

Research Article

Targeted yield of 100% Pure Thymol via Extraction over a mixture of terpenoids presents in essential oil of Ajwain (*Trachyspermum ammi*.) seeds

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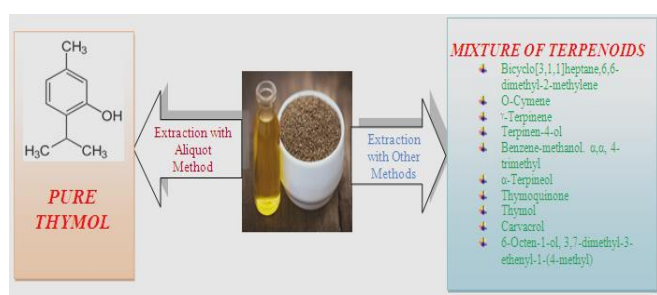


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Abstract—Thymol is an active constituent of ajwain essential oil to establish its preservative character and consequently their utilization in food and pharmaceutical industries. The extraction of ajwain essential oil from the natural source via hydro-distillation leads to a combination of terpenoids like thymol, carvacrol, γ -Terpene, o-cymene, α -pinene, terpinen-4-ol, caryophyllene, 4-carene, β -myecene etc. other extraction methods via organic solvent utilizes various toxic solvents such as methanol, ethanol, isobutyl alcohol etc. since this essential oil is the key replacement of chemical preservatives utilized in food and pharmaceutical industries as preservative, it is required to extract with safer method. Hydrodistillation is quite safe but due to presence of various isomeric forms of thymol in essential oil, its utilization is quite less as compare to pure thymol. As thymol is the key terpenoid responsible for antimicrobial and antioxidant properties of ajwain essential oil, it is necessary to extract pure thymol from its natural source. Present study represents the development of an aliquot (Water and methanol) extraction method via soxhlet apparatus by which Targeted yield of 100% Pure Thymol over a mixture of terpenoids presents in essential oil of Ajwain (*Trachyspermum ammi*.) seeds can be achieved.

Keywords—Thymol, preservative, Extraction, aliquot, ajwain essential oil, Antioxidant and antimicrobial properties

Graphical Abstract:



1. Introduction

Herbal plants have very significant value in present era because of their product i.e. essential oils. The products of herbal plants are used in food, cosmetic items and medical field etc.[1] The ajwain commonly known seed spice plant cultivated at most of the Asian countries such as Iraq, India, Iran, Pakistan and Afghanistan. It is originated from Egypt. In India it is cropped at Uttarpradesh, Maharashtra, Bihar, Rajsthan, Westbengal, Gujrat and Madhyapradesh. [2]

The root of ajwain plant is diuretic in nature and its seeds possess excellent aphrodisiac properties. The seeds contain 2–4.4% brown colored oil known as ajwain oil.[3] The main component of this oil is thymol, which is used in the treatment of gastro-intestinal ailments, lack of appetite and bronchial troubles. The oil exhibits fungicidal,[3] antimicrobial [3] and anti-aggregator effects on humans.[4] Ajwain is a traditional potential herb and is widely used for curing various diseases in humans and animals. The fruit possesses stimulant, antispasmodic and carminative properties. It is an important remedial agent for flatulence, atomic dyspepsia and diarrhea.[5] The seed of ajwain is bitter, pungent and it acts as anthelmintic, carminative, laxative, and stomachic. It also cures abdominal tumors, abdominal pains and piles.[6] Seeds contain an essential oil containing about 50% thymol which is a strong germicide, anti-spasmodic and fungicide. Thymol is also used in toothpaste and perfumery.[7] Ajwain essential oil has thymol as main active component. Seeds of ajwain are traditionally used to treat bowel disorder like indigestion, flatulence colic and diarrhea [8]. This essential oil consists of antibacterial, nematocidal, antilithiasis, cytotoxic, anthelmintic and antifilarial, antihistamine, anti-inflammatory, antispasmodic,

antifungal and antioxidant activities [9]. These pharmacological benefits allow the use of this tradition medicine i.e. ajwain essential oil in food industries as preservative. Their major chemical ingredients viz. thymol and carvacrol are considered safe by the European commission and the US-FDA as a flavouring agent [10]. It is also fruitful in the management of typhoid fever in combination with lemon juice [11].

Ajwain essential oil is having many biologically active components importantly thymol, phenolic compounds, p-Cymene, γ -terpinene, α - and β -pinene, carvacrol, α -terpinene and limonene [12].

In essential oil of ajwain seeds, thymol found in highest extent approx 30-50%. Thymol is used in toothpaste, deodorants, mouthwashes and other pharmaceutical preparations due to its antimicrobial, antifungal, antispasmodic antioxidant and germicidal properties [13].

Essential oils are generally considered as safe for their anticipated use by the U. S. Food and Drug Administration [14,15].

Various extraction methods such as Soxhlet extraction, steam distillation and low temperature distillation are used for the extraction of the essential oil of *Trachyspermum ammi* seeds with different organic solvents or in their combinations [16].

The quality of essential oil depends upon the method of extraction. Chemical composition of essential oil and other natural products present in essential oil are thermolabile and can easily be degrade during thermal extraction [17,18].

GCMS (Gas Chromatography-Mass Spectrometry) testing is the "gold standard" for analyzing the chemical composition of essential oils. It identifies and quantifies the various compounds present in an essential oil, providing a detailed chemical profile. This analysis helps verify the oil's authenticity, purity, and quality, ensuring it meets specific standards [19,20].

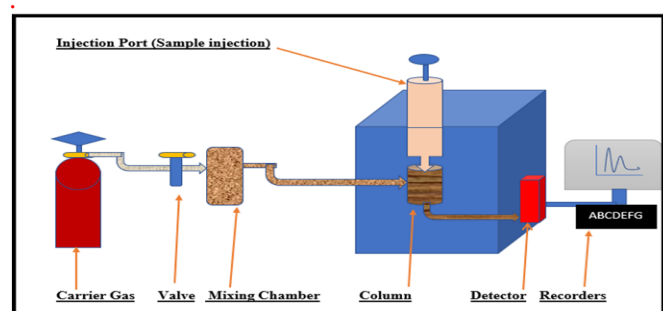


Figure 1. Scheme diagram for GC-MS

Thin-layer chromatography (TLC) can be used to analyze and identify the components of essential oils, including their antimicrobial activity and quality. TLC is a relatively quick and easy method that can be used to separate, visualize, and identify different compounds in a mixture, making it useful for analyzing essential oil

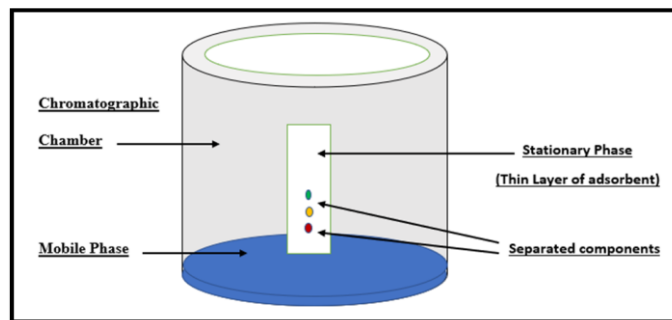


Figure 2. Scheme diagram for GC-MS

2. Related Work

Ajwain (*Trachyspermum ammi*) seeds were more effective as antimicrobial agent against gram positive strain as compared to gram negative strain.[21,22] As per the literature Antimicrobial Activity of essential oil extracted at 70°C was observed with the formation of zone of inhibition with diameter including disk, 18.3 ± 0.3 mm, and 14.36 ± 0.3 mm against *S. aureus* and *E. coli* respectively while essential oil extracted at 80°C, zone of inhibition was formed with diameter 18.68 ± 0.3 mm, and 15.30 ± 0.3 mm against *S. aureus* and *E. coli* respectively. The zone of inhibition with diameter 18.69 ± 0.3 mm, and 15.38 ± 0.3 mm against *S. aureus* and *E. coli* respectively were observed in the case of essential oil extracted at 90°C.[27] The diameter of zone of inhibition recorded including the size of filter paper discs. This showed that the essential oil had antimicrobial properties against various pathogenic and food spoilage microbes. Furthure more To scavage the effect of H_2O_2 , extracted essential oil at 70°C, 80°C and 90°C were used, which revealed that essential oil extracted at 90°C exhibited maximum antioxidant property due to the presence of highest amount of thymol.[24,25,26,27] This fact indicates the antioxidant activity of essential oil of *Trachyspermum ammi* seeds is due to thymol. As the chemical composition of essential oil and other natural products present in essential oil are thermolabile and can easily be degrade during thermal extraction [28]. It is required to extract them at low temperature with

The aim of study is to develop a simple, reliable and cheap method to extract 100% pure thymol from the seeds of *Trachyspermum ammi* i.e. ajwain while the extraction done at near the boiling point of methanol i.e. 64.7°C.

3. Experimental Method

3.1 Sample preparation and chemicals

Trachyspermum ammi i.e. Ajwain seeds were purchased from local market of Indore region. The essential oil from ajwain seeds was extracted by Soxhlet apparatus by using different aliquotes of water and methanol. Borosilicate soxhlet apparatus was used to obtain extract and rotator apparatus was used to separate essential oil from solvent. All chemicals used in qualitative and quantitative estimation were of AR grade, provided by Merck Specialties private Ltd. India. Methanol as solvent for sample preparation and Helium gas as mobile phase were used for GC-MS i.e. gas chromatographic

investigation. Borosilicate TLC chamber was used for analysis, ethanol for sample preparation, glass plate, Silica-gel, ethanol and water (9:1) to prepare silica-gel slurry to make thin layer over the glass plate, preheated (at 110°C) oven for plate activation, Benzene, Chloroform and UV-chamber.

3.2 Storage of essential oil

Essential oils are thermolabile and degraded or isomerized in presence of sunlight. It is prerequisite that it should be stored in suitable containers in appropriate condition to prevent its thermal or photochemical degradation before analysis. Small brown-colored bottles were used for the preservation of essential oil. Storage temperature for the storage of essential oil was 4°C.

3.3 Experimental work

Soxhlet extraction technique was used for the analysis by using two different solvents in different ratio such as 100%, 80%, 60%, 40%, 20%, methanol and water, further examined by GC-MS in order to achieve its chemical composition. Gas Chromatography is used to separate and identify the components of mixture from volatile liquids. Essential oils are volatile in nature hence these can be easily separated and identified by the gas chromatography [6]. Components got distributed according to their partition functioning between stationary phase (column) and gas mobile phase. Separation of components depends upon their structure, properties, size and affinity to bind with adsorbent surface in column so that they reach with different retention time to the detector ultimately produces a chromatogram [7]. It is a plot between concentration and volume of developer vs response time. This response time technically referred as retention time for the components of mixture. It is based on distribution of components between station phase and mobile phase [10].

$$R_f = \frac{M_t}{M_t + S_t} \quad \dots\dots\dots(3.1)$$

Where,

M_t = Time taken for mobile phase to pass through the column

S_t = Time taken by analyte retained in the stationary phase.

GC-MS analysis was done on "GC-MS QP 2010 ultra" mass spectrometer with gas chromatography at SIC, IIT Indore (MP). This GC-MS uses electron ionization (EI) and chemical ionization (CI) since it has capacity to perform positive and negative ionization for molecular weight information of more complex samples. Inert carrier gas used as mobile phase was Helium. This GC-MS instrument is very sensitive and able to detect up-to ppm level masses. Essential oil was dissolved in various organic solvents and water to check its solubility. It was found that all sample of ajwain essential oil extracted with different solvents are soluble in organic solvents like methanol, ethanol, chloroform, benzene, petroleum ether etc. but insoluble in water. Methanol was used to prepare sample for GC analysis.

Thin layer Chromatography utilizes a thin layer (approx. 0.25 mm) of adsorbent material used as a stationary phase. Silica gel or alumina or magnesium oxide can be used as adsorbent material due to its high surface area and porous nature. Components of mixture move upwards in influence of mobile

phase. The nature and binding sites of all molecules are different since different tendency to bind at adsorbent surface.

4. Results and Discussion

The colour of extracted essential oil of *Trachyspermum ammi* seeds is yellowish brown and having pungent smell. Essential oil of *Trachyspermum ammi* seeds is volatile in nature and their components were separated in gas chromatographic chamber, the presence of thymol is confirmed by TLC. Samples of essential oil for TLC analysis are prepared by dissolving the essential oil in ethanol while benzene and chloroform were used (taken in the ratio 3:1) as mobile phase and spots were identified in UV light. R_f Values found to be 0.66 This value is very close to standard R_f value of thymol i.e. 0.64, These results confirmed the presence of thymol as major constituent of essential oil of *Trachyspermum ammi*. Seeds.

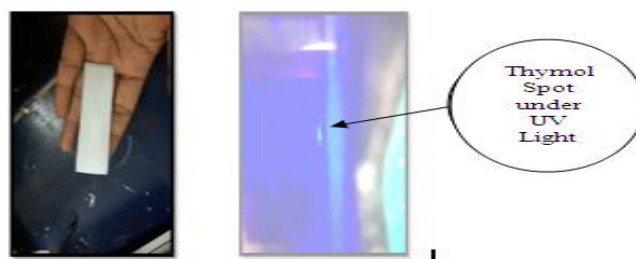


Figure:3 a. TLC Plate without uvlight b. TLC Plate under uv-light

Gas chromatogram of essential oil sample extracted via solvent of an aliquot 8:2 (Methanol: Water) revealed the presence of only thymol in essential oil sample with area under curve 100%. Results are indicated in Table-2. Only one peak is obtained in fig. 4 with retention time 11.817. The data compared with the literature in which Total 10 peaks were obtained with different areas under curve in which Thymol, was obtained with highest concentration with area under curve 67.77% retention time 11.817 from the start [27], which showed the presence of thymol with highest quantity as compared to other chemical components in essential oil.

Fig. 3 is indicating the TLC plate of essential oil sample thymol only can be observed in UV chamber and having the retention factor 0.64.

Table 1: Chemical composition (%) of essential oil of *Trachyspermum ammi* seeds with different extraction methods.

S. No.	Component	Method of Extraction & % Component			
		Hydro-distillation	Soxhlet app. With methanol	Soxhlet app. With methanol and water 8:2	Soxhlet app. With methanol and water 6:4
1	Bicyclo[3,1,1]heptane,6,6-dimethyl-2-methylene	1.25	0.73	-	-

2	O-Cymene	18.19	0.48	-	-
3	γ -Terpinene	1.85	2.72	-	-
4	Terpinen-4-ol	2.81	0.48	-	-
5	Benzene-methanol, α, α , 4-trimethyl	0.39	35.13	-	-
6	α -Terpineol	1.29	2.01	-	-
7	Thymoquinone	0.42	4.78	-	-
8	Thymol	67.66	48.06	100	-
9	Carvacrol	5.85	5.61	-	-
10	6-Octen-1-ol, 3,7-dimethyl-3-ethenyl-1-(4-methyl)	0.29	-	-	-

Table 1 indicating the variation in chemical composition (%) while they were extracted with different techniques. Temperature and partition coefficient of terpenoids may affect the concentration of terpenoids in essential oil sample.

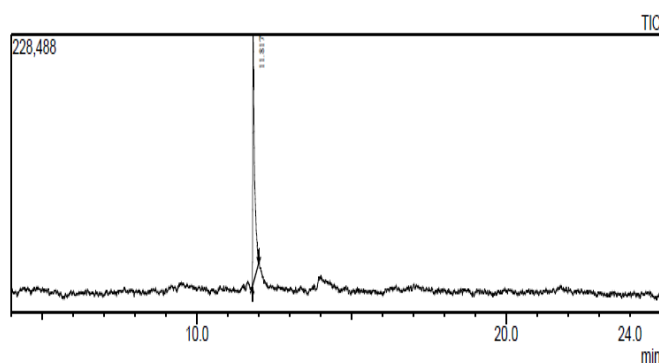


Figure 4. Chromatogram of essential oil extracted via Soxhlet apparatus by methanol and water 8:2 ratio

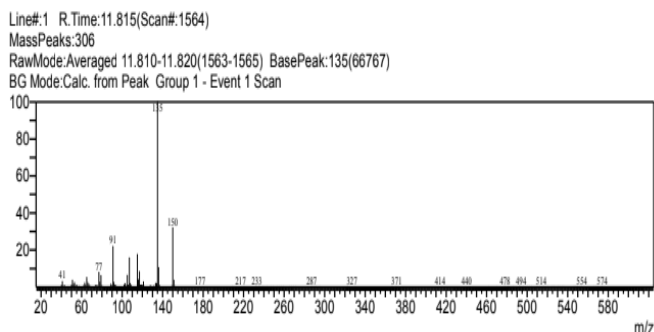


Figure 5. Mass spectra of Thymol

Table 2: Chemical composition (%) of essential oil of *Trachyspermum ammi* seeds extracted via Soxhlet apparatus by methanol and water 8:2 ratio

S.No.	R. Time	Area	Area%	Component
1	11.815	852443	100.00	Thymol
	11.815	852443	100.00	

Fig. 4 showing the chromatogram obtained from GC-MS analysis of essential oil sample extracted via soxhlet extraction method by taking methanol and water in the ration 8:2. This ratio is favourable to obtained thymol over various other isomeric form with retention time 11.815 min from the start. Thymol concentration and structure is confirmed by mass spectra of thymol which is having different fragmentations of the molecule with mass to charge ratio 135, 150, 91, 77 and 44. While Fig. 6 showing the chromatogram

obtained from GC-MS analysis of essential oil sample extracted via soxhlet extraction method by taking methanol and water in the ration 6:4. This ratio of solvents (methanol and water) completely diminishes the volatile components of essential oil samples hence cannot be used for isolation of essential oil of *trachyspermum ammi* seeds. To achieve the maximum pharmacological benefit as an antimicrobial and antioxidant agent solvent in soxhlet method dilution ratio 8:2 (methanol: water) is acceptable. This fact was supported by fig.4 which is indicating the 100% thymol extraction by taking methanol and water in 8:2 ratio. It has been proven that on increasing thymol concentration preservative properties of essential oil also increases.

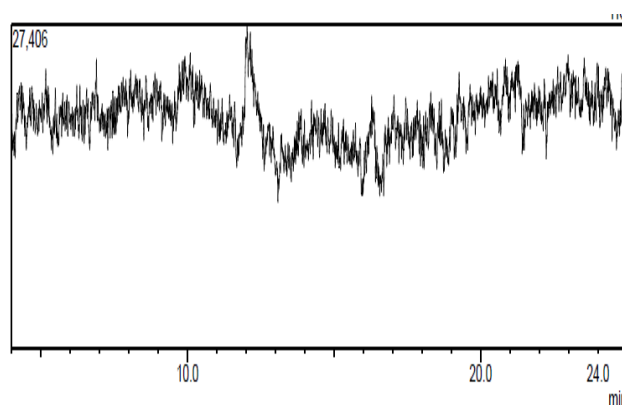


Figure 6. Chromatogram of essential oil extracted via Soxhlet apparatus by methanol and water 6:4 ratio

Table 3: Chemical composition (%) of essential oil of *Trachyspermum ammi* seeds extracted via Soxhlet apparatus by methanol and water 6:4 ratio

S.No.	R.Time	Hidgt%	Area%	Name of Component
01	0	0	0	-

Table 3 is showing that the ratio 6:4 is not as good to extract essential oil of *trachyspermum ammi* seed since volatile components. Further dilution of solvent lead to deterioration of the extraction of the terpenoids in essential oil. Since terpenoids are key antioxidants and having preservative character, the oil obtained at this dilution can not be used as an antimicrobial agent or an antioxidant agent in food and pharmaceutical industries.

5. Conclusion and Future Scope

It has been concluded that pure thymol can be obtained from its natural sources by using aliquot method. The above method is applicable in all contexts to extract pure thymol content with the use of soxhlet apparatus over various isomeric forms. Despite of the use of toxic solvents, an aliquot of water and methanol with the ratio 2:8 is suitable for the soxhlet extraction technique. Solvents from the essential oil can be separated by rotator apparatus and yield for the pharmacologically active compounds can be improved. Thymol can replace various toxic preservatives from food and pharmaceutical industries.

This technique can be utilized in the field of pharmaceutical industry to extract out 100% pure thymol from its natural sources. Chemically synthesized preservatives like sodium benzoate, potassium benzoate, benzoic acid, methyl paraben, sorbic acid, sulfites, sulfur dioxide, nitrites, butylated hydroxyl anisole (BHA), Butylated hydroxytoluene (BHT) and Tert-butyl hydro-quinone are commonly used as preservative in variety of food materials, Selection of preservative depends upon storage condition and thermal stability as well as their stability against microbial contamination of food and pharmaceutical materials. All chemical preservatives are having adverse and toxic effect and may damage human health either by creating acute or chronic defects in biological systems. But natural preservative like thymol from its natural source is quite safer as compare to other chemical preservatives. The method is applicable to extract pure components from their natural sources.

Data Availability- Not Applicable

Conflict of Interest-The author declares that she has no competing interests.

Funding Source- Not applicable

Authors' Contributions-Neha Joshi wrote the primary manuscript and crosschecked the results. She was associated with the planning phase of the study, experimental part and verified the results, also cross-checked the references after that approved the final version of the manuscript.

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