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Research Article

ESTIMATION OF ANDROGRAPHOLIDE IN HYDROALCOHOLIC EXTRACTS OF *ANDROGRAPHIS PANICULATA* AND NILAVEMBU KUDINEER CHURNAM BY HPTLC FINGERPRINTING METHOD

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ABSTRACT

Objective: An attempt has been made to develop a method by which to determine the chemical fingerprint of andrographolide present in *Andrographis paniculata* (*AP*) (Acanthaceae) and Nilavembu kudineer churnam (NVK), a siddha polyherbal formulation.

Method: High-performance thin layer chromatography (HPTLC) was used to analyze 50% methanol extracts of leaves of *AP* and coarse NVK churnam. A computerised densitometer was applied to the two-dimensional spectrographic image analysis of the HPTLC plates.

Result: The analyses showed that andrographolide concentration present 2.68% in *AP* and 0.82% in NVK in the 50% methanol extract.

Conclusion: These chromatograms may serve as a chemical fingerprint of the drug *AP* and NVK for quality control purposes and in the preparation of formulations based on the drug.

Keywords: *Andrographis paniculata*, Nilavembu kudineer churnam, Andrographolide, HPTLC fingerprint.

INTRODUCTION

Standardization of herbal preparation means confirmation of its identity and determination of its quality and purity and detection of nature of adulterant to meet the national and international regulations [1]. Phytochemical analysis is one of the latest tools to access the quality of herbal drugs, which include preliminary phytochemical screening, chemo profiling using modern analytical techniques. High-performance thin-layer chromatography (HPTLC) has been emerged as an important analytical tool for characterization of the phytoconstituents in herbal drugs. This involves developing TLC fingerprinting profiles. The concept of phytoequivalence was developed to ensure consistency of herbal products, where a chromatographic fingerprint of an herbal drug was constructed and compared with the profile of a reference product [2]. The concept of phytoequivalence could be utilized for addressing the problem in quality control of herbal drugs [3]. The analysis of a single plant drug represents an enormous challenge in setting standards for quality control and it finds more difficult to deal with are multi herbal preparations [1]. NVK is an herbal mixture contains in equal proportion of whole plant of *Andrographis paniculata (AP)* (Burm) Wall. Ex Nees (Acanthaceae), heartwood of *Santalum album Linn*. (Santalaceae), rhizomes of *Zingiber officinale Rose*. (Zingiberaceae), fruits of *Piper nigrum Linn* (Piperaceae), tubers of *Cyperus rotundus Linn.* (Cyperaceae), roots of *Vetiveria zizanioides* (Linn) Nash. (Poaceae), whole plant of *Hedyotis corymbosa* (Linn.) Lam. (Rubiaceae), roots of *Plectranthus vettiveroides* (Linn.) Nash. (Lamiaceae) and the whole plant of *Trichosanthes cucumerina Linn*. (Curcurbitaceae). All these plants are used traditionally in the treatment of fever, inflammation, arthralgia, arthritis, gastric ulcer, jaundice and general debility conditions, formulated with an intention to manage the chikungunya fever [4]. The presence of minimum number of ingredients facilitates in setting the parameters during quality control.

The active constituents of *AP*/NVK were reported in ancient literature. No scientific studies have been carried out in NVK, regarding HPTLC fingerprinting of andrographolide. Therefore, the present study aims to evaluate chemical fingerprint densitogram of andrographolide present in *AP*/NVK.

MATERIALS AND METHODS

Plant materials

Collection of Plant raw powder and authentication

Authentic standardized extract of *AP* and NVK were obtained as a gift sample from M/s. Amsar Pvt. Ltd, Goa, India and Arogya healthcare, Chennai, India, respectively. Both *AP* and NVK were supplied in the dry extract form by the dealer and claimed to contain an equal proportion of 9 ingredients of *AP, Vetiveria zizanioides, Cymbopogen jwarankusa, Santalum album, Trichosanthes cucumerina, Cyperus rotundas, Zingiber officinale, Piper nigrum* and *Mollugo cerviana* in NVK, respectively.

Preparation of the extract

The shade dried coarse powder of the *AP*/ NVK (50gm) is packed well in soxhlet apparatus and is subjected with 500ml of 50% methanol by hot extraction for about 48 hrs. The extract is filtered through whatmann filter paper and the filtrate is evaporated for total elimination of methanol in Rota flash vacuum evaporator at 90^{0}C. Further the filtrate is concentrated on a hot water bath. The extracts thus obtained (Dark Brown) are weighed and the percentage yield of *AP* and NVK is determined.

Preliminary phytochemical analysis of AP/ NVK churnam

The obtained extracts of *AP*/NVK were subjected to the preliminary phytochemical analysis following standard methods [5,9]. This is to identify the presence of the various phytoconstituents in a qualitative way.

HPTLC fingerprint analysis of andrographolide in *AP* /NVK

Standard solution

The andrographolide 5mg was procured from Natural Remedies Pvt. Ltd., Bangalore, India as a gift sample. An accurately weighed quantity of std. andrographolide (1mg) was dissolved in methanol (5-7 ml) and sonicate for 5 min. The volume was made up with methanol (10 ml) to give concentration (100 ng/µl). From this solution, 3 ml was further diluted to 10 ml to get final concentration (30 ng/µl).

Sample preparation

Prepare 50% v/v methanol (MeOH) solution. Weigh accurately 100 mg of *AP*/NVK and transfer into a 10 ml volumetric flask. Dissolve the contents with 5 ml of 50% MeOH, and sonicate until it got dissolved. Make up the volume to 10 ml with 50% MeOH. Sonicate the contents for another 10 minutes and filter using whatman filter paper and this solution was stored in 5 ml glass vials and used for HPTLC analysis.

HPTLC-Photodensitometry conditions and instrumentation

Instrument: CAMAG HPTLC (Switzerland)

Applicator: CAMAG linomat V

Syringe: Hamilton syringe (100 µl)

Scanner: CAMAG TLC scanner 3

Photo-documentation: CAMAG Reprostar 3 (winCATS software version 1.3.4)

Layer

Stationary phase: Pre coated silica gel GF_{254} on aluminium plates (Merck KGaA, Darmstadt, Germany)

Plate thickness: 0.2 mm

Plate size: 100 x 100 mm

Mobile phase

Andrographolide in chloroform: methanol (9:1)

AP in chloroform: methanol (9:1)

NVK in chloroform: methanol (9:1)

Solvent ratio: vol. / vol. (v/v)

Environmental condition

Temperature: 25±2°C

Relative humidity: 55–65%.

Sample application

Application rate: 10 µl s^{-1}

Table speed: 10 mm s^{-1}

Distance from starting: 15 mm

Distance from bottom: 10 mm

Volume applied: 2.5 – 10 µl

Band length: 10 mm

Distance between tracks: 10 mm

Development

Developing chamber: Twin trough glass chamber (20 x10 cm)

Developing solvent: 5 ml mobile phase/trough

Chamber saturation time: 1 hr

Developing mode: Ascending mode

Development distance: 80 mm

Detection reagent: Nil

Chromatographic condition

Sample: *AP* & NVK Churnam

Standard: Andrographolide

Sample & Standard prepared in: Methanol

Stationary phase: Silica gel GF_{254}

Mobile phase: Chloroform: Methanol (9:1)

Scanning wavelength: 220 nm

Sample concentration: Extract (10 mg/ml), Andrographolide (100 mcg/ml)

Applied volume : Track 1(4µl) Track 2 (8µl), Track 3 (2µl),

Track 4 (4 µl), Track 5 (6µl), Track 6 (8µl), Track7 (10µl), Track8 (4µl) and Track 9 (8 µl)

(Track 1& 2- *AP*)

(Track 3 – 7 - Andrographolide)

(Track 8 & 9 – NVK Churnam)

Development mode: Ascending mode

Detection of R_f value

Scanning wavelength : 254 nm and 366 nm

Slit dimensions : 5 mm × 0.45 mm

Documentation

$$\text{Calculation of } R_f = \frac{\text{Distance travelled by solute component}}{\text{Distance travelled by solvent front}}$$

Calibration curve of andrographolide

Fifty milligrams of standard *AP* was dissolved in 50 ml of methanol in a volumetric flask. From this stock solution we have taken 1 ml in a 10 ml volumetric flask and adjusted the volume to 10 ml with methanol (100 ng μl^{-1}). Then 2 µl, 4 µl, 6 µl, 8 µl, and 10 µl of the standard solution (200–1000 ng) was applied over on pre-coated silica gel GF_{254} TLC plate and developed the plate in the solvent system to a distance of 8 cm. The developed plate was dried in air and scanned at 220 nm. Calibration curve was constructed by plotting peak area vs. concentration.

RESULTS

Percentage yield of hydroalcoholic extracts of *Andrographis paniculata and* Nilavembu kudineer churnam

The hydroalcoholic extracts of *AP* and NVK were obtained as dark brown color and weighed. Their percentage yield was found to be 7.3% and 20.8% w/w respectively.

Preliminary phytochemical analysis of HEAP and HENC

The preliminary phytochemical analysis of the individual ingredients of HEAP and HENC was analyzed and the results indicated in **Table -1** showed the presence and absence of the phytoconstituents present. Both the extracts consist of phenol, triterpenoids, flavones, alkaloids, saponins, proteins, tannins and steroids. The number of positive signs indicates the intensity of the phytoconstituents concentration present in each extract.

Table 1: Determination of preliminary phytoconstituents of *AP* and NVK

S. No.	Chemical test	*AP*	NVK formulation
1	Phenol	+	+
2	Triterpenoids	+	+
3	Flavones	+	+
4	Alkaloids	+	+
5	Reducing sugars	-	-
6	Glycosides	-	-
7	Saponins	+	+
8	Quinones	-	-
9	Proteins	+	+
10	Tannins	+	+
11	Anthraquinones	-	-
12	Steroids	+	+

(+): present; (-) : absent

HPTLC fingerprinting analysis of andrographolide in *AP*/NVK

***Chromatogram evaluation and estimation*:**

The CAMAG TLC Scanner was used for chromatogram evaluation of HEAP and HENC against andrographolide. Plate was scanned at 254 nm and 366 nm (**Figure: 1**) See Figure & observation of HPTLC plate given in **Table -2**.

Quantification of andrographolide in AP/NVK

The HEAP and HENC extracts of were subjected to HPTLC fingerprinting. The results showing the R_f values and the percentage of the andrographolide in each extract are shown in **Table -2**. The HPTLC plates showing different bands for both the extracts are shown in **Figure: 1**. The HEAP and HENC at 4µl showed 7 peaks and at 8µl

showed 9 peaks. (**Figure 2A- 2I**). The andrographolide present in HEAP and HENC at 4µl with R_f values of 0.66 and 0.59 whereas at 8µl with R_f values of 0.64 and 0.6. Multiwavelengths scanning of HEAP, HENC and andrographolide were done for selection of wavelength (**Figure: 3**) and calibration curve was constructed by plotting peak area vs. concentration (**Figure: 4**). Concentration of andrographolide was found 2.68% in *AP* and 0.82% in NVK. The andrographolide can be further purified to find its potency for biological activities.

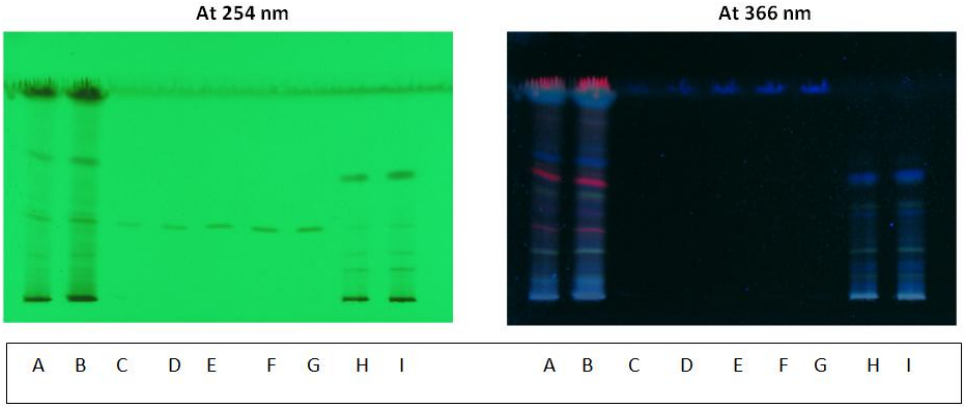
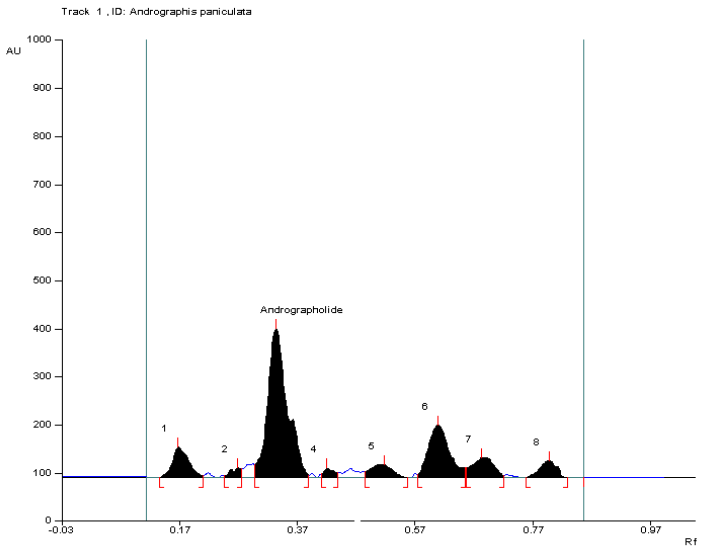


Fig. 1: Chromatogram of HEAP, HENC and andrographolide

A: Track 1 in 4µl HEAP
B: Track 2 in 8µl HEAP
C: Track 3 Standard andrographolide 2µl
D: Track 4 Standard andrographolide 4µl
E: Track 5 Standard andrographolide 6µl
F: Track 6 Standard andrographolide 8µl
G: Track 7 Standard andrographolide 10µl
H: Track 8 in 4 µl HENC
I: Track 9 in 8µl HENC

Table 2: Observation of HPTLC Plate of HEAP, HENC and andrographolide

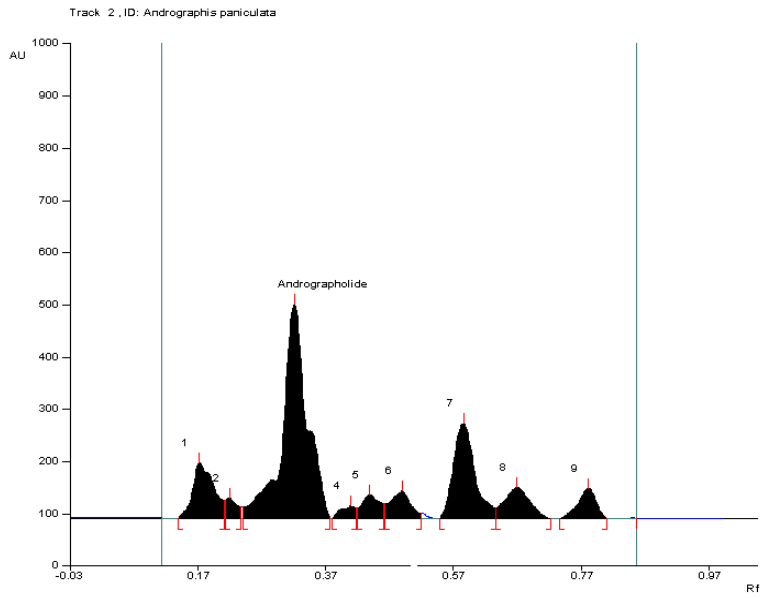
Track	Peak	Vol. (µl)	Max R_f for andrographolide	Area
1	3	4	0.66	9809.7
2	3	8	0.64	14381.6
3	1	2	0.61	4702.3
4	1	4	0.58	7310.6
5	1	6	0.59	8791.7
6	1	8	0.56	10032.8
7	1	10	0.57	10784.1
8	2	4	0.59	3011.5
9	2	8	0.6	4612.4



Max R_f	Max Height	Height%	Area	Area %
0.34	66.5	10.86	1557	8.86
0.43	10.4	1.69	100.7	0.57
0.66	**325.4**	**53.12**	**9809.7**	**55.79**
0.83	19.9	3.25	322.6	1.83
1.02	28.8	4.7	880.2	5.01
1.2	116.9	19.09	3465.5	19.71
1.35	44.6	7.28	1446.5	8.23

Fig. 2.A: HPTLC fingerprint profile of hydroalcoholic extract at 254 nm

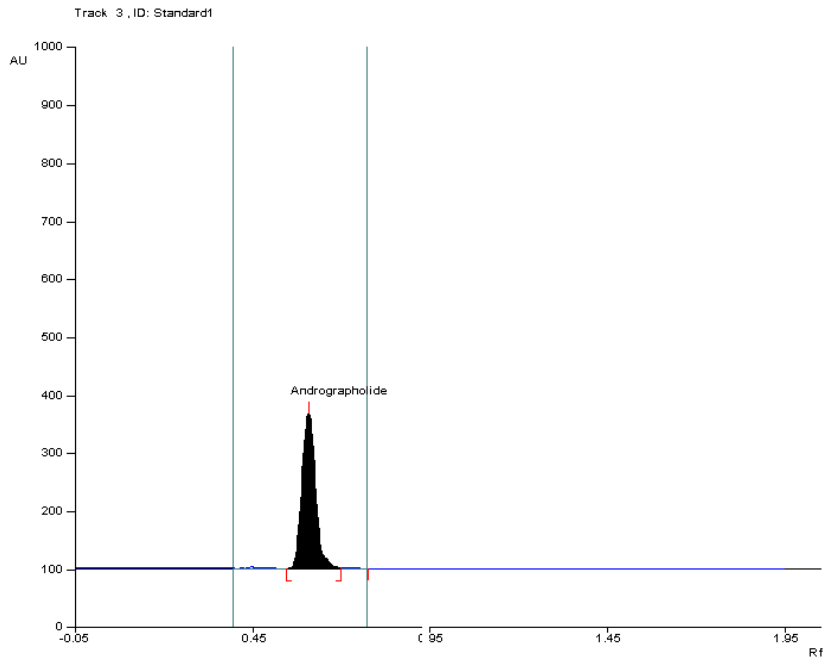
Track 1, HEAP (chloroform: methanol): 4µl



Max R_f	Max Height	Height%	Area	Area %
0.35	113.1	11.32	3172.9	10.71
0.44	43.1	4.31	704.1	2.38
0.64	**430**	**43.01**	**14381.6**	**48.55**
0.81	25.1	2.51	564.6	1.91
0.87	46.3	4.63	1037.6	3.5
0.97	54.8	5.48	1527.3	5.16
1.15	196.3	19.64	5715.3	19.29
1.32	64.6	6.46	2163.4	7.3
1.53	26.3	2.63	357.7	1.21

Fig. 2.B: HPTLC fingerprint profile of hydroalcoholic extract at 254 nm

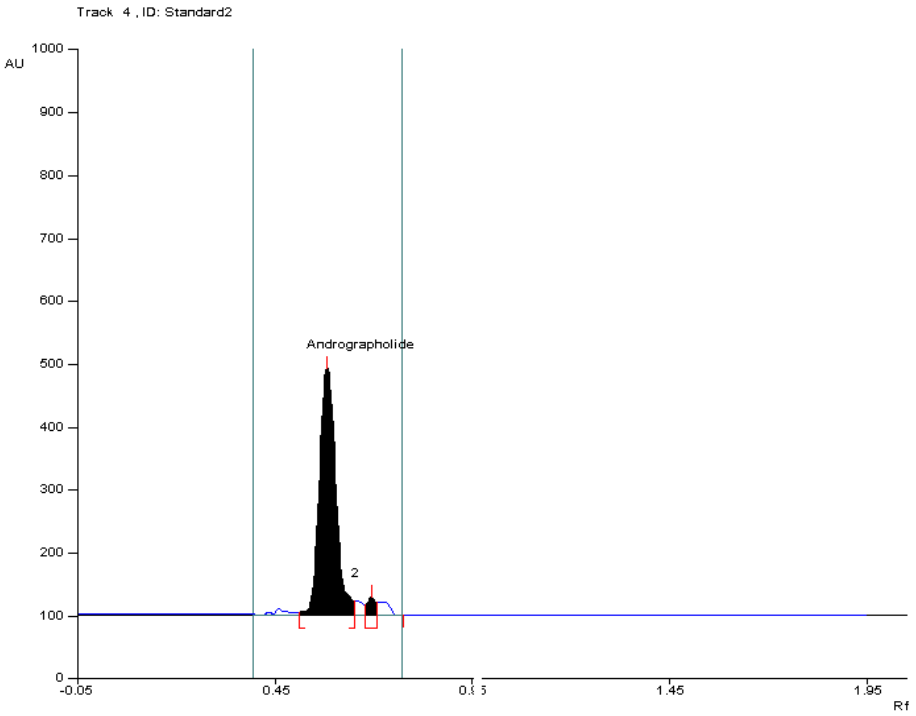
Track 2, HEAP (chloroform: methanol): 8µl



Max R_f	Max Height	Height%	Area	Area %
0.61	270.4	100	4702.3	100

Fig. 2.C: HPTLC fingerprint profile of hydroalcoholic extract at 254 nm

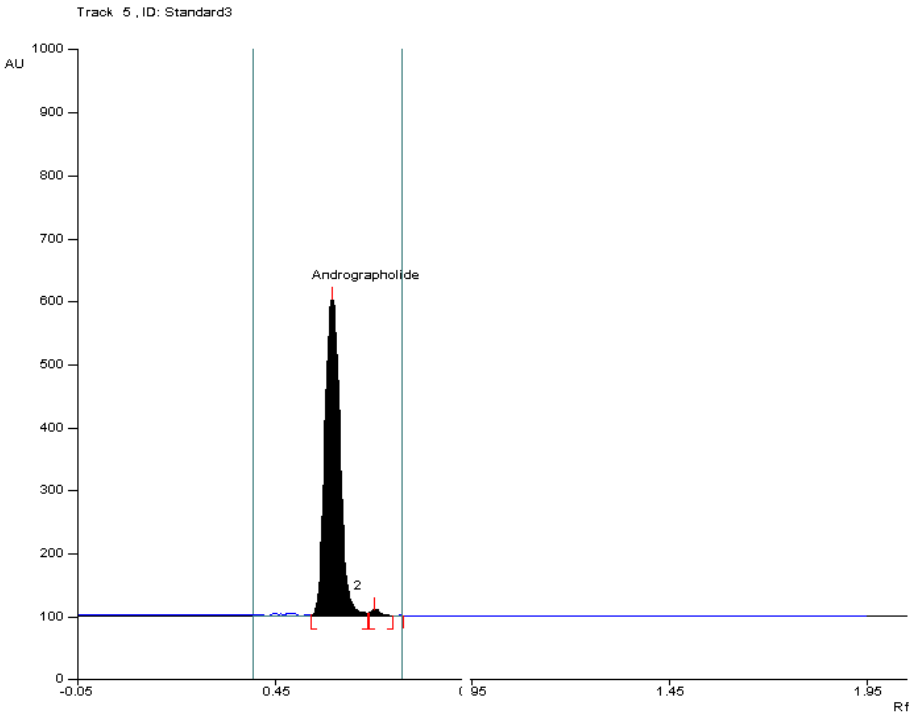
Track 3, andrographolide (chloroform: methanol): 2µl



Max R_f	Max Height	Height%	Area	Area %
0.58	394.2	93.18	7310.6	94.28
0.69	28.9	6.82	443.2	5.72

Fig. 2.D: HPTLC fingerprint profile of hydroalcoholic extract at 254 nm

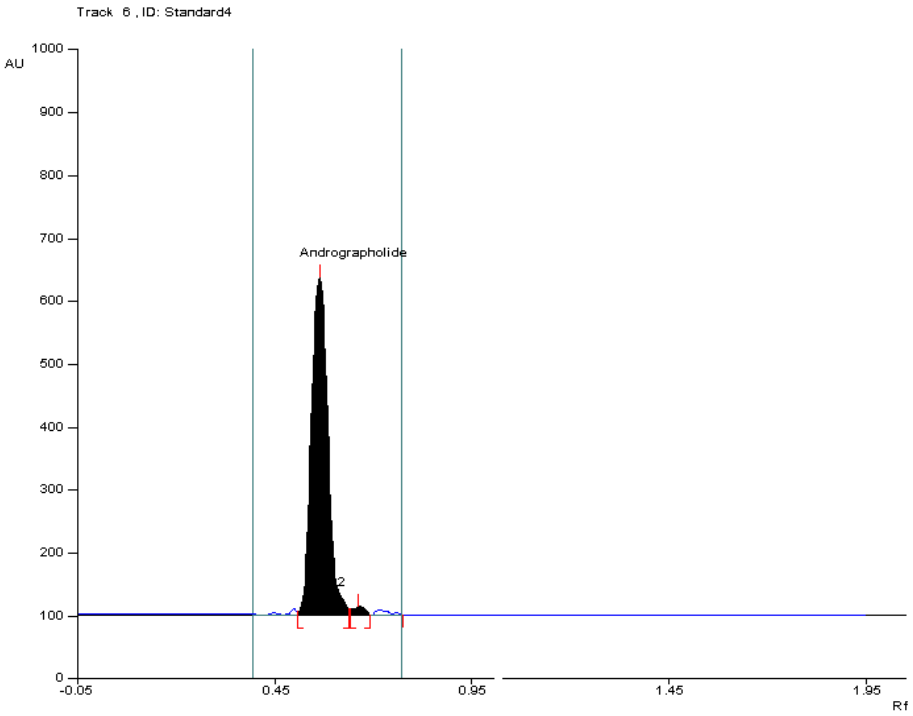
Track 4, andrographolide (chloroform: methanol): 4µl



Max R_f	Max Height	Height%	Area	Area %
0.59	510	97.24	8791.7	97.75
0.77	14.5	2.76	202.2	2.25

Fig. 2.E: HPTLC fingerprint profile of hydroalcoholic extract at 254 nm

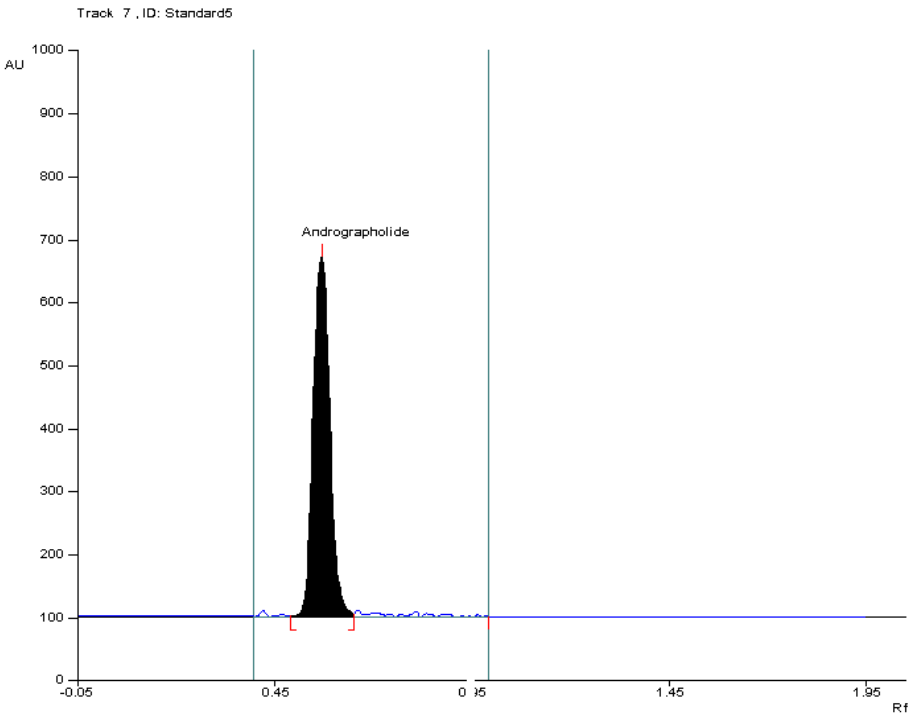
Track 5, andrographolide (chloroform: methanol): 6µl



Max R_f	Max Height	Height%	Area	Area %
0.56	540.2	97.34	10032.8	97.98
0.66	14.7	2.66	207.1	2.02

Fig. 2.F: HPTLC fingerprint profile of andrographolide at 254 nm

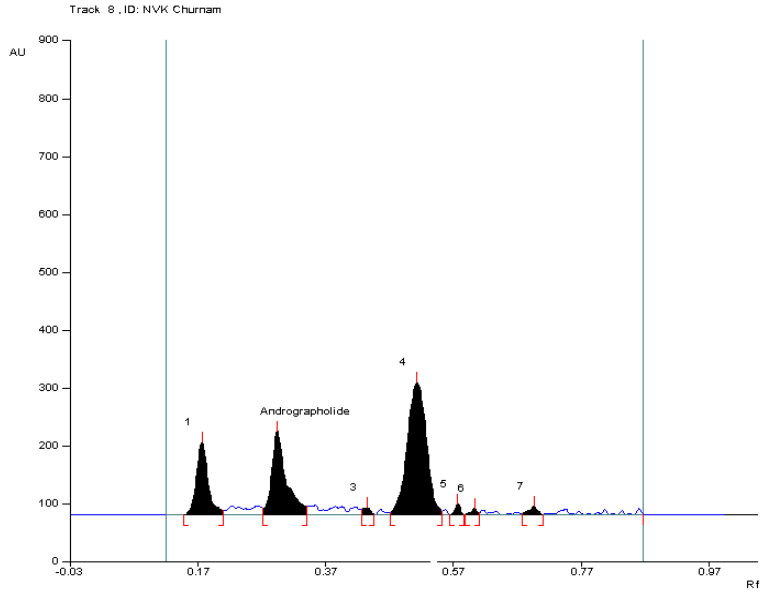
Track 6, andrographolide (chloroform: methanol): 8µl



Max R_f	Max Height	Height%	Area	Area %
0.57	573.6	100	10784.1	100

Fig. 2.G: HPTLC fingerprint profile of hydroalcoholic extract at 254 nm

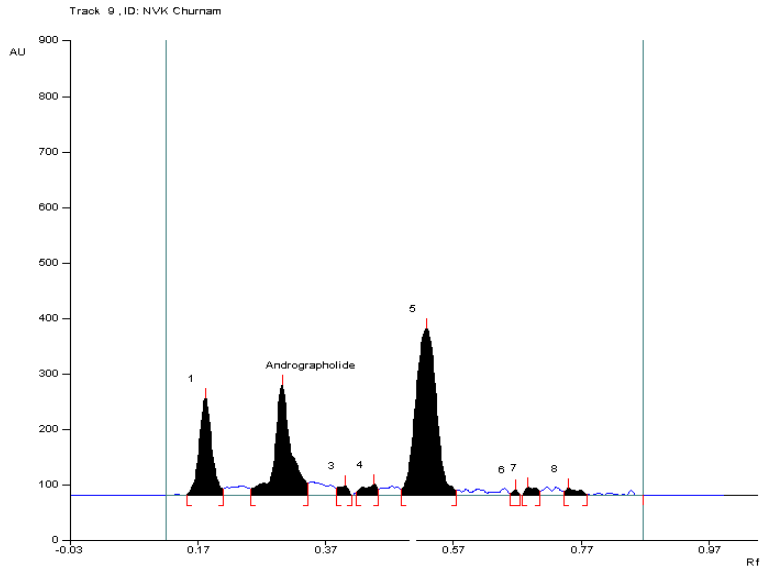
Track 7, andrographolide (chloroform: methanol): 10µl



Max R_f	Max Height	Height%	Area	Area %
0.36	134.7	22.64	2142.6	17.77
0.59	154	25.88	3011.5	24.97
0.86	12.8	2.16	154.6	1.28
1.01	247	41.5	6345.4	52.62
1.14	19.9	3.35	136.2	1.13
1.19	11.7	1.96	87.3	0.72
1.37	15	2.51	180.5	1.5

Fig. 2.H: HPTLC fingerprint profile of hydroalcoholic extract at 254 nm

Track 8, HENC (chloroform: methanol): 4µl



Max R_f	Max Height	Height%	Area	Area %
0.37	188.2	22.79	3115.8	17.19
0.6	212.3	25.71	4612.4	25.44
0.69	26.9	3.26	525.8	2.9
0.79	17	2.06	230.7	1.27
0.84	14.9	1.81	149.1	0.82
1.04	324.6	39.31	8921.5	49.21
1.31	11.7	1.42	85.4	0.47
1.35	15.7	1.9	245.6	1.36
1.48	14.4	1.74	242	1.33

Fig. 2.I: HPTLC fingerprint profile of hydroalcoholic extract at 254 nm

Track 9, HENC (chloroform: methanol): 8µl

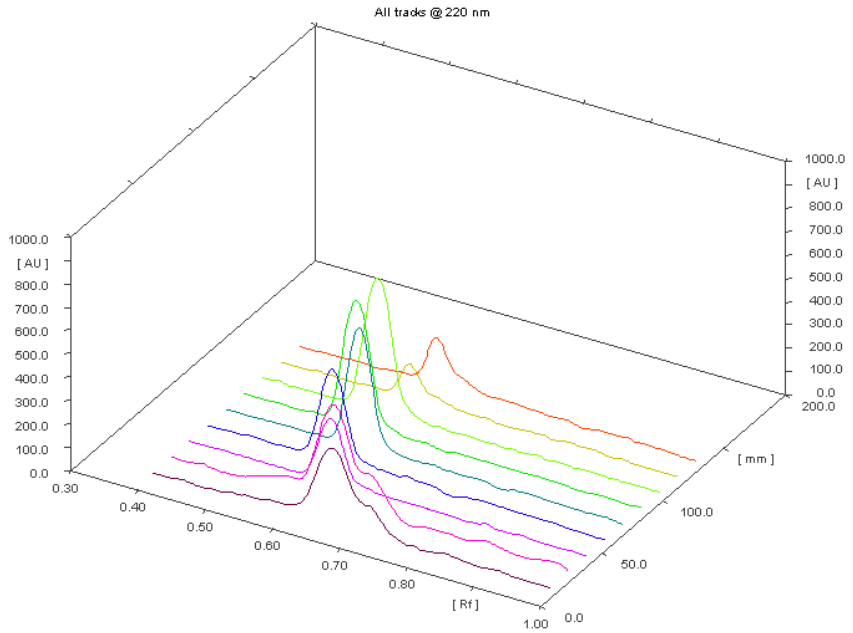


Fig. 3: Multiwavelengths scanning of HEAP, HENC and andrographolide for selection of wavelength

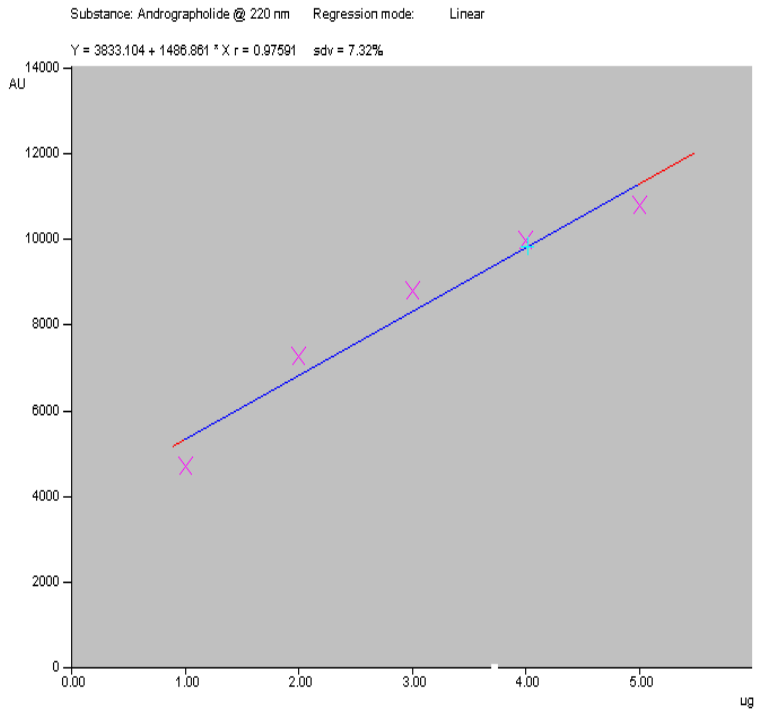


Fig. 4: Calibration plot of andrographolide (λ_{max} = 220nm).

Mobile phase - chloroform: methanol (9:1 v/v).

DISCUSSION

Percentage yield and preliminary phytochemical analysis of HEAP and HENC

Standardization of herbal drugs means confirmation of their identity and determination of their quality, purity and detects the nature of adulterant to meet the national and international regulations [1]. Phytochemical analysis is one of the latest tools to access the quality of herbal drugs, which include preliminary phytochemical screening, chemo profiling using modern analytical techniques. The determination of the percentage yield and the preliminary phytochemical profile of HEAP and HENC would help the researchers to authenticate and also to further investigate the presence of the total andrographolide concentration in HEAP and HENC by HPTLC fingerprinting method.

HPTLC fingerprinting analysis of andrographolide in HEAP and HENC

High-performance thin-layer chromatography (HPTLC) has been emerged as an important analytical tool for characterization of the

phytoconstituents in herbal drugs. This involved by developing TLC fingerprinting profiles. The concept of phytoequivalence was developed to ensure consistency of herbal products, where a chromatographic fingerprint of an herbal drug was constructed and compared with the profile of a reference product. The concept of phytoequivalence could be utilized for addressing the problem in quality control of herbal drugs[3]. The analysis of a single plant drug represents an enormous challenge in setting standards for quality control and it finds more difficult to deal with multi-herbal preparations [1].

Andrographolide is a diterpenoid constituent present in *AP* [6, 7, 8] and it was reported to possess anti-inflammatory activity. Therefore, we analyzed the HPTLC fingerprint of andrographolide in *AP* and NVK. The HPTLC fingerprinting showed maximum 9 peaks for HEAP and HENC. The concentration of andrographolide was found 2.68% in HEAP and 0.82% in HENC. Hence, the determination of andrographolide concentration in HEAP and HENC would help the researchers to authenticate and to fix up the physicochemical standards for *AP*/NVK.

SUMMARY AND CONCLUSION

The hydroalcoholic extracts of *Andrographis paniculata* (HEAP) and nilavembu kudineer churnam (HENC) was prepared and their percentage yield was calculated. The preliminary phytochemical analysis was done and the amount of andrographolide present in HEAP and HENC was calculated by the HPTLC fingerprinting method. It would like to fix up the physico-chemical standards for *AP*/NVK. HPTLC fingerprints developed for andrographolide could be used to document the quality of the raw material of *AP* or used in the formulation of NVK. The chromatogram of *AP*/NVK reveals the composition of the andrographolide and also, whether it has been changed during batch-to-batch production. The HPTLC method developed for *AP*/NVK and andrographolide, its individual ingredient was found to be simple and may be used for the routine quality control analysis and of simultaneous determination of phytomedicines containing andrographolide can be performed using this method.

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