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A HPTLC METHOD FOR THE QUANTITATIVE ESTIMATION OF *ADHATODA VASICA* IN RASNAIRANDADI KASHAYAM – AN AYURVEDIC POLYHERBAL FORMULATION

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ABSTRACT

The herbal products market in India is aggressively expanding. The standardisation of these herbal medications is necessary for the safety and effectiveness of herbal goods. There is a need for more sophisticated standardisation approaches because the conventional methodology is insufficient for the contemporary herbal industry. With this view the present study was undertaken to develop a standardisation procedure viz., Q-HPTLC profiling for a poly herbal formulation-rasnairandadi kashayam. Initially, the ingredients of rasnairandadi kashayam was procured. Among all the ingredients necessary for the preparation of rasnairandadi kashayam, the present study focussed on quantitative estimation of an ingredient, *Adhatoda vasica* root composition. The selected ingredient was standardized according to WHO guidelines 1992 and IP, 1996. Simultaneously, extraction and phytochemical screening of the selected ingredient was done. Next, by using all the purchased ingredients, different concentration of rasnairandadi kashayam formulation was prepared as per the procedure in Sahasrayoga Kashaya Prakarna. 428. The prepared formulations were subjected to phytochemical screening. Two brands (Brand A & B) of rasnairandadi kashayam available in the market of Kerala was purchased. The samples from all the four different concentrations of rasnairandadi kashayam prepared and two brands (Brand A & B) purchased were analysed for the phytoconstituents. Finally, QHPTLC evaluation were done to confirm the presence of ingredient in the formulation as per the guidelines of API. From this study it was concluded that the sample B contains a slightly excess concentration of *A. vasica*. Summarily, through this present study, it was able to develop a sophisticated method for quantification of individual ingredient present in poly-herbal Ayurvedic preparations.

KEYWORDS: Rasnairandadi kashayam, *Adhatoda vasica* root, Q-HPTLC profiling.

INTRODUCTION

The application of herbs for medicinal purposes represents the most ancient method of healthcare recognized by humanity, utilized across all cultures throughout history. Primitive humans acknowledged their reliance on nature for maintaining health, and since that era, humanity has relied on the variety of plant resources for sustenance, clothing, shelter, and medicinal remedies to cure numerous ailments. The understanding of plant-derived medications evolved over time and was transmitted through generations, thereby establishing the foundation for numerous traditional medicine systems globally. In certain communities, herbal medicine remains a vital component of their healthcare practices. Medicinal plants are found extensively across the globe, with the highest concentration in tropical regions.[1]

India has a lengthy, secure, and uninterrupted history of utilizing numerous herbal medicines within the officially acknowledged alternative health systems, namely Ayurveda, Yoga, Unani, Siddha, Homeopathy, and Naturopathy. These systems have justifiably coexisted alongside allopathy and are not in "the domain of obscurity". A millions of Indians consistently utilize herbal medications in various forms, including spices, home remedies, health foods, and over-the-counter options for self-medication, as well as medications prescribed within non-allopathic systems. Over 500,000

non-allopathic practitioners have received training in the medical colleges (more than 400) associated with their respective health systems and are officially registered with the councils that oversee professionalism. Therefore, these systems should not be regarded as mere folklore or traditional herbal practices. They are founded on fundamental principles that contribute to a logical and systematic framework for understanding pathogenesis and diagnosis, which also serves as a basis for therapeutic interventions.[2]

In the meantime, humans place the utmost importance on quality in every aspect of life. It is essential that medications for humans are of superior quality, as they are employed to safeguard the well-being of humanity. The quality control of pharmaceuticals derived from synthetically manufactured chemicals is governed by stringent rules and regulations. They are required to successfully complete various tests and meet quality standards prior to being marketed and used by patients and consumers. This stringent regulatory process guarantees the safety and efficacy of pharmaceutical products by confirming that the quality of synthetically produced medications meets the necessary standards.[3-7]

Currently, it is quite challenging to ascertain the presence of all the ingredients as stated in a formulation for herbal preparations.[8] Hence standardization of herbal formulations is essential in order to assess the quality of drugs, based on the concentration of their active principle, physical, chemical, physcio-chemical standardization and in vitro, *in-vivo* parameters.[9]

Regarding with standardization of herbal medicines, it is the process of prescribing a set of standards or inherent characteristics, constant parameters, definitive qualitative and quantitative values that carry an assurance of quality, efficacy, safety and reproducibility. It is the process of developing and agreeing upon technical standards. Specific standards are worked out by experimentation and observations, which would lead to the process of prescribing a set of characteristics exhibited by the particular herbal medicine. Hence standardization is a tool in the quality control process. However, proper standardisation of poly-herbal ayurvedic formulation is one of the serious challenges faced by the present scenario of herbal treatment technology in order to prove its safety and efficacy.[8] With this background, the present work aims at the standardisation of an herbal ingredient in an ayurvedic poly-herbal formulation, *Rasnairandadi kashayam*.

MATERIALS AND METHODS

Procurement of ingredients of Rasnairandadi kashayam

Ingredients of Rasnairandadi kashayam was procured from Hema Ayurvedic Centre located at No. AP X/1391, Thazhamel, Anchal, Kollam District of Kerala.

Standardization of procured ingredients

The procured ingredients were standardized according to WHO guidelines 1992 and IP, 1996. The procedures recommended in IP, 1996 and WHO guidelines 1992 were followed to calculate the physicochemical constants.

Ash values

Total Ash

The total ash was determined by incinerating 2-3g accurately weighed and air dried coarsely powdered drug in a tarred silica crucible which was previously ignited and cooled before weighing, at a temperature not exceeding 450°C. The ignition was repeated and the percentage of ash with reference to air-dried drug was calculated.

Water soluble ash

The total ash was boiled for 5min with 25ml of water. The residue was washed with hot water, ignited for 15min at a temperature not exceeding 450°C, cooled and weighed. This weight was subtracted from the weight of ash; the difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to air-dried drug.

Acid insoluble ash

The ash obtained was boiled with 25ml of dilute hydrochloric acid for 5min and filtered through an ash less filter paper. The residue was washed with hot water, ignited, cooled in a desiccator and weighed. The percentage of acid insoluble ash was calculated with reference to air dried drug.

Sulphated ash

The sulphated ash was determined by incinerating 1g of accurately weighed air dried coarsely powdered drug in a tarred silica crucible which was previously ignited and cooled before weighing at a temperature not exceeding 450°C. The residue was moistened with 1ml of concentrated sulphuric acid, ignited at 800±25°C until all black particles had disappeared. It was then cooled; again sulphuric acid was added and ignited. It was cooled and the percentage of sulphated ash was calculated with reference to air dried drug.

Extractive values

Ethanol soluble extractive

Dried coarse powder (5g) was weighed and macerated with 100ml of 90% ethanol in a closed flask for 24h, shaken frequently during 6h and allowed to stand for 18h. Filtered immediately taking precautions against loss of ethanol and 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish. The residue was dried at 105°C and weighed. The percentage of ethanol soluble extractive was calculated with reference to air dried drug.

Water soluble extractive

Coarse powder (5g) was weighed and dissolved in 100ml

of water in a stoppered flask, heated at 80°C, shaken well and allowed to stand for 10min. It was cooled; 2g of kieselghur was added and filtered. 5ml of the filtrate was transferred to a tarred evaporating dish and the solvent was evaporated on a water bath. The percentage of water soluble extractive was calculated with reference to air dried drug.

Ether soluble extractive

Coarse powder (5g) was macerated with 100ml of diethyl ether in a stoppered flask for 24h, shaken frequently during the first 6h and allowed to stand for 18h. Thereafter, that was filtered rapidly without loss of solvent and 25ml of the filtrate was evaporated to dryness in a tarred evaporating dish and solvent was evaporated on a water bath dried at 105°C. The percentage of ether soluble extractive value was calculated with reference to the air dried drug.

Determination of volatile oil in drug

50g of the fresh drug was weighed and boiled with water in a Clavenger's apparatus. The isolation was continued till no more oil was collected in the graduated tube. The volume of oil was measured and percentage of volatile oil present in the plant was calculated.

Determination of loss on drying

Glass stoppered shallow bottle was weighed that had been dried in the same conditions to be employed in the determination. About 1g of the sample was transferred to the bottle and distributed evenly by gently side wise shaking to a depth not exceeding 10mm, placed the loaded bottle in a drying chamber (the stopper was removed and left in the chamber). The sample was dried to a constant weight and allowed to cool. The bottle along with the content was weighed and process was repeated until the successive weights differed not more than 0.5mg (drying to constant weight). The percentage loss of weight was calculated with reference to the air-dried drug.

Extraction and preliminary phytochemical evaluation

Powdering, extraction (Soxhlet extraction using the solvents such as petroleum ether, chloroform, ethyl acetate, methanol and water) and preliminary phytochemical evaluation (test for alkaloids, glycosides, phenolic compounds and tannins, flavonones and flavonoids, carbohydrates, proteins and aminoacids, terpenoids, saponins, and sterol) were done in reference with the standard procedure.[10-17]

Preparation of Rasnairandadi kashayam

Four different concentrations (N-Std; Q-Std; H-Std; D-Std) of rasnairandadi kashayam was prepared. For the preparation, all the ingredients mentioned in the Table 1 was cleaned and crushed to form a coarse powder and add it to 16 times water and boil it till it reduces to 1/4th its actual amount and get it off the flame and let it cool. Filter this decoction and keep the filtrate in tinted glass bottles.

Table 1: Ingredients of Rasnairandadi Kashayam.

No	Ingredients	Scientific name	Family	Plant part used
1	Rasna	*Alpina galanga*	Zingiberaceae	Root/leaf
2	Ikshura	*Asteracantha longifolia*	Acanthaceae	Root
3	Vasa	*Adhatoda vasica*	Acanthaceae	Root
4	Sahachara	*Barleria prionitis*	Acanthaceae	Whole plant
5	Ghana	*Cyperus rotundus*	Cyperaceae	Rhizome
6	Shati	*Hedychium spicatum*	Zingiberaceae	Rhizome
7	Vishwa	*Zingiber officinale*	Zingiberaceae	Rhizome
8	Vari	*Asparagus racemosus*	Asparagaceae	Root
9	Dusparsha	*Tragia involucrata*	Euphorbiaceae	Whole plant
10	Bala	*Sida cordifolia*	Malvaceae	Root
11	Amruta	*Tinospora cordifolia*	Menispermaceae	Stem
12	Dehahva	*Cedrus deodara*	Pinaceae	Hard wood
13	Ativisha	*Aconitum heterophyllum*	Ranunculaceae	Root tuber
14	Eranda	*Ricinus communis*	Euphorbiaceae	Root

Preparation of N-Std was done as per the procedure mentioned in Sahasrayoga Kashaya Prakarna. 428. For the preparation of Q-Std, 1/4th of the standard quantity of the ingredients mentioned in the Sahasrayoga Kashaya Prakarna. 428 were taken and the preparation was done as per the procedure stated above. Likewise, the H-Std was prepared by using ½ of the standard quantity and D-Std was prepared by using double the amount of standard quantity of ingredients recommended by the Sahasrayoga. All the four prepared formulations were stored properly for further studies.

Procurement of marketed brands of rasnairandadi kashayam

Two brands (Brand A & B) of rasnairandadi kashayam available in the market of Kerala was purchased from the Kollam district of Kerala and preserved properly for further studies.

Phytochemical analysis of the formulations prepared and purchased

The samples from all the four different concentrations of rasnairandadi kashayam prepared and two brands (Brand

A & B) purchased were analysed for the phytoconstituents viz., alkaloids, glycosides, phenolic compounds and tannins, flavanones and flavonoids, carbohydrates, proteins and amino acids, terpenoids, sterols, saponins etc.

HPTLC evaluation

In the HPTLC evaluation, the ingredient No. 3 in Table 1 was analyzed comparatively with the normal standard of rasnairandadi kashayam prepared to confirm the presence of that ingredient in the preparation. In the next level, the quantitative HPTLC evaluation was done in which the selected ingredient was subjected to comparative evaluation with four different concentrations of the prepared rasnairandadi kashayam and the two purchased (Brand A & B) formulations from the market. The instrument details such as system setup and chromatographic data are presented in Table 2. The mobile phase used in HPTLC analysis was Ethyl acetate : Methanol : Ammonia in the ratio of 8 : 2 : 1. The Rf value was found at 254nm.

Table 2: HPTLC and Chromatography specifications.

Parameter		**Specification**
System setup	Software	Server Desktop-60R112G, Version 3.2.23095.1
	Linomat 5	S/N: 241506
	TLC scanner 4; TLC visualizer 2	S/N: 241072; S/N: 241537
Plate layout	Stationary phase	Merck, HPTLC Silica gel 60 F_{254}
	Plate format; Application type	100 × 100mm; Band
	Application	PositionY:8.0mm;Length: 8.0mm; Width:0mm
	Track	I^{st} position X: 15.0mm Distance: 11.4mm (In quantitative HPTLC, I^{st} position X: 20.0mm)
	Solvent front position	70mm
Sample solvent type: Methanol; **Dosage speed**: 150nL/s; **Pre-dosage vol**.: 0.20µl		
Chamber	Tank; Saturation time	20 × 10; 20min
	Use saturation pad;	true
	Use smartALERT	False
	Vol. front through; Vol. rear through	10mL; 20mL
	Drying time & Temp.;	5min; Room temperature
Image developed (plate 1a)	Quantity; RT white; R254; R366	Std.; Auto, level 85% Band
Scan developed (plate 1b & 1c)	Scanner type & Speed	Single λ; 20mm/sec
	Optimization for; Measurement mode	Resolution; absorbance
	Detector mode; Data resolution	Automatic; 100µm/step
	Slit	5 × 0.2mm; micro
	Lamp	For plate 1b: Deuterium & Tungsten; For Plate 1c: Mercury
	Wavelength	For plate 1b: 254nm; For plate 1c: 366nm

RESULTS AND DISCUSSION

Among the total of thirteen ingredients necessary for the preparation of rasnairandadi kashayam, the present study focussed on quantitative estimation of an ingredient, *Adhatoda vasica* root composition in the four different concentrations (N-Std; Q-Std; H-Std; D-Std) of rasnairandadi kashayam prepared and two brands (Brand A & B) of rasnairandadi kashayam available in the market of Kerala. Initially, the physicochemical constants such as ash values, extractive values, volatile oil content and loss on drying were assessed and their results are shown in Table 3.

Table 3: Analysis of Physicochemical Constants of *Adhatoda Vasica Root* Powder – An Ingredient of Rasnairandadi Kashayam.

Physicochemical constants		***Adhatoda vasica root* powder**
Ash value (%w/w)	Total ash	13.5
	Water soluble ash	3.55
	Acid insoluble ash	0.94
	Sulphated ash	5.20
Extractive value (%w/w)	Ethanol soluble extractive	5.63
	Water soluble extractive	34.50
	Ether soluble extractive	4.25
Volatile oil (%w/w)	--	0.096
Loss on drying (%w/w)	--	3.40

Presence of alkaloids, glycosides, tannins, flavonoids, terpenoids, sterol, saponin and carbohydrate were found in the preliminary phytochemical evaluation of the extracts of *Adhatoda vasica root* powder (Table 3). Particularly, the alkaloids, tannins and carbohydrates were found in the ethyl acetate, methanol and aqueous extract, but the glycosides, flavonoids, terpenoids, sterol and saponin were found in all the tested extracts.

Table 4: Preliminary Phytochemical Evaluation of the *A. Vasica Root* Powder Extracts.

Constituents	***Adhatoda vasica***				
	1	**2**	**3**	**4**	**5**
Alkaloids	–	–	+	+	+
Glycosides	+	+	+	+	+
Tannins	–	–	+	+	+
Flavonoids	+	+	+	+	+
Terpenoids	+	+	+	+	+
Sterol	+	+	+	+	+
Saponin	+	+	+	+	+
Carbohydrate	–	–	+	+	+
Protein & Amino acids	–	–	–	–	–

1-Petroleum ether extract; 2-Chloroform extract; 3-Ethyl acetate extract; 4-Methanol extract; 5-Aqueous extract.

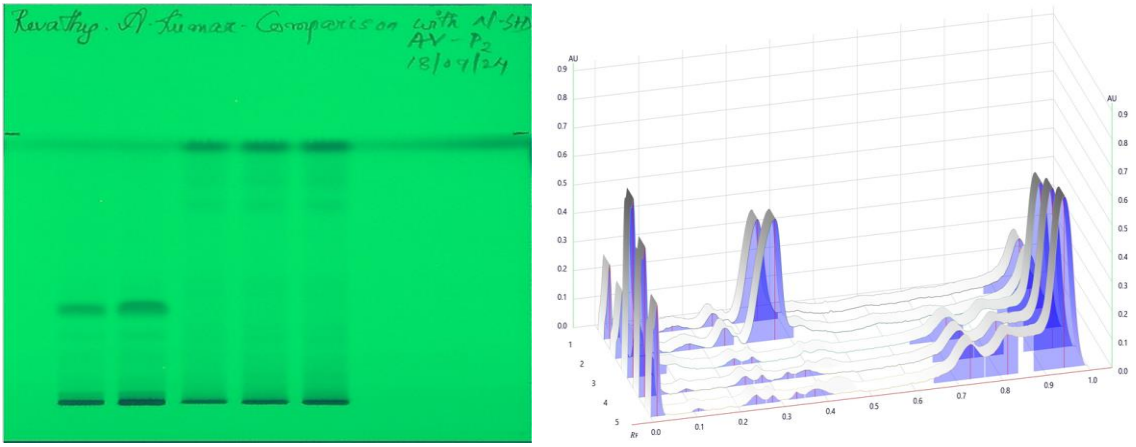
In the preliminary phytochemical evaluation of all the four prepared concentrations of rasnairandadi kashayam and also the purchased rasnairandadi kashayam both brand A & B showed the presence of above stated phytoconstituents. The presence of *A. vasica*, and also the other ingredients present in the formulation might reasoned for the results obtained here.

In the present study, it was able to identify the presence of alkaloids widely in the tested extracts. Literature indicates that plant alkaloids have considerable biological activity. Due to their potent biological activity, approximately 12000 known alkaloids have been exploited as pharmaceuticals, stimulants, narcotics and poisons. The use of alkaloid-containing plants as dyes, species, drugs or poisons can be traced back almost to the beginning of civilization. Alkaloids are also known to be anti-arrythmic effects, antihypertensive effects, anticancer and antimalarial activity. Likewise, alkaloids, presence of flavonoids were found widely. Flavonoids are a group of polyphenolic compounds. They are ubiquitous in photosynthesizing cells and are commonly found in fruits, vegetables, nuts, seeds, stems, flowers, tea, wine and honey. They are known to have medicinal properties and play a major role in the successful medical treatments from ancient times, and their use has persevered till date. Flavonoids have been reported to have anti-hyperglycemic and anticancer effect. Flavonoids are known to improve cardiac function, decrease anginas and lowers cholesterol levels. Presence of tannins were observed in tested extracts. Tannins may participate in the management of glucose level in blood. Tannins such as ellagic acid and resveratrol are known to effectively inhibit skin tumorgenesis in mice. Terpenoids, another one phytochemical found in the tested extracts. Terpenoids have antioxidant properties and also interact with most regulatory proteins. Terpenoids also improve the skin tone, increases the concentration of antioxidants in wounds, and restore inflamed tissues by increasing blood supply. Terpenoids also improve lung function. Terpenoids have shown to reduce diastolic blood pressure and lower the sugar level in blood in hypertensive and diabetic patients respectively. Regarding with saponins, another one compound found in the tested extracts. These phytochemicals are known to have hypo cholesterolaemic, immunostimulant, hypoglycemic effect and anticarcinogenic properties. Saponins are believed to act as adjuvants in enhancing antibody production and in the stimulation of cell mediated immune system. Saponins are believed to lower the risk of cancer and other chronic diseases. Regarding with sterol, another one compound found in the tested extracts. They have decreased serum cholesterol level as reported in several studies. Plant sterols are industrially important chemical sources for steroid compounds, insecticides, antioxidant and anticancer drugs.[18,19]

In case of HPTLC analysis of *Adhatoda vasica*, to confirm the Rf value, the prepared normal standard and pure extract of *A. vasica* was run consecutively at different concentration over five tracks as mentioned in Table 5 and Figure 1. All the five tracks showed similar spots with Rf value 0.23 and 0.35, but in track 3 the *A. vasica* was spotted 1.5µl concentration only, so that the spot of Rf 0.35 was not observed significantly.

Table 5: Comparison of *Adhatoda vasica* extract with prepared rasnairandadi kashayam (N-std.).

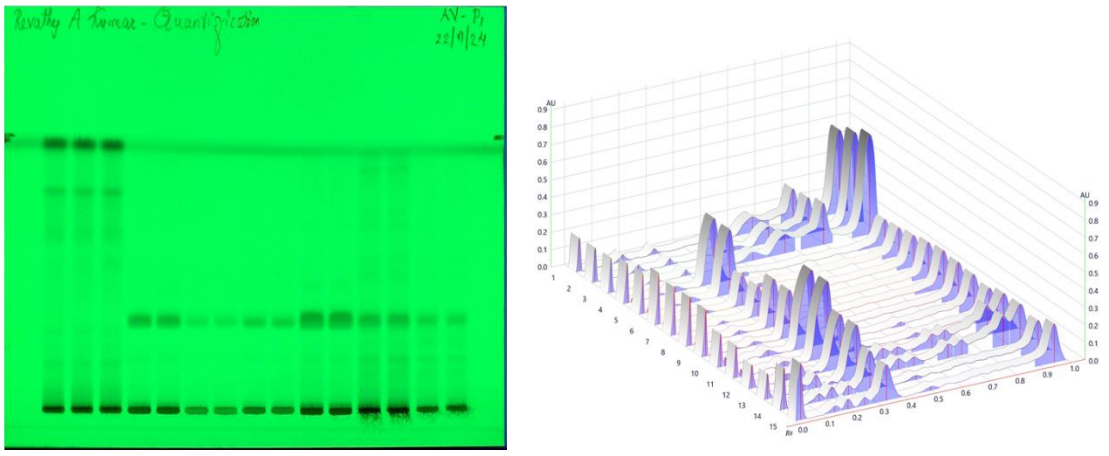
Track no.	Sample name	Volume (µl)	Rf
1	N-std.	1.5	0.23; 0.35
2	N-std.	2.5	0.23; 0.35
3	N-std.	1.5	0.23
4	AV	2	0.23; 0.35
5	AV	2.5	0.23; 0.35



A B

Figure 1: Comparative study of *A. vasica* extract with N-Std. of prepared rasnairandadi kashayam – A) Chromatogram development; B) 3D image

Next to comparative study, the quantification of *A. vasica* was done at two spots with Rf value 0.19 and 0.33 which were commonly found in all the 15 tracks. But the peak at Rf 0.33 was found prominent and easy to quantify. The Table 6 revealed that the sample A contains *A. vasica* concentration as per the standards of API. But the spot obtained in sample B was showed a slightly greater area than prepared N. Std., but less than D-Std. Hence it was concluded that the sample B contains a slightly excess concentration of *A. vasica* (Figure 2-17).



A B

Figure 2: Quantitative HPTLC analysis of pure extract of *A. vasica* with different concentration of rasnairandadi kashayam prepared and purchased brands - A) Chromatogram development; B) 3D image.

Table 6: QHPTLC analysis of *A. vasica* extract and different concentration of rasnairandadi kashayam prepared and purchased brand (Sample A & B)

Track no.	Sample name	Volume (µl)	Rf	Area
1	AV	3	0.19	0.00118
			0.33	0.00185
2	AV	3	0.19	0.00121
			0.33	0.00190
3	AV	3	0.19	0.00119
			0.33	0.00178
4	N-std	3	0.19	0.00147
			0.33	0.01880
5	N-std	3	0.19	0.00144
			0.33	0.01872
6	Q-std	3	0.19	0.00042
			0.33	0.00658
7	Q-std	3	0.19	0.00053
			0.33	0.00648
8	H-std	3	0.19	0.000785
			0.33	0.01018
9	H-std	3	0.19	0.000772
			0.33	0.01012
10	D-std	3	0.19	0.00296
			0.33	0.03670
11	D-std	3	0.19	0.00290
			0.33	0.03699
12	Sample A	3	0.19	0.00157
			0.33	0.01767
13	Sample A	3	0.19	0.00162
			0.33	0.01846
14	Sample B	3	0.19	0.00187
			0.33	0.02145
15	Sample B	3	0.19	0.00174
			0.33	0.02041

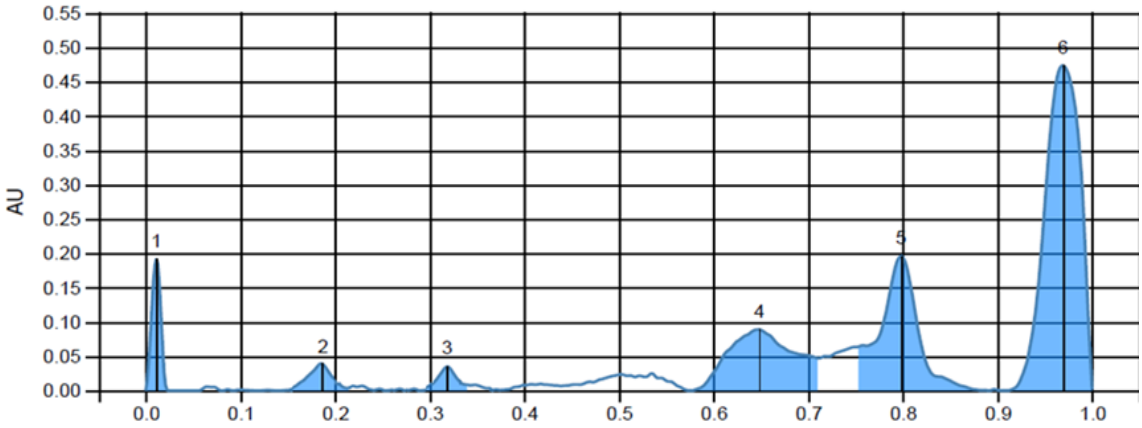


Figure 3: QHPTLC analysis of *A. vasica* extract (Track 1).

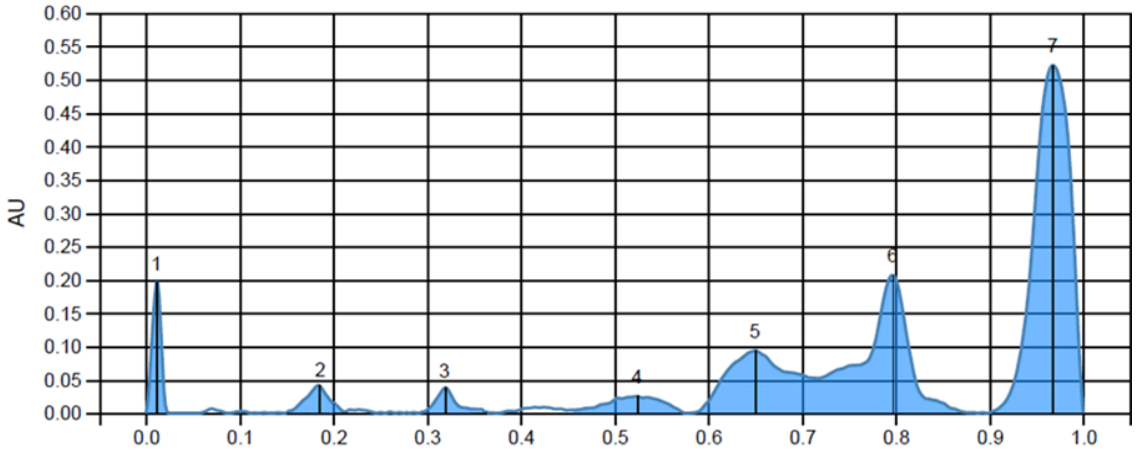


Figure 4: QHPTLC analysis of *A. vasica* extract (Track 2).

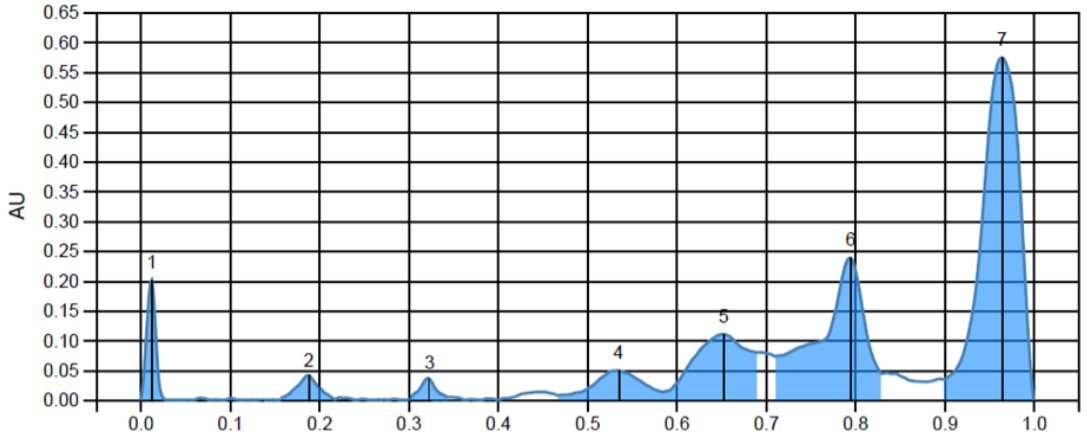


Figure 5: QHPTLC analysis of *A. vasica* extract (Track 3).

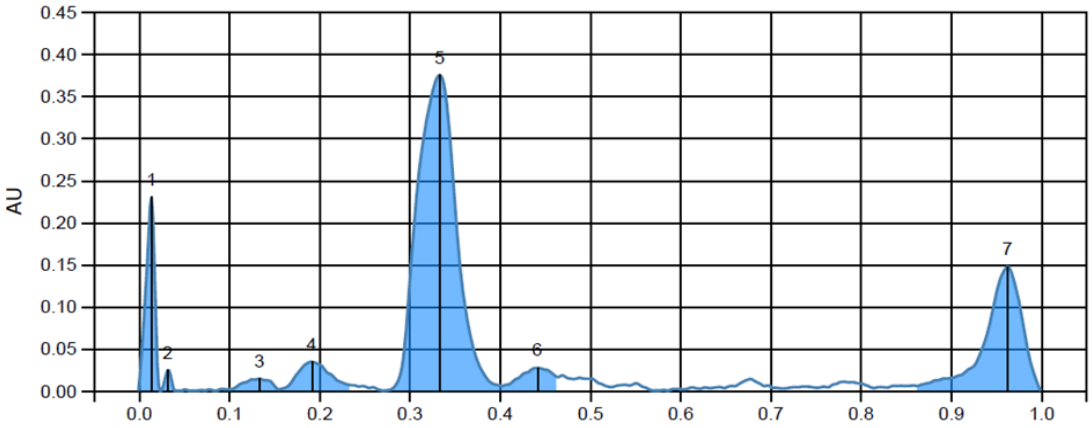


Figure 6: *A. vasica* - QHPTLC analysis of prepared rasnairandadi kashayam (N-Std.; Track 4)

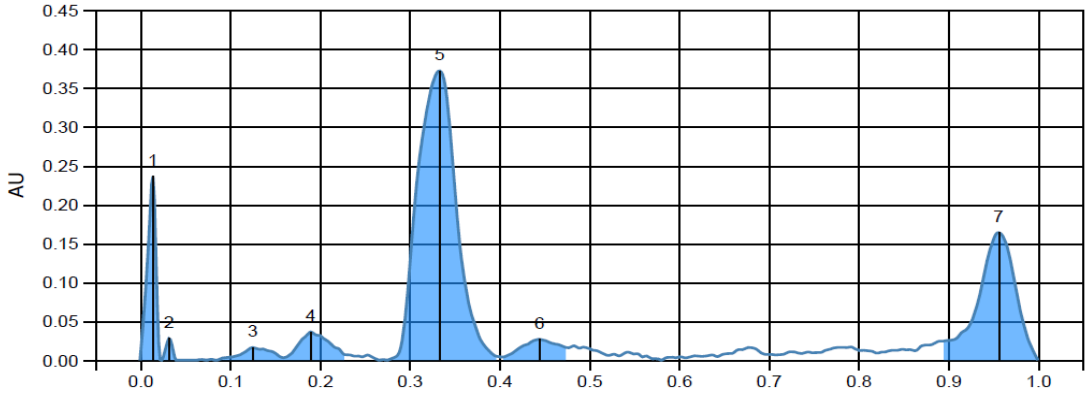


Figure 7: *A. vasica* - QHPTLC analysis of prepared rasnairandadi kashayam (N-Std.; Track 5).

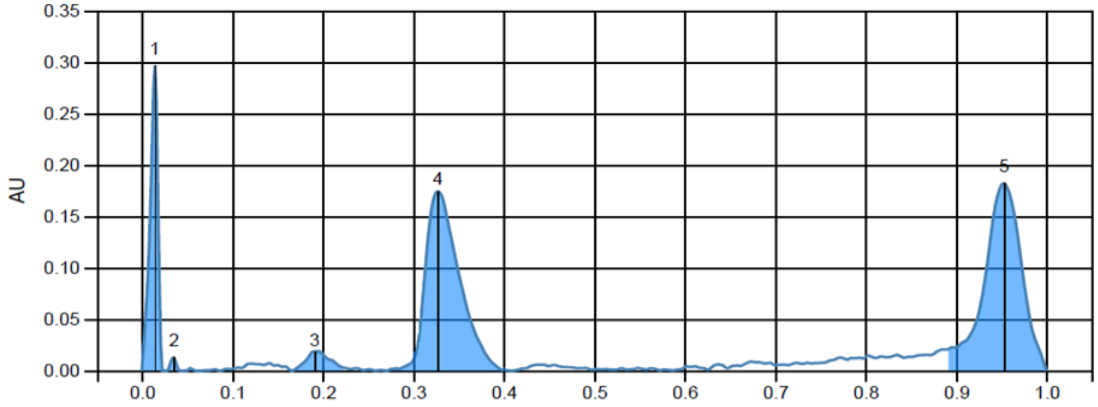


Figure 8: *A. vasica* - QHPTLC analysis of prepared rasnairandadi kashayam (Q-Std.; Track 6).

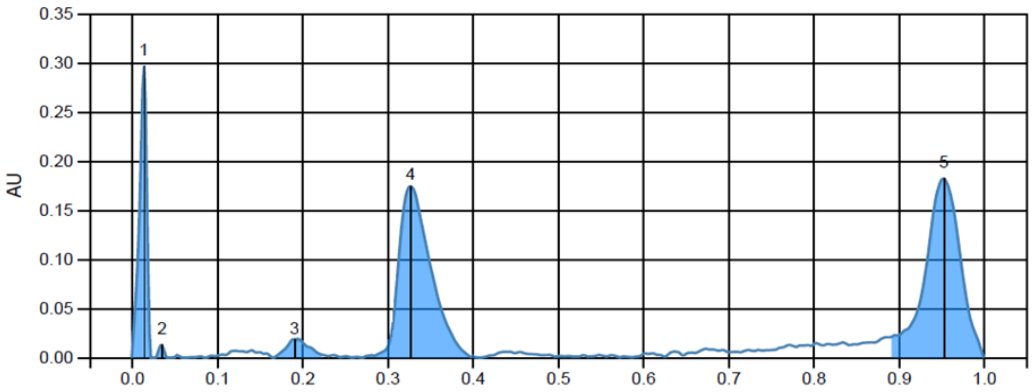


Figure 9: *A. vasica* - QHPTLC analysis of prepared rasnairandadi kashayam (Q-Std.; Track 7).

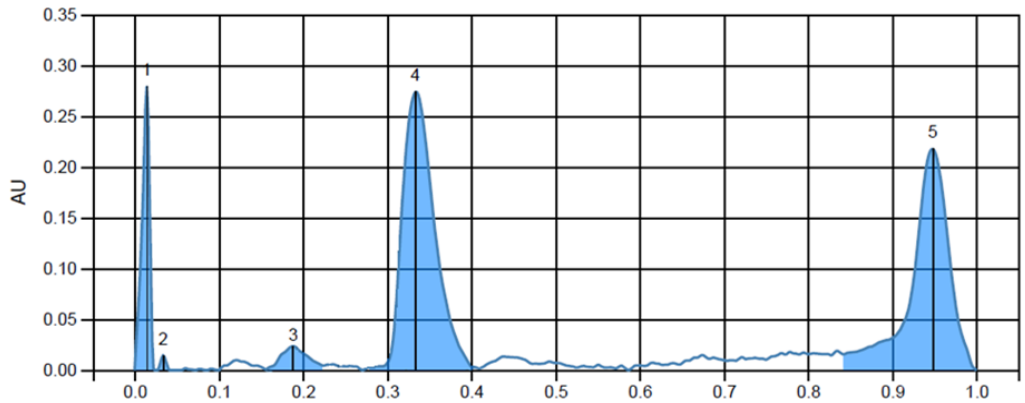


Figure 10: *A. vasica* - QHPTLC analysis of prepared rasnairandadi kashayam (H-Std.; Track 8).

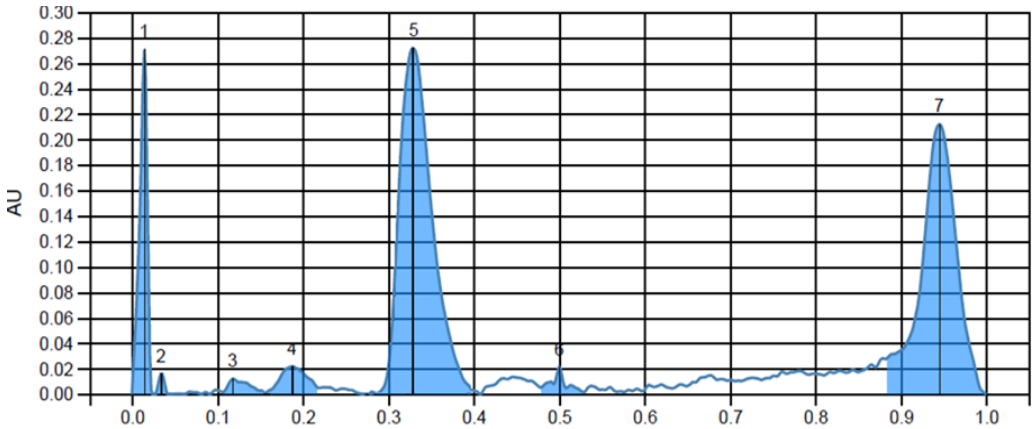


Figure 11: *A. vasica* - QHPTLC analysis of prepared rasnairandadi kashayam (H-Std.; Track 9)

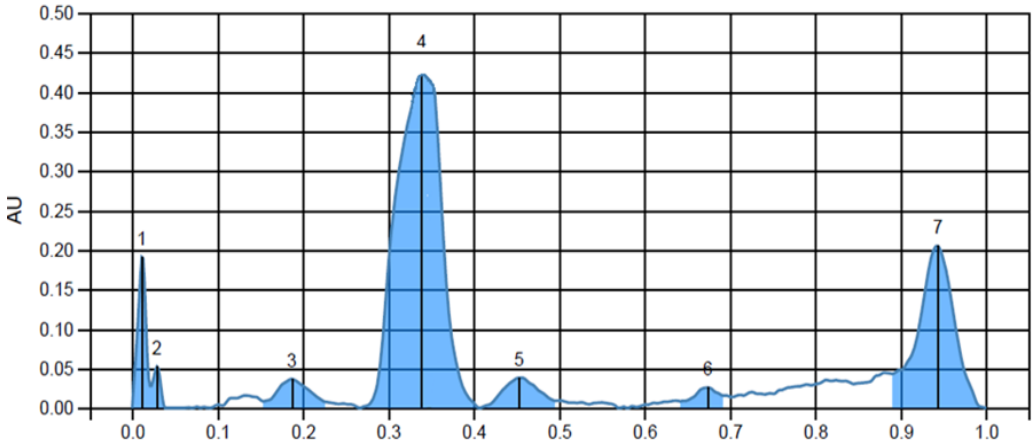


Figure 12: *A. vasica* - QHPTLC analysis of prepared rasnairandadi kashayam (D-Std.; Track 10).

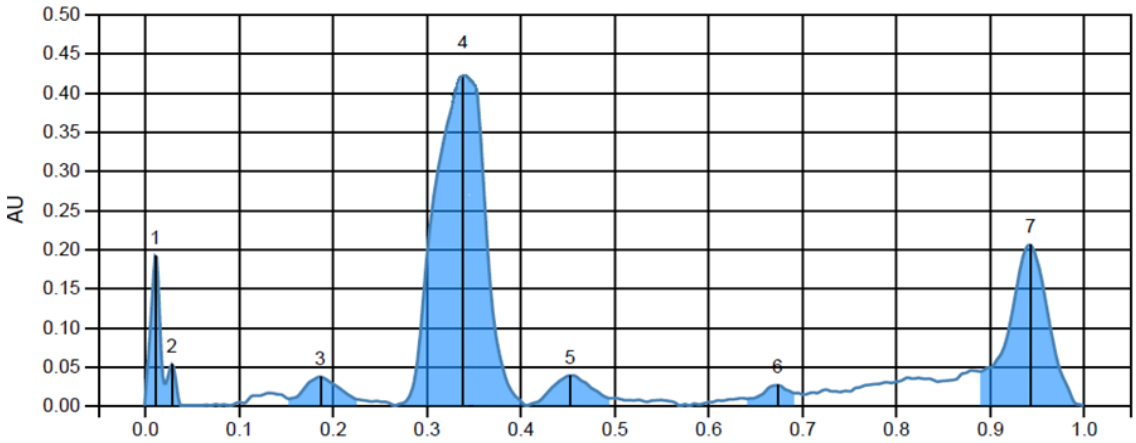


Figure 13: *A. vasica* - QHPTLC analysis of prepared rasnairandadi kashayam (D-Std.; Track 11)

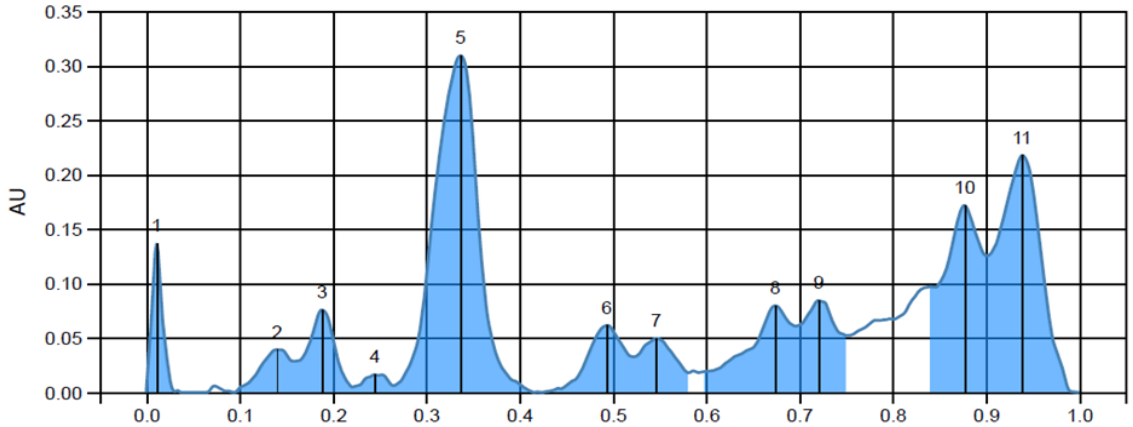


Figure 14: *A. vasica* - QHPTLC analysis of rasnairandadi kashayam purchased brand (Sample A., Track 12).

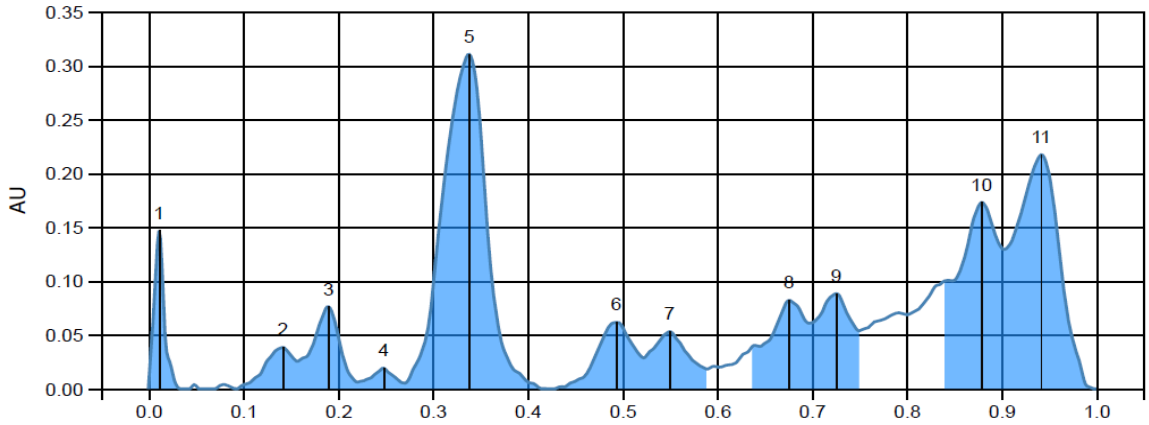


Figure 15: *A. vasica* - QHPTLC analysis of rasnairandadi kashayam purchased brand (Sample A., Track 13)

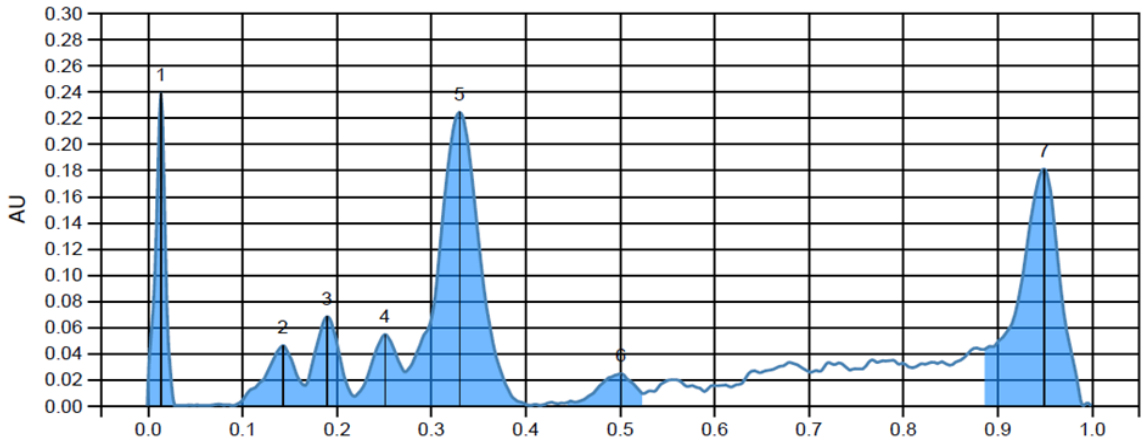


Figure 16: *A. vasica* - QHPTLC analysis of rasnairandadi kashayam purchased brand (Sample B., Track 14).

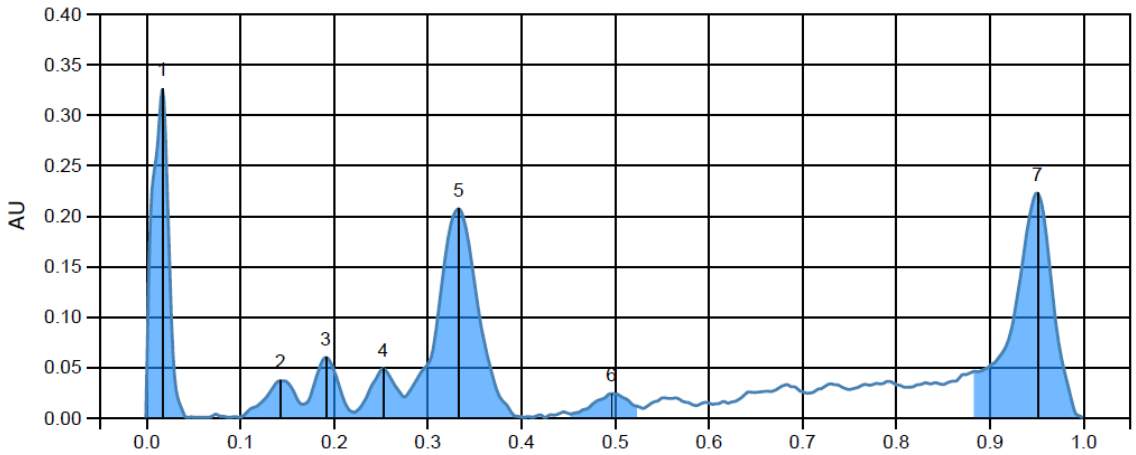


Figure 17: *A. vasica* - QHPTLC analysis of rasnairandadi kashayam purchased brand (Sample B., Track 15).

CONCLUSION

In the present study, the quantitative estimation of *Adhatoda vasica* in rasnairandadi kashayam**,** an ayurvedic polyherbal formulation was done successfully. From the results of present study, it was able to identify a slightly excess concentration of *A. vasica* in sample B. Of course, several factors may contribute to these minor variations in active constituent. Shortly, the results of the present study have strongly stressed the need of standardisation of Ayurvedic formulations and also laid a stone for the development of quantification procedures for the standardisation of Ayurvedic formulations in the future.

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