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Gas and high performance thin layer chromatographic based-determination method for quantification of thymol in semisolid traditional dosage forms

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ABSTRACT

The control and standardization process of herbal products is a critical point in preparation of those medicaments. In Traditional Persian Medicine (TPM) literature, out of all the different pharmaceutical dosage forms, *Jawarish* is a semisolid gastrointestinal dosage form with positive related effects. *Jawarish-e-Khuzi*, including *Zataria multiflora*, *Lepidium sativum, Trachyspermum ammi, Terminalia chebula,* ferrous sulfate, and also honey is one of the popular mentioned traditional oral formulations. However, there have been no noticeable and proven control and standardization for this formulation. In this study, *Jawarish-e-Khuzi* was prepared based on one of the pharmaceutical textbooks of Traditional Persian Medicine (TPM). Using gas chromatography/mass spectroscopy (GC/MS), the volatile composition of this formulation was analyzed. Subsequently, Gas chromatography/flame ionization detector (GC/FID) and High-Performance Thin-Layer Chromatography (HPTLC) techniques were employed to determine the main component. The GC/MS results showed thymol as the main constituent. In the content determination process via GC/FID, thymol was proved to be 0.02% of the whole preparation. The outcome of HPTLC method also corresponded with that of GC/FID. Based on the method validation parameters, both GC/FID and HPTLC methods are useful for the volatile content determination of semisolid dosage forms.

Keywords: *Jawarish-e-Khuzi; Standardization; HPTLC; GC/MS; GC/FID.*

1. INTRODUCTION

For thousands of years, medicinal plants have been employed for various ailments by mankind. In contrast to current pharmaceutical agents, the pharmacological assessment of natural medicaments has been relatively neglected and related human trials involving herbal medicines are still infrequently performed. Lack of attention to the pharmaceutical analysis of medicinal plants and herbal preparations has contributed to concerns with availability of herbal remedies, poorly defined chemical composition and inadequate knowledge regarding the active markers. Consequently, natural health products might often be produced with unknown pharmaco-active components. Moreover, the determination of toxicity and the propensity for interaction with pharmaceutical agents are often neglected. Without adequate description and standardization of an herbal remedy understudy, further clinical research becomes unreliable due to inherent inconsistencies in the substance (s) that are being studied [1].

Plants synthesize a large number of bioactive compounds which can be a candidate to be a source of new drug [2]. The most important purpose of using traditional medicine is to discover and present new drugs for the pharmaceutical market around the world [3]. About 25% of medicines in modern pharmacopeias have been derived from plants [4].

Traditional medicine in Iran has a rich history from ancient Persia [5]. Actually, Traditional Persian Medicine (TPM), as an ancient great school, provided plants-based resources for clinical studies [6]. In order to achieve valuable information about the application of medicinal herbs, reviews of historical literature in TPM can be more useful [7].

In pharmaceutical textbooks of TPM, the process of compounding the natural medicaments, administration, warnings and precautions, uses, dosage forms and target organs has been mentioned. These types of textbooks are called "*Qarābadin*" [8].

Gastrointestinal (GI) diseases are prevalent among human. Although there have been restricted treatment options for GI disease, herbal drugs have been used widely in GI problem [9]. In TPM, it is supposed that digestive system has a notable effect on the other organs in the body. So, in regard to treatment various kinds of diseases, there has been specific attention to gastric function [10]. Medical literature of TPM has mentioned a large number of GI diseases and ailed treatment [11].

Jawarish formulations are herbal drugs that have been used for improving gastrointestinal problems [12]. *Jawarish-e-Khuzi* is one of the dosage forms that has been mentioned in the *Teb-e-Akbari*. This semi-solid traditional formulation, included 5 parts (*Zataria multiflora*, *Lepidium sativum, Trachyspermum ammi, Terminalia chebula,* ferrous sulfate and honey).

Zataria multiflora Boiss. (ZM), traditionally named *Avishan-e-Shirazi* (Shirazi thyme), belongs to the Lamiaceae family, cultivated and grows in Iran, Afghanistan and Pakistan [13]. In Iranian folk medicine, ZM has been used as antiseptic, anti-flatulence, intestine-soothing, and pain relieving [14]. Thymol, carvacrol, ρ-cymene and linalool are the main volatile components of this plant [15].

Terminalia chebula Retz. (*T. chebula*), commonly called the "Haritaki", belongs to the Combretaceae family. This herbal is used in Ayurveda, Unani , Siddha and homeopathy medicines.

Haritaki possesses a wide variety of phytochemicals such as polyphenols, terpene, anthocyanins and flavonoids, alkaloids, and glycosides. It has several traditional uses as antimicrobial, antioxidant, anti-viral, anti-carcinogenic, hypocholesterolemia, radio-protective, and anti-spasmodic properties [16].

Lepidium sativum Linn. (L.S), generally known as garden cress, belongs to the Brassicaceae, broadly cultivated in many countries of the world [17]. Alkaloids, flavonoids, tannins, glucosinolates, sterols, triterpenes, saponins, anthracene glycosides, carbohydrates, proteins and phenolics are the chief bioactive component of garden cress [18]. This plant has various pharmacological effects such as antibacterial activity, antifungal activity, antioxidant activity, cytotoxic activity, diuretic activity, hepatoprotective activity, hypoglycemic activity, antiosteoporotic activity, antiasthmatic activity, anti-carcinogenic effect, cardiotonic activity, smooth and skeletal muscles contraction activity, fracture healing property, chemo-protective effects, and hemagglutination. Also, in Indian folk medicine, it has been prescribed for menstrual cycle regulation, gastrointestinal problems (diarrhea and constipation) and to increase milk production [19].

Trachyspermum ammi (L.) Sprague (T.A), commonly called Ajowan, belongs to the Apiaceae. This plant is widely cultivated all over the World. Thymol is the major constituent of Ajwain seeds essential oil. Ajowan seeds have possess aphrodisiac, diuretic, antimicrobial, antiviral, antiulcer, anti-inflammatory, analgesic, and bronchodilatory, as well as antitumor and antioxidant properties [20, 21].Among most investigations, *Jawarish-e-Khuzi* with those ingredients, has never been evaluated, standardized and reformulated. In this regard, the current study was conducted to introduce this semisolid dosage form and parallelly determined in regard to a major volatile constituent via Gas Chromatography/ Flame Ionization Detector (GC/FID) and High-Performance Thin-Layer Chromatography (HPTLC) techniques to compare these two methods additionally.

2. MATERIALS AND METHODS

2.1. Plant studied, Manufacturing and preparation of the *Jawarish-e-Khuzi.*

As mentioned by the *Teb-e-Akbari* book, the *Jawarish-e-khuzi* is a semi-solid formulation made with the following ingredients in the constitution as shown in Table 1. All ingredients of this formulation were purchased from a popular medicinal plant market in Shiraz and brought to the Department of Phytopharmaceuticals (Traditional Pharmacy), Shiraz School of Pharmacy for authentication and specification of a voucher number.

Table 1. Ingredients of the *Jawaiesh-e-Khuzi* formulation.

Common name	Scientific name	Voucher number	Quantity
Avishan-E-Shirazi	*Zataria multiflora* Boiss.	PM1122	300g
Haritaki	*Terminalia chebula* Retz.	PM1096	200g
Ajwain	*Trachyspermum ammi* L.	PM1098	300g
Cress	*Lepidium sativum* L.	PM1097	500g
Ferrous sulfate	-	-	100g
Honey	-	-	1300g

According to *Teb-e-Akbari* instruction, L.S was dried on a hot plate for 10 minutes at 140 ˚C. Other plants were ground via electric miller and sieved through 30 British mesh, separately. Finally, 300 g of Z.M and T.A, 200 g of T.C and 500 g of L.S and 100 g of ferrous sulfate were mixed together (final weight of the product was 1300 g). Then, the honey with the same amount of powder was added to the finished product and mixed well.

2.2. Hydro-distillation and sample preparation.

The *Jawarish-e-khuzi* in dry form was hydro-distilled in a Clevenger apparatus for 4 hours. For this purpose, 300 g of *Jawarish* powder was weighed and soaked in a certain amount of distilled water for 24 hours, then poured into the Clevenger apparatus. After finishing the extraction process, the yield of essential oils was calculated on a dry weight basis. Then, the whole of essential oil was poured in the test tubes with screwed caps. The essential oils after drying over anhydrous sodium sulfate, were stored in the refrigerator at -20°C until GC/MS analysis.

2.3. GC/MS analysis.

GC/MS analysis was performed via an Agilent GC-MSD system (model 7890A). HP-5MS capillary column (phenyl methyl siloxane, L × I.D. 30 m × 0.25 mm, with 0.25-µm film thickness) was used with Helium as a carrier gas at 1 mL/min flow rate. GC oven temperature was scheduled from 60 (0 min) to 220°C (heating rate of 5°C/min) and then kept fixed for 10 min at 220°C. The mass spectrometer (Agilent technologies 5975 C) employed at 70 eV. The mass range was documented from 30 to 600 m/z and injected temperature was adjusted at 280°C.

In order to identify the essential oil components, the Kovats indices were calculated via using retention times of synchronically injected normal alkanes (C_9 – C_{24}) as well as their mass spectra with Willey (nl7) and Adams libraries spectra [22].

2.4. GC/FID analysis.

GC/FID analysis was performed via an Agilent GC-FID system (model 7890A) supplied with a HP-5 column (phenyl-methyl siloxane, L × I.D. 25 m × 0.32 mm, with 0.52-µm film thickness) and flame ionization detector (FID). Nitrogen (5^{th} grade), as a carrier gas, was used at 1 mL/min flow rate. The column temperature was programmed from 60 (0 min) to 250 °C at the heating rate of 5 °C/min and then kept stable for 10 min at 250 °C. The injector and detector were modified at 270°C and 300°C, respectively. The stoke solution of thymol (the main bioactive component in the essential oil) as a reference compound was prepared in methanol (with 99-101% purity). The calibration curve is needed for the quantification of the specific marker. In order to plot the calibration curve, dilutions of thymol (0.04, 0.1, 0.5, 0.1 mg/ml) prepared by methanol and about 1µg of each one sample injected to GC/FID for three times. Also, the essential oil yielded from *Jawarish* was prepared (0.02 mg/ml) and injected to GC/FID three times a day in three days to determine the difference of inter-day, intraday and Relative Standard Deviation. The limit of detection (LOD) and limit of quantification (QOD) was determined.

2.5. High-performance thin-layer chromatography (HPTLC) analysis.

HPLC analysis was performed using the CAMAG TLC system equipped with ATS 4 (automatic TLC sample 4) and an automatic developing chamber (ADC2). The stationary phase was a silica gel plate 60F254 (10×10 cm, Merck, Germany) and the used mobile phase was Toluene: Ethyl acetate (9:1; v/v).

A stock solution containing 6 mg/ml thymol and decreasing serial dilutions from the stock solution in the range of 3, 1.5, 0.75, 0.375 and 0.187 mg/ml was prepared in methanol to set the calibration curve. Also, 4 mg of *Jawarish* essential oil was diluted via 1 ml ethanol. Then, four samples (2, 1, 0.5, 0.25 and 0.125 mg/ml) were prepared and each one was injected three times.

Finally, the chromatographic spots were visualized first with ultraviolet lamps emitting at 254 and 365 nm and then anisaldehyde-sulfuric acid reagent. All chemicals and solvents were purchased as the analytical grade from Merck (Germany) or Sigma Aldrich (USA).

3. RESULTS

3.1. Essential oils and Yield determination.

The amount of dried sample, essential oil and yield are shown in Table 2.

Table 2. Essential oil extraction.

Jawarish-e-Khuzi	Weight (g)	Essential oil (ml)	Yield (%)
	600	4.5	0.75

3.2. GC/MS Essential oil analysis.

About 1 µl of dehydrated *Jawarish* sample without honey was injected into GC/MS device and their components were analyzed. Table 3 and Figure 1 are shown the chemical composition and the GC/MS chromatogram of *Jawarish-e-Khuzi* essential oil, respectively. According to GC/MS analysis, thymol is the main volatile composition in the *Jawarish-e-Khuzi*.

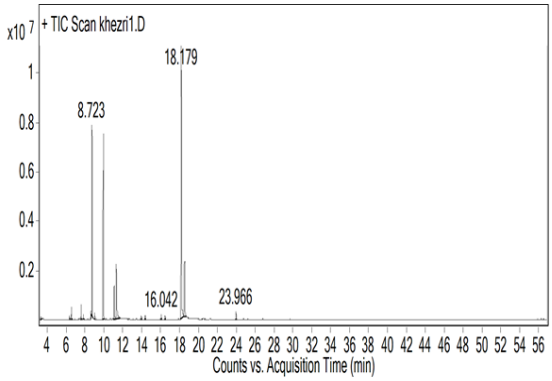

Figure 1. GC chromatogram of the *Jawarish-e-Khuzi.*

3.3. Determination of the amount of thymol via HPTLC method GC/FID method.

GC/FID method was applied to determine the exact amount of thymol in *Jawarish-e-Khuzi* formulation. For this purpose, the standard curve of thymol was plotted and the related equation was calculated according to different concentrations of the standard (Figure 2). Also, table 4 represented the mean ± SD area and RSD for every 4 standard concentrations of thymol, separately. The linearity of the protocol was confirmed by R^2 (R^2=0.99). Then, for determining the exact amount of thymol in the mentioned formulation, 1µl of prepared essential oil was injected to GC/FID for 3 times a day in three days. The intra-day and inter-day variation was calculated and showed in table 5. Finally, Table 6 represented the exact amount of thymol in *Jawarish-e-Khuze* formulation.

Table 3. Chemical composition of the *Jawarish-e-Khuzi* Essential oil.

No.	Component	Product	KICAL	MS_KI	Reference
1	*α*-Pinene	1.16	934	934	**[17]**
2	*β*-Pinene	1.29	975	975	**[23]**
3	*β*-Myrcene	0.48	985	985	**[24]**
4	*α*-terpinolene	0.4	1042	1045	**[25]**
5	*o*-Cymene	16.9	1015	1017	**[26]**
6	*Cis*-Sabinene hydrate	0.73	1027	1036	**[27]**
7	γ-Terpinene	18.1	1050	1050	**[17]**
8	Linalool	4.01	1085	1085	**[28]**
9	l-4-Terpineol	0.47	1162	1160	**[25]**
10	*α*-Terpineol	0.49	1172	1171	**[28]**
11	Thymol	36.5	1286	1284	**[29]**
12	Carvacrol	1.03	1291	1290	**[30]**
13	Durenol	7.38	1276	1281	**[26]**
14	Caryophyllene	0.99	1412	1410	**[31]**
15	% Identification	89.93	-	-	-

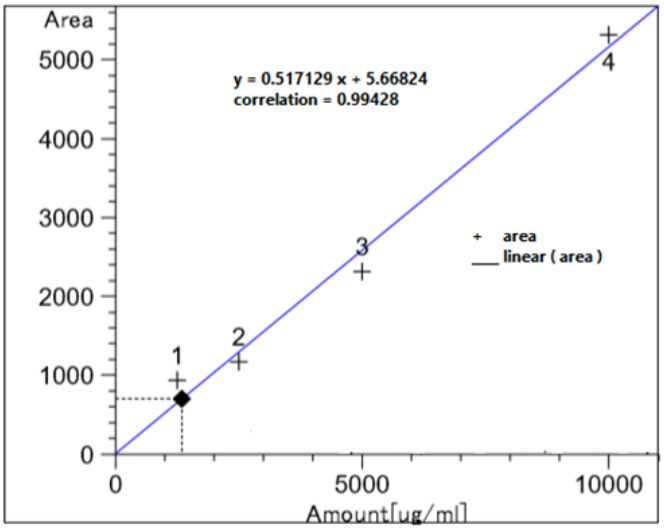

Figure 2. Standard curve of thymol.

Table 4. the Mean ± SD area and RSD for standard concentrations of thymol.

Concentration (mg/ml)	Number	Area	Mean	SD	RSD (%)
0.04	1 2 3	864.8 760.9 688.5	771.4	86.6	**11.22**
0.1	1 2 3	2589.7 2524.3 2590.9	2568.3	33.9	**1.31**
0.5	1 2 3	9610.0 9907 9310.0	9609	298.5	**3.2**
1	1 2 3	25995 25172 27128	26098	978.7	**0.03**

Table 5. Inter-day and intra-day variation.

Day	Area			Mean ± SD (intra-day)	RSD% (intra-day)	RSD% (inter-day)
	A_1	A_2	A_3			
1	2273.4	2386	2325	2328±56.36	2.42	5.7
2	2589.7	2524.3	2590.9	2568±38.1	1.4	
3	2599.5	2517.2	2712.8	2609.83±98.2	3.7	

Table 6. Determined exact amount of thymol in *Jawarish-e-Khuzi.*

No.	Area	Concentration (mg/ml)	Mean ± SD	RSD%	LOD (mg/ml)	LOQ (mg/ml)
1	703.11	1.34	1.41±0.07	4.96	0.006	0.02
2	732.4	1.40				
3	789.1	1.51				

3.4. Quantification of the amount of thymol via HPTLC method.

With the intention of verifying the results of the GC/FID method, the presence and the amount of thymol in the jawarish essential oil, has been detected via HPTLC method. At first, different concentrations of Jawarish essential oil (4, 2, 1, 0.5, 0.25 and 0.125mg/ml) and primary thymol stoke (6, 3, 1.5, 0.75, 0.375 and 0.187mg/ml) were prepared. After loading these concentrations on the HPTLC plate, the plate was submerged in a tank with Toluene: Ethyl acetate (9:1; v/v) as the mobile phase. Followed by the development of the spots on the plate, and drying at room temperature, derivatization of spots were carried out via treatment with sulfuric acid/anisaldehyde reagent. The spots accordingly resembled under heat induction. The spots were photographed at white (245nm) and UV (366nm) lights, before and after treatment with sulfuric acid/anisaldehyde reagent. Figure 3 has exhibited the HPTLC plate at visible light, after submerging in the tank.

Also, in order to calculate the exact concentration of the thymol, the area under the curve of different concentrations of the standard (thymol), were calculated. The results have been shown in table 7. Subsequently, a calibration curve was plotted and exhibited in figure 4. Centered on the calibration curve, the linearity of the protocol was confirmed by R^2 (0.96).

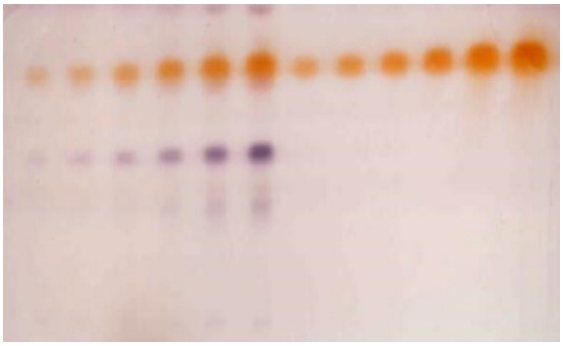

Figure 3. The HPTLC plate at visible light, after derivatization.

Then, the area under the curve of different concentrations of Jawarish essential oil was calculated (Table 8). Eventually, after considering the dilution factor and essential oil percentage, the thymol concentration was determined as 2.33 mg per100g Jawarish.

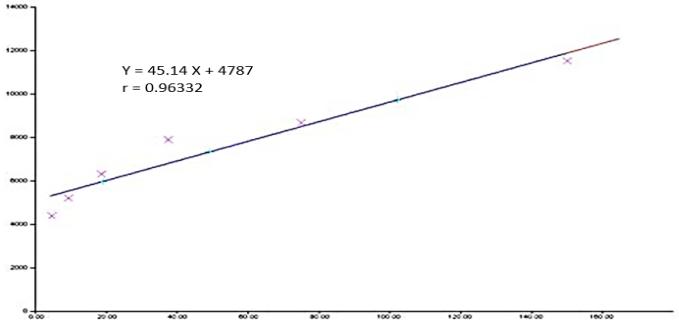

Figure 4. The calibration curve of thymol.

Table 7. The area under the curve of different concentrations of the standard (thymol).

Conc. (mg/ml)	Number	Area	Mean	SD	RSD%
6	1 2 3	11201.7 10578.0 11081.1	10953.6	270.1	**2.46**
3	1 2 3	8355.0 7413.0 8226.9	7998.3	417.2	**5.21**
1.5	1 2 3	7555.7 7115.9 7507.9	7393.1	197.0	**2.66**
0.75	1 2 3	5995.5 5574.2 5960.9	5843.5	190.9	**3.26**
0.375	1 2 3	4889.8 4552.4 4809.5	4750.5	143.9	**3.02**
0.187	1 2 3	4053.3 3800.3 4020.1	3957.9	112.3	**2.83**

Table 8. The area under the curve of different concentrations of Jawarish essential oil.

Sample number	Area	Conc. (mg/ml)	Mean ± SD	RSD%
1	7001.7	1.4	6494.03±528.56	8.13
2	5765.0	1.2		
3	6715.3	1.3		

4. CONCLUSIONS

Herbal remedies have been frequently employed for the treatment of human disease for thousands of years. Although the new drugs have been approved in current medicine, the use of the medical herb has not been reduced [8]. Many people believe that the use of herbal drugs has not any risk but some significant reactions subsequent the administration of herbal medicine have been reported. Then, it seems that the standardization of herbal medicine is necessary [32]. Plus, standardization as a critical process, could help formulators gain repetitive drug responses elsewhere.

GI disorders are frequent in the general population. Herbal medicine has an important role in treatment of GI disorder. There is a growing appeal for the prescribe and use of herbal medicaments for these disorders. *Jawarish* formulations are the set of herbal compound drugs that have been prescribed for improving GI problems. *Jawarish-e-Khuzi* as a kind of *Jawarish*, includes *Zataria multiflora*, *Lepidium sativum, trachyspermum ammi, Terminalia chebula,* and ferrous sulfate and also honey. This formulation widely prescribed by traditional physicians in Iran. Till now, there have been no noticeable and proven control and standardization or any pharmacognosy studies on this formulation. The current study aimed to introduce some specific and reliable parameters to standardization of *Jawarish-e-Khuzi* as a semi-solid herbal medicine in treatment of GI diseases.

To the aim of this study, *Jawarish-e-Khuzi* was prepared based on the reliable instruction from *Teb-e-Akbari* book. Then, essential oil was extracted via Clevenger apparatus. In order to assess the containing of the formulation, GC/MS was employed.

According to GC/MS analysis, thymol is the main component from yielded essential oil. Also, following content determination via GC/FID and HPTLC, the exact amount of thymol was evaluated and the result of GC/FID method has been verified by HPTLC analysis. The validity of these methods, confirmed by RSD (<10%). Regarding the quantification of thymol in *Jawarish-e-Khuzi* formulation, hydrodistillation via Clevenger apparatus confirmed as an appropriate and easy method to extract active volatile components from semisolid formulation.

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