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FLAVONOIDS – CHEMICAL CONSTITUENTS OF *WITHANIA SOMNIFERA* DUNAL THROUGH TLC AND HPTLC

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ABSTRACT:

Withania somnifera Dunal (Ashwagandha) is important medicinal plant. Plant tissue culture is very useful to increase the production of secondary metabolites. Callus was obtained on MS supplemented with 2 mg/l 2, 4-D and 0.2 mg/l KIN. Chromatography is the second important technique for analysis and isolation of these herbal drugs. Qualitative and quantitative analysis was done through TLC and HPTLC. TLC of *in vitro* suggested quercetin, kaempferol, 14 unidentified free flavonoids and 22 unidentified bound flavonoids were obtained in *in vitro* materials. HPTLC of *in vitro* materials was done and scanned at different wavelengths (200 nm, 254 nm, 366 nm). Calibration spectrum, Rf values and amount of particular spot were recorded at particular wavelength through scanning. Total 7 flavonoids at 200 nm, 7 at 254 nm, 4 at 366 nm were analyzed. Absorption spectra of each substance were also prepared at 200 nm. Some of these are confirmed while others are unidentified.

KEY WORD: *Steroids, Withania somnifera, TLC, HPTLC.*

INTRODUCTION:

Plants are very important commercial source of chemical compounds including primary and secondary metabolites. Ayurveda practitioner employ these plant to cure swelling, poultices lesions, tubercular ulcers, scabies, ophthalmia, muscular pain, dropsy, rheumatism, diabetes and even cancer. These properties are mainly because of its primary and secondary metabolites of drugs. This drug is naturally present in the parts like leaf, stem, root, seed or some times in the whole plant. These parts can be artificially cultured on the media and can get maximum amount of drugs. For it, Murashige and Skoog's, 1962 (MS) medium is used as a basal nutrient medium. Different types of growth promoters like 2, 4-D, IAA, IBA, NAA, Kinetin, BAP are used to prepare hormonal medium. Even it can be prepared with alone auxin or cytokinin or in combination of both (Vasil, 1984). Amount of drugs, which are artificially produced in 8 week old callus can be compared with naturally occur in plant (whole plant). Plant tissue

culture approach has been found to be advantageous as it provides a continuous and reliable source of artificial product year around without the destruction of entire plant. With the help of tissue culture, high quantities of desired compounds can be obtained.

Flavonoids have 15-carbon in their basic skeleton. They are widely spread, soluble phenolic derivatives that are variously coloured. They are restricted to the vacuole though some are found in chloroplast and chromoplast. Flavonoids are glycosides and their glycones vary depending upon the flavan structure, which comprises two aromatic rings joined in a chroman structure by a 3-carbon unit (C_6-C_3-C_6). Free glycones are found in dead woody tissue and formed from free flavonoids through hydrolysis and are variously classified depending upon the state of oxidation of the C_3 unit in the molecule. The flavonoids, chalcones etc. are chief classes of flavonoids, which contribute to the colour scheme of the plants. On the contrary, flavonols and flavones do not contribute to the colour of various plant organs. Bioflavonoids have as many as C_{30} atoms in their basic skeleton and are obtained from dimerisation of flavones by C-C linkage. They are found in the gymnosperms. Flavonoids directly form the C_6-C_3 portion of phenylpropanoids. Flavonoids are distinguished by various increasing state of the C_3 chain. Glycosides involving fine aglycones were isolated from parsley cell suspension culture. The flavones (apigenin, luteolin, chrysoniol) occurred either as 7-0-glycoside or 7-0-apiglycosides while the flavonols (quercetin, isorhamnetin) are 3-0-monoglycoside or 3-7-diglycosides. β-glycosidase enzyme for degradation of flavonol-3-glycoside but each cell has not this enzyme so degradation of flavonoid is not so common. Based on the number of carbon atoms in their skeleton, phenolics are divided into several major groups (Hess, 1975) (1) Simple phenolics (2) Phenol carboxylic acid (3) Flavan derivatives. Hence, the present investigation was taken up for qualitative and quantitative analysis of *in vitro* produced flavonoids through TLC and HPTLC.

PLANT MATERIAL:

Scientific name **:** *Withania somnifera* Dunal

Family **:** Solanaceae

Common names **:** Asandh, Asagandh, Ashwagandha (Nadkarni, 1954)

Known chemical constituents:

Alkaloids: Withanine, Withaninine, Somniferine, Tropeltigloate, Somniferinine, Somninine, Nicotine, Visamine, Withasomine

Salts: Cuscohygrine, Anahygrine, Tropine, Pseudotropine, Anaferine

Steroidal Lactones: Withaferin-A, Withanone, WS-1, Withanolide E $C_{28}H_{38}O_7$, Withanolide F $C_{28}H_{38}O_6$, Withanolide G $C_{28}H_{36}O_4$, Withanolide H $C_{28}H_{36}O_5$, Withanolide I $C_{28}H_{36}O_5$, Withanolide J $C_{28}H_{36}O_5$, Withanolide K $C_{28}H_{36}O_5$, Withanolide L $C_{28}H_{36}O_5$, Withanolide M $C_{28}H_{36}O_6$

Nitrogen containing compounds: Withanol $C_{25}H_{34}O_5$, Somnisol $C_{32}H_{46}O$, Somnitol $C_{33}H_{46}O_7$

Steroids:Cholesterol, β-sitosterol, Stigmasterol, Diosgenin, Stigmastadien, Sitoinosides VII, Sitoinosides VIII, Sitoinosides IX, Sitoinosides X

Flavonoids: Kaempferol, Quercetin

Medicinal properties and uses: Leaves and roots of this plant are abortifacient, aphrodisiac, diuretic, nervine tonic, alterative, narcotic, sedative, astringent, growth promoter and anthelmintic. It has anti-arthritic, anti-bacterial, anti-dote for scorpion sting, anti-stress, anti-tumour and anti-cancer activities. It is used in toning of uterus, consumption, dropsy, leucoderma, impotence, rheumatism, debility from old age, ulcer, sexual and genital weakness, assumption, rheumatic swelling, loss of memory, loss of muscular energy, spermatorrhoea, syphilis, sterility of women, blood discharge, leucorrhoea, anemia with emaciation, nervous exhaustion, multiple sclerosis, neoplasia, cancer and fatigue. Fruits and seeds are diuretic and used in coagulation of milk (Nadkarni, 1954).

MATERIALS AND METHODS:

(A) TISUE CULTURE

1. Preparation of nutrient medium
   - Preparation of stock solutions
   - Mix the solutions and stir it
   - Dissolve sugar and add agar-agar
   - Heat the above solution till agar-agar dissolve
   - Add stock solutions of PGRs and make up the volume
   - Adjust pH with HCl or NaOH to 6.8
   - Pour the medium into culture vials
   - Plug the vials with non-absorbent cotton
   - Autoclave for 15 minutes at 120^0 C and cool it
2. Inoculation and Culturing of the explants
   - Explants should be washed in soap water, tap water and finally rinsed with distilled water
   - Under aseptic condition treatment with 0.1% $HgCl_2$ is given for few minutes and finally rinsed with DW
   - Explants are introduced into the vial over a flame to avoid microbial contamination

- Immediately plug the vial

3. Maintaining the culture vials under controlled light and temperature conditions
4. Periodic observations of the cultured explants
5. Maintaining the culture through subculture
6. Conclude and make inferences about the experiment conducted / field trial of *in vitro* raised plantlets

(B) THIN LAYER CHROMATOGRAPHY (TLC)

The operation performed in TLC is essentially the same as in paper chromatography. This technique involves several steps:

1. Preparing thin layer (e.g. silica gel G.)
2. Choice of solid for support (e.g. glass plate)
3. Sample application

Extraction of flavonoids: (Subramanian and Nagarajan, 1969)

Procedure**:** 100 mg dried, powdered callus was extracted with methanol in soxhlet apparatus. Petroleum ether was mixed with methanolic extracts. Both the layer was separated with the help of separating funnel. Methanolic fraction was several times washed with petroleum ether. Petroleum ether fraction (fraction I) was discarded due to the presence of fatty substances. Solvent ether was added to the first methanolic part in the ratio of 1:1. This fraction (fraction-2) was further analysed for free flavonoids. Ethyl acetate was added to the methanolic part in the ratio of 1:1. This fraction (fraction-3) was further analysed for bound flavonoids.

Sample extract: Fraction-1 was discarded**.**

Fraction-2 was redissolved in solvent ether

Fraction-3 was redissolved in ethyl acetate

4. Choice of solvent (mobile phase)

Benzene : Acetic Acid (125:25) for flavonoids

5. Development of chromatograph
6. Detecting or spraying reagent
7. Identification and calculation of Rf value

$$Rf = \frac{\text{Distance traveled by sample}}{\text{Distance traveled by solvent}}$$

(C) HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) (Sethi, 1996)

- Formulation : Herbal drugs
- Classification : Alkaloids / Steroids / Flavonoids
- Dosage form : Liquid
- Sample preparation

Extraction of flavonoids: (Subramanian and Nagarajan, 1969)

Procedure: 100 mg dried, powdered callus was extracted with methanol in soxhlet apparatus. Petroleum ether was mixed with methanolic extracts. Both the layer was separated with the help of separating funnel. Methanolic fraction was several times washed with petroleum ether. Petroleum ether fraction (fraction I) was discarded due to the presence of fatty substances. Solvent ether was added to the first methanolic part in the ratio of 1:1. This fraction (fraction-2) was further analysed for free flavonoids. Ethyl acetate was added to the methanolic part in the ratio of 1:1. This fraction (fraction-3) was further analysed for bound flavonoids.

Sample extract: Fraction-1 was discarded.Fraction-2 was redissolved in solvent ether Fraction-3 was redissolved in ethyl acetate

Mobile phase: Benzene : Acetic Acid (125:25) for flavonoids

- Conditions of Chromatography:

1. Test plate : HPTLC precoated plate, silica gel 60 F_{254} – aluminium (Merk)
2. Format : 10 X 10 cm^2
3. Thickness : 250 µm
4. Spotting volume : 2 µl,4 µl,6 µl, 8 µl (Linomat IV)
5. Separation technique : Ascending
6. Development chamber : Twin-trough glass chamber (10 X 10 cm^2) (Camag) (saturate for 10 minutes prior to development)
7. Mobile phase : Benzene : Acetic Acid (125:25) for flavonoids
8. Spraying reagent : 0.1 % $AlCl_3$ in alcohol
9. Relative Humidity : 52%
10. Temperature : 24° C
11. Migration distance : 80 mm
12. Migration time : (30 minutes for flavonoids)
13. Detection : UV

- Densitometric Scanning (Camag Scanner III)
  1. Wavelength and Mode
     a. 200 / 254 nm - Absorbance / Reflectance
     b. 366 nm – Florescence / Reflectance
  2. Slit Dimension : 6 X 0.45 mm

RESULTS AND DISCUSSION:

Table (1): Tissue culture results of *Withania somnifera*

Explants	Level of Auxin		Level of cytokinin		Callus initiation (in weeks)				Characters
	Name	Concentration (mg/l)	Name	Concentration (mg/l)	1	2	3	4	
Nodal region with leaf	2,4-D	1	BAP	1	-	-	+	+	Green coloured, compact and nodular callus
		2		2	-	+	+	+	
		3		3	-	+	+	++	
		4		4	+	++	++	++	
Leaf	2,4-D	2.0	KIN	0.5	-	++	++	++	Whitish green coloured callus, less friable
		2.0		0.25	+	++	+++	+++	

Table (2): TLC analysis of *Withania somnifera* flavonoids from 8 week old callus

Plants material	Compounds	Spray treatment		Iodine treatment	
		Rf	Colour	Rf	Colour
Free Flavonoids					
Callus	Unidentified 1	0.65	Brown	0.65	Brown
	Unidentified 2	0.67	Brown	0.67	Brown
	Unidentified 3	0.69	Brown	-	-
	Unidentified 4	0.73	Brown	0.73	Brown
	Unidentified 5	0.84	Brown	-	-
	Kaempferol	0.88	Yellow	0.88	Yellow
Bound Flavonoids					
	Unidentified 1	-	-	0.05	Brown
	Unidentified 2	-	-	0.07	Brown
	Unidentified 3	-	-	0.09	Brown
	Unidentified 4	-	-	0.15	Brown
	Unidentified 5	-	-	0.23	Brown
	Unidentified 6	-	-	0.27	Brown
	Unidentified 7	0.34	Light Brown	0.33	Brown
	Unidentified 8	-	-	0.47	Brown

Callus	Unidentified 9	0.55	Brown	-	-
	Unidentified10	0.56	Brown	-	-
	Unidentified 11	0.59	Brown	-	-
	Unidentified 12	0.62	Green Brown	-	-
	Unidentified 13	0.64	Green Brown	-	-
	Unidentified14	0.66	Green Brown	-	-
	Unidentified 15	0.69	Green Brown	-	-
	Unidentified 16	0.71	Green Brown	-	-
	Unidentified 17	0.73	Green Brown	-	-
	Unidentified18	0.75	Yellow green	-	-
	Quercetin	0.78	Yellow	0.78	Brown
	Unidentified 19	0.82	Dark Brown	-	-
	Unidentified 20	0.85	Dark Brown	0.86	Brown
	Unidentified 21	0.89	Dark Brown	0.89	Brown
	Unidentified 22	0.91	Dark Brown	0.91	Brown

Table (3): HPTLC results of Flavonoids of 8 week old callus of *Withania somnifera*

Secondary metabolite	Spot number	Scanning at different wavelengths					
		200 nm		254 nm		580 nm	
		Rf	Area %	Rf	Area %	Rf	Area %
Flavonoids	Spot 1	0.02	55.50	0.02	56.15	-	-
	Spot 2	-	-	-	-	0.04	83.27
	Spot 3	0.13	13.55	0.13	17.52	-	-
	Spot 4	-	-	-	-	0.34	5.62
	Spot 5	-	-	-	-	0.40	4.64
	Spot 6	0.46	14.94	0.47	2.26	-	-
	Spot 7	0.52	3.75	0.52	6.37	-	-
	Spot 8	0.60	1.79	0.60	1.64	-	-
	Spot 9	0.82	4.22	0.83	5.06	-	-
	Spot 10	0.91	6.86	0.91	11.00	0.92	6.47
Total		**7**		**7**		**4**	

Plant tissue culture or the aseptic culture of cells, tissues and organs is an important tool in both basic and applied studies. Callus was obtain on MS medium supplemented with combinations of 2,4-D + BAP and 2,4-D + KIN in *Withania somnifera*. The satisfactory result was obtained on MS + 2mg/l 2,4-D + 0.25mg/l KIN **(Table-1)**. TLC results suggested that Total 5 unidentified free flavonoids, kaempferol in callus and 22 unidentified bound flavonoids, quercetin in callus were analysed from *in vitro* (8 week old callus) *W. somnifera.* Some of secondary metabolites were identified with iodine treatment only **(Table 2)**. HPTLC results suggested that *In vitro* materials (8 week old callus) of *W. somnifera* were scanned at different wavelengths (200 nm, 254 nm, 366 nm). Calibration spectrum, Rf values and maximum absorbance at particular wavelength of each substance were recorded through scanning. Total 7 flavonoids at 200 nm, 7 at 254 nm, 4 at 580 nm were scanned from *in vitro* (8 week

old callus) *W. somnifera.* Absorption spectra of total 6 flavonoids at 190 nm were obtained from *W. somnifera* **(Table-3, Figure 1, 2, 3, Photo-1, 2, 3, 4).** Kundu et al., 1979 have given the chemistry of withanolides. Moza and Singh, 1967 have estimated total alkaloids. Gupta et al., 1996 have used TLC densitometry for withaferin-A. Bhar and Hansel, 1982; Vitali et al., 1996; Bhattacharya et al., 1997 showed immuno-modulatory and anti-oxidant activities through steroidal lactones – withanolides of *W. somnifera.* Asthana and Raina, 1989 have given the pharmacology of *W. somnifera.* Saxena et al (2012) had deeply studied few medicinal plants and its chemical constituents with the help of TLC and HPTLC at Botany department, Gujarat University, Ahmedabad, Gujarat.

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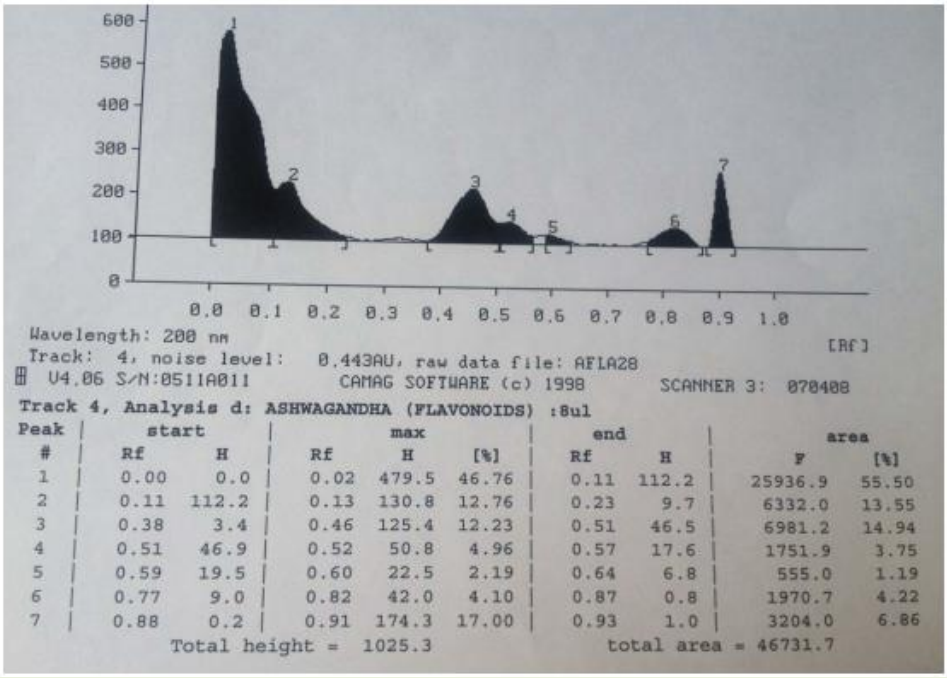
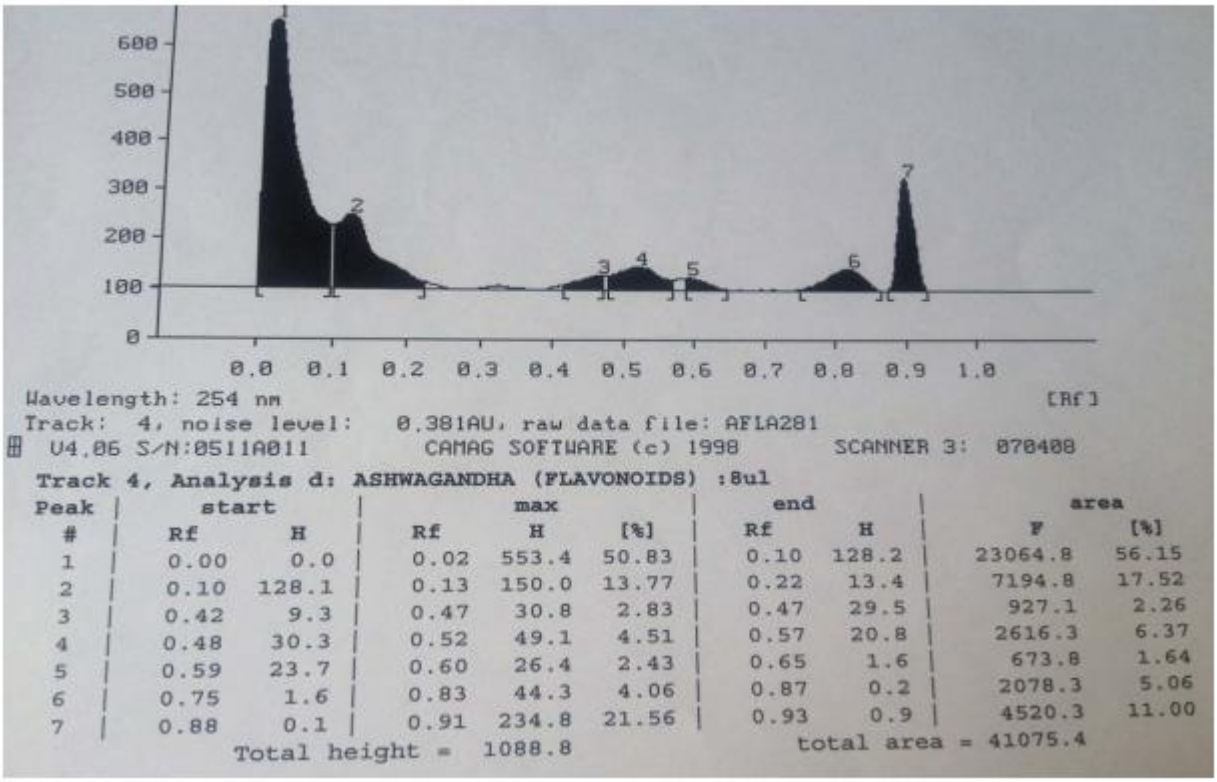
Figure 1: quantitative estimation at 200 nm through HPTLC



Peak #	start Rf	start H	max Rf	max H	max [%]	end Rf	end H	area F	area [%]
1	0.00	0.0	0.02	479.5	46.76	0.11	112.2	25936.9	55.50
2	0.11	112.2	0.13	130.8	12.76	0.23	9.7	6332.0	13.55
3	0.38	3.4	0.46	125.4	12.23	0.51	46.5	6981.2	14.94
4	0.51	46.9	0.52	50.8	4.96	0.57	17.6	1751.9	3.75
5	0.59	19.5	0.60	22.5	2.19	0.64	6.8	555.0	1.19
6	0.77	9.0	0.82	42.0	4.10	0.87	0.8	1970.7	4.22
7	0.88	0.2	0.91	174.3	17.00	0.93	1.0	3204.0	6.86

Total height = 1025.3 total area = 46731.7

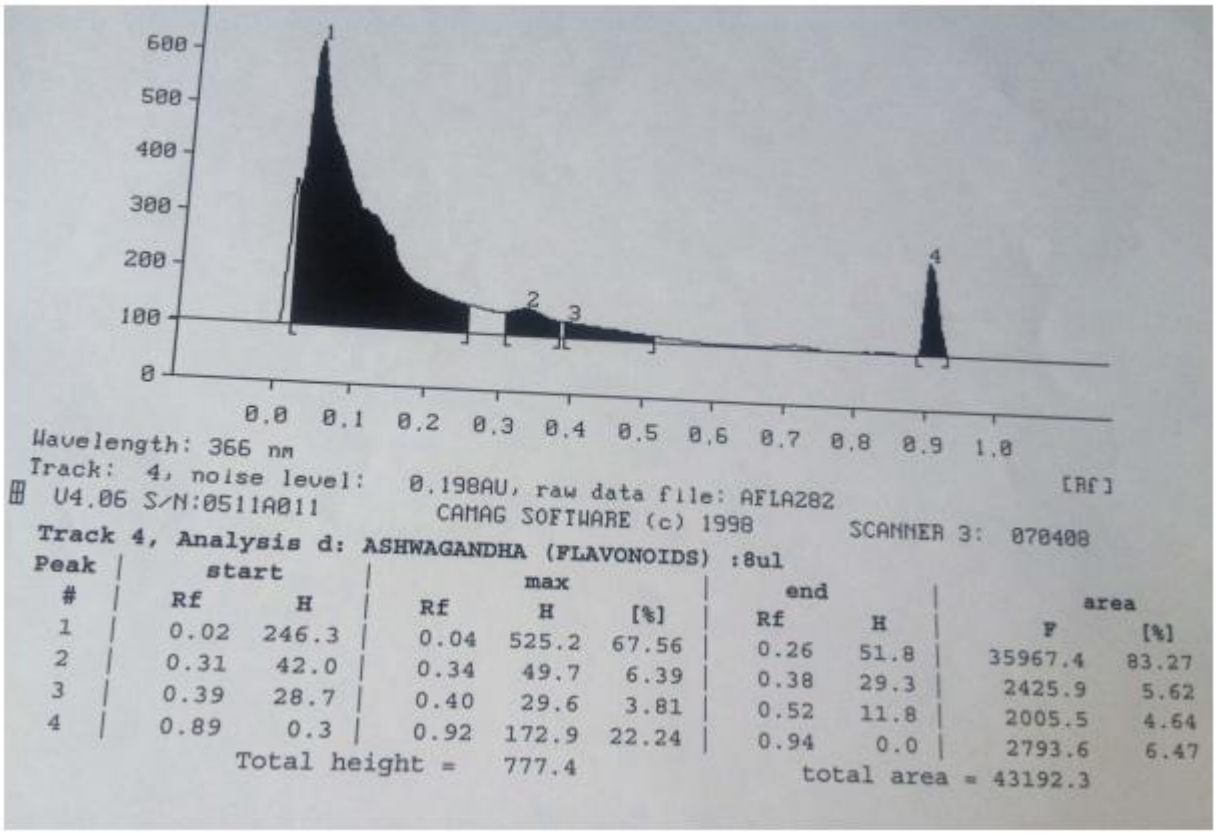
Figure 2: quantitative estimation at 254 nm through HPTLC



Peak #	start Rf	start H	max Rf	max H	max [%]	end Rf	end H	area F	area [%]
1	0.00	0.0	0.02	553.4	50.83	0.10	128.2	23064.8	56.15
2	0.10	128.1	0.13	150.0	13.77	0.22	13.4	7194.8	17.52
3	0.42	9.3	0.47	30.8	2.83	0.47	29.5	927.1	2.26
4	0.48	30.3	0.52	49.1	4.51	0.57	20.8	2616.3	6.37
5	0.59	23.7	0.60	26.4	2.43	0.65	1.6	673.8	1.64
6	0.75	1.6	0.83	44.3	4.06	0.87	0.2	2078.3	5.06
7	0.88	0.1	0.91	234.8	21.56	0.93	0.9	4520.3	11.00

Total height = 1088.8 total area = 41075.4

Figure 3: quantitative estimation at 580 nm through HPTLC



Peak #	start Rf	start H	max Rf	max H	max [%]	end Rf	end H	area F	area [%]
1	0.02	246.3	0.04	525.2	67.56	0.26	51.8	35967.4	83.27
2	0.31	42.0	0.34	49.7	6.39	0.38	29.3	2425.9	5.62
3	0.39	28.7	0.40	29.6	3.81	0.52	11.8	2005.5	4.64
4	0.89	0.3	0.92	172.9	22.24	0.94	0.0	2793.6	6.47

Total height = 777.4 total area = 43192.3

Photo 1: HPTLC photo under fluorescent light

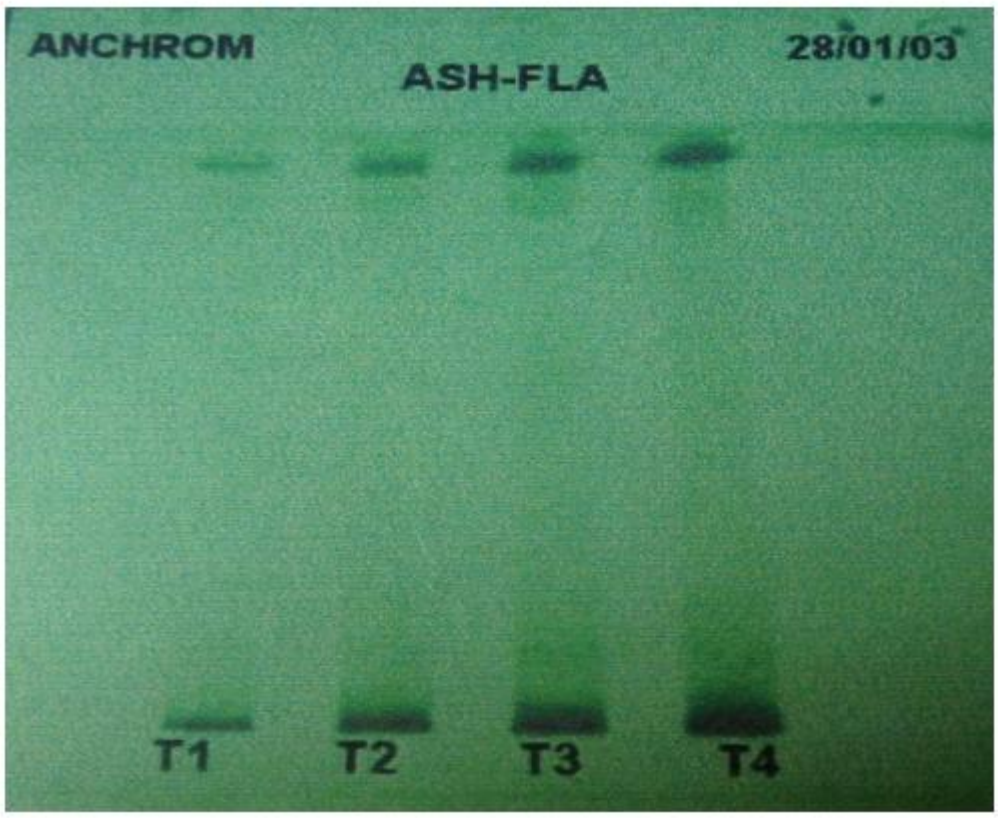


Photo 2: HPTLC photo under UV light without stain

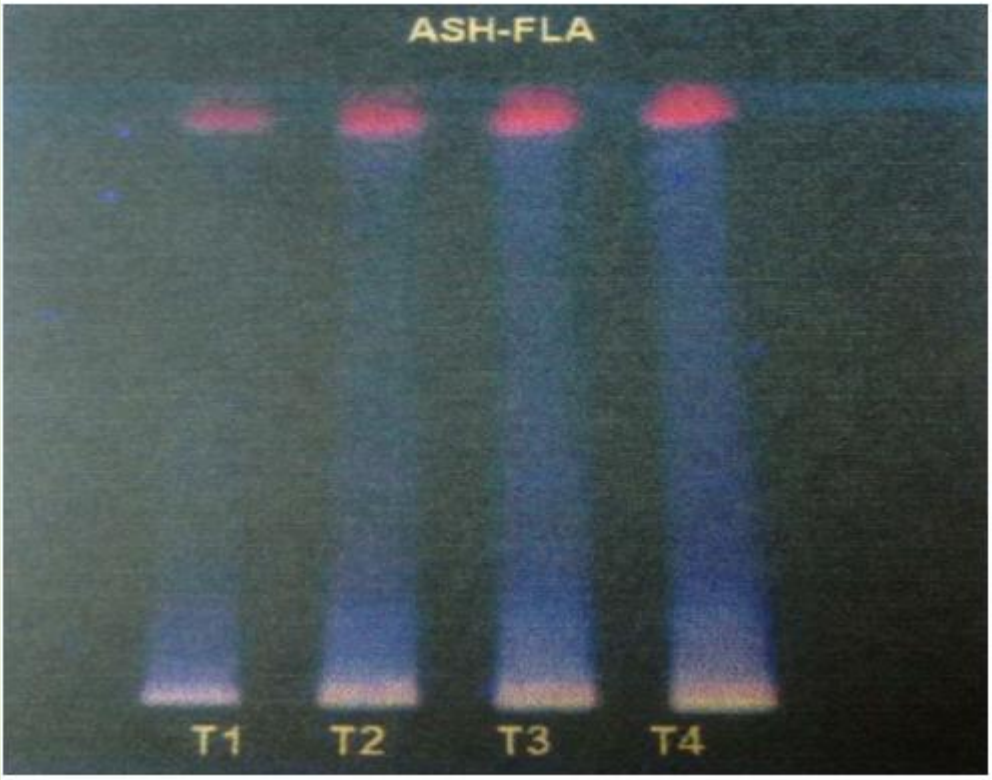


Photo 3: HPTLC photo under UV light with stain

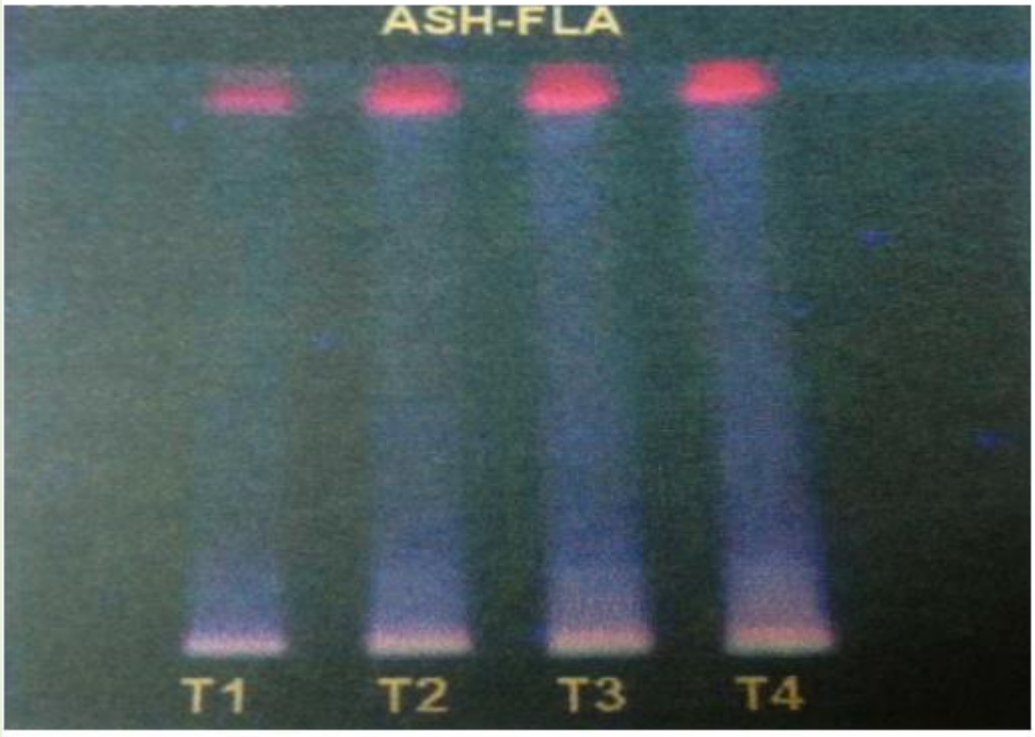


Photo 4: HPTLC photo under visible light