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Research Article

DEVELOPMENT AND VALIDATION OF HPTLC METHOD FOR THE DETERMINATION OF ANDROGRAPHOLIDE IN KALMEGH NAVAYAS LOHA- AN AYURVEDIC FORMULATION

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ABSTRACT

A simple, rapid, selective and quantitative HPTLC method has been developed for determination of Andrographolide in *Andrographis paniculata* (Whole Plant) and its formulation-Kalmegh Navaya Loha. The alcoholic extract of *Andrographis paniculata* (Whole Plant) and its ayurvedic formulation-Kalmegh Navayas Loha samples were applied on TLC Aluminium plate pre coated with Silica gel60 GF_{254} and developed using Toluene : Ethyl acetate : Formic acid (5:4.5:0.5) v/v as a mobile phase. The plate was sprayed (derivatized) with Anisaldehyde- Sulphuric Acid reagent followed by heating at 110^0C for 10 minutes and detection and quantification were carried out densitometrically using an UV detector at wavelength of 235 nm. Content of marker compound- Andrographolide found in the *Andrographis paniculata* (Whole Plant) and its formulation-Kalmegh Navaya Loha were 0.7746% w/w and 0.1155 % w/w respectively.

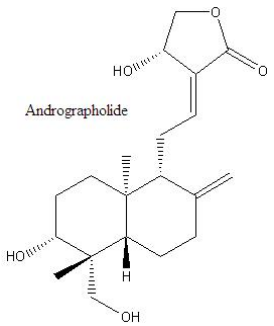
Keywords: Andrographolide, *Andrographis paniculata* (Whole Plant), Kalmegh, Bhunimba, Chiretta, King of Bitter, HPTLC, Kalmegh Navayas Loha, Ayurvedic formulation.

INTRODUCTION

Kalmegh is vernacularly known as Kirata, Chrayetah, Kiryat, Nila vembu, Karyatu, Kreata, Nelaberu, Mahatita, Chiretta, Creat, King of Bitter etc. It is chiefly found in the plains throughout India from Himachal to Assam and Mizoram, West Bengal and all over south India.

Kalmegh is used mainly for liver disorders and jaundice. A decoction or infusion of leaves is used in general debility and dyspepsia and a tincture of the root as a tonic, stimulant and aperients. The macerated leaves and juice, together with carminative spices such as cardamom, clove and cinnamon, may be made into pills and prescribed for gripe and other stomach ailments in infants. The leaves and roots also find use as an adjunct in the treatment of diabetes, malaria, cholera dysentery, enteritis, gastritis, pneumonia, pyelonephritis, diarrhea and even rabies [1-6]. The plant, especially the leaves, has been used to treat dhatoora (datura) poisoning, maggots in wounds, worms in the eyes and abdomen, liver fluke, glossitis, holes in the hard palate, constipation, tuberculosis, leeches in the nostrils, contagious abortion, retention of placenta, tetanus and scabies [7].

The plant contains bitter glucosides: andrographolide, neoandrographolide, panaculoside, flavnoids, andrographonin, panicalin, apigenin 7-4-dimethyl ether4]; diterpenoids- 14-deoxy-11-oxo- andrographolide; 14-deoxy-11,12-didehydroandro-grapholide, 14-deoxyandrographolide, neo- androgra-pholide and andrographolide. The roots gave flavones-apigenin-7,4'-di-O-methyl ether, 5-hydroxy-7,8,2'3'-tetramethoxyflavone, andrographonin and panicolin and α-sitosterol. Leaves contain homoandrographolide, andrographosterol and andrographone[2-12].



Literature survey reveals that the TLC and HPTLC methods are not reported yet for the determination of Andrographolide in *Andrographis paniculata* (whole plant). This method can be used for phytochemical profiling of *Andrographis paniculata* whole plant and quantification of Andrographolide.

With increasing demand for herbal products in medicines and cosmetics there is an urgent need for standardization. So the aim of the work is to develop a simple, rapid, selective and cost effective HPTLC method for the quality evaluation of herbal products containing *Andrographis paniculata* (Whole plant) in market samples.

MATERIAL AND METHOD

Material

(i) Kalmegh whole plant was Locally collected sample from Ghaziabad. It was identified and authenticated by the Botanists of Pharmacopoeial Laboratory for Indian Medicine, Ghaziabad.

(ii) An herbal product KALMEGH NAVAYAS LOHA**,** B. No. Nil containing *Andrographis paniculata* (Whole plant) was procured from the Local Market, Ghaziabad.

Label claimed- Each pills contains:

Badi Haran (Terminalia chebula), Bahera (Terminalia belerica), Kalimirch (Piper nigrum), Pippali (Piper longum), Sonth (Zingiber officinale), Amla (Embelica officinalis), Chitrak (Plumbago zeylanica), Baybidang (Embelica ripes), Nagarmotha (Cyprus rotundus) each equal parts; Kalmegh Kwath (Andrographis paniculata) Q.S. and Loha Bhasma(Farrum-calcined) – 9parts.

H.P.T.L.C. (High Performance Thin Layer Chromatography)

Equipment

A Cammag (Switzerland) HPTLC system equipped with a sample applicator Linomat V, Twin trough glass Chamber (20x10 cm^2) with SS lid, TLC Scanner III, Reprostar III and Wincats an integrated Software 4.02 (Switzerland), Rotavapour.

Chemicals and reagents

Analytical grade; Alcohol, Toluene, ethyl acetate, Formic acid, Chloroform, Methanol, Anisaldehyde, Sulphuric acid and n-Hexane were used; obtained from S.D. Fine Chem. Ltd. (Mumbai, India). TLC Aluminium pre coated plate with Silica gel 60 GF_{254} (20x10 cm^2; 0.2 mm thick) used were obtained from E. Merck Ltd. (Mumbai, India).

Reference standard- Andrographolide procured from Natural Remedies Pvt. Ltd., Bangalore (CAS No. 5508-58-7).

Sample and standard preparation

Sample preparation

(1) 1g of coarsely powdered drug sample (Kalmegh Navayas Loha) was extracted with 10 ml absolute alcohol for 24 hours by cold extraction method. The extract was filtered by Whatmann filter paper and make up to 10 ml in a volumetric flask. Filtrate was concentrated to 2 ml and used for T.L.C.

(2) 1g of coarsely powdered drug sample (Kalmegh whole plant) was extracted with 10 ml absolute alcohol for 24 hours by cold extraction method. The extract was filtered by Whatmann filter paper and make up to 10 ml in a volumetric flask.

Standard preparation

5mg of standard Andrographolide dissolved in 5ml of absolute alcohol and made up to 5ml in standard volumetric flask.

Chromatography

TLC Aluminium pre coated plate with Silica gel60 GF_{254} (20x10 cm^2; 0.2 mm thick) was used with Toluene : Ethyl acetate : Formic acid (5:4.5:0.5) V/V as mobile phase. absolute alcoholic extract of samples and Andrographolide standard solution applied on plate by using Linomat V applicator. Cammag Twin Trough Glass Chamber (20x10 cm^2) with SS lid was used for development of TLC plate. The Twin Trough Glass Chamber was saturated with mobile phase for 30 minutes. TLC plate was developed to 8 cm distance above the position of the sample application. The plate was removed from the chamber and air dried at room temperature. This plate was sprayed (derivatized) with Anisaldehyde- Sulphuric Acid reagent followed by heating at 110^0C for 10 minutes and HPTLC finger print profile was snapped by Cammag Reprostar III, before deivatization under UV 254 nm, 366 nm and after derivatization (Fig.1). The plate was scanned before derivatization using Camag TLC Scanner III at wavelength 235nm. Wincats an integrated Software 4.02 was used for the detection as well as for the evaluation of data.

Method validation and recovery study

To study the accuracy and precision of the proposed method, recovery experiment was carried out. To a fixed amount of absolute alcoholic extract of samples, the standard solution of Andrographolide was added (ratio 9:1 v/v) and total amount of standard Andrographolide were determined. Percent recovery was calculated from the amount of Andrographolide found via graph (Table No. 4).

Linearity of detector response, assay and recovery

In order to establish linearity, standard solution of Andrographolide (1mg/ml) applied on TLC Aluminium pre coated plate with Silica gel60 GF_{254} (20X10 cm^2; 0.2 mm thick), 2.5µl, 5µl, 10µl on Track No. S1, S2 & S3 respectively and for assay, absolute alcoholic extract of both samples applied on Track No. T1 & T2 and for recovery study, the absolute alcoholic extract of both samples were spiked with standard Andrographolide solution (ratio 9:1v/v) and applied on Track No. T3 & T4 on the same plate. TLC plates was developed to 8 cm and was scanned immediately before derivatization using Camag TLC Scanner III at wavelength 235nm. It was observed that Andrographolide appeared at R_f. 0.38 (dark grey colour). The peaks, graph and spectra obtained were given in Fig.2 and 3 and R_f. values, colour of bands (Table No.2), quantity of Andrographolide linearity, standard deviation & regression coefficient found via graph (Table No.3) and calculated quantity of Andrographolide & % recovery were given in Table No. 4.

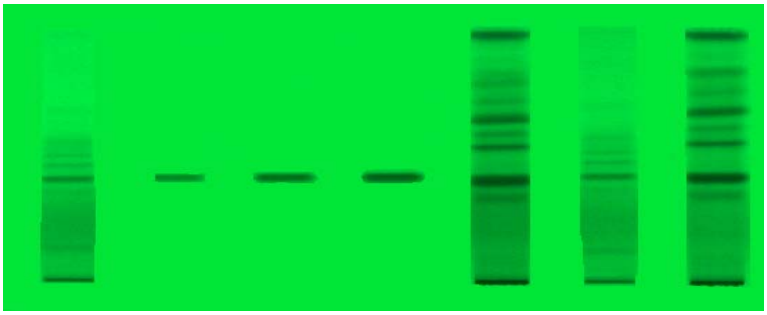
Table 2: HPTLC chromatogram

Detection/ visualization	Kalmegh whole plant (Track No. T1 and T3)		Standard- Andrographolide (Track No. S1, S2 and S3)		Kalmegh Navayas Loha (Track No. T2 and T4)	
	R_f. Values	Colour of band	R_f. values	Colour of band	R_f. values	Colour of band
Under UV 254 nm	0.10	Gery	0.38	dark grey	0.30	grey
	0.38	dark grey			0.38	dark grey
	0.47	grey			0.52	dark grey
	0.52	grey			0.55	grey
	0.59	grey			0.59	dark grey
	0.73	grey			0.67	grey
	0.87	grey			0.73	grey
					0.77	grey
					0.90	dark grey
Under UV 366 nm	0.10	green	-	No significant band	0.10	green
	0.45	bright sky blue			0.35	blue
	0.52	blue			0.40	blue
	0.59	blue			0.45	bright sky blue
	0.64	red			0.55	blue
	0.73	red			0.59	blue
	0. 82	red			0.64	red
	0.90	red			0.67	red
					0.73	red
					0.82	red
					0.90	red
After derivatiz-ation	0.21	light violet	0.38	dark violet	0.21	light violet
	0.38	dark violet			0.30	light blue
	0.52	blue			0.38	dark violet
	0.64	violet			0.45	light violet
	0.73	violet			0.52	blue
	0.82	violet			0.59	grey
					0.73	violet
					0.77	brown
					0.90	violet

Table 3: Results of HPTLC

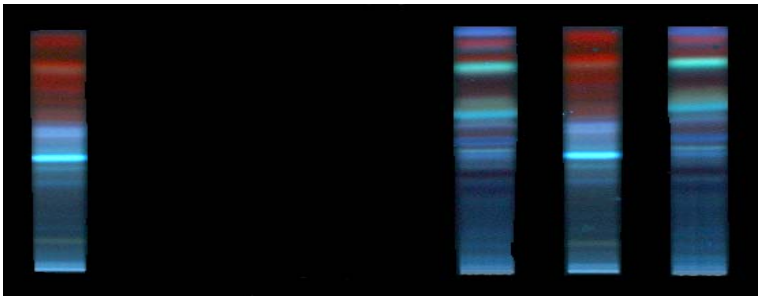
Sr. No.	Track No.	Volume applied on plate	Quantity applied on plate	Quantity of Andrographolide via graph	Linearity & Regression Coefficient and Standard deviation via graph
1.	T1	9µl	800µg	6.973µg	
2.	S1	2.5µl	2.5µg	2.500µg	
3.	S2	5.0µl	5.0µg	5.000µg	Y = 1882.118 + 9.875 * X
4.	S3	10.0µl	10.0µg	10.000µg	
5.	T2	11µl	5500µg	6.357µg	r = 0.99902 sdv = 3.09%
6.	T3	(9+1)µl	900µg +1µg	7.969µg -1µg = 6.969µg	
7.	T4	(11+1)µl	5500µg +1µg	7.351µg -1µg = 6.351µg	

T1- Alcoholic extract of Kalmegh whole plant, Locally collected from Ghaziabad
S1- Andrographolide standard solution
S2- Andrographolide standard solution
S3- Andrographolide standard solution
T2- Alcoholic extract of Kalmegh Navayas Loha, Local Market Sample, Ghaziabad
T3- Alcoholic extract of Kalmegh whole plant, Locally collected from Ghaziabad
T4- Alcoholic extract of Kalmegh Navayas Loha, Local Market Sample, Ghaziabad



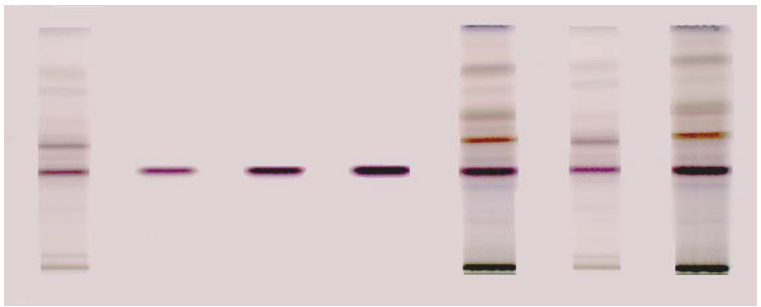
T1 S1 S2 S3 T2 T3 T4

UV-254 nm



T1 S1 S2 S3 T2 T3 T4

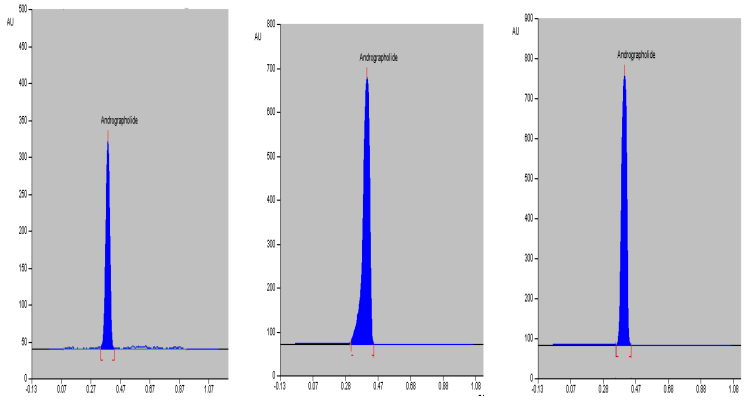
UV-366 nm



T1 S1 S2 S3 T2 T3 T4

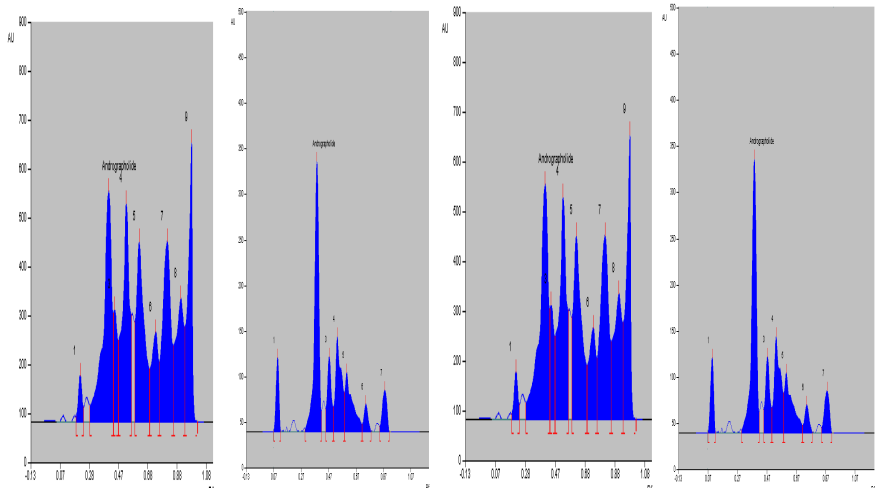
After derivatization

Fig. 1: HPTLC finger print of Kalmegh Navayas Loha



(Track S1) **(Track S2)** **(Track S3)**

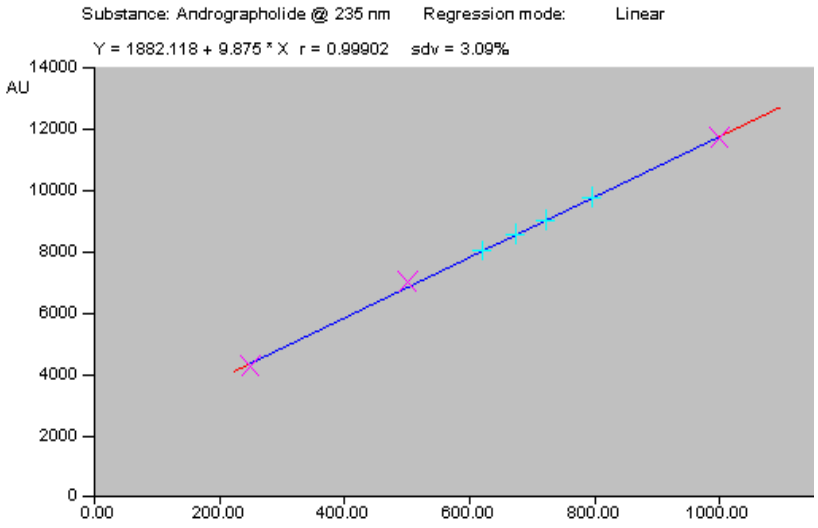
Peaks of Andrographolide (@ 235nm



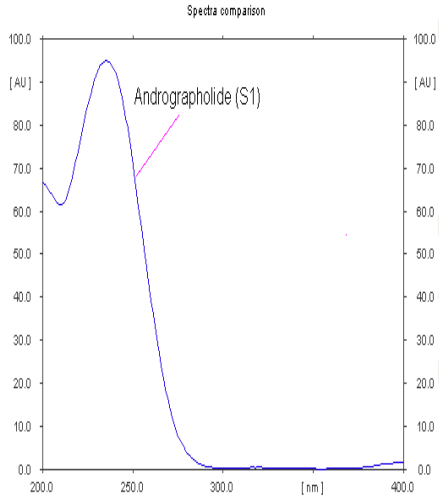
(Track T1) **(Track T2)** **(Track T3)** **(Track T4)**

Peaks of Kalmegh (W.P.) extract @ 235nm (Track T1& T3); Peak of Kalmegh Navayas Loha extract @ 235nm (Track T2& T4)

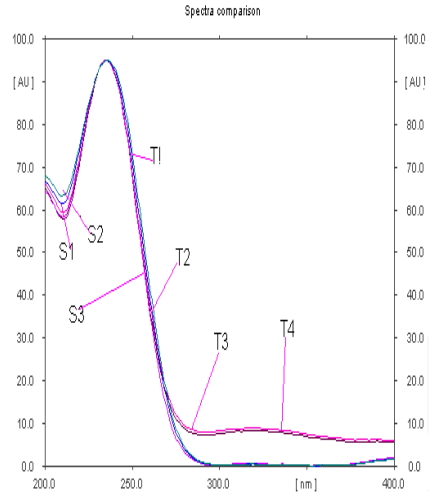
Fig. 2: Peaks of Kalmegh Navayas Loha in all tracks



Graph Area vs AU



Spectra of Andrographolide @ 235 nm



Spectra of Andrographolide in all tracks @ 235 nm

Fig. 3: Graph and Spectra of Kalmegh Navayas Loha

Table 4: Quantity of Andrographolide

Sr. No. ↓	Sample →	Kalmegh whole plant	Kalmegh Navayas Loha
1.	Quantity of Andrographolide in 1gm	7.746mg	1. 155 mg
2.	% Andrographolide	0.7746% w/w	0.1155 % w/w
3.	% Recovery	99.94% w/w	99.90% w/w

RESULTS AND DISCUSSION

Of the various mobile phases tried, the mobile phase containing Toluene : Ethyl acetate : Formic acid(5:4.5:0.5) v/v and the active principle Andrographolide resolved as a dark grey colour band at R_f. 0.38 very efficiently from the other components in alcoholic extract of *Andrographis paniculata* Linn. (whole plant) and Kalmegh Navayas Loha (Fig.1). Sharp peaks of Andrographolide (Standard and samples) were obtained when the plate was scanned at wavelength 235nm (Fig.2). Quantity of Andrographolide found in samples were obtained automatically (Table No.3) via graph (Fig.3) and % Andrographolide found in samples and % recovery were calculated (Table No.4). Quantity of Andrographolide found in Locally Collected Sample, Ghaziabad (U.P.) is 7.746mg in 1g drug sample (0.7746% w/w) and quantity of Andrographolide found in Kalmegh Navayas Loha is 1.155mg in 1g drug sample (0.1155%w/w). The % recovery of Andrographolide in Locally Collected Sample, Ghaziabad (U.P.) is 99.94% w/w and 99.90%w/w in Kalmegh Navayas Loha. The mean % recovery was 99.92%.

The accuracy and reproducibility of the method was established by means of recovery experiment. The mean recovery was close to 100% which indicates the accuracy of the method.

The robustness of the method was studied, during method development, by determining the effect of small variation, of mobile phase composition (±2%), chamber saturation period, development distance, derivatization time, and scanning time (10% variation of each). No significant change of R_f. or response to Andrographolide was observed, indicating the robustness of the method.

CONCLUSION

The proposed HPTLC method is simple, rapid, accurate, reproducible, selective and economic and can be used for routine quality control analysis of Kalmegh Navayas Loha powder and quantitative determination of Andrographolide in Kalmegh Navayas Loha powder.

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