtained from methanol extracted root powder of Withaniasomnifera with the spectra obtained from standard Withaferin-A. By using toluene:ethyl acetate (8:2) as the mobile phase,

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Withaferin-A chromatography was attained with symmetrical and repeatable peaks. The band was visualized by tacking image at white light, 277nm and 366nm by TLC visualizer 2 before derivatization and at 366nm after derivatization as shown in Fig1.

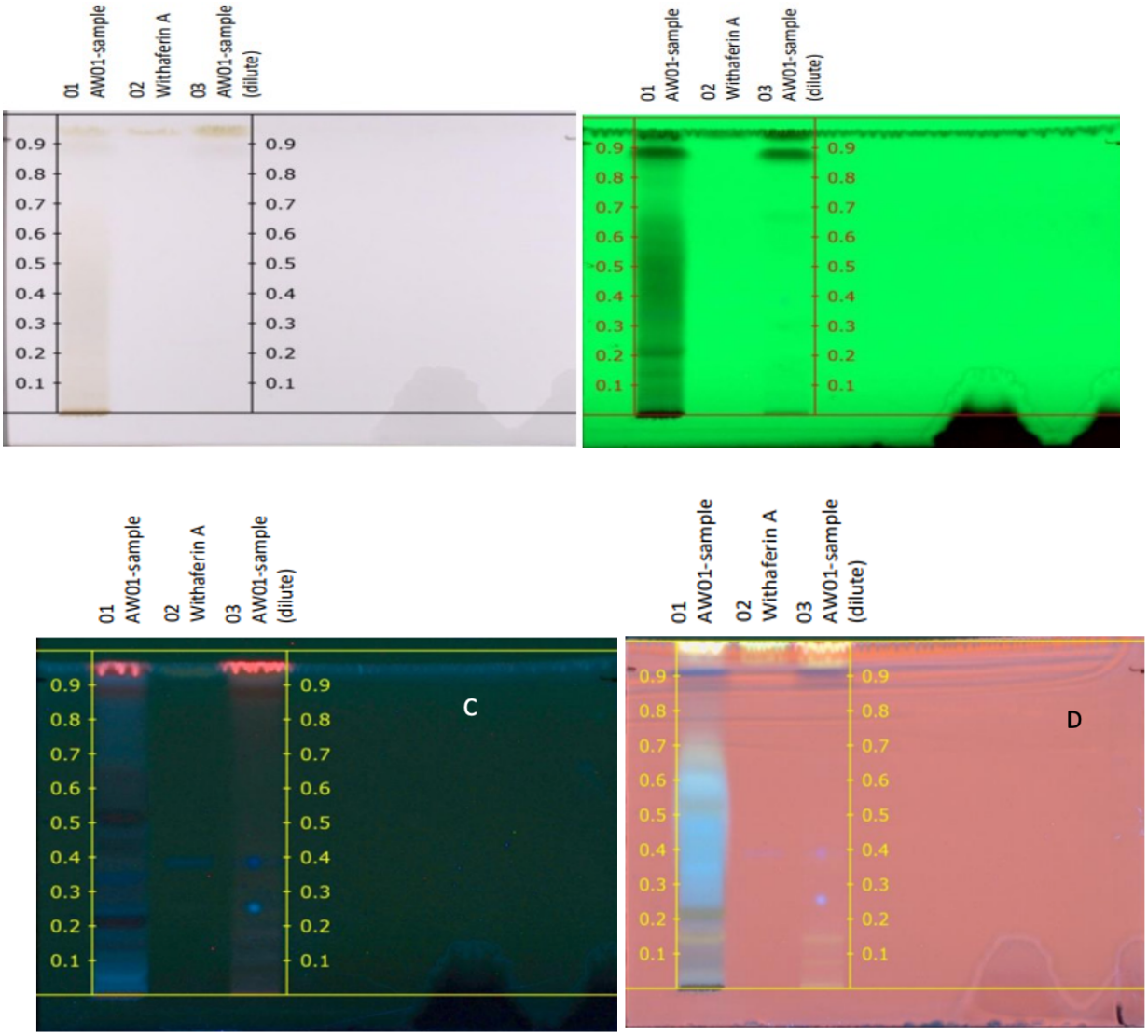

Fig1 – HPTLC profile of methanol extracted withaferin-A from root extract of Withaniasomnifera before derivatization (A, B and C) at white light, 277nm and 366nm and after derivatization (D) at 366nm.Track 01: AW01 sample , Track 02: Withaferin-A, Track 03: AW01 sample (dilute)

Fig 2, the calibration plot, was expressed as follows: y = 0.0065x + 0.0082, where x is the amount and y is the withaferin-A response. 0.9555 was the correlation coefficient.

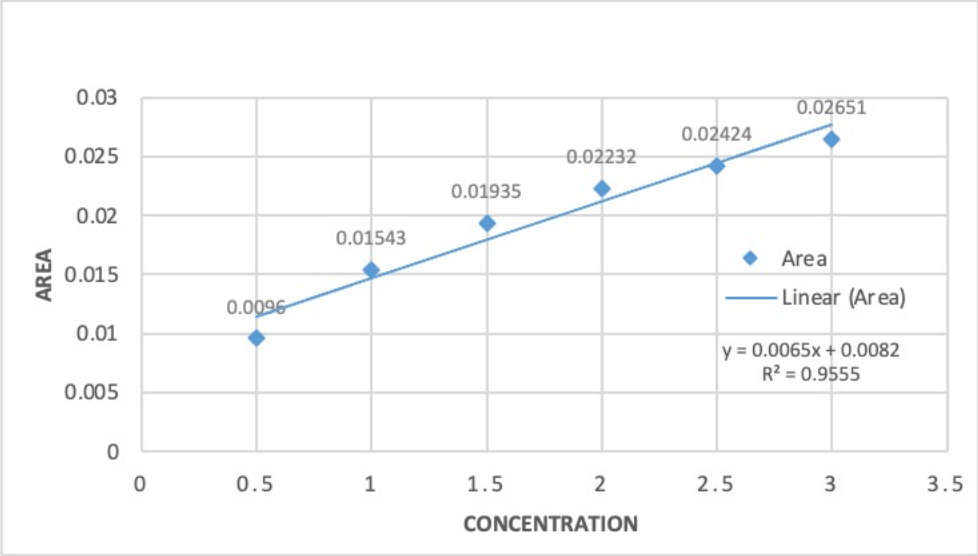

Fig 2 – Calibration curve standerd (withaferin A)

Fig 3, 4, 5, 6, 7, 8 and 9 each displays the peak area for the following amounts: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5µl.

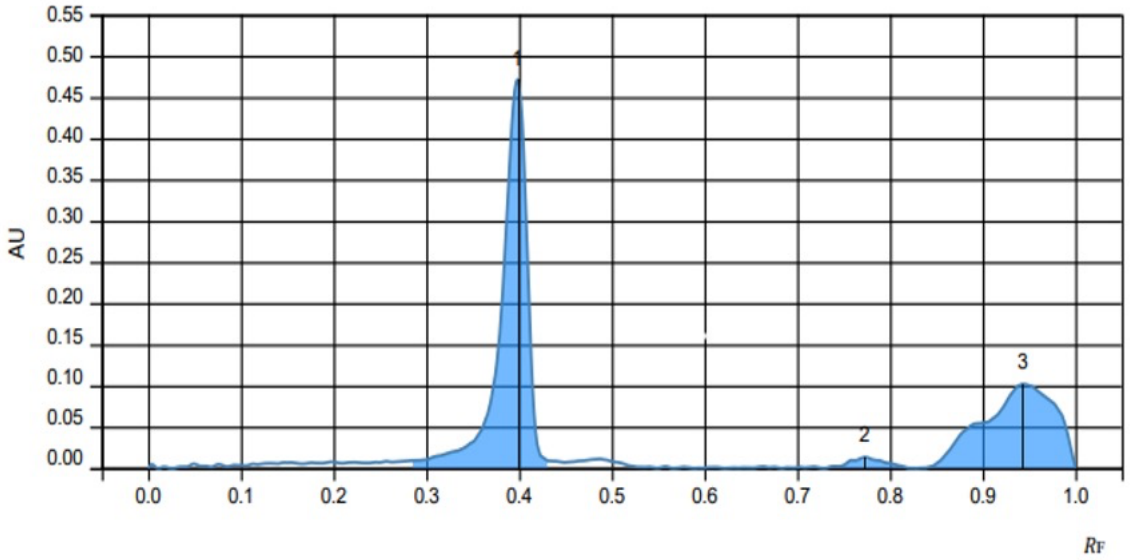

Fig 3 – Peak at 0.5µl volume of withaferin-A

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

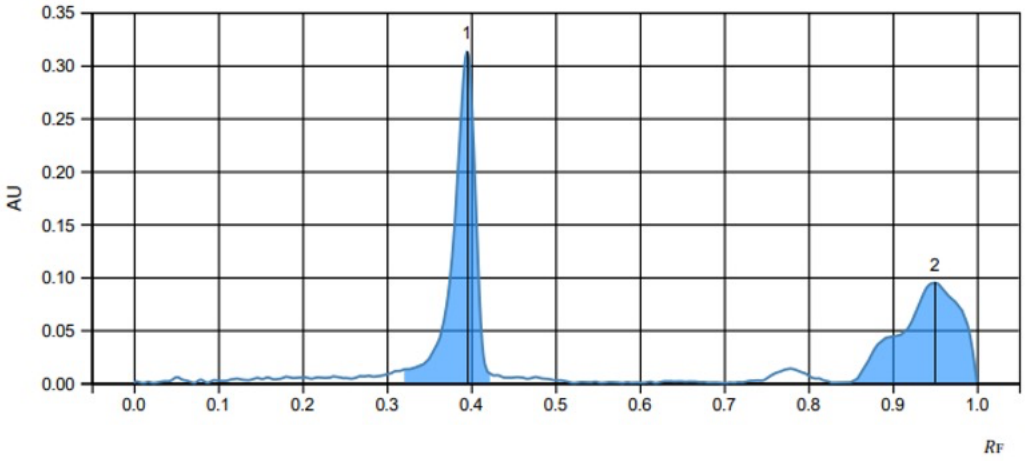

Fig 4– Peak at 1.0µl volume of withaferin-A

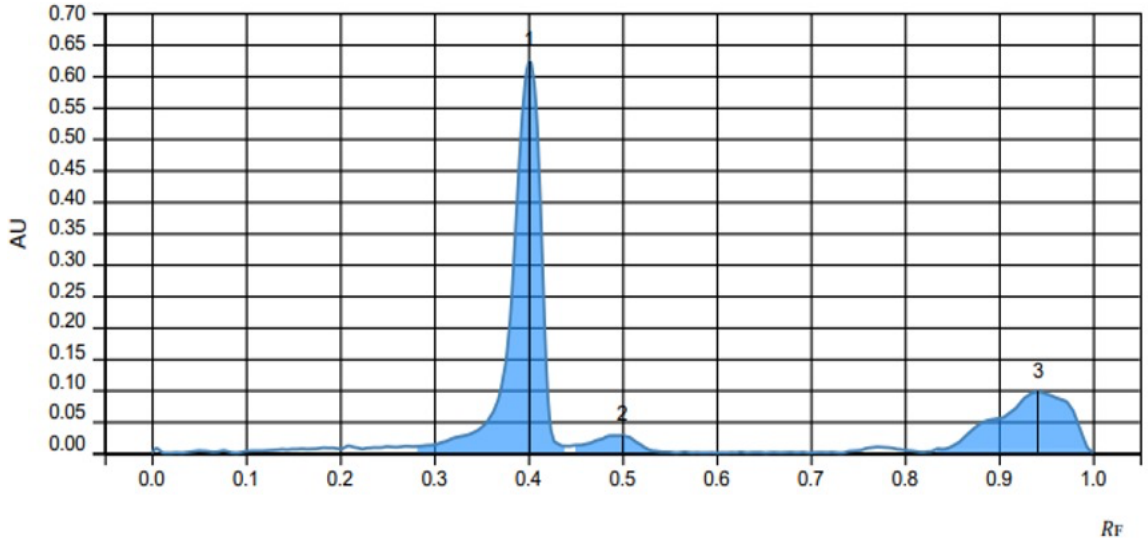

Fig 5 – Peak at 1.5µl volume of withaferin-A

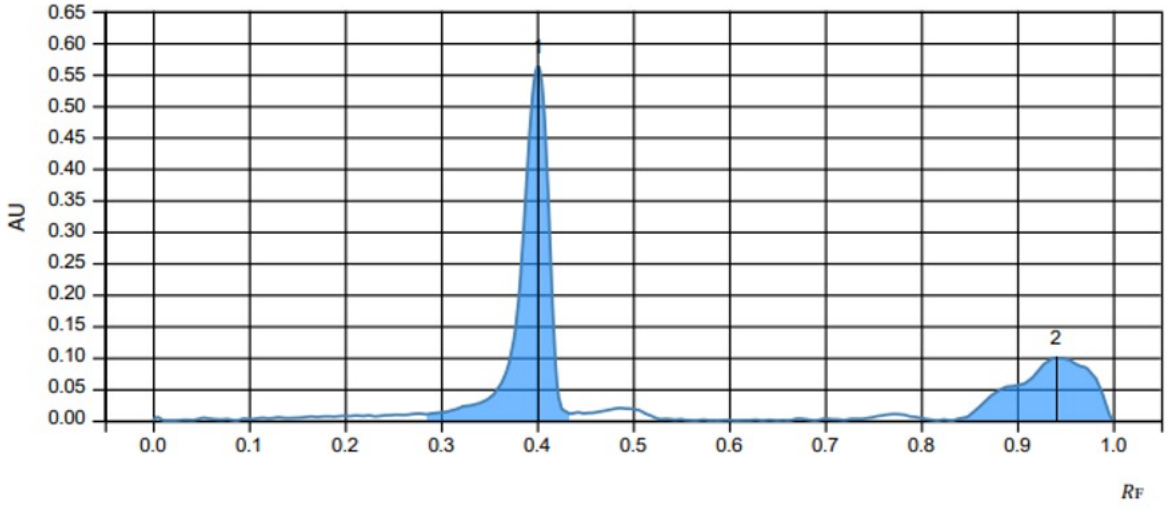

Fig 6 – Peak at 2.0µl volume of withaferin-A

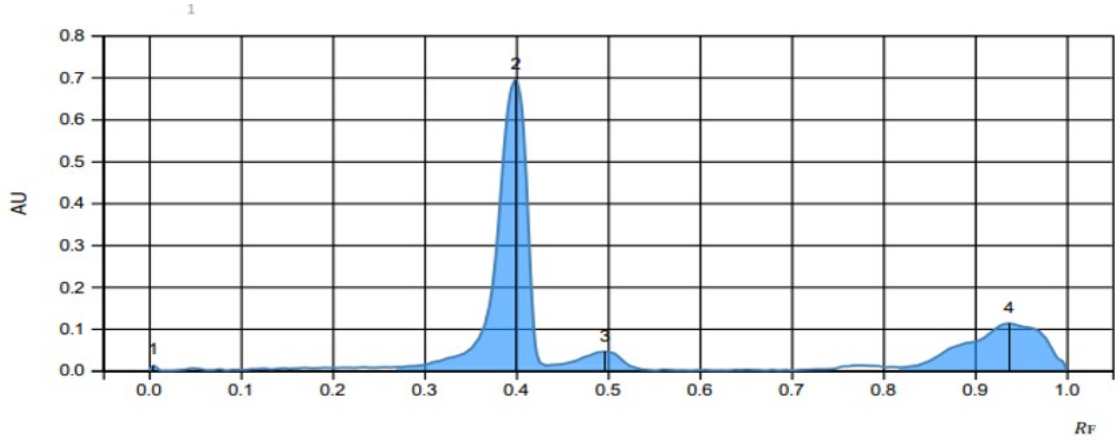

Fig 7– Peak at 2.5µl volume of withaferin-A

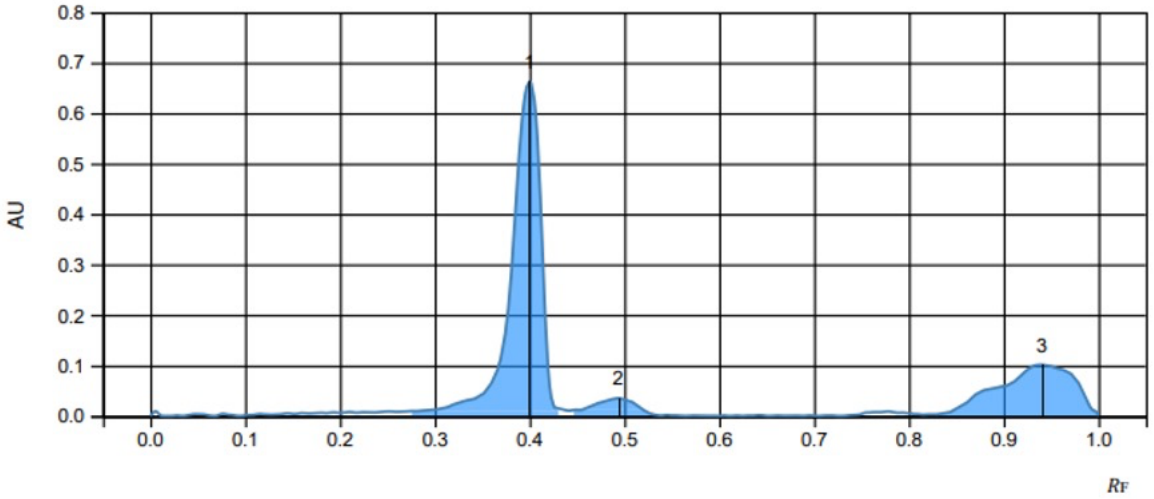

Fig 8 – Peak at 3.0µl volume of withaferin-A

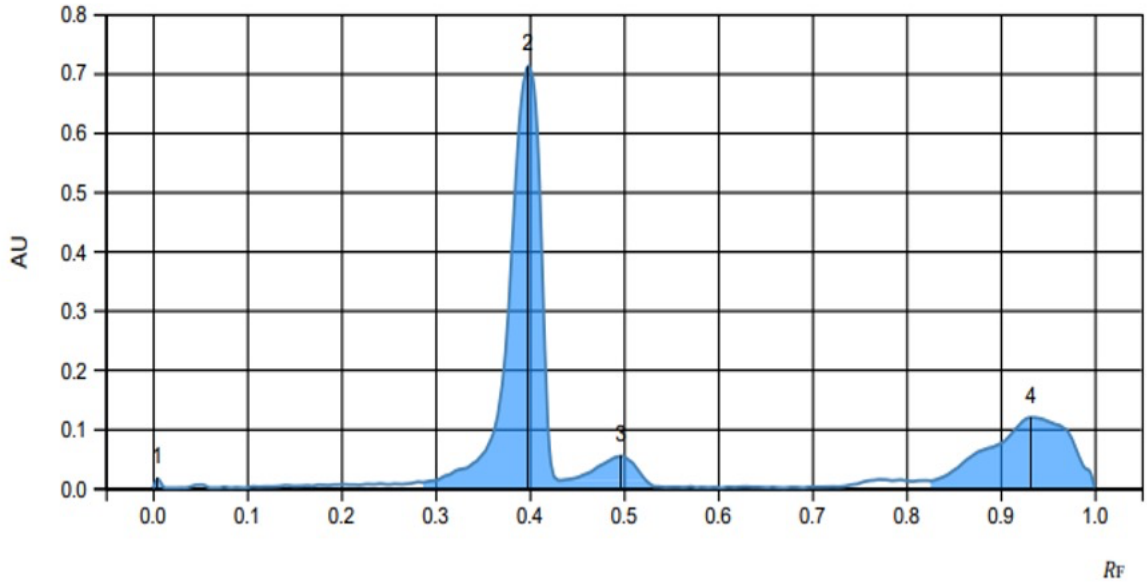

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Fig 9 – Peak at 3.5µl volume of withaferin-A

To get the optimum Withaferin-A resolution, different compositions of solvent were utilized. The utilisation of toluene–ethyl acetate (8:2) as the mobile phase enabled the chromatography of Withaferin-A to produce symmetrical and repeatable peaks. Withaferin-A's RF under these circumstances was 0.40, and the substance was clearly separated from the extract's other constituents. By correlating the UV-visible spectra of withaferin-A obtained from the separation of a Withaniasomnifera extract with that obtained from a standard, the peak purity of withaferin-A was determined.

For the quantitative determination of Withaferin A in extract total 2 tracks are used. The track 1and track 2. Track 1 chromatogram shown in Figure 10. Track 2 chromatogram shown in figure 11. For that figure 10 and figure 11the RF value and peak area of Withaferin A are shown in Table 3 and 4 respectively. In Table 3 at RF 0.498 the area obtained for Withferain A in extract isolated from soxhlet is 0.00515. Table 4 at RF 0.494 the area obtained for Withaferin A in extract isolated from soxhlet is 0.0049.

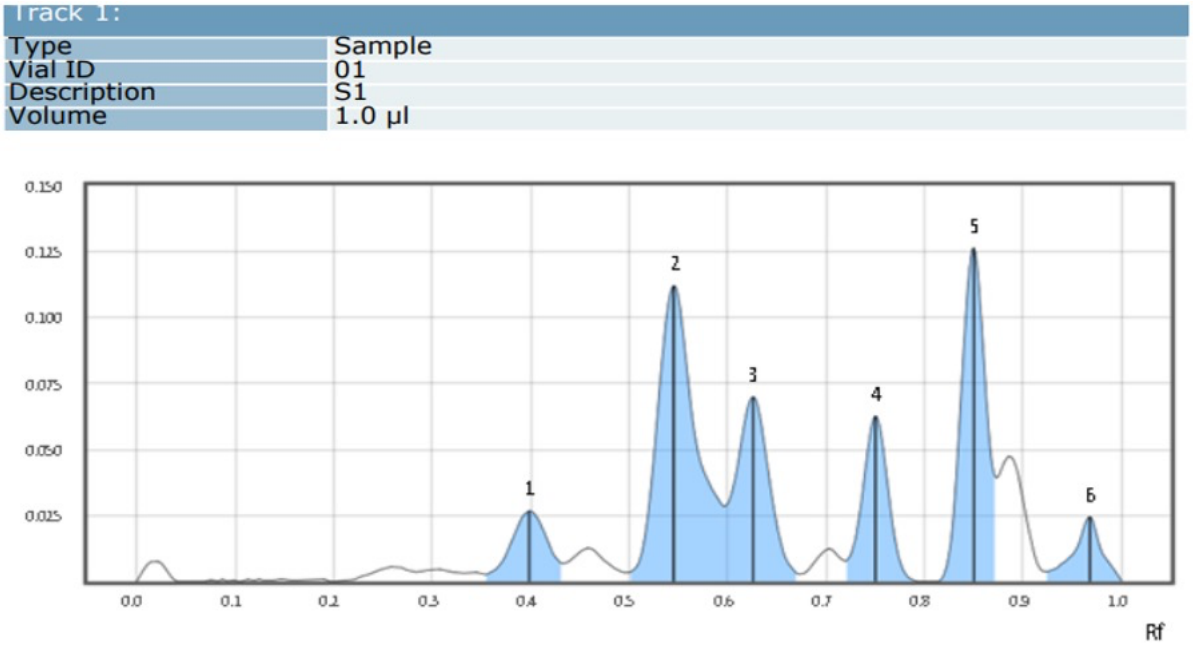

Fig 10 – Track 1 HPTLC fingerprint profile of methanol extracted Withaniasomniferaroot powder

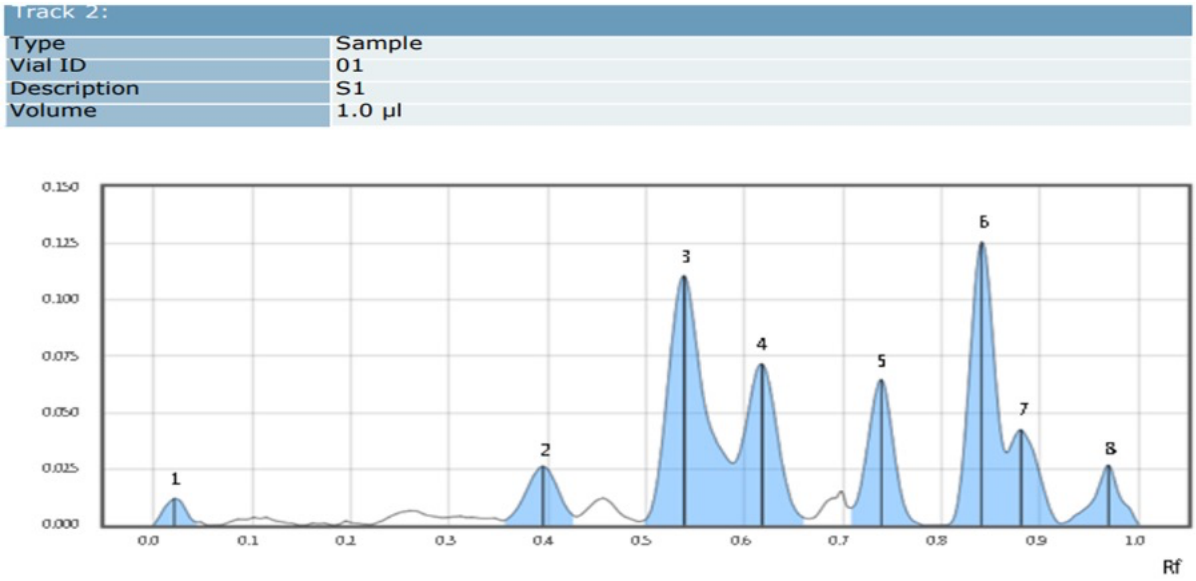

Fig11 – Track 2 HPTLC fingerprint profile of methanol extracted Withaniasomnifera root powder.

Table3: RF value and peak area of withaferin-A in methanol extracted Withaniasomnifera root powder of track 1.

Peak	Start		Max			End		Area
	RF	H	RF	H	%	RF	H	
1	0.355	0.0026	0.398	0.0267	6.34	0.432	0.0067	0.00115
2	0.498	0.0033	0.545	0.1119	26.53	0.595	0.0287	0.00515
3	0.597	0.285	0.626	0.0699	16.55	0.673	0.0027	0.00278
4	0.719	0.0077	0.75	0.0627	14.85	0.794	0	0.00191
5	0.813	0	0.85	0.1263	29.92	0.873	0.0393	0.00365
6	0.923	0.0037	0.968	0.0245	5.81	1	0.0002	0.00081

Table 4: RF value and peak area of withaferin-A in methanol extracted Withaniasomnifera root powder of track 2.

Peak	Start		Max			End		Area
	RF	H	RF	H	%	RF	H	

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1	0	0	0.021	0.0119	2.48	0.044	0.0014	0.0003
2	0.355	0.002	0.395	0.0262	5.48	0.429	0.0038	0.00103
3	0.494	0.0015	0.539	0.1106	23.07	0.587	0.0275	0.0049
4	0.587	0.0275	0.618	0.0715	14.92	0.665	0.0028	0.00283
5	0.708	0.0077	0.739	0.0646	13.49	0.782	0	0.00198
6	0.8	0	0.84	0.1255	26.18	0.866	0.0322	0.00371
7	0.866	0.0322	0.881	0.0423	8.82	0.921	0.0008	0.00139
8	0.921	0.0008	0.969	0.0267	5.57	1	0.0003	0.00083

CONCLSION

A quick, reliable, precise, and selective technique for separating withaferin-A in Withaniasomnifera is HPTLC densitometry. It is clear from the study that HPTLC performed both the qualitative and quantitative estimation with great ease.Further, Withaniasomnifera roots are enriched with withaferin-A, a major constituent of many herbal preparations. The benefit of HPTLC is the high sample throughput which results from the small amount of sample preparation required and the simultaneous quantification samples.The technique shown here considerably enhances the Withaferin-A analysis in Withaniasomnifera. It meets all requirements for a qualitative and quantitative assessment of Withaferin-A in addition to facilitating direct, quick, and accurate analysis of Withaferin-A. The suggested technique is useful for both commercial and scientific purposes and can be used to standardize Withania species based on varying dates of planting by using withaferin-A as a marker chemical.

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CONFLICT OF INTEREST

There is no conflict of interest among authors.

REFERENCES

1. Nayak P, Upadhyaya S, Upadhyaya A. HPTLC method for analysis of withaferin-A in ashwagandha (Withaniasomnifera). J Planar Chromatogr - Mod TLC. 2009;22(3):197–200.
2. Mishra LC, Singh BB, Dagenais S. Scientific basis for the therapeutic use of Withaniasomnifera (ashwagandha): A review. Altern Med Rev. 2000;5(4):334–46.
3. Shohat B, Gitter S AA, Lavie D, Lavie' S articles by 'D, D L. Antitumor activity of withaferin A (NSC-101088). Cancer Chemother Reports, 01 Sep 1967, 51(5)271-276.
4. Jayaprakasam B, Zhang Y, Seeram NP, Nair MG. Growth inhibition of human tumor cell lines by withanolides from Withaniasomnifera leaves. Life Sci. 2003;74(1):125–32.
5. Dar NJ, Hamid A, Ahmad M. Pharmacologic overview of Withaniasomnifera, the Indian Ginseng. Cell Mol Life Sci. 2015;72(23):4445–60.
6. Vitali G, Conte L, Nicoletti M. Withanolide composition and in vitro culture of Italian Withaniasomnifera. Planta Med. 1996;62(3):287–8.
7. Vitalini S, Tomè F, Fico G. Traditional uses of medicinal plants in Valvestino (Italy). J Ethnopharmacol. 2009;121(1):106–16.
8. Hossain AM NM. Biochemical Profiling and Total Flavonoids Contents of Leaves Crude Extract of Endemic Medicinal Plant Corydylineterminalis L. Kunth. Phcog J 2011; 3 25-30 [Internet]. 2011;3(24):25–30. Available from: https://doi.org/10.5530/pj.2011.24.5
9. Gurib-fakim A. Medicinal plants : Traditions of yesterday and drugs of tomorrow. 2006;27:1–93.
10. Shanbhag DA JS. Application of HPTLC in standardization of homoeopathic mother tincture Andrographispaniculata and its comparison with market products. Asian J Chem. 2008;4:155–159.
11. Sharma V, Gupta AP, Bhandari P, Gupta RC, Singh B. A validated and densitometric HPTLC method for the quantification of withaferin-A and withanolide-A in different plant parts of two morphotypes of Withaniasomnifera. Chromatographia. 2007;66(9–10):801–4.
12. R Asthana MR-I drugs. Pharmacology of Withaniasomnifera (L.) Dunal-a review. 1989;
13. Devi PU. WithaniasomniferaDunal (Ashwagandha): potential plant source of a promising drug for cancer chemotherapy and radiosensitization. Indian J Exp Biol. 34(10):927–32.
14. S Singh SK-. Central Institute of Med and Arom Plants, p 293. 1998;298.
15. Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazón J. Steroidal lactones from Withaniasomnifera, an ancient plant for novel medicine. Molecules. 2009;14(7):2373–93.
16. Scartezzini P, Speroni E. Review on some plants of Indian traditional medicine with antioxidant activity. J Ethnopharmacol. 2000;71(1–2):23–43.
17. Sharada AC, Solomon FE, Devi PU, Udupa N, Srinivasan KK. Antitumor and radiosensitizing effects of withaferina on mouse ehrlich ascites carcinoma in vivo. ActaOncol (Madr). 1996;35(1):95–100.
18. Ziauddin M, Phansalkar N, Patki P, Diwanay S, Patwardhan B. Studies on the immunomodulatory effects of Ashwagandha. J Ethnopharmacol. 1996;50(2):69–76.

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19. RD Budhiraja SS. Review of biological activity of withanolides. J SciInd Res (India). 1987;46:488–91.
20. Ray AB GM. Withasteroids, a Growing Group of Naturally Occurring Steroidal Lactones. ProgChem Org Nat Prod. 1994;63:1–106.