VALIDATED HPTLC ANALYSIS METHOD FOR QUANTIFICATION OF THYMOL CONTENT IN *TRACHYSPERMUM AMMI* AND POLYHERBAL UNANI FORMULATION *ARQ ZEERA*

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ABSTRACT

A new, simple, sensitive, precise and accurate stability-indicating high-performance thin layer chromatographic (HPTLC) method was developed for the quantification of thymol in herbal extract (*Trachyspermum ammi),* prepared authentic formulations (PAF) and marketed formulation (MF) Arq Zeera and validated as per ICH guidelines. Analysis of thymol was performed on TLC aluminium plates pre-coated with silica gel 60 F_{254} as stationary phase. Linear ascending development was carried out in twin trough glass chamber saturated with toluene: ethyl acetate (9: 3 v/v) at room temperature (25 ± 2 °C). Photodensitometric analysis was performed by UV-visible absorbance at 513 nm after spraying with vanillin sulphuric acid. The thymol content was found to be 2.1 % w/w, 0.784 % w/w, and 0.167 % w/w, in *Trachyspermum ammi,* prepared authentic formulations and marketed formulation Arq Zeera respectively. This system was found to have compact spot of thymol at R_f value of 0.72 ± 0.02. For the proposed procedure, linearity (r^2 = 0.994 ± 0.054), limit of detection (0.039 μg/spot), limit of quantification (0.118 μg/spot), recovery (ranging from 98.99% – 101.12%), and precision (< 1.00%) were found to be satisfactory. Statistical analysis of the data showed that the method is reproducible and selective for estimation of thymol. The proposed HPTLC method can be used for the quality control of the raw materials as well as formulations.

Keywords: HPTLC, Validation, Thymol, *Trachyspermum ammi,* Arq Zeera.

INTRODUCTION

Thymol (2-isopropyl-5-methylphenol**)** is a natural monoterpene phenol derivative of cymene, $C_{10}H_{14}O$, isomeric with carvacrol. It has been reported the many plants which contains thymol as major component likes *Trachyspermum ammi* (Ajwain), *Monarda didyma, Monarda fistulosa, Origanum dictamnus Origanum compactum Origanum dictamnus Origanum onites, Origanum vulgare, Thymus glandulosus, Thymus hyemalis, Thymus vulgaris, Thymus zygis, Satureja hortensis* etc[1-4]. It is a white crystalline substance of pleasant aromatic odor and strong antiseptic properties. This molecule has a broad range of activities including antioxidant, anti-microbial, antiseptic, antitussive, expectorant and antispasmdic properties[5-12]. It is reported that Ajwain oil contains thymol (39.1%) as a major component along with *p*-cymene (30.8%), γ-terpinene (23.2%), β- pinene (1.7%), terpinene-4-ol (0.8%) [13]. Arq Zeera is a herbal liquid formulation used extensively as treatment of obesity in Unani System of Indian Medicine. It is prepared from four herbs Ajwain (*Trachyspermum ammi*), Zangabeel (*Zingiber officinale), Zeera Siyah (Carum carvi), and Zeera Safaid (Cuminum cyminum)* by simple distillation[14]. The Pharmacopeial standards in Ayurvedic, Siddha and Unani are not adequate enough to ensure the quality of formulations. Analysis of marker compounds is necessary to maintain the quality and identity of the formulations. No work has been carried out in the estimation of markers compounds in the Arq Zeera till now. It contains mainly volatile chemical constituents. Essential oils are usually analyzed by gas chromatography (GC), because of its high sensitivity, other techniques, for example high-performance liquid chromatography, are rarely used. Thin-layer chromatography is used for analysis of essential oils from vegetable matrices when the concentration is sufficiently high. Such analyses are widely reported in the literature[15]. 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 formulations by estimation of markers compound[16]. The objective of the present work was to develop identification, accurate, specific and reproducible method for the estimation of thymol in bulk drug as well as polyherbal formulation. The developed method is also utilized to determine the purity and quality of the market sample by comparing with the prepared authentic formulation.

MATERIALS AND METHODS

Plant materials

All the ingredients were procured from Unani raw drug dealers with the knowledge of Unani physician, identified by Dr. H. B. Singh from National Institute of Science Communication and Information Resources (NISCAIR), New Delhi. Voucher specimen and identification certificate reference number NISCAIR/RHMD/Consult/2011-12/1753/53 was obtained and kept in the department for future reference. All the ingredients were taken of Pharmacopeial quality and quantity.

Chemicals and reagents

The thymol standard was procured from Sigma (Aldrich) and Assigned purity: 98%. The vanillin reagent used for visualization was from Merck (Germany), and the solvents (toluene and ethyl acetate) were from Sigma (Aldrich). All chemicals used were of analytical grade. The vanillin sulphuric acid agent was prepared accordance with Wagner et al[17].

Extraction of dried Ajwain fruits for analysis

The dried fruits of Ajwain (5g) were extracted by reflux using 100 mL of HPLC grade methanol for minimum of 2 hours at temperature 70°C and then filtered through Whatman No# 42. Extracts obtained after the reflux were concentrated on hot water bath (HH-6 Digital thermostatic, Jintan medical instrument) and finally the volume was adjusted to 100 mL with methanol for HPTLC analysis. 10 μl of the filtered solution was applied on the TLC plate followed by development, derivatizing with vanillin sulphuric acid agent and scanning at 513 nm.

Preparation and extraction of formulations for analysis

Crushed the cleaned and dried all the four ingredients viz: *Zingiber officinale rhizome*, *Carum carvi, Cuminum cyminum and Trachyspermum ammi* fruits (7g) in an iron mortar to obtained coarse powder and soaked in 96 ml purified water as above given ratio (Table 1). Transferred the soaked all four ingredient to the distillation plant along with purified water. Distilled the same at 100° C for about five and half hrs and collected the 60 ml of Araq Zeera. 30 ml of chloroform was added to formulation Arq Zeera and frequently shaken for 1 hour and allowed to stand for 24 hrs. Transferred the content in separating funnel and mixed

thoroughly. The chloroform layer was separated and rest of the solution was again extracted with more quantities of chloroform at least three times. Combined chloroform extract was evaporated by rotatry evaporator and residue was redissolved in 30 ml methanol. The resulting solution (233 mg/ml) was filtered by Whatman filter paper and volume was reduced to 10 ml in volumetric flask. This solution was used for the analysis of thymol in Arq Zeera. 10 µl of the filtered solution was applied on the TLC plate followed by development, derivatizing with vanillin sulphuric acid agent and scanning at 513 nm. The analysis was repeated six times. Similarly marketed formulation (10 µl) was used for the analysis of Thymol.

Table 1: Formulation composition

S. No.	Unani name	Botanical /English name	Parts used	Quantity
1	Ajwain Desi	*Trachyspermum ammi*	Fruit	2 g
2	Zanjabeel	*Zingiber officinale*	Rhizome	1 g
3	Zeera Siyah	*Carum carvi*	Fruit	1 g
4	Zeera Safaid	*Cuminum cyminum*	Fruit	3 g
5	Water			96 ml

TLC instrumentation & conditions

The chromatography was performed for extracts as per method reported earlier from in our lab[18]. In brief, the samples were spotted in the form of bands of width 3mm with a Camag micro litre syringe on precoated silica gel aluminium plate 60 F_{254} (20 cm × 10 cm with 0.2 mm thickness; E. Merck, Darmstad, Germany) using a Camag Linomat V (CAMAG, Muttenz, Switzerland). A constant application rate of 150 nL/s was employed and space between two bands was 4 mm. The slit dimension was kept at 4 mm × 0.1 mm, and 20 mm/s scanning speed was employed. These parameters were kept constant throughout the analysis of samples. The mobile phase consisted of toluene and ethyl acetate in a ratio of 9: 3 *v/v*. Plates were developed in ascending order with a CAMAG twin trough glass tank which was pre-saturated with the mobile phase for 15 min; the length of each run was 8 cm. The TLC runs were performed under laboratory conditions of (Temp: 25 ± 2°C and % RH: 60 ± 5). The plates were then dried in air. Densitometric analysis was performed at 513 nm with a Camag TLC scanner III operated by Win CATS software (Version 1.2.0). The source of radiation utilized was deuterium and tungsten lamp. The composition of the mobile phase for TLC was optimized using different solvents of varying polarity and good resolution was achieved using toluene: ethyl acetate (9: 3 *v/v*) as mobile phase. The R_f value for thymol was found to be 0.72 ± 0.02. The scanning wavelength selected was 513 nm, the absorption maxima of the thymol spot.

Calibration curve of thymol

A stock solution of thymol (1 mg/mL) was prepared in methanol. 1, 2, 3, 4 and 5 µL from the stock solution were spotted on TLC plate in six (*n* = 6) to obtain final concentration range of 1000–5000 ng/spot. The data of peak area versus drug concentration were treated by linear least-square regression. QC samples chosen for the study were 1000, 3000 and 5000 ng/ spot.

Method validation

The developed method is validated as per the ICH guidelines[19]. The developed method was validated in terms of Linearity, precision, accuracy, limits of detection (LOD), limits of quantification (LOQ), and recovery.

Precision and accuracy

The intra-day precision and accuracy of the assays were evaluated by performing replicate analyses (*n* = 6) of QC samples (1000, 3000 and 5000 ng/spot). The inter-day precision and accuracy of the assay was determined by repeating the intra-day assay on three different days. Precision was expressed as the percentage coefficient variation (CV %) of measured concentrations for each calibration level, whereas accuracy was expressed as percent recovery.

Sensitivity and linearity

In order to estimate detection (LOD) and quantification (LOQ) limits, we spotted blank methanol (*n* = 6) following the same method as explained under the section of chromatographic conditions and the standard deviation (σ) of the magnitude of analytical response was determined. The LOD was expressed as (LOD = 3.3 σ/slope of thymol calibration curve), whereas LOQ was expressed as (LOQ = 10 σ/slope of thymol calibration curve).

Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for thymol in sample was confirmed by comparing R_f and spectra of spot with that of standard. The peak purity of thymol was assessed by comparing 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

Initially toluene: ethyl acetate in varying ratios was investigated. The mobile phase Toluene: ethyl acetate (9:3.5: *v/v*) gave good resolution with R_f value of 0.72 ± 0.02 for thymol but typical peak nature was missing (Fig 1). The volume ratios of ethyl acetate of over said system was varied to determine the effect on R_f and on the response to thymol. Finally, the mobile phase consisting of Toluene: ethyl acetate (9: 3 *v/v*) gave a sharp and well-defined peak at R_f value of 0.72 ± 0.02 (Fig 2). Well-defined spots were obtained when the chamber was saturated with the mobile phase for 15 min at room temperature.

For determination of the linearity curves of area vs concentration, different amounts of stock solution of thymol was applied on the HPTLC plate and analyzed.

Calibration curve

Calibration was linear in the concentration range 1000–5000 ng. The linear regression equation was Y = 238.750 + 1225.318 X, for thymol, while the correlation coefficient (r2) was 0.994, with high reproducibility and accuracy (Table 2).

Detection limit of thymol was determined by plotting a series of concentrations on the plate and scanning at 513 nm. The lowest amount of thymol which could be detected (LOD), was 0.039 μg/spot. The lowest amount of thymol which could be quantified (LOQ), was found to be 0.118 μg/spot.

Validation of method

Recovery studies

The proposed method, when used for estimation of thymol after spiking with 50, 100 and 150% of additional drug, afforded recovery ranging from 98.99% – 101.12% for thymol was obtained as listed in (Table 3).

Precision and accuracy

The intra- and inter-day precision, as coefficient of variation (CV, %) and accuracy of the assay determined at thymol concentration of 1000, 3000 and 5000 ng/spot has been summarized in [Table 4]. The intra-day precision (*n* = 6) was < 1.00%. The inter-day precision over three different days was≤ 0.1%. The intra -day and inter-day

accuracy were in the range of 99.32% – 100.71% and 98.55% – 99.43%, respectively. The repeatability of the method was studied by assaying six samples of thymol at same concentration under the same experimental conditions. The values were within the acceptable range and so we concluded that the method was accurate, reliable and reproducible.

Robustness of the Method

The standard deviation of peak areas was calculated for each parameter and RSD was found to be in the acceptable range .The low values of SD (<2.0) and % RSD (<1.33) obtained after introducing small deliberate changes in the developed HPTLC method indicated the robustness of the method (Table 5).
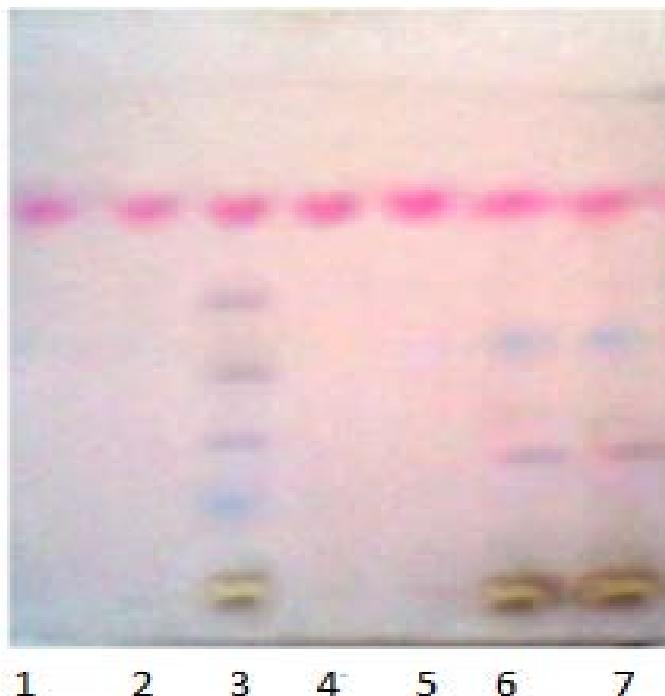

Fig. 1: Developed HPTLC Plate at Vanillin sulphuric acid agent, 1, 2, are standard thymol (1 µg/spot), 3 is herbal extract (1 µg/spot), 3, 4 standard thymol (2 µg/spot), 6 is prepared formulation extract (1 µg/spot), 7 is marketed formulation extract (1 µg/spot).
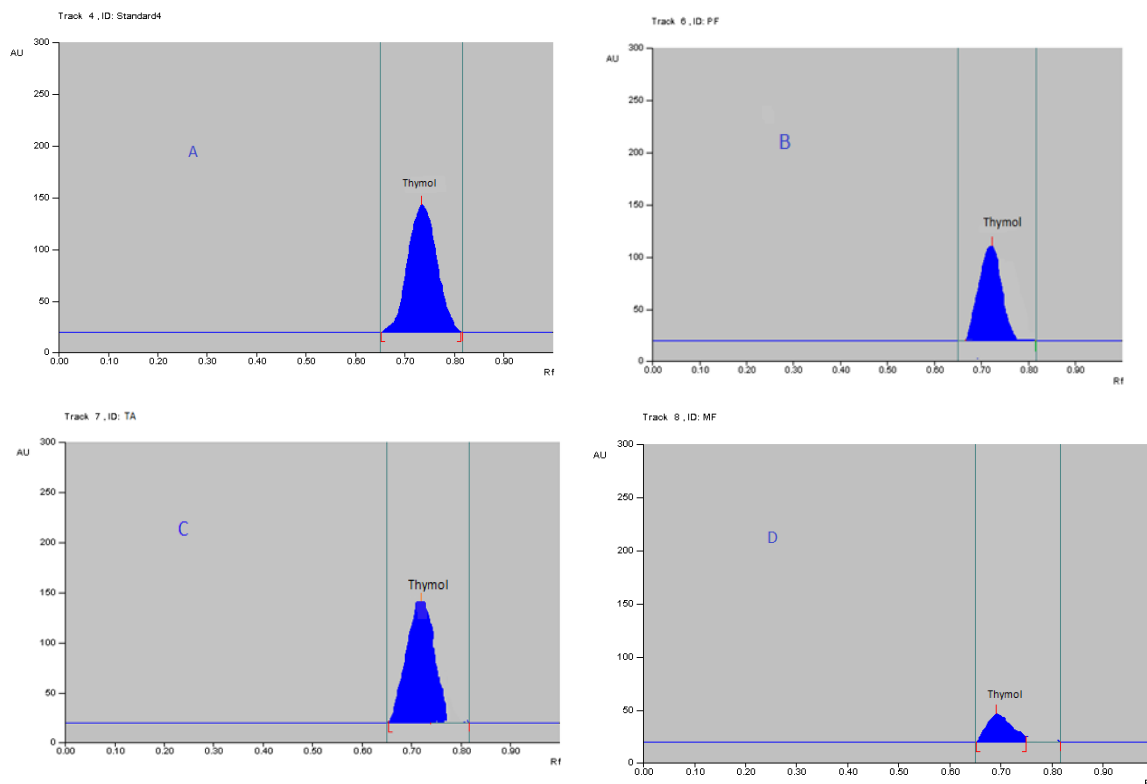

Fig. 2: HPTLC chromatograms of thymol standard (A), PF extract (B), TA extracts (C), and MF extracts (D).

Table 2: Linear regression data for the calibration curves (Y = 238.750 + 1225.318 X)

Linearity range (ng/spot)	r^2	Slope	Intercept
1000–5000	0.994	1225.318	238.750

Table 3: Recovery studies (n = 6)

Excess drug added to the analyte (%)	Theoretical content (ng /spot)	Recovery (%)	Mean
0	1000	98.99	
50	2000	100.12	100.93 ± 0.8751
100	3000	99.89	
150	4000	101.12	

Table 4: Intra- and inter-day precision of HPTLC method (n = 6)

Amounts (ng/spot)	Intra-day precision			Inter-day precision		
Thymol	Mean of peak area	S.D of areas	R.S.D.%	Mean of peak area	S.D of areas	R.S.D.%
1000	1549.33	5.508	0.355	1550.66	1.523	0.098
3000	4402.53	2.991	0.06	4402.78	2.513	0.057
5000	5812.28	2.257	.04	5811.47	1.09	0.018

Table 5: Robustness testing (n = 6)

Parameter	S. D.[a] of peak area	R.S.D.[a]%
Mobile phase composition	1.57	1.12
Amount of mobile phase	1.26	1.02
Temperature	1.94	0.84
Relative humidity	1.52	1.33
Plate pre-treatment	0.90	0.61
Time from spotting to chromatography	0.77	0.45
Time from chromatography to scanning	0.89	0.37

[a] Average of three concentrations 1000, 3000 and 5000 ng/spot.

LOD and LOQ

The calibration curve in this study was plotted between amount of analyte versus average response (peak area) and the regression equation was obtained Y = 238.750 + 1225.318 X over the concentration range 1000–5000 ng/spot with respect to the peak area with a regression coefficient of 0.994. Limit of detection and limit of quantification was calculated by the method as described in validation section and was found to be 0.039 and 0.118 μg respectively, which indicates the ample sensitivity of the method.

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 apex, and peak end was compared and found superimposed. This indicated that standard thymol and sample peaks were not merging with any other components or 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 positions (Fig 3). A good resolved single spot of thymol was observed at R_f value 0.72 ± 0.02 in the chromatogram of the samples extracted from Ajwain fruits and formulations. The thymol content in Ajwain fruits, PAF and MF was observed and calculated (Table 6).

A validated HPTLC method has been developed for the determination of thymol in bulk drug (Ajwain) and its formulation Arq Zeera. The proposed method is simple, precise, specific, accurate, less time consuming and cost effective. Statistical analysis proved that the method is evitable for the analysis of thymol. The developed HPTLC method will help the manufacturer for quality control and standardization of herbal formulations. In this experiment the contents of thymol was found slightly higher in PAF as compared to MF. The method established in this study could be used for the quality control of herbal medicines derived from different species thymol containing plant.
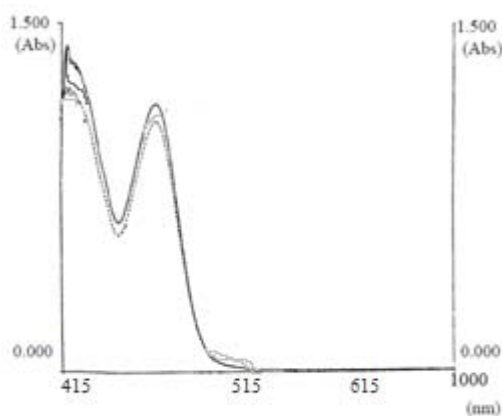

Fig. 3: Overlay spectra of standard and samples (bulk and formulations) in absorption mode in the UV range, taken on the CAMAG TLC scanner 3.

Table 6: The content of thymol in bulk drug and formulations

Thymol	Average Thymol content (% w/w), n = 6
(a) Analysis of the herbal extract	2.1
(b) Analysis of the formulation	0 .784
(c) Analysis of the marketed formulation	0.167

CONCLUSION

In this research, an antiobesity polyherbal formulation Arq Zeera in Unani system of medicine was identified as thymol and confirmed by TLC and UV. The high performance thin chromatographic method developed to quantify the thymol in Ajwain, PAF and MF Arq Zeera was shown to be rapid, reliable and accurate. The method was found to be useful in detecting the geniuses of the formulation. In the present study, though both formulations can be used for therapeutic activity, the quality of marketed formulation is not up to the level of prepared laboratory formulation.

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