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VALIDATED HPTLC ANALYSIS METHOD FOR QUANTIFICATION OF THYMOL CONTENT IN AJWAIN (*TRACHYSPERMUM AMMI)* FRUIT

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ABSTRACT

A simple, sensitive and accurate HPTLC method has been develop and validated for the quantitative estimation of Thymol in Ajwain (*Trachyspermum ammi*) collected from local market.The method employed TLC Aluminum plate Precoated with silica gel $^{60}F_{254}$ as stationary phase with mobile phase as Hexane : EtOAc (8:2; v/v). Thymol showed mean R_f value of 0.46 with λ_{max} at 278 nm. The method was validated in terms of linearity (200-600ng/spot) precision and accuracy (100.91%) recovery. The Thymol content was found to be 330.63ng/gm in *Trachyspermum ammi* obtained from marketed sample. In this procedure, linearity (r^2 =0.99998) ,limit of detection (50 ng/spot), limit of quantification (200 ng/spot) were found to be satisfactory. The proposed HPTLC method can be used for the quality control of the raw materials and for routine analysis.

KEYWORDS: HPTLC, Validation, Thymol, *Trachyspermum ammi*, Quantification.

INTRODUCTION

Trachyspermum ammi (L.) Sprague,syn. *Carum copticum* Benth. et hook., commonly known as ajwain or Brishop's Weeds is an erect, aromatic, annual herb with striate stem white flowers and small brownish fruits. It belongs to the family Apiaceae. Ajwain is grown in Iran, Egypt, Afghanistan, and India[2] (largely in Uttar Pradesh, Bihar, Madhya Pradesh, Punjab, Rajasthan, West Bengal, Tamil Nadu and Andhra Pradesh). The fruits posses characteristic aromatic odour and pungent taste due to the presence of an essential oil mainly composed of Thymol (50%), α-cadinol, δ- cadinene, β-caryo Phyllene and Carvacrol.[3,4] Thymol (2-isopropyl -5- methyl phenol) is a natural monoterpene phenol derivative of cymene, $C_{10}H_{14}O$, isomeric with carvacrol. It is a white crystalline substance of present aromatic odor and strong antiseptic properties.This molecule has a broad range of activities including antioxidant, antimicrobial, antiseptic, expectorant and antiplasmodic properties.[5,6]

High Performance Thin Layer Chromatography is one of the modern sophisticated techniques that can be used for wide diverse applications. It is a simple and powerful tool for high resolution chromatography and trace quantitative analysis is made possible. It is most widely used for quick and easy determination of quality authenticity and purity of the crude drugs and market formulation. Literature survey reveals that there are very few validated HPTLC methods for the estimation of thymol. Hence an attempt was made to develop and validate HPTLC method for evaluation of thymol. The validated method was proposed to the applied for estimation of the marker (Thymol) compound obtained from the sample (Ajwain) collected from local market to study the % of thymol in it.

MATERIAL AND METHOD

Plant Material

Thymol standard was procured from Process Chemical Industries Kol- 700006 and raw material Ajwain was purchased from local market of Kolkata and authenticated by Dr. M.N. Das, Department of Pharmacognosy, National Research Institute of Ayurvedic Drug Development, Kolkata.

Pharmacognostic studies

Microscopy

Simple hand sections **(**transverse section) of fruit was done by blade and mounted in 50% glycerin to form temporary slides for microscopical study under compound light microscope

(Olympus OIC, 07964) and diagrams were drawn by using standard Camera Lucida technique.[7,8,9] External morphological evaluation are shown in Fig. 1.

Fig. 1: External morphology of fruit of *Trachyspermum ammi* (L.) Sprague

Powder microscopy

Few mg of powder was washed in water, excess water was removed by decantation. Washing process was repeated to clear all extraneous and interfering materials and plant debris was isolated as much as possible; a few mg in 50% glycerin was mounted; a few mg of the washed material was treated in saturated chloral hydrate solution for 3-5 hrs; washed in water and mounted in 50% glycerin; another few mg of plant debris was stained in iodine (IKI) and mounted in glycerin. Different cell components were observed under compound light microscope ((Olympus OIC, 07964)) and diagrams were drawn by using standard Camera Lucida technique.[7,8]

Powder analysis: Presence of unicellular warty trichome as protuberances, profuse oil globules of different sizes, and groups of endosperm cells, characterized, filled with profuse oil globules and few aleurone grains, groups of polygonal mesocarp cells and few spiral vessels with fibre.[10,11] Pictures of powder analysis of fruit are shown in Fig.2.

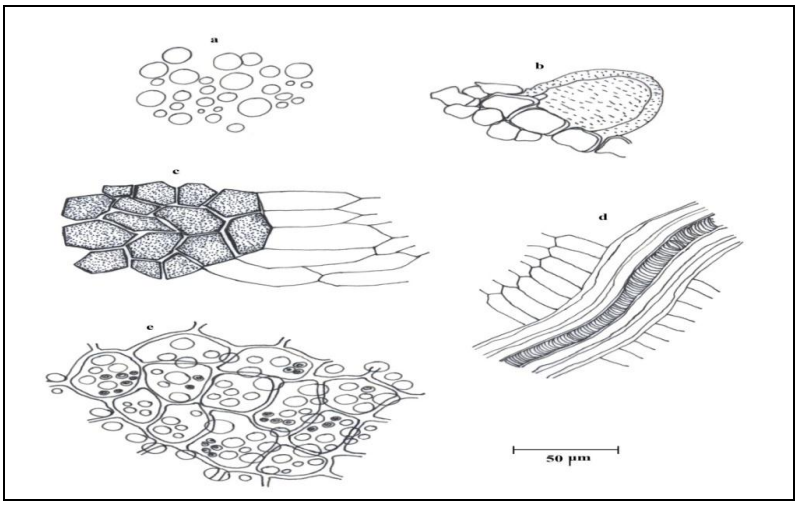


Fig.2 : Powder analysis of fruit

***a**: Oil globules of different sizes , **b**: Unicellular warty trichomes as protuberances, **c**: Groups of polygonal mesocarp cells, **d**: Spiral vessel with fibre, **e**: Group of endosperm cells filled with profuse oil globules and few aleurone grains.*

Method Development

A CAMAG HPTLC system (Switzerland) comprising CAMAG Linomat 5 applicator, CAMAG TLC scanner 3, CAMAG Wincats software, version 1.44, Hamilton syringe (100µl), CAMAG Reprostar 3, CAMAG TLC plate heater, CAMAG UV Cabinet were used for the study. Silica gel $^{60}F_{254}$ Aluminum plates (Merck) was used as stationary phase. Hexane : Ethyl acetate (8:2; v/v). was used as mobile phase. Methanol was used as solvent.

PREPARATION OF STANDARD AND SAMPLE DRUG SOLUTION

Preparation of Standard solution

Accurately weighed 1 mg of Thymol was dissolved in 10 ml of methanol in a volumetric flask. This stock solution containing 100 mcg / ml thymol was prepared in methanol and this solution was used as calibration solution in the range of 2.0, 3.0, 4.0, 5.0 and 6.0 µl volumes gave a series of spots covering the range 200 - 600 ng of thymol.

Preparation of Sample solution

1gm fine dust of Ajwain (*Trachyspermum ammi*). was gently refluxed in 60ml methanol for 2hrs. and filtered through whatman filter paper (No. 41, pore size : 20-25µm). The residue was refluxed again with 40ml methanol for 2hr. and filtered it same as before. The combined filtrates were evaporated to make it a final volume of 10ml. Now 1ml of this solution was diluted to 10 ml with methanol and this was used for estimation of the thymol in the plant.

HPTLC method and Chromatographic conditions

The chromatographic estimation was performed using the following conditions. Stationary phase was precoated silica gel $^{60}F_{254}$ aluminium sheets (10x10cm) and the mobile phase used was Hexane: Ethylacetate (8:2; v/v). The chamber saturation time employed was 20 mins and the developing distance was 8 cm. scanning wavelength of 278 nm with a slit dimention of (6.0x 0.45)mm.micro and scanning speed of 20mm/s were employed.

METHOD VALIDATION

The developed method is validated as per the ICH guidelines.[12] The developed method was validated in terms of linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and recovery.

Precision and accuracy

The precision of the method in terms of intra-day precision (%RSD) was determined by analysing thymol standard solutions in the range (200-600ng/spot) three times on the same day. Inter-day precision (%RSD) and accuracy of the assay assessed by analyzing these solutions (200-600ng/spot) on three different days over a period of one week. Accuracy was expressed as percent recovery.

Specificity

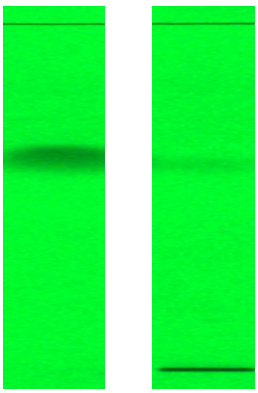
Thec specificity of the method was as curtained by analyzing standard drug and the sample. The spot for Thymol in sample was confirmed by compairing R_f and spectra of spot with that of standard. The peak purity of Thymol was assessed by compairing the spectra at three different levels i.e, peak start, peak apex and peak end positions of the spot.

RESULTS AND DISCUSSION

Selection and optimization of mobile phase

A wave length of 278nm was chosen for quantification .The R_f value of Thymol after development with mobile phase Hexane: Ethylacetate (8.0:2.0v/v) was 0.46 and shown in Fig3.

Observed at 254nm



Thymol Ajwain

Fig. 3 Photography of Thymol with the sample Ajwain

Calibration curve

The validated calibration range was 200-600 ng/spot , (r^2 =0.99998). The spots were scanned at 278nm which is the λ_{max} , shown in Fig.4 and the values are shown in the Table No. 1

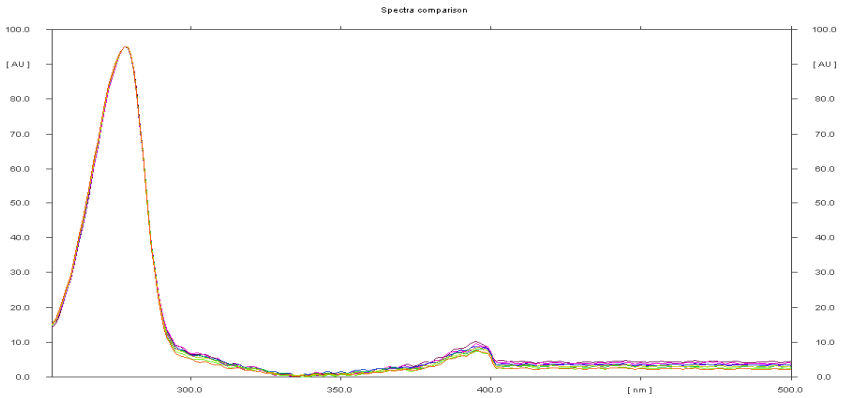


Fig. No.4 Typical HPTLC Chromatogram of the λ_{max} of the thymol by HPTLC method.

Table No.1 Values of λ_{max}

Track No.	R_f	Assined substance	Max. signal
1	0.46	Thymol	131AU @278nm
2	0.46	Thymol	141AU @278nm
3	0.46	Thymol	167AU @278nm
4	0.46	Thymol	182AU @278nm
5	0.46	Thymol	194AU @278nm
6	0.46	Thymol	201AU @278nm
7	0.46	Thymol	182AU @278nm
8	0.46	Thymol	210AU @278nm

The calibration was linear in the concentration range of 200-600ng/spot. The linear regression equation was Y = -232.4 +4.236 X, where 'Y' is the peak area and 'X' is the concentration of thymol were shown in Table No. 2

Table 2: Characteristic parameters for the proposed HPTLC method

Parameters	HPTLC
	Thymol
Calibration range (ng/spot)	200 – 600
Detection wave length	278nm
Mobile phase (Hexane : Ethyl acetate)	8.0/2.0
R_f value	0.46
Regression equation (y^*)	Y=-232.446 + 4.236*X
Slope(b)	4.236
Intercept (a)	-232.446
Correlation coefficient (r^2)	0.99998
Limit of detection (ng/spot)	50
Limit of quantification (ng/spot)	200

Linearity of the Standard

For determination of the linearity curves of area vs. concentration , different amounts of stock solution of Thymol was applied on the HPTLC plate and analysed.The calibration curve of standard Thymol is shown in Fig. 5.

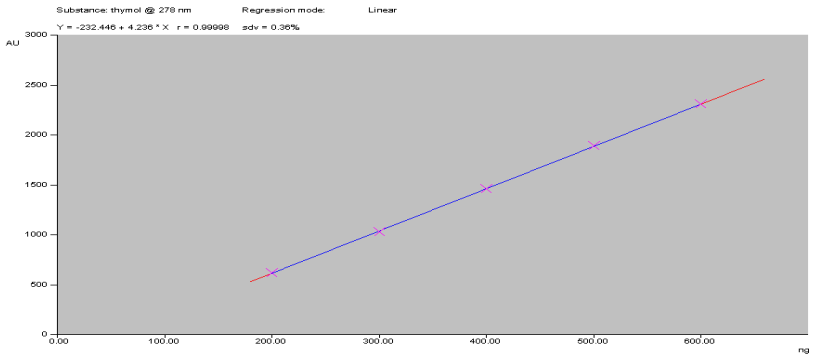


Fig. 5 : Calibration curve of thymol by HPTLC method

The linearity was found in the concentration range of 200-600ng/spot. The correlation co-efficient was found to be 0.99998.The results are presented as in Table 3.

Table 3: Calibration data of thymol by HPTLC method

S.NO	Amount in ng/Spot	Rf values	Peak area
1	200	0.46	619.2 AU
2	300	0.46	1031.5 AU
3	400	0.46	1416.3 AU
4	500	0.46	1889.2 AU
5	600	0.47	2308.3 AU

3D- display of calibration curve of Thymol by HPTLC method is shown in Fig. 6.

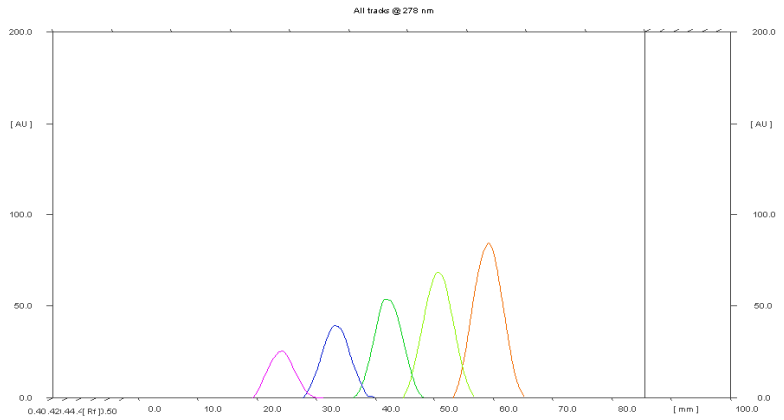


Fig..6: 3D-Display Calibration curve of thymol by HPTLC method

The drug peak area was calculated for each concentration level and a graph was plotted of drug concentration against the peak area and shown in Fig. 7

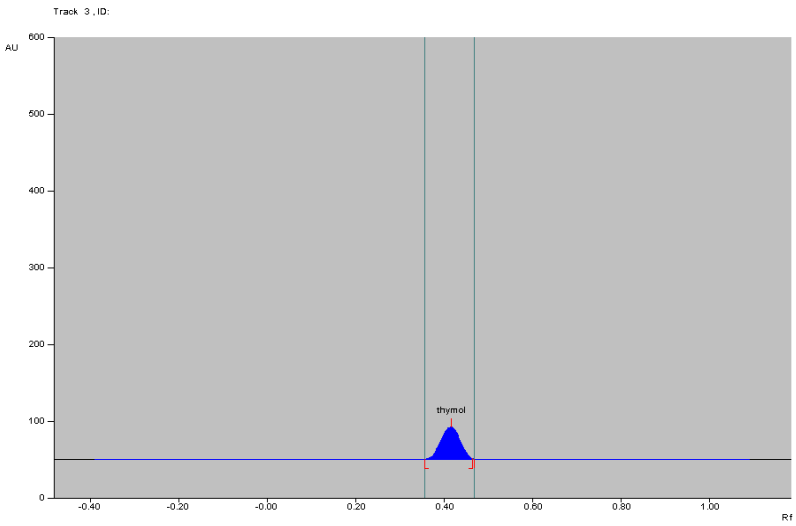


Fig. 7: Typical HPTLC Chromatogram of Thymol by HPTLC method

The calibration curve of thymol with market sample , Ajwain (*Trachyspermum ammi*) is shown in Fig. 8.

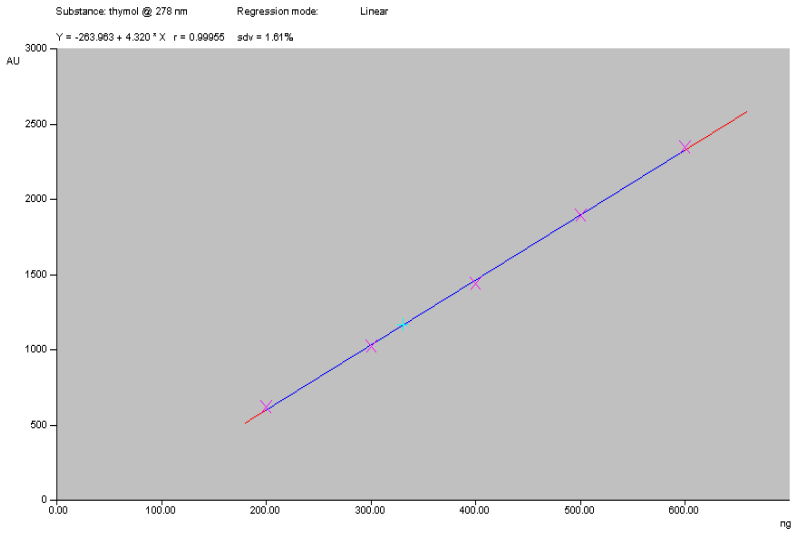


Fig. 8: Calibration curve of thymol with sample , Ajwain (*Trachyspermum ammi*) by HPTLC method

Calibration data of thymol along with the sample by HPTLC method

S.NO	Amount in ng/Spot	Rf values	Peak area
1	200	0.46	621.60 AU
2	300	0.46	1023.03 AU
3	300	0.46	1164.47 AU
3	400	0.46	1435.63 AU
4	500	0.46	1894.69 AU

5	600	0.47	2343.95 AU

Quantification of thymol in the sample

The regression equations were used to estimate the amounts of thymol present in the sample Ajwain (*Trachyspermum ammi*). The content of thymol present in the sample is shown in Table: 4.

Table 4: Content of thymol in the sample

S.NO	Ajwain (*Trachyspermum ammi*)	Thymol
1	Sample used	1gm
2	Concentration	1gm/100ml
3	Amount applied on plate	3.0µl
4	Area of the spot	1164.47 AU
5	Amount of calculated thymol	330.63ng
6	Standard deviation of the calibration curve including sample	1.61
7	Sample present in the plant, Ajwain (*Trachyspermum ammi*) after calculation	1.102 %

VALIDATION OF METHOD

Recovery studies

The proposed method, when used for determination of thymol from market sample , afforded recovery ranging from 99.52% - 100.91% for thymol.

Precision

The precision of the method in terms of intra – day precision (% RSD) was determined by analyzing Thymol standard solutions in the range (200 -600 ng / spot) three times on the same day. Inter - day precision (% RSD) was assessed by analyzing these solutions (200 - 600 ng / spot) on three different days over a period of one week. The results of the precision studies are shown in.

Table 5: Precision of Thymol by HPTLC method

	Intraday precision			
	5µl/spot (in ng)	(Area)	Std. Dev	% RSD
1st set	200	1085.75	18.53	1.71 %
	200	1094.97		
	200	1059.26		
2nd set	300	1851.62	4.05	0.22 %
	300	1859.64		

	300	1854.62		
3rd set	400	2662.85	1.73	0.07%
	400	2665.57		
	400	2662.35		
	Interday precision			
	5µl/spot (in ng)	(Area)	Std. Dev	% RSD
1st day	200	1056.51	8.02	0.77 %
	200	1045.92		
	200	1050.62		
	200	1052.63		
	200	1035.65		
2nd day	200	1082.53	20.95	1.95 %
	200	1033.69		
	200	1078.52		
	200	1081.48		
	200	1079.02		
3rd day	200	1042.62	8.63	0.82 %
	200	1055.69		
	200	1060.03		
	200	1061.24		
	200	1044.88		

Accuracy

Determination of method accuracy by the standard addition method at three concentration levels are shown in **Table: 6**

Table 6: Recovery studies of thymol by HPTLC method

S.No	Sample	Initial amount (ng/spot)	Amount added (ng/spot)	Total amount after addition (ng/spot)	Amount recovered (ng/spot)	Recovery (%)
1	Ajwain (*Trachyspermum ammi*)	330.63	50	380.63	378.82	99.52%
			50	380.63	380.63	
			50	380.63	380.63	
		330.63	100	430.63	448.48	100.91%
			100	430.63	429.55	
			100	430.63	425.65	
		330.63	150	480.63	480.63	98.94%
			150	480.63	480.63	
			150	480.63	480.63	

Robustness

The robustness of the HPTLC method was evaluated by analyzing the system suitability parameters after varying the detection wave length (±2%), mobile phase volume (±2%). None of these alterations caused a significant change in peak area RSD. So the system is robust.

Limit of Detection and Limit of Quantitation

The LOD and LOQ of piperine was found to be 12.6AU (50ng/spot) and 36.7 AU(200ng/spot) respectively.

Specificity

The specificity of the proposed method was determined by comparing the sample and standard peak for its R_f and UV spectra. Three point peak purity i,e peak start , peak apexand peak end was compared and found superimposed. This indicated that standard thymol and market sample peaks were not merging with any other components and impurities.

The peak purity of thymol was assessed by comparing the spectra at three different levels, i,e peak start, peak apex and peak end position. Shown in Fig. 9

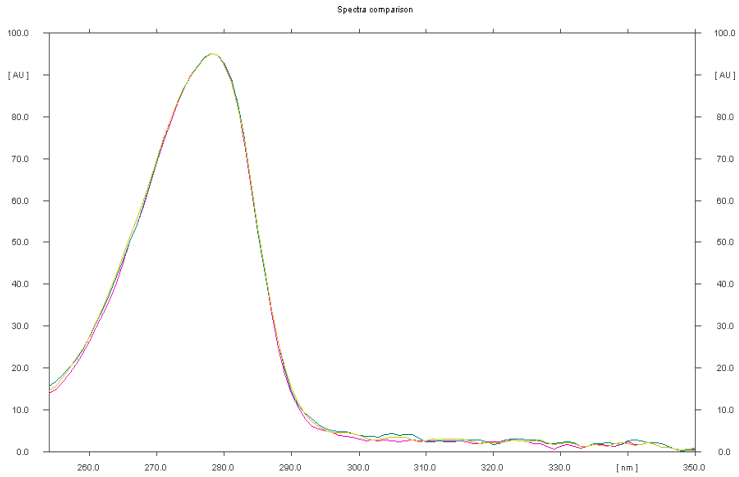


Fig. 9: Overlay spectra of Thymol(standard)and Ajwan(sample) in absorption mode in the UV range taken on the CAMAG TLC scanner 3.

CONCLUSION

A rapid, simple, accurate, sensitive and specific HPTLC method developed and validated. This developed and validated method was used for quantitative estimation and macro and micro fingerprinting analysis of thymol from different geographical sources of Ajwain (*Trachyspermum ammi*) and its formulation. Also this study can be employed for the rutine quality control analysis of thymol in ajwain.

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