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RESEARCH ARTICLE

High Performance Thin Layer Chromatographic Method for Simultaneous Estimation of Vitamin C, Thymoquinone and Thymol in Plant Extracts

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Abstract

Background: Lack of an effective HPTLC simultaneous estimation method for vitamin C, thymoquinone and thymol in plant extracts. **Aim:** The present study involves the development of an accurate, precise, specific, and specific HPTLC method for the identification and quantification of three phytomarkers thymol and thymoquinone in *Nigella sativa (kali jiri)* seed extract and vitamin C in *Hylocereus polyrhizus* (dragon fruit) extract. **Methods:** Using an aluminum plate pre-coated with silica gel 60 F254 and methanol-n hexane-ammonia (15%) (8.5:1.5:0.2 v/v/v) as the mobile phase, thin layer chromatographic development was performed. **Results:** For each of the three markers, densitometric quantification was carried out at the isobestic point of 271 nm. Vitamin C, thymol, and thymoquinone bands were separated chromatographically at R_f values of 0.66, 0.35, and 0.19, respectively, by using developed mobile phase. For thymol, thymoquinone, and vitamin C, linearity range was 2000-8000 ng/band. The three markers showed 99.39%–99.91% recovery for thymol, 99.22%–99.89% recovery for thymoquinone, and 99.19%–99.69% recovery for vitamin C. **Conclusion:** The optimized method was used to quantify three thymol and thymoquinone in *N. sativa* (*kali jiri*) seed extract and vitamin C in *H. polyrhizus* (dragon fruit) extract.

Keywords: Anti-hypertensive, Dragon Fruit, *Hylocereus polyrhizus, Kali Jiri, Nigella sativa,* Seed Extract

1. Introduction

The main risk factor for the onset of heart failure, stroke, myocardial infarction, and chronic renal disorders is hypertension. For millions of years, herbal medications have been used to manage and treat hypertension with little to no negative side effects[1]. Numerous herbal remedies, including ginseng, *kali jiri*, *arjuna*, garlic, ginger, barberry, rauwolfia, and *punarnava*, can be taken safely to treat hypertension[2].

Global interest in the use of herbal medicines has grown steadily and occasionally exponentially during the last three decades. In the international market, it is clear that the use and acceptance of herbal medicines is growing. Herbal remedies, encompassing both completed herbal goods and the raw ingredients needed to produce them, such as herbal preparations, medicinal plants, and herbal materials.

Herbal dosage forms are becoming more prevalent in worldwide trade and international business, which is indicative of their growing economic significance[3]. *N. sativa*, often known as black cumin seed, has long been used to treat a wide range of illnesses, including various respiratory disorders, persistent headaches, back pain, diabetes, paralysis, infections, inflammations, and high blood pressure[4].

The sesquiterpene longifolene, α-pinene, 4-terpineol, p-cymene, thymoquinone, thymol, carvacrol, dithymoquinone, t-anethol, and other chemical and biological marker substances found in

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N. sativa seeds[5]. Trace levels of other chemicals are also present in black seeds. Black cumin seed's quinine components, of which thymoquinone and thymol are the most prevalent, are primarily responsible for its pharmacological effects. These compounds have demonstrated a promising role in hypertension by their ability to block calcium channels and inhibit acetyl choline esterase[6,7].

Several natural antioxidants, including betalains, polyphenols, vitamin C, calcium, and phosphorus, are found in good amounts in dragon fruit[8]. Its higher medical values include the reduction of diabetes and hypertension. Its antioxidant activity is higher[9]. Additionally, it strengthens teeth and bones, improves the metabolism of carbohydrates, and aids in the development of heart tissue. In order to raise the level of erythrocytes and hemoglobin, blood production balance and the composition of total ascorbic acid, pectin total dietary fiber, and iron[10]. Moreover, it lessens aortic stiffness. Dragon fruit controls blood pressure using a method that is higher than that of pomegranate, rambutan, mangosteen, banana, and coconut in terms of its half maximal inhibitory concentration for radical scavenging activity[11,12].

"Marker based standardization", which is predicated on the examination of phytochemical markers using chromatographic techniques like HPTLC and HPLC, is one of the most extensively recognized approaches. Chromatographic methods for standardizing herbal products have been approved by a number of pharmacopoeias and drug regulatory bodies; these methods are listed in the monographs for herbal goods. Genetically homogeneous monocultures of the source plant under completely standardized circumstances are necessary for botanical medications in order to guarantee biochemical consistency and maximize safety and efficacy for batch-to-batch manufacture.

A review of the literature indicated that thymoquinone in black seed oil has been determined using chromatographic techniques such as HPTLC, and that thymoquinone in black seed and its nano formulation has been quantified using HPTLC[13].

Densitometric HPTLC method for the quantification of thymoquinone in *N. sativa* seed extract and its commercial formulation[14]. The densitometric HPTLC method for simultaneous quantification of thymol and linalool in essential oils and have been published[15]. Thymoquinone, thymol, and dithymoquinone levels in black seed oil were measured using a TLC technique[16]. Quantitative determination of vitamin C in fruit juice[17]. HPTLC- Densitometric Method for Determination of Ascorbic Acid, Paracetamol and Guaifenesin in Presence of Their Toxic Impurities[18]. Reports on the quantitative estimation of vitamin C using HPTLC in various Amla varieties19 have been made.

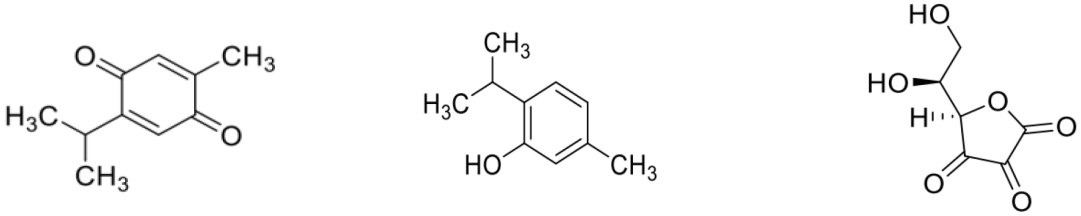
Standardization of *N. sativa* and dragon fruit extract with phytomarkers thymol (Figure 1), thymoquinone (in *N. sativa* extract) (Figure 1) and vitamin C (in dragon fruit extract) (Figure 1) by simultaneous HPTLC method has not been reported to date.

In order to estimate the phytomarkers thymol, thymoquinone (found in *N. sativa* seed extract), and vitamin C (found in *H. polyrhizus* fruit extract) in their respective extracts, the current study develops a simultaneous HPTLC method.

2. Materials and Methods

2.1 Reagents and Materials

Sigma aldrich provided analytically pure phytomarkers (≥98%), including vitamin C, thymoquinone, and thymol. We bought the extracts of *N. sativa* seeds and *H. polyrhizus* (dragon fruit) from Yucca enterprises in Mumbai and Vital Herbs in Delhi.



Thymoquinone Thymol Vitamin C

Figure 1. The chemical structures of marker compound thymoquinone, thymol from *N. sativa (kali jiri)* seed extract and vitamin C from *H. polyrhizus* (dragon fruit) extract.

2.2 Instrumentation

In the current work, CAMAG TLC Scanner IV, CAMAGLinomat V sample applicator, a CAMAG HPTLC system (Muttenz, Switzerland) with a twin-trough developing chamber (10 × 10 cm) and CAMAG TLC visualize was utilized along with a UV cabinet with a dual-wavelength UV lamp and CAMAG win CATS software. The calculations for extract quantification and validation parameter assessment were performed using Microsoft Excel 2019.

2.3 Chromatographic Condition

2.3.1 Pre-treatment of HPTLC Plates

Methanol used as the mobile phase and HPTLC plates were stored in a twin troughs glass chamber. The methanol was allowed to move in an ascending manner to the upper edge of the plate. After being taken out of the oven and allowed to dry for five minutes at 110°, the plates were utilized immediately for the experiment.

2.3.2 Chromatographic Separation

On HPTLC plates, chromatographic separation was achieved using n-hexane:methanol:ammonia (8.5:1.5:0.2 v/v) as the mobile phase. Using a linomat V applicator fitted with a 100µl syringe, samples were applied to the plates as 6mm bands, maintaining 14mm distance between bands, 8 mmdistancefrom the plate side edges, and 15 mm distance from the bottom of the plate. The plates were prepared in a 20 cm by 10cm camag twin-trough chamber, lined with filter paper, and after being saturated for 20 minutes at room temperature (25 ± 2°C) with mobile phase vapor. Plates were developed at a distance of 8 cm. The plates that were taken out of the room were dried with hot air. With TLC Scanner IV and winCATS software, the developed plates were scanned at λ = 200 nm–400 nm. 100 nm/s of scanning speed and a slit size of 6.00 x 0.45 mm were used.

2.4 Mobile Phase Development

2.4.1 Preparation of the Mobile Phase

To prepare the mobile phase for several trials, different ratios of mobile phases were mixed together. Before being used, the mobile phase was kept in a twin-trough glass container that was covered with a lid and saturated for 20 minutes.

2.4.2 Preparation of the Mobile Phase (for Optimized Condition)

The mobile phase was prepared by mixing 8.5ml of n-hexane, 1.5ml of methanol, and 0.2ml of ammonia. Prior to usage, the mobile phase kept in a twin-trough container that was covered with a lid and saturated for 20 minutes.

2.4.3 Preparation of Standard Solution

A precisely weighed 10mg of standard thymol, thymoquinone, and vitamin C were dissolved in 10ml of methanol to produce a stock solution of approximately 1000 µg/ml each in a 10 ml volumetric flask. To obtain a 100 µg/ml concentration of solutions for each phytoconstituent, 1 ml of this solution was pipetted out and the volume was diluted with methanol up to 10ml.

2.4.4 Preparation of Working Standard Solution for Method Development

From standard solutions of thymol (10 µl corresponding to 1000 ng/band), thymoquinone (10 µl corresponding to 1000 ng/band), vitamin C (10 µl corresponding to 1000 ng/band), were applied to the plate for method development by changing mobile phase ratio.

The mobile phase, which was n-hexane: methanol:ammonia (8.5:1.5:0.2) ml v/v/v/v, was used to develop the TLC plates. Linear ascending development was performed using a twin-trough container that was equilibrated with the mobile phase vapors for a duration of 20 minutes. For every development, a 10-milliliter aliquot of the mobile phase was used, and it was let to travel eight centimeters apart. Once the development was finished, the HPTLC plates were fully dried.

2.5 Preparation of Standard Solutions of Thymol, Thymoquinone and Vitamin C

A 1000µg/ml stock solution was prepared by dissolving 10mg of standard thymol, thymoquinone and vitamin C in methanol in three separate 10 ml volumetric flasks. Appropriate aliquots of thymol, thymoquinone and vitamin C stock solution were transferred to a 10 ml three separate volumetric flasks to obtain 10 ml of final working standard solutions with thymoquinone concentrations of 100 µg/ml and thymol concentrations

of 100µg/ml and vitamin C concentrations of 100µg/ml. These solutions were stored at 4–6 degrees. Calibration curves was five replicas spanning a range of 2000–8000 ng/band for thymol, thymoquinone, and vitamin C at 271 nm, the linearity of the method observed. Peak area *vs* concentration was plotted to create the calibration curves.

The peak area observed were reported in terms of %RSD, and the developed method precision was evaluated using repeatability and intermediate precision studies. Three duplicates of three distinct concentrations (2000, 4000, and 6000 ng/band for thymol, thymoquinone, and vitamin C) were carried out on the same day. Likewise, an intermediate precision investigation was conducted on various days.

The recovery studies were used to check 80%, 100%, and 120% by adding a known amount of standard to the extracts and analyzing them in triplicate using the proposed method. The formulas LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the SD (Standard Deviation) of the response, peak area, and S is the slope of the calibration curve, were used to calculate the LOD and LOQ of markers based on the SD(Standard Deviation) of the response, peak area, and slope of the calibration curve.

The standard marker and extracts were analyzed to ascertain the suggested method's specificity. Peak purity was determined by comparing the spectra at three distinct levels: Peak start (S), peak end (E), peak apex (M). By comparing the R_f and spectra of the band with those of the standard, the extract's band for thymol, thymoquinone, and vitamin C was verified. The robustness of HPTLC method on R_f and peak area was evaluated using small changes in method conditions, such as saturation duration (20 ± 2 min), maximum wavelength (271 ± 2 nm), and change in mobile phase (10 ml±0.05 ml).

2.6 Fingerprinting and Analysis of the *N. sativa Seed* and *Dragon Fruit Extracts*

Sample of *N. sativa* seed extract 1: After precisely weighing 100 mg of *N. sativa* seed extract and the mixture were dissolved in 5ml methanol, sonicated for 15 minutes, and then diluted with methanol in 10ml volumetric flask. The mixture filtered using whatman filter paper. Sample of the *N. sativa* seed extract 2 was made using the same method as previously mentioned. Sample of dragon fruit extract 1: 100mg of the extract was precisely weighed, then put into a 10ml volumetric flask. It was then dissolved in 5ml methanol, sonicated 15 minutes, then diluted with methanol to the desired consistency. Finally, the mixture was filtered using whatman filter paper. Sample of dragon fruit extract 2 was made using the same method as previously mentioned.The plate was developed by spotting 10 µl of the filtered sample solution and 15 µl of the standard phytomarker solutions. The bands at the corresponding Rf value were scanned to detect the peak of thymol, thymoquinone, and vitamin C at 271 nm.

3. Results and Discussion

3.1 Optimization of Mobile Phase and Detection Wavelength

Spectra of the thymol, thymoquinone and vitamin C were recorded in range of 200–800 nm by using the CAMAG TLC scanner IV, where thymol exhibited max absorbance at 271 nm thymoquinone exhibited max absorbance at 282nm, while vitamin C exhibited absorbance at 266 nm. The detection wavelength for all phytomarkers was selected as 271 nm (isobestic point) as all markers gives good results on 271 nm. In order to optimize the mobility phase, a variety of solvents, including methanol, ethyl acetate, toluene, n-hexane, and chloroform, were investigated in variable concentrations in order to produce well-resolved spots of markers and extracts. The first HPTLC trial, which used a mobile phase methanol, hexane, and chloroform with ratio of 2:5.5:2.5 (v/v/v), unable to separate all three markers. Further, the modification of mobile phase composition with the removal of chloroform and ammonia was added to avoid tailing observed in the vitamin C band showed the separation of three marker compounds with acceptable R_f values. n-hexane:methanol:ammonia (15%) (8.5:1.5:0.2) v/v/v/v was selected as the optimized mobile phase as it provided a good resolution of bands at R_f of 0.66, 0.35, and 0.19 for thymol, thymoquinone and vitamin C respectively (Figure 2), vitamin C (1000 ng/spot), thymoquinone (1000 ng/spot) and thymol (1000 ng/spot).

3.2 HPTLC Fingerprinting (Quantification of Thymol and Thymoquinone in *N. sativa* Seed Extracts and Quantification of Vitamin C in Dragon Fruit Extracts)

HPTLC fingerprint of *N. sativa* seed extracts indicated an intense band at 271 nm for thymol (R_f 0.66), thymoquinone (R_f 0.35), and dragon fruit extracts indicated an intense band at 271 nm for vitamin C (R_f 0.19). A reference standard band was compared to the marker compounds identified in both extracts using the HPTLC fingerprint. NS sample 1 or *N. sativa* seed extract sample 1 is *N. sativa* seed extract purchased from vital herbs Delhi and NS sample 2 or *N. sativa* seed extract sample 2 is *N. sativa* seed extract purchased from Yucca enterprises, Mumbai. DF sample 1 or dragon fruit extract sample is dragon fruit extract purchased from vital herbs Delhi and DF sample 2 or dragon fruit extract sample 2 is dragon fruit extract purchased from Yucca enterprises, Mumbai.

Thymol and thymoquinone were quantified in *N. sativa* extracts (NS sample 1 and NS sample 2) by comparing the band with bands of standard thymol and thymoquinone (Figures 3 and 4). Vitamin C was quantified in dragon fruit extracts (DF sample 1 and DF sample 2) by comparing the band with bands of standard vitamin C (Figure 5 and 6). The content of thymol and thymoquinone was found to be 2.3 % and 2.5 % in *N. sativa* extract sample 1. The content of thymol and thymoquinone was found to be 2.1 % and 2 % in *N. sativa* extract sample 2. The vitamin C content was found to be 3.5 % in dragon fruit extract sample 1. The vitamin C content found to be 3.2 % in dragon fruit extract sample 2.

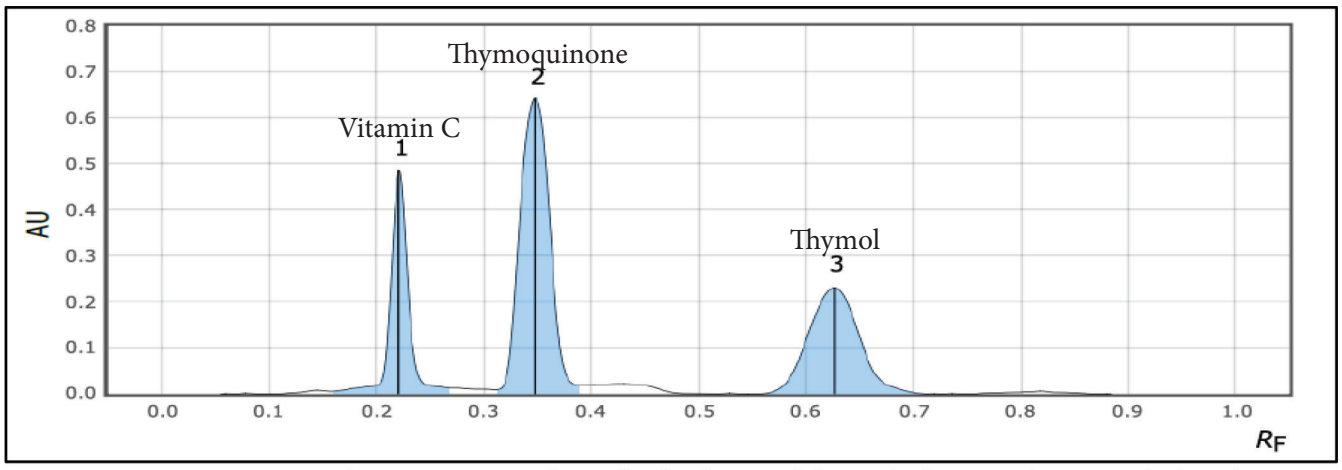


Figure 2. HPTLC chromatogram of standard mixers of thymol, thymoquinone and vitamin C.

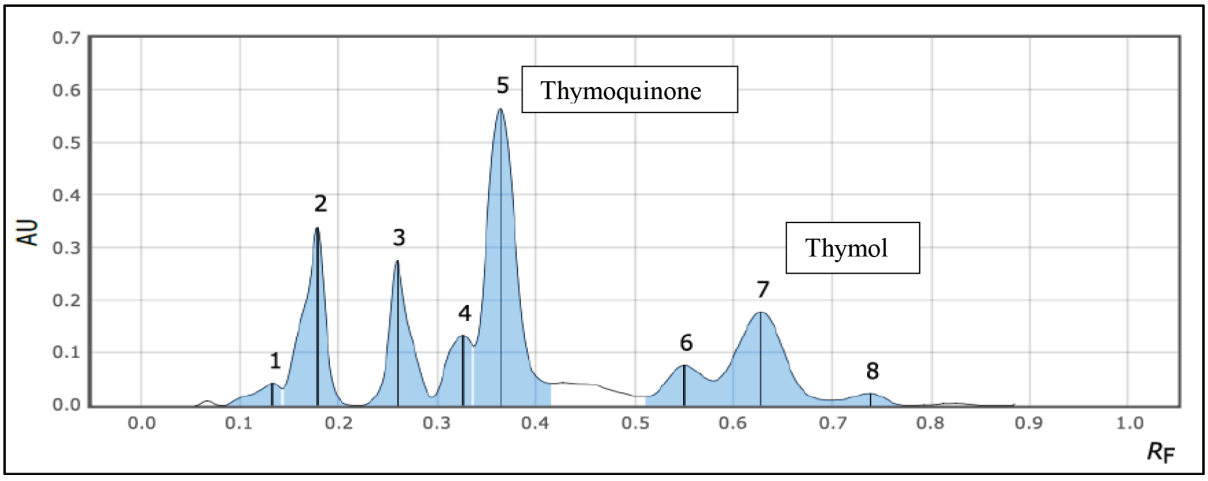


Figure 3. HPTLC chromatogram of *N. sativa* seed extract sample 1.

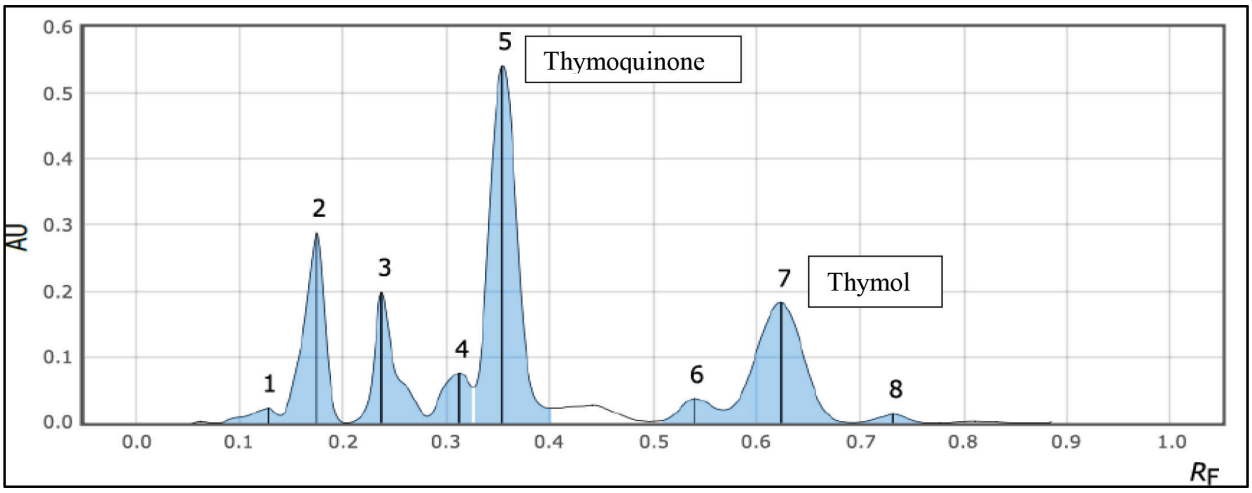


Figure 4. HPTLC chromatogram of *N. sativa* seed extract sample 2.

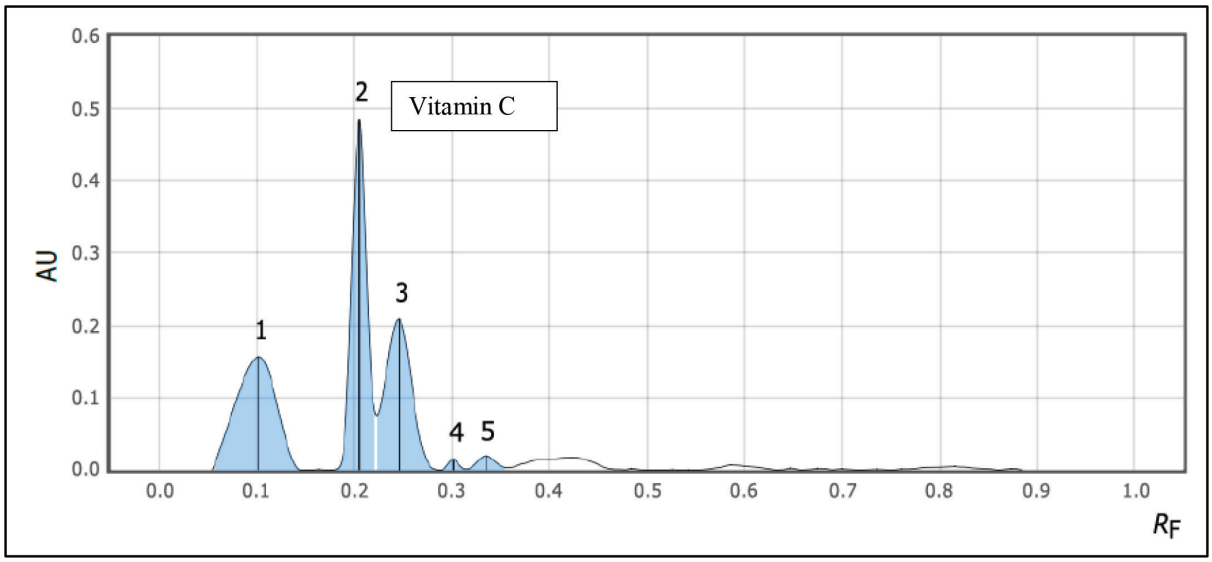


Figure 5. HPTLC chromatogram of dragon fruit extract sample 1.

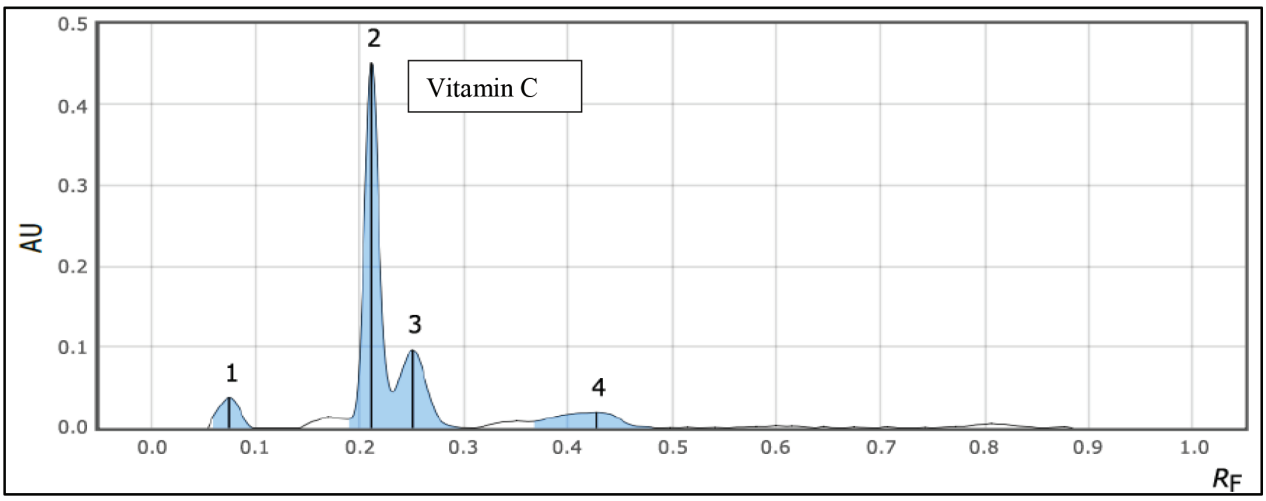


Figure 6. HPTLC chromatogram of dragon fruit extract sample 2.

3.3 Method Validation

By using ICH Q2 (R1) guidelines[20]. The calibration curve were plotted in the range of 2000-8000 ng/band for thymol, thymoquinone and vitamin C. Detection limits for thymol, thymoquinone and vitamin C were found to be 158.7, 317.38, and 184.06 ng/spot, respectively, while quantitation limits were 480.9, 961.76, and 557.77ng/spot. A prequantified sample solution was spiked with known concentrations of thymol (1900, 2375, 2850 ng/spot), thymoquinone (2064, 2580 and 3096 ng/spot), and vitamin C (2832, 3540 and 4248 ng/spot) in order to conduct recovery studies. Recoveries for the three markers were found to be 99.39%–99.91% for thymol, 99.22%–99.89% for thymoquinone, and 99.19%–99.69% for vitamin C (Table 1). Fingerprinting analysis of extract was performed using the optimized method and the densitogram show band at R_f of 0.66, 0.35, and 0.19 for forthymol, thymoquinone and vitamin C, respectively. Comparing the overlay spectra at the spot's peak start, peak end positions, peak apex allowed us to determine the purity of peak of each marker in the extracts. The % RSD was found to be less than 2, which indicates that the method was robust. The validation parameters and quantification of thymoquinone, thymoland vitamin C are summarized in Tables 2 and 3.

Table 1. Recovery studies for the determination of thymol, thymoquinone and vitamin C by HPTLC-densitometric method

Level	Amount of Sample Taken (ng/spot)	Amount Spiked (ng/spot)	Drug Recovered (ng/spot)	% Re covered
Thymol				
80%	2375	1900	4271	99.91
100%	2375	2375	4721	99.39
120%	2375	2850	5210	99.71
Thymoquinone				
80%	2580	2064	4639	99.89
100%	2580	2580	5120	99.22
120%	2580	3096	5640	99.37
Vitamin C				
80%	3540	2832	6351	99.67
100%	3540	3540	7023	99.19
120%	3540	4248	7764	99.69

Table 2. Summary of validation parameters

Parameters	Thymol	Thymoquinone	Vitamin C
Linearity	2000-8000 ng/band	2000-8000 ng/band	2000-8000 ng/band
LOD (ng/band)	158.7	317.38	184.06
LOQ (ng/band)	480.9	961.76	557.77
Accuracy	99.39% -99.91%	99.22%-99.89%	99.19%-99.69%
Solution Stability	Stable for (24 hr).	Stable for (24 hr).	Stable for 24 hr.
Specificity	Specific	Specific	Specific
Precision (% RSD)			
System Suitability	1.52	0.28	0.61
Repeatability	1.5	1.0	1.0
Intraday(n=3)	0.4-1.3	0.4-1.4	1.3-1.4
Interday (n=3)	0.5-1.6	1.1-1.8	0.9-1.5
Robustness (% RSD) Robust			
Change in chamber saturation time	0.8	0.4	0.3
Change in wavelength	0.2	0.9	0.1
Change in mobile phase	0.2	1.1	0.2

Table 3. Quantification of thymoquinone and thymol in *N. sativa* (*kali jiri*) seed extract and vitamin C in *H. polyrhizus* (dragon fruit) extract

Extracts	Company (Vendor)	Marker	Area	Conc.(ng/spot) Found±RSD	µg/mg of Dried Extract±%RSD
N. sativa Extract	Vital Herbs, Delhi	Thymol	0.01170	2375 ±1.2	23.75±1.2
	Yucca Enterprises, Mumbai	Thymol	0.01050	2155 ±1.0	21.55±1.0
	Vital Herbs, Delhi	Thymoquinone	0.01496	2580 ±0.2	25.80±0.2
	Yucca Enterprises, Mumbai	Thymoquinone	0.01411	2075 ±1.3	20.75 ±1.3
Dragon Fruit Extract	Vital Herbs, Delhi	Ascorbic acid	0.01622	3540 ±0.2	35.40 ±0.2
	Yucca Enterprises, Mumbai	Ascorbic acid	0.01520	3200 ±0.9	32 ±0.9

4. Conclusion

HPTLC method were developed and validated for the separation and quantification of three phytomarkers thymol and thymoquinone in *N. sativa (kali jiri)* seed extract and vitamin C in *H. polyrhizus* (dragon fruit) extract.

Chromatographic development by using an HPTLC aluminum plate, pre-coated with silica gel (60 F_{254}) with n hexane-methanol- ammonia (8.5:1.5:0.2 v/v/v) as the mobile phase. Vitamin C, thymol, and thymoquinone bands were separated chromatographically at R_f values of 0.66, 0.35, and 0.19, respectively, as a result of the optimized mobile phase. For thymol, thymoquinone, and vitamin C, the method was found to be linear in range of 2000-8000 ng/band. Recoveries for the three markers were found to be 99.39%–99.91% for thymol, 99.22%–99.89% for thymoquinone, and 99.19%–99.69% for vitamin C. The novel aspect of this method is the optimization of the mobile phase achieve the separation of all three markers thymol, thymoquinone and vitamin C, which had not previously been reported in *N. sativa* extracts and dragon fruit extracts. The developed method resulted in a well-resolved spot of markers and extracts, with good resolution of bands at specific R_f values in both extracts. Thymol 2.3% and 2.1% were found in *N. sativa* extract sample 1 and 2 respectively. Thymoquinone 2.5% and 2% were found in and *N. sativa* extract sample 1 and 2 respectively. Vitamin C 3.5% and 3.2% were found in dragon fruit extract samples 1 and 2 respectively. The method used for the standardization of both extracts and further, it can be used in the standardization of traditional medicines and nutraceuticals.

5. References

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