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A HPTLC Method for Determination of Anethole in Essential Oil, Methanolic Extract of *Foeniculum vulgare* and Commercial Herbal Products

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Authors' contributions

This work was carried out in collaboration among all authors. Authors Prawez Alam, MHA and AIF designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors Pravej Alam and MG managed the analyses of the study. Author MG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A rapid and feasible method of HPTLC is standardized for quantification of anethole in essential oil's extract and from herbal formulations of fennel seed. The developed densitometric HPTLC method was performed to estimate the existence of anethole in the essential oil, extract and herbal formulations of fennel with the optimized concentration of hexane: Ethyl acetate (8:2%, *v*/*v*, mobile phase) on glass coated silica gel 60 F_{254} plates (20 × 10 cm) scanned with the absorbance of λ_{260} nm under densitometric condition. The Linearity of regressions revealed a satisfactory relationship between peak area and concentration of anethole in between the range of 100-600 ng/spot. This reliable method was validated as per the ICH guidelines to fulfill the necessary parameters such as

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accuracy and robustness. The amount of anethole in essential oil (0.098 ± 0.002%), extract (0.101 ± 0.004%) and three herbal formulations A (0.024 ± 0.004%), C (0.019 ± 0.002%) while anethole is not detected in B formulations from fennel seed was completely estimated by the developed method. The standardized methods and its validation gave new insights of HPTLC based detection and quantification of anethole in other aromatic plants as well as in other pharmacological formulations.

Keywords: Anethole; HPTLC; validation; quantitative.

1. INTRODUCTION

Aromatic plants, usually used for flavoring, seasoning and imparting aroma in foods having important classes of valuable metabolites showed different biological activities, e.g. antioxidant and antimicrobial activity. *Foeniculum vulgare* (family Apiaceae; common name fennel) is a cherished medicinal plant used for formulation of herbal drugs industries and traditional systems of medicine [1,2]. The plant is originated from southern Mediterranean province and now introduced and cultivated in most of the world. The plant is presently used for antioxidant, antispasmodic, hepatoprotective anti-inflammatory, analgesic activities either the particular part of the plants or its essential oils [3]. In other studies of fennel, the seed has also shown the anticancer activity and along with used in respiratory disorder reported that [4,5]. All these pharmacological activities are may be due to the medically active compounds, e.g. flavonoids, fatty acids, phenolics, and high complex protein known as phytochemicals [6,7]. The essential oil of fennel has composed ~ 87 volatile compounds [7-8]. The main volatile compounds have reported earlier from *F. vulgare* are anethol, methyl chavicol, fenchone and limonene as well as piperitenone and piperitenone oxide [9]. Among them, a GC/MS study revealed that anethole is the most abundant compound present in fennel essential oil [6]. The anethole is a monoterpene, a naturally existing aromatic compound isolated in fennel, tarragon, and basil [10]. Due high concentration and its volatility of anethole (80% in fennel,), the plants used in fragrance and other industries e.g. pharmaceuticals, cosmetics, and a food [10].

Recent past, many researchers have been conducted to identify and quantify the exact concentration of bioactive molecules in the essential oil of fennel from different origins or regions have different concentration [1,6,11]. Due to adulteration or their geographical origin the active metabolites are either metabolites are not detected or found in low amount of anethole which affect their quality. Due to this reason, we have to standardize the HPTLC method for *Foeniculum vulgare* to isolate and quantify the anethole to check the quality of herbal products present in market.

2. MATERIALS AND METHODS

2.1 Plant Material

The *Foeniculum vulgare* (fennel) seeds and formulations were procured from local market of AL-Kharj-Riyadh governate. The specimen seeds were identified with voucher specimen repository available at College of Pharmacy, Prince Sattam bin Abdulaziz University, Al Kharj, Saudi Arabia.

2.2 Standard and Chemicals

All the AR and GR grade chemical used in this study like acetone, methanol, ethyl acetate and known compounds of anethole used as a standard were procured from Sigma-Aldrich USA.

2.3 Preparation of Methanolic Extract and Herbal Formulations

10 g of fennel seeds were made powdered and extracted overnight in methanol at room temperature. The methanolic extract was then transferred to 50 ml volumetric flasks to maintain the volume of 50 ml. Similarly, the herbal formulations (A–C) procured from local market were separately dissolved in methanol and extracted at same condition and same w/v followed the method of extraction of seeds as above at room temperature. The extracts were filtered under 0.25 µm Millipore filter and evaporated using rotary vacuum evaporator under reduced pressure. The formulations extracts (A-C) were transferred to 10 ml volumetric flasks to maintain the volume of 10 ml. All the extraction process was repeated thrice.

2.4 Preparation of the Volatile Oil

The volatile oil of fennel seeds was obtained by hydro distillation method followed the standard method of Egyptian Pharmacopoeia [12]. 200 gm of fennel seeds were used for oil extraction under Clevenger trap apparatus. 1000 mL of water with fennel seeds mixed for 8 hr distillations. The oil layer and water separation were trapped with dichloromethane (3X50 mL). Further, the organic layer was concentrated under rotary vacuum evaporator to get the pure essential oil.

2.5 Optimization of Standard Solution

1 mg/ml of anethole (purity >=98%, HPLC grade) dissolved in methanol was used as a stock solution for further study. Six concentration of working solution (100, 200, 300, 400, 500 and 600 ng) were made from stock solution and used for HPTLC calibrations using the peak height and areas against the consistent amount per spot.

2.6 Chromatographic Conditions

HPTLC separation was carried out on HPTLC plates (glass backed, 10 × 20 cm) coated with silica gel 60 F_{254} (0.2 mm Merck). The six concentration of standards (solution (100, 200, 300, 400, 500 and 600 ng) were applied to the TLC plates (6 mm band) for the generation of standard curves and using HPTLC system (CAMAG Automatic TLC Sampler 4) with sample applicator (Switzerland). Similarly, the essential oil, extract of herbal formulations of fennel seeds was also analyzed under the above parameters in HPTLC systems with constant rate of 150 nl/s. The developed plates with the distance of 8 cm in a liner manner was carried out in a CAMAG Automatic Developing Chamber 2 (ADC2) having mobile phase (Hexane: ethyl acetate 8: 2 (%, *v*/*v*) as) for 30 min at 23 ± 2°C and 30% relative humidity.

2.7 Method Validation

The developed method was validated [13] in term of the accuracy, percentage recovery and robustness under densitometric TLC by the optimizing in composition of hexane and ethyl acetate (mobile phase) concentration, duration, saturation and glass backed activation of HPTLC for the determination of anethole keeping the values of detection and qualification limit i.e. LOD and LOQ under the formula of LOD = 3.3× SD /S; LOQ = 10× SD /S, respectively.

2.8 Quantification of Anethole in Essential Oil, Extract and Herbal Formulations of Fennel Seed

The activated silica backed glass plate was screened and densitometric quantification was observed at the absorbance of 260 nm. Linear regression analysis revealed a good linear relationship between peak area and amount of anethole in the range of 100-600 ng/band. The essential oil, extract (A-C) formulations were analyzed on the basis of standard curves of anethole. The peak area of anethole corresponding to the R_f value with anethole used as standard was analyzed to calculate the exact amount from the regression equation with the help of standard curve.

3. RESULTS

3.1 Method Validation

The compositions and concentrations of mobile phase play an important role to establish a reliable protocol for separation of plant metabolites for the established of densitometric HPTLC methods. In this regard, the hexane: ethyl acetate 8: 2 (%, *v*/*v*) were tested and found the effective mobile phase with the compactness and symmetry (0.58 ± 0.01; R_f value; Fig. 1). The maximum absorbance of UV spectra was recorded and maximum band showed at 260 nm.

3.2 Calibration Curve

In this study, the observed linear regression graph confirmed the good linear relationship with standard curve of anethole with peak area against concentrations (100-600 ng/spot; Table 1). The correlation coefficient (R^2) was recorded 0.9995 with highly significant P value (<0.05).

3.3 Method Validation

3.3.1 Accuracy

The linearity equation y = 15.917x + 79.027 found to be satisfactory with the accuracy of 98.22-99.30%, with RSD (0.68-1.23, Table 2).

Table 1. Linear regression data for the calibration curve of anethole (n=6)

Linearity range (ng/spot)	100-600
Regression equation	Y = 15.917 + 79.027
Correlation coefficient	0.9995
Slope ± SD	15.917 ± 0.245
Intercept ± SD	79.027 ± 54.18
Standard error of slope	0.05743
Standard error of intercept	2.43
95% confidence interval of slope	4.578 – 5.287
95% confidence interval of intercept	789- 878.2

Table 2. Accuracy of the proposed method (n=6)

Excess drug added to analyte (%)	Theoretical content (ng)	Conc. found (ng) ± SD	% Recovery	% RSD
0	200	196.67 ± 2.16	98.33	1.10
50	300	294.67 ± 3.61	98.22	1.23
100	400	395.17 ± 4.12	98.79	1.04
150	500	496.50 ± 3.39	99.30	0.68

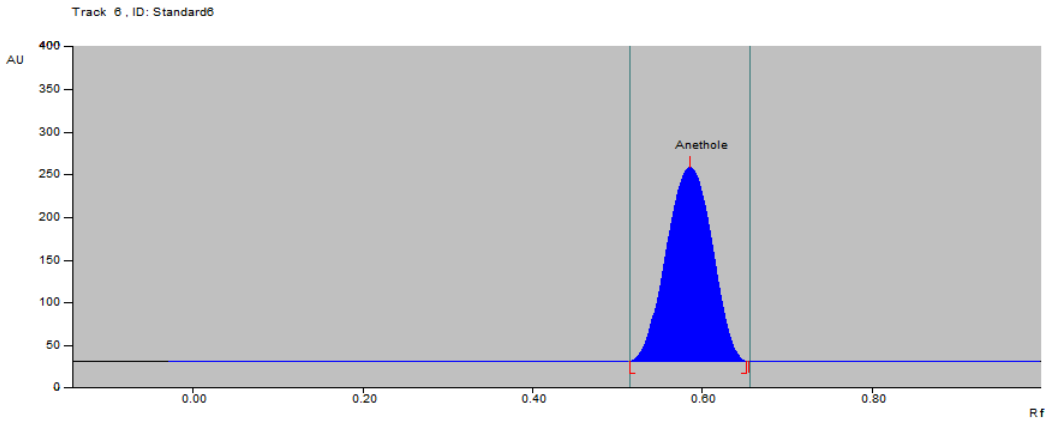

Fig. 1. HPTLC densitogram of standard Anethole

3.3.2 Precision

The % variation coefficient (CV) of precision was measured for each calibration level to confirm the validation of the method under the RSD repeatability and intermediate precision ranged 0.34-0.80 and 0.45-1.02 respectively Table 3. It is now confirmed that this method is highly precise and reliable for resolution of anethole by obtaining low values. Therefore, these findings confirmed the accuracy and validation of the HPTLC method.

3.3.3 Robustness of the method

Similarly, the robustness of % RSD (0.38-0.87) were obtained after introducing trace thoughtful change into the densitometric TLC process revoked the robustness of the developed method for anethole.

3.3.4 Limit of detection and limit of quantification

8.11 and 26.07 ng/spot, limit of detection and quantification was also observed and indicated that the proposed method can be used in wide range for detection and quantification of anethole effectively by using the formula mentioned in methods sections. The anethole purity with reference to the obtained chromatogram (peak) was evaluated by equating the overlapped spectra at beginning of peak, peak apex and end of the spot positions. The overlapped spectra of anethole standards and samples were given in Fig. 5.

Table 3. Precision of the proposed method

Conc. (ng/spot)	Repeatability (Intraday precision)			Intermediate precision (Interday)		
	Area ± SD (n = 6)	Standard error	% RSD	Area ± SD (n = 6)	Standard error	% RSD
300	4898.20 ± 39.14	15.98	0.80	4900.40 ± 49.96	20.40	1.02
400	6532.80 ± 33.67	13.75	0.52	6545.80 ± 36.43	14.88	0.56
500	7942.00 ± 27.23	11.12	0.34	7945.80 ± 35.95	14.68	0.45

Table 4. Robustness of the proposed HPTLC method

Conc. (ng/spot)	Mobile phase composition (Hexane: ethyl acetate)			Results		
	Original	Used		Area ± SD (n = 6)	% RSD	R_f
400	8: 2	7.8: 2.2	+0.2	6528.00 ± 25.07	0.38	0.60
		8: 2	0.0	6560.60 ± 36.30	0.55	0.58
		8.2: 1.8	-0.2	6535.80 ± 57.04	0.87	0.56

Table 5. Contents of anethole in essential oil, extract and herbal formulations of fennel seed (n=3)

Samples	Contents mean ± SD (% w/w)
Extract	0.101 ± 0.004
Essential oil	0.098 ± 0.002
A	0.024 ± 0.004
B	------
C	0.019 ± 0.002

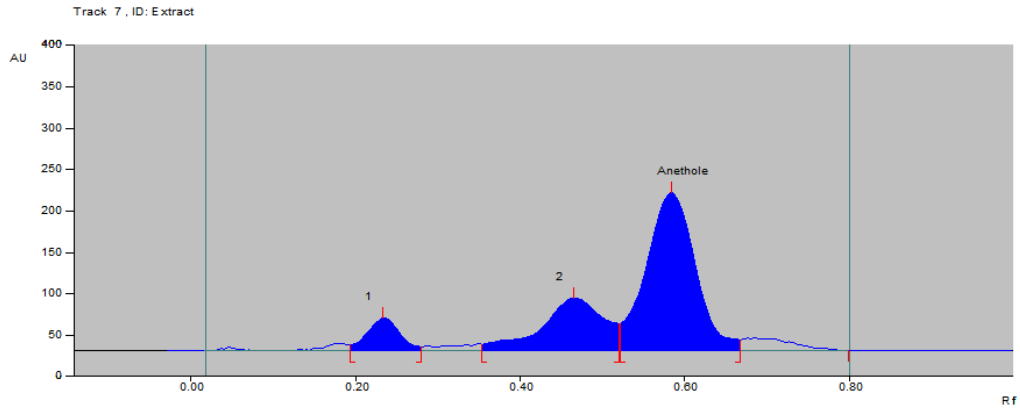

Fig. 2. HPTLC densitogram of extract of *Foeniculum vulgare*

3.3.5 Quantification of anethole in essential oil, extract and herbal formulations of fennel seed

Anethole peaks from essential oil, extract and herbal formulations of fennel seed were estimated with the help of its single spot of R_f values obtained during the method development of the anethole standard solutions at the identical circumstances (Figs. 2-4). The anethole concentration present in fennel seed essential oil, extract and herbal formulations further estimated using the developed standard linear regression equation. The concentration for the analysis anethole essential oil (0.098 ± 0.002%), extract (0.101 ± 0.004%) and three herbal formulations A (0.024 ± 0.004%), C (0.019 ± 0.002%) while anethole is not detected in B formulations listed in Table 5.

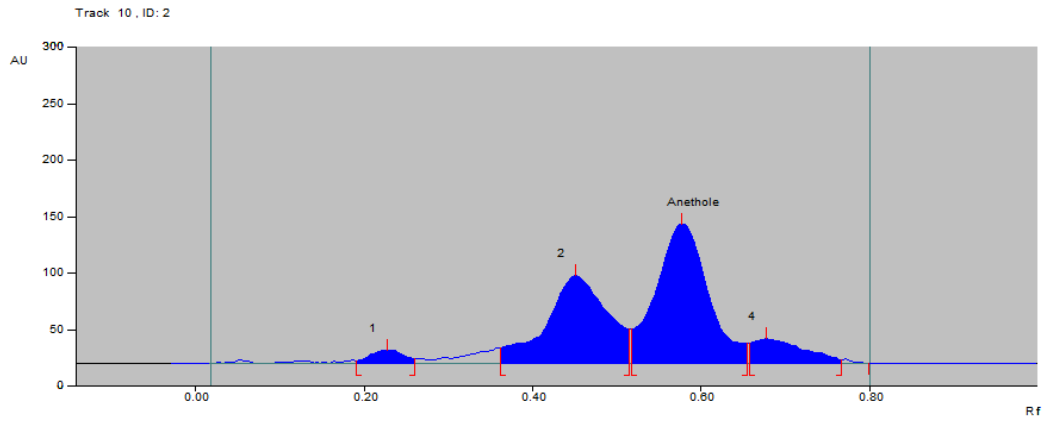

Fig. 3. HPTLC densitogram of oil of *Foeniculum vulgare*

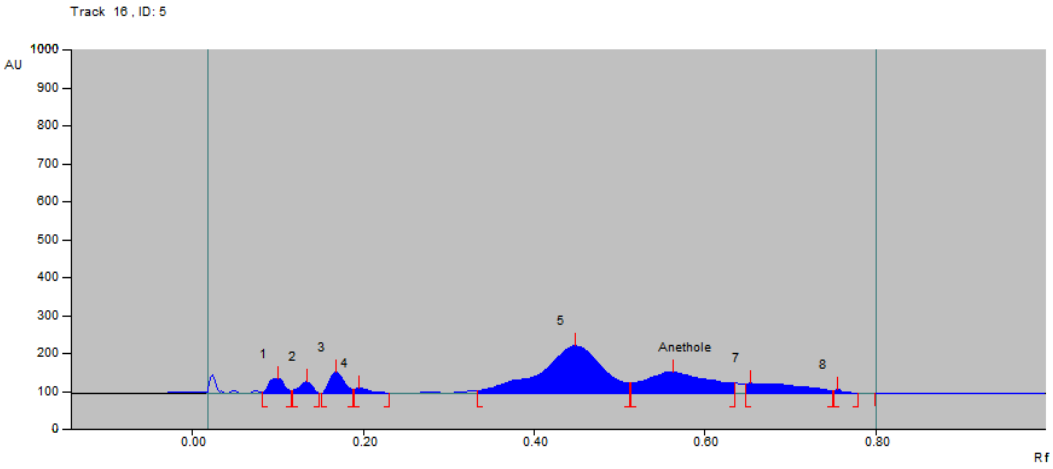

Fig. 4. HPTLC densitogram of formulation containing *Foeniculum vulgare*

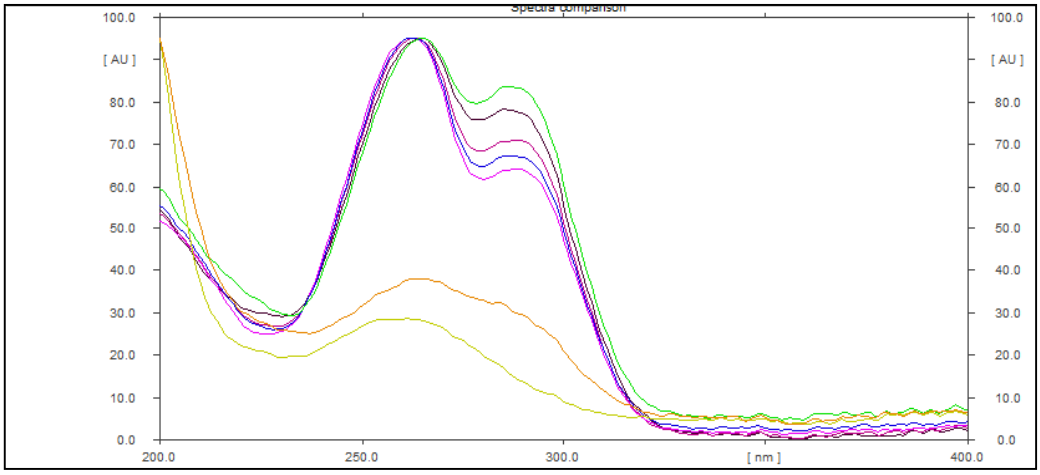

Fig. 5. Overlay UV absorption spectra of standard anethole, extract, oil and different herbal formulations

4. DISCUSSION

Keeping the all chromatographical conditions, the method was found very sensitive and adequate for the anethole estimation. The results obtain in this study have shown good resolution and did not fused with any contamination with the other metabolites present in the samples. Due to short time span, robust, less expensive, this method is more effective as compared to other for anethole estimation [14,15]. This could be due to small amounts of mobile phase and other chromatographic parameters optimized by us. This is the 1st report which based on densitometric separations of anethole.

5. CONCLUSION

The developed densitometric method for estimation of anethole could be used for determination of anethole in other aromatic plants. It is more reproducible, robust, accurate and more sensitive than other methods for the estimation of anethole concentration in a short span showing cost effectiveness.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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