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HPTLC METHOD FOR THE ESTIMATION OF *COLEUS VETTIVEROIDES* IN *PATOLADI GHRITA*- AN AYURVEDIC POLYHERBAL FORMULATION

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ABSTRACT

The quantitative estimation of crude drugs present in an Ayurvedic formulation still remains a big challenge. A quantitative High Performance Thin Layer Chromatography (HPTLC) method was developed for the estimation of *Udicya (Coleus vettiveroides)*, one of the herbal ingredients present in the Ayurvedic Polyherbal formulation '*Patoladi Ghrita'*. The formulation '*Patoladi Ghrita'* is a medicated ghee originally described in the Ayurvedic book *'Ashtangahridaya'* and is reproduced in the Ayurvedic Formulary of India, The methanol extracts of the crude drug *Udicya* and the formulation *Patoladi Ghrita* were run using a mobile phase Isopropyl alcohol : Water :: 9 : 1 (v/v) on a silica G 60 F_{254} stationary phase. Methanol extract of *Udicya* gave a spot with R_f value of 0.73. The methanol extract of *Patoladi Ghrita* also gave a spot with R_f value of 0.73. The chemical equivalency of these two spots was revealed by the same pattern of the UV spectrum. The estimation was done graphically by preparing Quarter Standard, Half Standard, Normal Standard, and Double Standard of the formulation. The methanol extract of the genuine drug and that of the four standards and four marketed samples were run using the same mobile phase. The reference spots on each track were scanned and integrated to get the AUC. A calibration plot was drawn between the concentration of standards and AUC of the reference spots. The concentration of *Udicya* in the samples was found from the corresponding AUC obtained for the reference peak of the samples. A regression coefficient of 0.951 was obtained. The developed HPTLC method is a simple and accurate one and can be used as a tool for the estimation of each ingredient in a Polyherbal formulation.

INTRODUCTION

Ayurveda, the Indian system of medicine, is the oldest system of medicine and dates back to about 5000 years.[1] Before independence this system was the first choice of an average Indian. With independence, the modern system of medicine became the first choice and Ayurveda the second. Presently the system is gradually gaining its glory. In many of the countries, Ayurveda has been accepted as an alternate system of Medicine.[1,2] The major limitation to its acceptance is the lack of proper scientific backing. The modern concept of reductionism does not work on Ayurveda. The therapeutic property or the usefulness of a formulation cannot be correlated to the individual molecular entities present in a formulation. Thus the modern research methodologies based on reproducibility, repeatability, dose-response relationship etc. are not strictly applicable to this system.[3,4] Amidst of these intricacies, it has become necessary to develop some analytical techniques for the estimation of individual crude drugs present in an Ayurvedic formulation.

Indian Scenario

The necessity of standardization of Ayurvedic products is immensely felt and the Department of AYUSH has taken initiatives in this direction. Now majority of the crude drugs used in Ayurveda are made official and monographs are available in the various volumes of Ayurvedic Pharmacopoeia of India (API). Similarly the majority of the conventional Ayurvedic formulations also got standardized. Now in the official monograph, one can find the composition of the Ayurvedic product, its method of preparation and various standards for the quality control of the products. Thus uniformity is brought among products specified in the official monograph which helps in the control over the production and sale of Ayurvedic products.

Though Ayurvedic Pharmacopoeia of India have set standards for crude drugs and formulations, serious limitations are felt especially in the case of formulations. The official monograph quite often prescribes limits to the bulk property of the formulations such as specific gravity, refractive index, optical rotation and chemical constants such as acid value, iodine value, ester value, peroxide value, Polanski value, saponification value etc.[5- 8] With these parameters it is impossible to ascertain precisely the quantity of a crude drug present in a formulation. In a very few formulations certain biomarkers are identified and quantified. Although such a step is acceptable to the scientific community, it is confined to very few crude drugs and formulations only.[9 -11]

Presently it is very difficult, rather impossible to determine exactly the amount of crude drug present in an Ayurvedic formulation. Many Ayurvedic formulations in the market do not contain all the ingredients they should contain. If at all, all the ingredients are present in a formulation each one may not be present to the required amount. This situation is alarming, and there should evolve very specific methods for the accurate estimation of each ingredient present in a formulation. HPTLC is a method developed in this line and is found to be an effective tool in addressing the above limitations.[12 - 14]

MATERIALS AND METHODS

- **Chemicals**

Methanol, Isopropyl alcohol and HPLC grade Water. All chemicals used were HPLC grade and purchased from Merck Specialties Mumbai.

- **Crude Drugs**

Trichosanthes dioica, Azadirachta indica, Picrorhiza kurroa, Coscinium fenestratum, Vetiveria zizanioides, Terminalia chebula, Terminalia belerica, Eblica officinalis, Adhatoda zeylanica, Tragia involucrata, Bacopa monnieri, Oldenlandia corymbosa, Cyperus rotundus, Solanum indicum, Glycyrrhiza glabra, Holarrhena anti-dysenterica, Coleus vettiveroides, Santalum album, Piper longum, Cow's ghee.

All the drugs except sandal wood, were procured from a crude drug shop at Karunagapally, Kollam District, and Kerala State, India. A genuine sample of *Santalum album* (Sandal wood or '*Candana')* was purchased from the

"*Vanasree"* outlet of the Forest Department, Government of Kerala.

All the crude drugs were found to comply with the official standards.

- **Apparatus**

CAMAG Linomat IV Autosampler, CAMAG TLC Scanner-III, UV Cabinet, TLC Plate Heater, 100µl Syringe (Hamilton Switzerland), Twin Trough Chamber, Silica Gel $60F_{254}$ Plates (Merck), Soxhlet Apparatus.

- **Methodology**

The HPTLC method for the quantification of *Udicya (Coleus vettiveroides)* in the Ayurvedic formulation *Patoladi Ghrita* is undertaken in the present study. In Malayalam, the drug *Udicya (Coleus vettiveroides)* is known as *'Iruveli'*. *Udicya* consists of the whole plant of the species *Coleus vettiveroids* belonging to the family, Labiatae. It is a small profusely branched succulent aromatic herb. Leaves are glandular and hairy, broadly ovate with dentate margins and prominent veins. These are widely cultivated in South India through vegetative cuttings and the whole plant is used in the medicine. Pictures of the live plant and the dried plant are shown in Fig-1.

Coleus vettiveroids (live plant) *Coleus vettiveroids* (dried plant)

Fig. 1. Photograph of *Udicya (Coleus vettiveroids)*

Preparation of Methanol extract of crude drug

25 g of the coarse powder of authenticated sample of *Udicya (Coleus vettiveroids)* was packed in a thimble made of filter paper. The thimble was inserted into a Soxhlet extractor and extracted with 50 ml of methanol, 5 cycles a day for 3 days. The volume was reduced by evaporation and made up to 10 ml.

Preparation of standard formulation

The Ayurvedic formulation *Patoladi Ghrita* is originally described in *Astangahridaya*.[15] Official formula for the preparation of *Patoladi Ghrita* according to API is given in (Table 1). A standard preparation of the Ayurvedic formulation was made strictly as per the procedures described in the Ayurvedic Formulary of India.[16] This is called the 'Normal Standard (N-Std)'. Three standard preparations namely 'Quarter Standard (Q-Std)', 'Half Standard (H-Std)' and 'Double Standard (D-Std)' were also prepared by taking quarter, half and double the quantities of individual ingredients present in the Standard formulation.

Preparation of methanol extracts of standard and samples

Four marketed samples of *Patoladi Ghrita* manufactured by four different manufacturers were purchased from the local market. Methanol extracts of the four standards and the four samples were prepared from 25 g each of the standards and samples by solvent extraction using 50 ml of methanol by refluxing on a boiling water bath for 3 hours. The volume was reduced by simple evaporation and made up to 10 ml.

HPTLC Chromatogram development and scanning

The chromatograms were developed on 10 x 20 cm HPTLC Silica gel $60F_{254}$ plates previously activated by heating at 110°C on a TLC Plate Heater for 15 minutes. On this plate, 2µl of the methanol extracts of the crude drug, that of the different standards and samples were spotted sequentially from 1 to 9, using the applicator Linomat-IV of CAMAG. The plate was developed in a Twin Trough chamber using the mobile phase, Isopropyl alcohol: Water: 9: 1 (v/v). The solvent front was allowed to run about 9 cm. The plate was taken out, dried and visualized under the UV cabinet. The photograph of such a chromatogram is shown in (Fig.2). Each track on the plate was

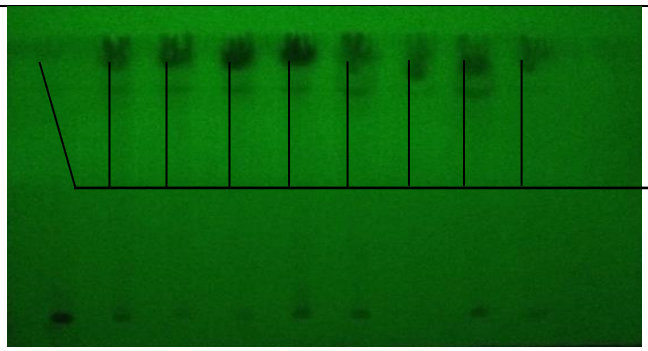
scanned by CAMAG TLC Scanner. The same procedure was done in triplicate.

Table 1. Formula for the preparation of 768g of *Patoladi Ghrita*

Sl. No.	*Sanskrit Name*	*Plant part **	*Malayalam name*	*Botanical name*	*Qty.*
KVATHA					
1	Patola	Pl.	Padavalam	*Trichosanthes dioica*	48 g
2	Nimba	St. Bk.	Veppu	*Azadirachta indica*	48 g
3	Katuka	Rt/Rz.	Katukurohini	*Picrorhiza kurroa*	48 g
4	Darvi	St.	Maramanjal	*Coscinium fenestratum*	48 g
5	Sevya	Rt.	Ramacham	*Vetiveria zizanioides*	48 g
6	Haritaki	P.	Kadukka	*Terminalia chebula*	48 g
7	Bibhitaki	P.	Thannikka	*Terminalia belerica*	48 g
8	Amalaki	P.	Nellikka	*Emblica officinalis*	48 g
9	Vasa	Rt.	Adalodakam	*Adhatoda zeylanica*	48 g
10	Dhanvayasa	Pl.	Kodithoova	*Tragia involucrate*	48 g
11	Trayanti	Pl.	Brahmi	*Bacopa monnieri*	48 g
12	Parpata	Pl.	Parpatakapullu	*Oldenlandia corymbosa*	48 g
13	Amalaki	P.	Nellikka	*Emblica officinalis*	768 g
14	Water for decoction Volume reduced to			12.288 L 3.072 L	
15	Goghritha	-	Pasuvin Neyyu	*Cow's Ghee*	768 g
KALKA					
16	Musta	Rz.	Muthanga	*Cyperus rotundus*	24 g
17	Bhunimba/Brhati▪	Rt.	Putharichunda	*Solanum indicum*	24 g
18	Yasti	Rt.	Irattimadhuram	*Glycyrrhiza glabra*	24 g
19	Kutaja(Indrayava)	Sd.	Kutakappalayari	*Holarrhena antidysenterica*	24 g
20	Udicya	Rt.	Iruveli	*Coleus vettiveroides*	24 g
21	Candana	Ht. Wd	Chandanam	*Santalum album*	24 g
22	Pippali	Fr.	Thrippali	*Piper longum*	24 g

* Pl: Whole Plant, P: Pericarp, St. Bk: Stem Bark, St: Stem, Rt: Root, Rz: Rhizome, Sd: Seed, Ht. Wd: Heart Wood and Fr: Fruit

▪As per the Ayurvedic Pharmacopoeia of India, the drug *Bhunimba* is *'Kiriyath',* the botanical name of which is *Andrographis paniculata.* In North India this drug is used for *Bhunimba*, but in Kerala *'Putharichunda*' is used instead. The Sanskrit name of *'Putharichunda*' is *Brhati* and its botanical name is *Solanum indicum.* For the present research work *Solanum indicum* is used. Its use in the formulation is authorized by The Pharmacopoeia (Malayalam) published by the Kerala State Ayurvedic Publications, Trivandrum, 1996.[17]



Track- a b c d e f g h i

Track- a : Pure drug *Udicya*

Track- b : Q- Std
Track- c : H- Std
Track- d : N- Std
Track- f : Sample-A
Track- g : Sample-B
Track- h : Sample-C

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Track- e : D- Std Track- i : Sample-D

Fig. 2. Photograph of HPTLC plate of *Udicya*

The reference spot on each track with R_f value of 0.73 was integrated to get the AUC. The chromatogram of the plant extract, the four standards and the four samples are shown below.

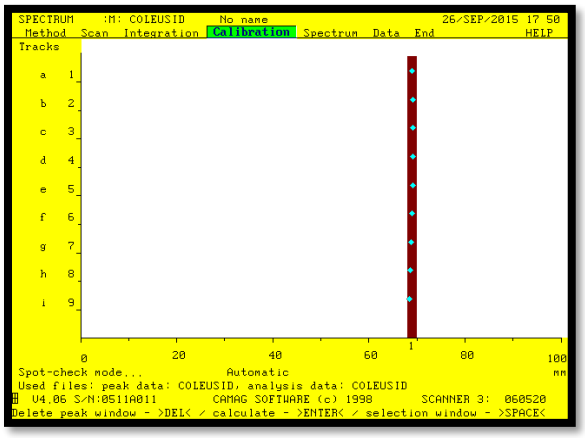

Fig. 3. Chromatogram of *Udicya*, standards and marketed formulations

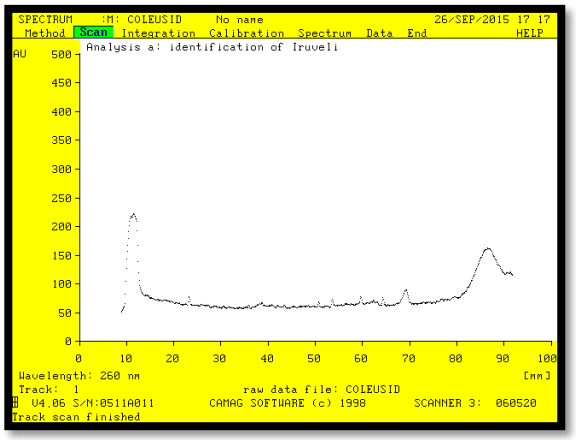

Fig.3(a.1)- Chromatogram of plant extract

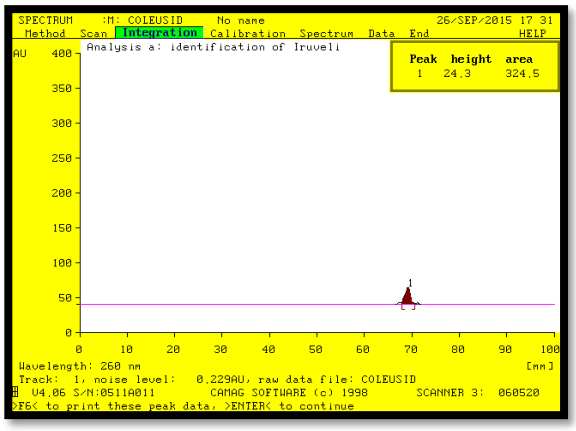

Fig.3(a.2)- Integrated chromatogram of *Udicya*

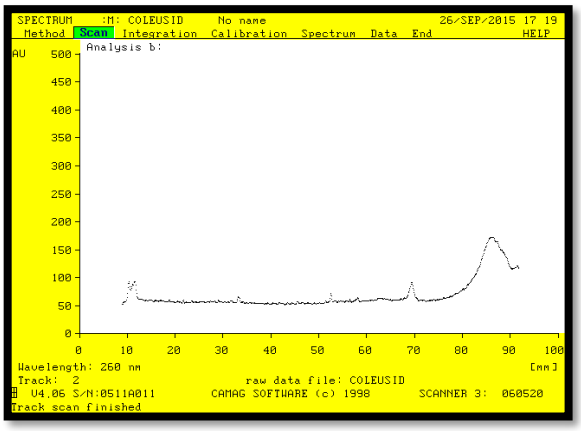

Fig.3(b.1)- Chromatogram of Quarter standard

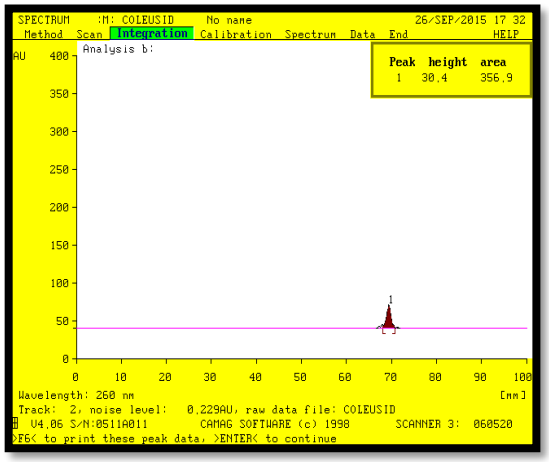

Fig.3(b.2)- Integrated chromatogram

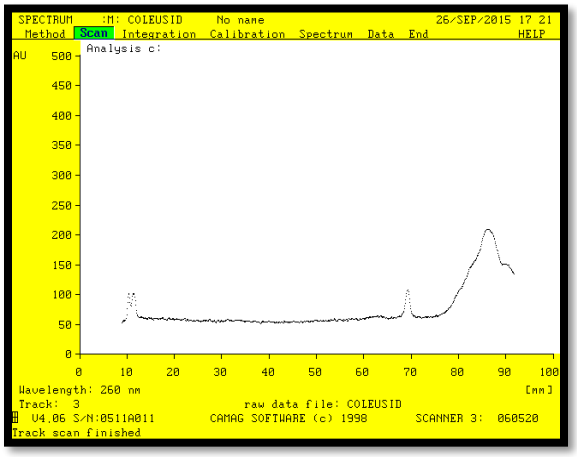

Fig.3(c.1)- Chromatogram of Half standard

 DOI- 10.22270/jmpas.v9i1.909

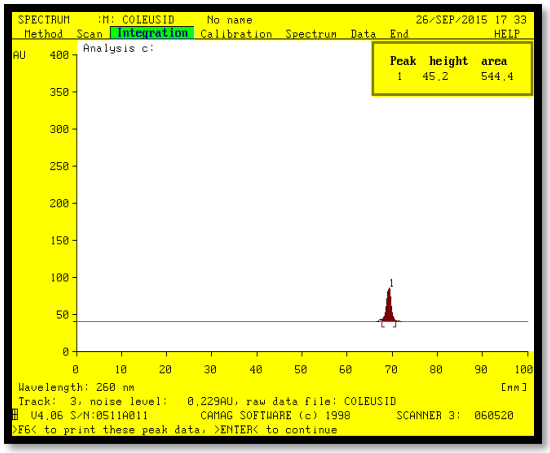

Fig.3(c.2)- Integrated chromatogram

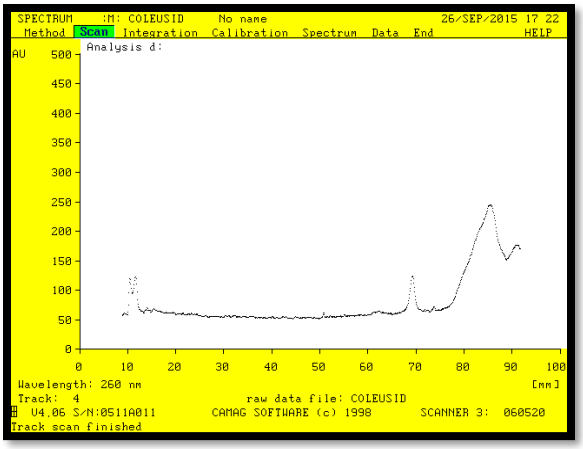

Fig.3(d.1)- Chromatogram of Normal standard

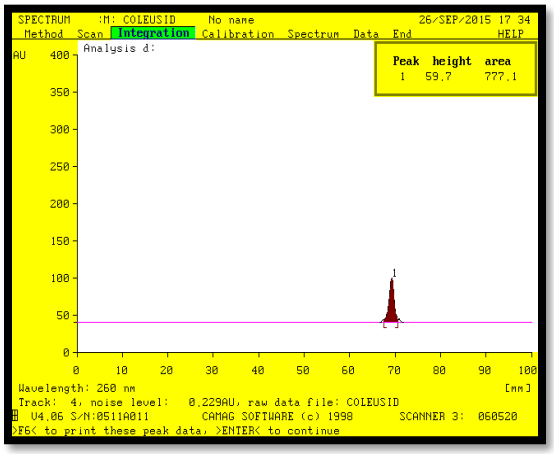

Fig.3(d.2)- Integrated chromatogram

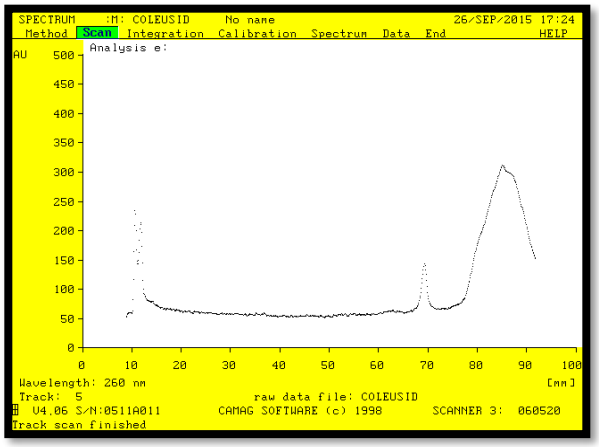

Fig.3(e.1)- Chromatogram of Double standard

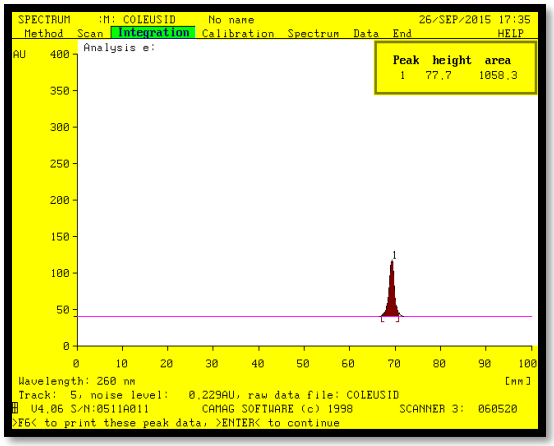

Fig.3(e.2)- Integrated chromatogram

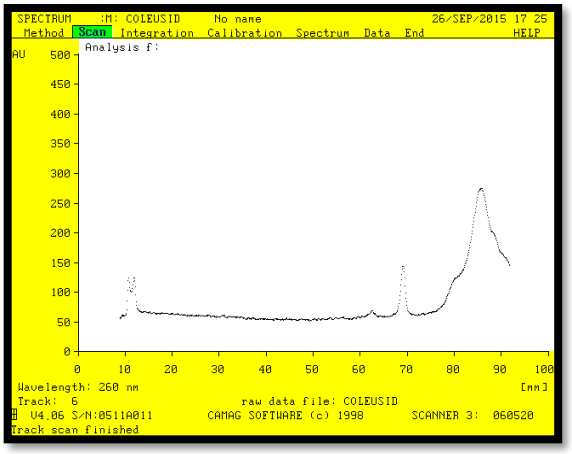

Fig.3(f.1)- Chromatogram of Sample A

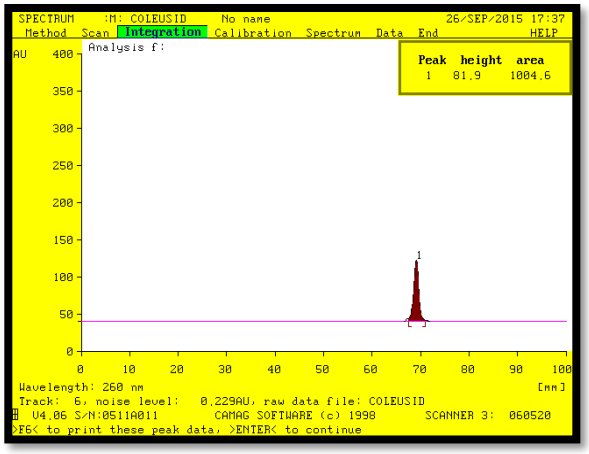

Fig.3(f.2)- Integrated chromatogram

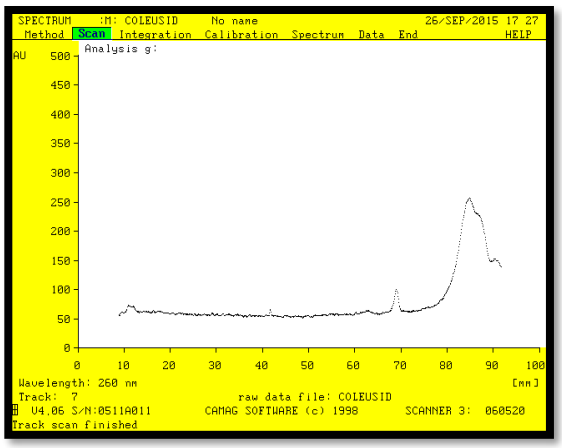

Fig.3(g.1)- Chromatogram of Sample B

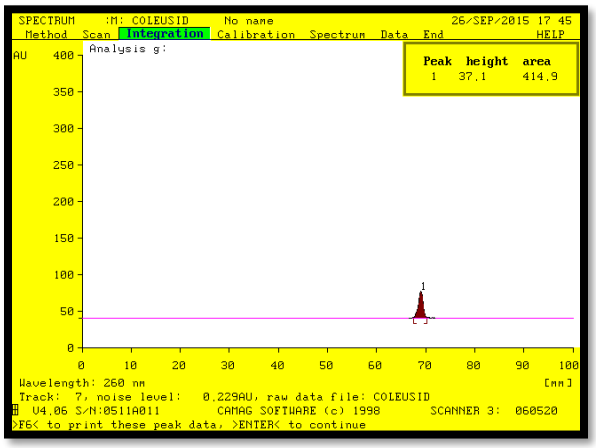

Fig.3(g.2)- Integrated chromatogram

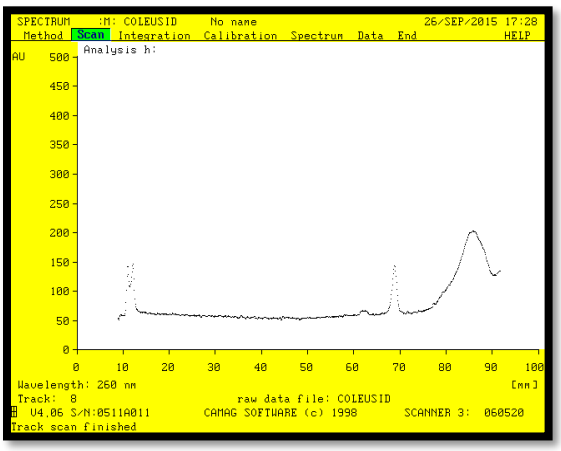

Fig.3(h.1)- Chromatogram of Sample C

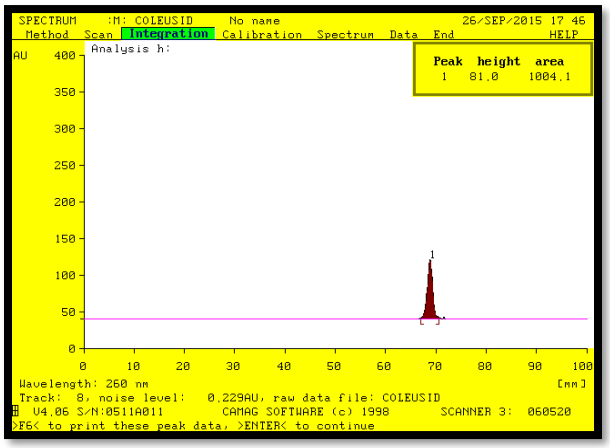

Fig.3(h.2)- Integrated chromatogram

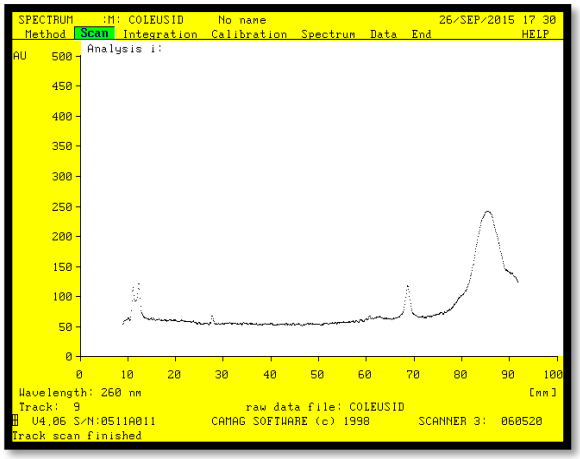

Fig.3(i.1)- Chromatogram of Sample D

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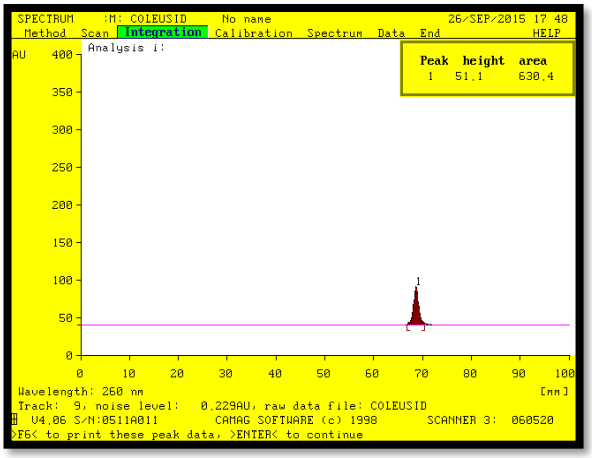

Fig.3(i.2)- Integrated chromatogram

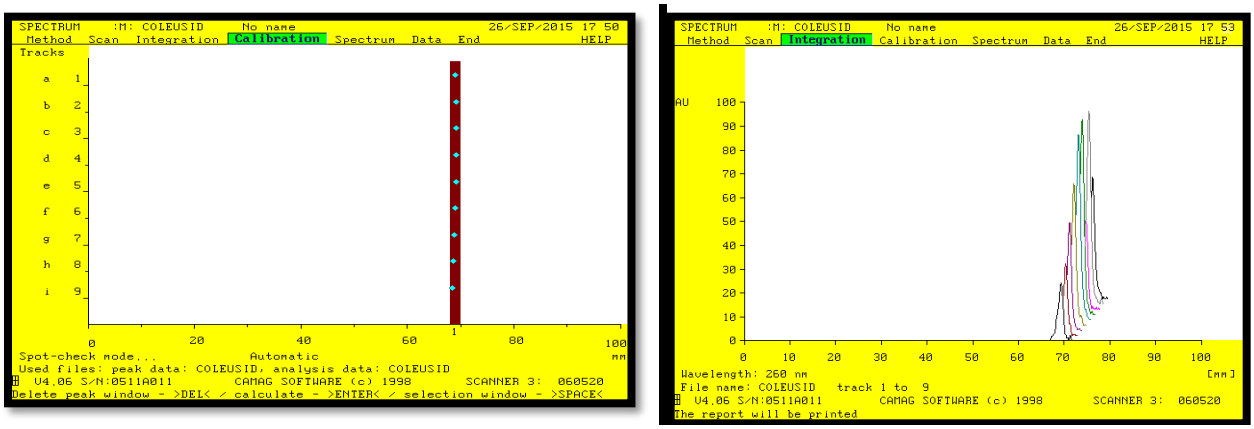

Fig. 4. The condensed chromatogram of Trial-1 (*Udicya*)

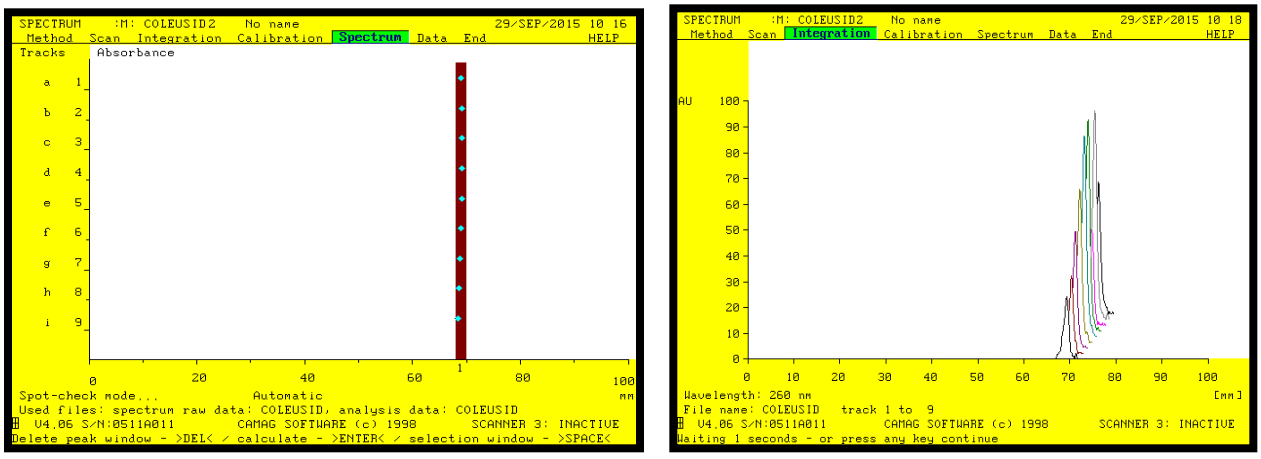

Fig. 5. The condensed chromatogram of Trial-2 (*Udicya*)

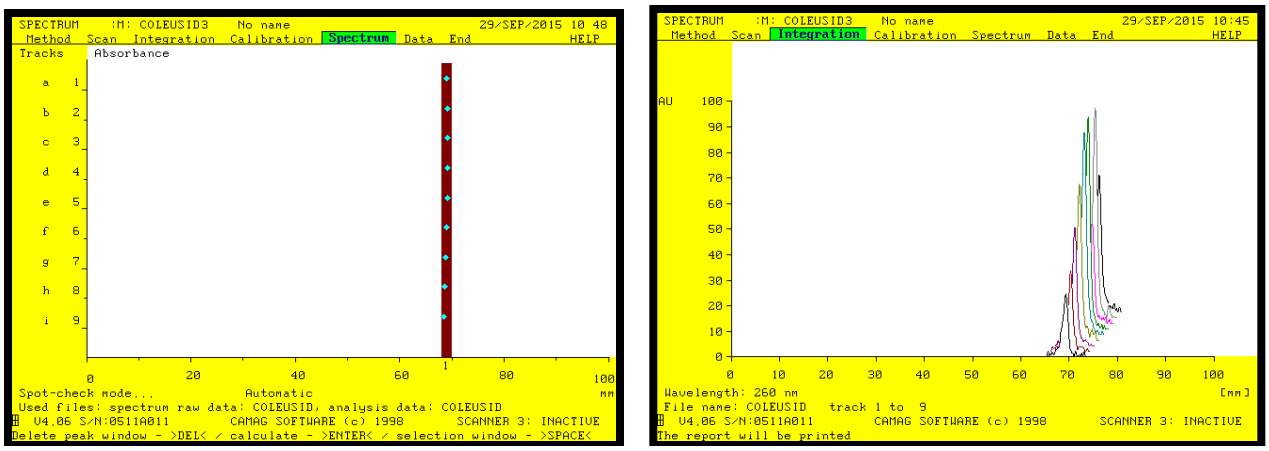

Fig. 6. The condensed chromatogram of Trial-3 (*Udicya*)

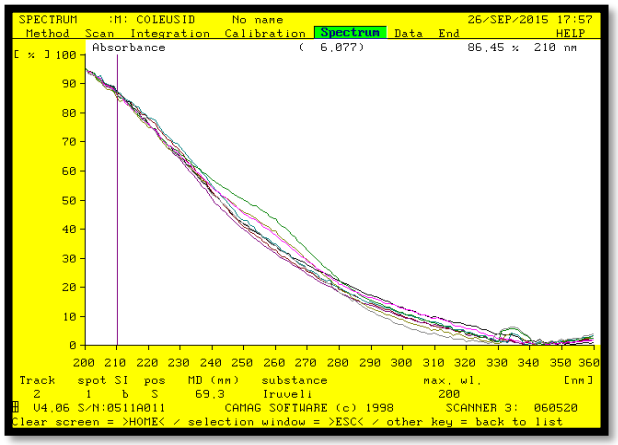

Fig. 7. The overlaid UV spectrum of the reference spots with R_f value 0.73

RESULTS AND DISCUSSION

Quantitative estimation of one of the herbal ingredients *Udicya* present in the Polyherbal formulation *Patoladi Ghrita* was carried out by HPTLC method. For the optimization of the method, different mobile phase compositions were used to get a good separation. Among the various mobile phases tried, Isopropyl alcohol: Water:: (9: 1 v/v) showed a good resolution. The methanol extract of the crude drug *Udicya*, methanol extracts of standard preparations of *Patoladi Ghrita* and the methanol extracts of the marketed samples of *Patoladi Ghrita*, gave dark brown spots with an R_f value of 0.73 when observed through the UV cabinet. Photograph of the developed HPTLC plate is given in (Fig.2). The chemical equivalency of the spots was confirmed by the UV surface scanning. The overlaid UV spectrum of the spots is given in (Fig.7).

Table 2: AUC of spots obtained in the HPTLC analysis of *Udicya*

Sl. No.	Track identity	AUC of the peak			Mean Value ± SD
		Trial-1	Trial-2	Trial-3	
1	Track-1: Methanolic extract of Udicya	**324.5**	**372.5**	**348.9**	**348.53±24.00**
2	Track-2: Methanolic extract of Q- Std.	**356.9**	**443.3**	**375.1**	**391.77±45.55**
3	Track-3: Methanolic extract of H- Std.	**544.4**	**595.9**	**602.3**	**580.87±31.74**
4	Track-4: Methanolic extract of N-Std.	**777.1**	**816.8**	**800.9**	**798.27±19.98**

5	Track-5: Methanolic extract of D- Std.	**1058.3**	**1038.6**	**1054.8**	**1050.57±10.51**
6	Track-6: Methanolic extract of Sample A	**1004.6**	**1028.5**	**1012.6**	**1015.23±12.17**
7	Track-7: Methanolic extract of Sample B	**414.9**	**442.5**	**475.3**	**444.23±30.24**
8	Track-8: Methanolic extract of Sample C	**1004.1**	**1029.7**	**1025.9**	**1019.90±13.81**
9	Track-9: Methanolic extract of Sample D	**630.4**	**665.5**	**699.2**	**665.03±34.40**

Table 3: Concentration vs AUC of *Udicya*

Concentration (Mass units)	AUC (Average of 3 values)
1	**391.77**
2	**580.87**
4	**798.27**
8	**1050.57**

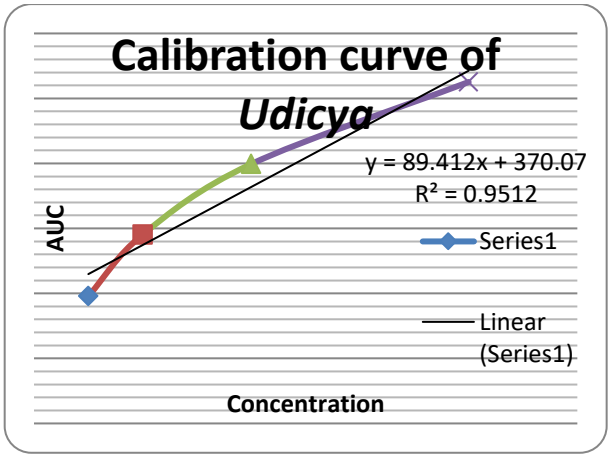

Fig 8 : Calibration Curve of *Udicya*

The AUC obtained for reference spots of the four standard tracks were used to plot a graph to establish the linearity between the concentration and AUC (Fig.8). The correlation coefficient obtained was within the acceptable limits. Thus it could be possible to obtain the quantities of the drug in question used in the different samples. From the graph the concentration of *Udicya* present in the four marketed samples of the formulation namely Sample- A, B, C and D (Table 4) were found out.

Table 4. Concentration of *Udicya* estimated in various marketed formulations by HPTLC method

Sample	AUC (Average of 3 values)	Theoretical Concentration (Mass units)	Practical Concentration (Mass units)	Percentage label claim of *Udicya* in the formulation (% w/w)
Sample-A	**1015.23**	4	7.22	180.50
Sample-B	**444.23**	4	0.83	20.75
Sample-C	**1019.90**	4	7.27	181.75
Sample-D	**665.03**	4	3.30	82.50

The percentage of drug present in the four marketed samples ranged from 20.75 to 181.75. The variation though very wide, is expected from a biological system because of the inherent variability. In the case of allopathic formulations the limit of assay is usually in the range of ± 5 to 10 %. In the case of Polyherbal formulations, taking in to account the inconsistency and complexity of the biological system, a wider range of ± 20 to 30 % may be prescribed. It is possible to achieve better range by a careful and rigorous

selection of ingredients and adhering to Good Manufacturing Practices.

The advantage of this method is that no marker compounds are required for the estimation of the phytochemicals. HPTLC method of quantification of some phytochemicals whose reference standards (marker compounds) are available is described in the literature.[18-21] Though many marker compounds are now available, not all crude drugs have it. More over the marker compounds are generally very costly. In the present study, the chemical constituent generating the reference spot need not be identified. However the chemical homogeneity of the reference spots in the standard and sample is established by the same R_f value and the spectral pattern.

CONCLUSION

The HPTLC technique is a very scientific method for the quantification of individual ingredients used in Polyherbal formulations. The HPTLC fingerprinting can be used as a reliable method for the identification of individual crude drugs in a formulation. Having established official standards for the crude drugs and Ayurvedic formulations, it now becomes possible to fix a range for all the ingredients present in a formulation. The HPTLC method described for *Patoladi Ghrita* can be extended to other type of formulations with suitable modifications. For example, in the case of liquid oral preparations, a Chloroform extract may be prepared instead of the methanol preparation.

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