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Changes in essential oil composition of *Thymus vulgaris* under different storage conditions and its antimicrobial activity

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Abstract. *Thyme (Thymus vulgaris* L.*) has been used for centuries in traditional medicine due to its various health benefits, and it is widely used today in aromatherapy, cosmetics, and even as a culinary herb. This study aimed to investigate how the chemical compositions and antimicrobial activity of essential oils extracted from the aerial parts of T. vulgaris were affected by storage at different temperatures. The essential oils were obtained by hydrodistillation of air-dried samples and analyzed using gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). The study observed changes in the essential oil's composition when stored in a refrigerator (4 °C) and at room temperature (25 °C) for three months. The results revealed that the proportions of compounds with lower boiling temperatures such as β-myrcene (2.29–0.20%) and α-pinene (2.74–0.24%) along with γ-terpinene (7.84–4.81%) and p-cymene (10.93–5.61%) as thymol and carvacrol precursors, were significantly decreased when stored at room temperature. However, the amounts of thymol and carvacrol increased by 51.64 and 21.81%, respectively, after three months storage period, indicating a rise in the oil quality index. Storing the essential oil in a refrigerator resulted in minimal changes to the essential oil composition and maintained its primary quality. In addition, the antimicrobial activity of the essential oils was tested using the broth microdilution method and demonstrated that the essential oils from both storage methods retained their antimicrobial activity compared to freshly extracted ones. In summary, these findings are beneficial for essential oil producers and consumers in the pharmaceutical and cosmetic industries.*

Keywords: *Thymus vulgaris, essential oil, storage conditions, antimicrobial activity, thymol, carvacrol*

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ФИЗИКО-ХИМИЧЕСКАЯ БИОЛОГИЯ

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Изменение состава эфирного масла *Thymus vulgaris* при различных условиях хранения и его антимикробная активность

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Аннотация. *Благодаря своим целебным свойствам чабрец (Thymus vulgaris* L.*) веками использовался в традиционной медицине, а сегодня он широко применяется в ароматерапии, косметологии и даже в качестве кулинарной добавки. Данное исследование посвящено изучению влияния хранения при различных температурах эфирных масел, извлеченных из надземных частей T. vulgaris, на химический состав и антимикробную активность. Эфирные масла были получены путем гидродистилляции высушенных на воздухе образцов и проанализированы с использованием газовой хроматографии (ГХ) и газовой хроматографии/масс-спектрометрии (ГХ/МС). В исследовании наблюдались изменения в составе эфирного масла при хранении в холодильнике (4 °C) и при комнатной температуре (25 °C) в течение трех месяцев. Результаты показали, что доли соединений с более низкими температурами кипения, такие как β-мирцен (2,29–0,20%) и α-пинен (2,74–0,24%), наряду с γ-терпиненом (7,84–4,81%) и п-цимолом (10,93–5,61%) в качестве предшественников тимола и карвакрола значительно снижались при хранении при комнатной температуре. Однако количество тимола и карвакрола увеличилось на 51,64 и 21,81% соответственно после трех месяцев хранения, что указывает на повышение индекса качества масла. Хранение эфирного масла в холодильнике привело к минимальным*

изменениям состава эфирного масла и сохранению его исходного качества. Кроме того, противомикробная активность эфирных масел была проверена с использованием метода микроразведения бульона, а также было продемонстрировано, что эфирные масла обоих способов хранения сохраняли свою противомикробную активность по сравнению со свежеэкстрагированными. Таким образом, полученные результаты полезны для производителей и потребителей эфирных масел в фармацевтической и косметической промышленности.

Ключевые слова: *Thymus vulgaris, эфирное масло, условия хранения, антимикробная активность, тимол, карвакрол*

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INTRODUCTION

Essential oils are concentrated hydrophobic liquid extracts that are derived from various parts of plants, including flowers, leaves, stems, roots, and fruits [1, 2]. They are highly concentrated and possess a distinct aroma that is characteristic of the plant species from which they are derived. In recent years, scientific research has confirmed the potential therapeutic benefits of essential oils and their bioactive components. For example, many essential oils have been shown to possess antimicrobial activity against a range of pathogens [1, 3]. They have also been shown to possess antioxidant properties, which can help protect against oxidative stress and associated diseases such as cancer, cardiovascular disease, and neurodegenerative disorders [2, 4, 5]. The biological activity of essential oils is attributed to the presence of various bioactive compounds such as terpenes, phenolics, and flavonoids, which are known to have pharmacological properties [1, 2]. These compounds interact with specific receptors in the body, triggering a cascade of biochemical reactions that result in the observed biological effects [1, 2].

Thymus vulgaris L., commonly known as Thyme, is an aromatic herb belonging to the Lamiaceae family [6]. It is native to the Mediterranean region and is now widely cultivated throughout the world. *T. vulgaris* is also known for its medicinal properties and has been used for centuries to treat various ailments [7–9]. The essential oil extracted from *T. vulgaris* has been extensively studied for its antimicrobial, antioxidant, anti-inflammatory, and antitumor properties [9, 10]. Thymol and carvacrol are the major active components of *T. vulgaris* essential oil, responsible for its biological activities [10]. *T. vulgaris* essential oil has been reported to exhibit broad-spectrum activity against pathogenic microorganisms, making it a potential candidate for use in pharmaceutical and food industries [10].

However, the quality and efficacy of essential oils can be influenced by various factors, such as storage conditions [11–13]. Essential oils are highly volatile and can be easily degraded by heat, light, and oxygen exposure [11]. Changes in the chemical composition of essential oils can lead to alterations in their therapeutic properties, which can affect their overall efficacy. It is essential to understand how different storage conditions can affect the chemical composition and biological activities of essential oil. Therefore, the aim of the present study was to investigate the influence of storage conditions on the chemical compositions and antimicrobial activity of *T. vulgaris* essential oils.

MATERIALS AND METHODS

Plant material and isolation procedure. The aerial parts of *Thymus vulgaris* were obtained from Da Lat, Vietnam in July 2022. The plants were air-dried for two weeks at room temperature (25 °C). After that, the essential oils were extracted from the dried samples using hydrodistillation for 4 h using a Clevenger-type apparatus, following the method suggested by the Vietnamese Pharmacopoeia [14], as previously stated [15, 16]. The distilled oils were dried using anhydrous sodium sulfate and transferred to sealed dark vials for further analysis.

Essential oils storage conditions. To study how the compositions of distilled oils were affected by various storage conditions, the oil samples were stored at different temperatures: in a refrigerator (4 °C) and at room temperature (25 °C). The analysis of all the stored oils was carried out after three months. Additionally, to accurately determine how the storage conditions affected the compositions of essential oils during the entire experiment period, the freshly extracted oil was analyzed right after extraction.

Essential oil analysis. The essential oils were examined using gas chromatography (GC) and gas chromatography-mass spectrophotometry (GC-MS), following the same methods as previously described [17, 18]. The GC analysis was performed using an Agilent Technologies 7890A GC, which was equipped with a flame ionization detector (FID) and an HP-5MS chromatographic column (i.d. 0.25 mm × 30 m, 0.25 μm film thickness). The GC-MS analysis was conducted using an Agilent GC 7890A chromatograph, with the same column used in the GC analysis, and coupled with an HP 5973 MSD mass spectrometer. The essential oil components were identified by their GC retention time in comparison to known compounds, and by comparing their mass spectra with those in the computer data bank [19] and published spectra [20]. To determine the percentage composition, peak area normalization was used without employing any correction factors.

Antimicrobial assay. To evaluate the antimicrobial activity of essential oils, five different strains of microorganisms were used: two strains of Gram-positive bacteria (*Bacillus cereus* ATCC 14579 and *Staphylococcus aureus* ATCC 25923), two strains of Gramnegative bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853), and one strain of yeast (*Candida albicans* ATCC 10231). The

minimum inhibitory concentration (MIC) of the essential oils was determined using the broth microdilution susceptibility method, as previously described [17, 18]. The bacteria were cultured in Mueller-Hinton broth (MHB) and *C. albicans* was cultured in Sabouraud broth (SB). The essential oils were dissolved in 1% dimethylsulfoxide (DMSO) and diluted to the highest concentration. Serial doubling dilutions were made in a 96-well microtiter plate. Overnight broth cultures of each strain were prepared and the final concentration in each well was adjusted to 5×10^5 CFU/mL for bacteria and 1×10^3 CFU/mL for *C. albicans*. The bacteria and *C. albicans* were then incubated for 24 h at 37 and 30 °C, respectively. Positive controls of Streptomycin for bacteria and Nystatin for *C. albicans*, as well as a negative control of the vehicle (DMSO), were prepared under the same experimental conditions. The MIC values were determined as the lowest concentration of the essential oil at which no visible growth of the microorganism was observed [21].

RESULTS AND DISCUSSION

Changes in the composition of essential oils. Currently, there is a lack of research on the storage of plant secondary metabolites, particularly essential oils, due to their volatile nature and susceptibility to potential alterations under various storage conditions [12]. In this study, the compositions of essential oils of *T. vulgaris* were determined at two different storage temperatures: refrigerator (4 °C) and room temperature (25 °C). In total, 27 compounds were identified, representing 99.43–99.65% of the total essential oils (Tab. 1).

Table 1. Composition of *Thymus vulgaris* essential oils stored at the refrigerator and room temperature compared with freshly extracted

Таблица 1. Состав эфирных масел *Thymus vulgaris*, хранящихся в холодильнике и при комнатной температуре, по сравнению со свежеэкстрагированными маслами

Compound*	RI**	Relative peak area (%)		
		After distillation	Refrigerator	Room Temperature
α-Thujene	931	0.33	0.24	–***
α-Pinene	938	2.74	2.06	0.24
Camphene	954	0.17	0.14	–
1-Octen-3-ol	978	0.12	–	–
β-Pinene	980	0.23	0.27	0.13
β-Myrcene	991	2.29	0.94	0.20
α-Phellandrene	1005	0.67	0.95	0.10
α-Terpinene	1018	0.14	0.13	–
p-Cymene	1028	10.93	9.84	5.61
Limonene	1031	0.19	0.22	0.17
Eucalyptol	1036	1.17	1.14	1.19
γ-Terpinene	1062	7.84	7.69	4.81
Terpinolene	1088	0.27	0.26	0.13
Linalool	1098	4.82	4.97	6.84
Camphor	1143	0.97	1.12	1.04
Borneol	1165	0.24	0.28	0.26
α-Terpineol	1189	0.21	0.26	0.27
Thymol methyl ether	1235	1.78	1.82	1.98
Carvacrol methyl ether	1244	0.95	0.97	1.34
Geraniol	1255	0.14	0.14	0.15
Thymol	1290	45.78	47.12	51.64
Carvacrol	1298	16.05	17.24	21.81
β-Caryophyllene	1419	0.85	0.88	0.79
Germacrene D	1480	0.15	0.15	0.14
γ-Cadinene	1512	0.11	0.10	–
δ-Cadinene	1524	0.17	0.16	0.14
Caryophyllene oxide	1581	0.34	0.36	0.45
Monoterpene hydrocarbons		25.50	22.74	11.39
Oxygenated monoterpenes		72.11	75.06	86.52
Sesquiterpene hydrocarbons		1.28	1.29	1.07
Oxygenated sesquiterpenes		0.34	0.36	0.45
Others		0.12	–	–
Total identified		99.65	99.45	99.43

Note. * – elution order on HP-5MS column; ** – retention indices on HP-5MS column; *** – not identified.

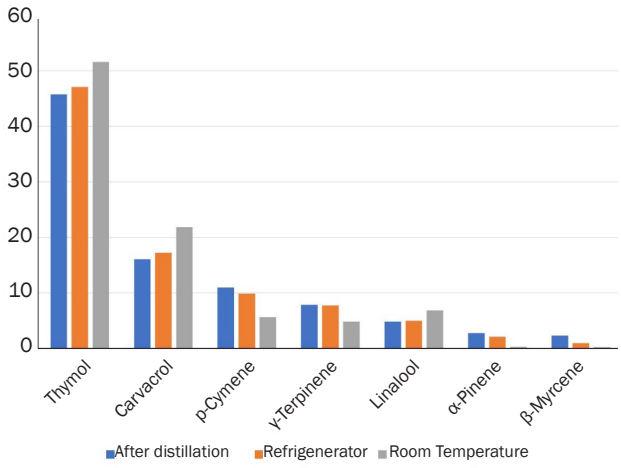
Essential oils of *T. vulgaris* were characterized by a very high percentage of oxygenated monoterpenes (72.11–86.52%). The main components of the essential oils were similar across storage methods, including thymol, carvacrol, *p*-cymene, *γ*-terpinene, and linalool. It can be seen that the main components of essential oil samples in this study are similar to those of previous studies [22–26].

Although all essential oils extracted from *T. vulgaris* contain the same main components, a comparison indicated that the amounts of main compounds were drastically changed during storage at room temperature compared to those of corresponding conditions (Figure and Tab. 1). Our research discovered that the concentration of essential oil constituents with lower molecular weights decreased, particularly when stored at room temperature. This decrease could be attributed to various factors such as evaporation, oxidation, and other undesirable alterations that occurred during the storage period [12, 17]. Notably, after three months of storage, the levels of lower boiling compounds significantly decreased. The decrease was observed in both refrigerated and room temperature conditions, but it was more pronounced in the latter. For example, the changes in the amounts of some components are as follows: *α*-pinene initially accounted for 2.74% of the oil content but decreased to 2.06% when stored in the refrigerator and 0.24% when stored at room temperature. The second component which showed the same trend was *β*-myrcene which is a monoterpene. This component was 2.29% at the beginning of the experiment and decreased to 0.94 and 0.20% when stored at the refrigerator and room temperature, respectively. The *p*-cymene was the third component that showed a decrease after three months of storage. The quantity of this component was 10.93% at the time of oil extraction and then its amounts were 9.84 and 5.61% when stored in the refrigerator and at room temperature, respectively. Another important constituent that showed an interesting alteration trend was *γ*-terpinene. As can be seen in Tab. 1, after three months stored at room temperature, the quantity of *γ*-terpinene drastically decreased by 4.81%. The amount of this compound at the time of oil extraction was 7.84%.

The most important results of the present study were the increasing trend in the quantities of thymol and carvacrol after three months of storage, particularly at room temperature. The thymol was 45.78% at the time of oil distillation, then increased to 47.12% when stored in the refrigerator and 51.64% when stored at room temperature. Carvacrol also represented the same trend as thymol. This compound showed an increase to 17.24 and 21.81% when stored at the refrigerator and room temperature, respectively. The quantity of carvacrol was 16.05% at the time of oil extraction. The findings of this research indicated that the ratios of carvacrol and thymol, which are the primary compounds, had a different change pattern compared to their precursors (*p*-cymene and *γ*-terpinene) after three months of storage. At this period, the amounts of carvacrol and thymol increased in all conditions, especially at room temperature, while the quantities of their precursors declined. This contrast in the trends can be observed in Figure. To explain this problem, previous research has shown that *γ*-terpinene can be converted into *p*-cymene through aromatization, and *p*-cymene can be transformed into carvacrol or thymol through hydroxylation, which may occur during storage [28, 29]. Despite being aromatic, thymol has been identified as a terpenoid biosynthetic product [30]. In the late 1970s, experiments were conducted in which radioactively labeled monoterpenes, including *γ*-terpinene and *p*-cymene, were fed to thymol [31]. Based on the results of this study, it was suggested that the biosynthesis of thymol and its chemical isomer, carvacrol, begins with *γ*-terpinene as the initial monoterpene substrate and proceeds via the intermediate aromatic *p*-cymene.

In addition, after storage of the essential oil, an increase in the concentration of oil components such as *α*-phellandrene, limonene, linalool, camphor, borneol, thymol methyl ester, *β*-caryophyllene, and carvacrol methyl ester was observed. This could be explained by the fact that essential oils, being stored in sealed vials, may retain some of their volatile components as well as undergo chemical reactions over time, leading to an increase in concentration. Storing the oils in a dark and cool place, such as sealed dark vials in a refrigerator, may slow down the degradation process and preserve the essential oil components for a longer period of time [13]. Further studies may be necessary to determine the exact mechanisms underlying the observed changes in essential oil composition after storage.

Antimicrobial activity of essential oils. Tab. 2 displays the minimum inhibitory concentrations (MICs) of the essential oils from *T. vulgaris*, which were evaluated using the microdilution broth susceptibility test for their antimicrobial effects against four bacterial strains and one yeast. The study's findings showed that the antimicrobial activity of essential oils from *T. vulgaris* remained similar when stored at room temperature or in the refrigerator for three months, compared to freshly extracted oils, against *B. cereus* (MIC = 25 μg/mL), *E. coli* (MIC = 50 μg/mL),



Changes in the main components of *Thymus vulgaris* essential oils stored at the refrigerator and room temperature compared with freshly extracted

Изменения основных компонентов эфирных масел *Thymus vulgaris* при хранении в холодильнике и при комнатной температуре по сравнению со свежеэкстрагированными маслами

Table 2. Antimicrobial activity of *Thymus vulgaris* essential oils stored at the refrigerator and room temperature compared with freshly extracted

Таблица 2. Антимикробная активность эфирных масел *Thymus vulgaris*, хранящихся в холодильнике и при комнатной температуре, по сравнению со свежеэкстрагированными маслами

Microorganisms	Minimum inhibitory concentration – MIC (μg/mL)		
	After distillation	Refrigerator	Room Temperature
Bacillus cereus ATCC 14579	25	25	25
Staphylococcus aureus ATCC 25923	50	25	50
Escherichia coli ATCC 25922	50	50	50
Pseudomonas aeruginosa ATCC 27853	100	50	50
Candida albicans ATCC 10231	50	50	50

and *C. albicans* (MIC = 50 μg/mL). However, the essential oil stored in the refrigerator had greater activity against *S. aureus* with a MIC of 25 μg/mL, whereas the MIC of the freshly extracted oil and oil stored at room temperature was 50 μg/mL. Furthermore, the newly extracted essential oil showed activity against *P. aeruginosa* with a MIC of 100 μg/mL, whereas the MIC for the oil preserved in the two methods was 50 μg/mL. This difference may be due to the content and quantity of compounds present in the analyzed essential oil samples. In general, essential oils stored in the refrigerator and at room temperature have retained their antimicrobial properties compared to freshly extracted. These results are consistent with previous studies that have demonstrated the selective growth-inhibitory effects of *T. vulgaris* essential oils on various microorganisms [32–34].

Overall, the antimicrobial properties of essential oils from *T. vulgaris* are primarily linked to their composition, especially oxygenated monoterpenes that are present in large quantities. The differences in antimicrobial activity among the essential oils may be due to their major constituents, such as thymol, carvacrol, *p*-cymene, γ-terpinene, and linalool [22, 23]. However, due to the complex nature of essential oils, it is challenging to attribute their overall antimicrobial activity to one or a few components. In this study, it was discovered that Gram-positive bacteria were more susceptible to the essential oils than Gram-negative bacteria, which are frequently reported to be resistant to essential oils and their components due to the presence of cell wall lipopolysaccharides that can act as a barrier [35–37]. To fully comprehend the relationship between chemical constituents and antimicrobial properties, additional research is necessary to accurately account for their effects.

CONCLUSIONS

The primary process involved in storing essential oils is the evaporation of compounds with lower boiling temperatures, particularly mono hydrocarbons. The results of this study suggest that storing the essential oil of *T. vulgaris* in a refrigerator for three months preserves its original quality better than storing it at room temperature. Generally, storing *T. vulgaris* essential oil at low temperatures limits the concentration of oil components from increasing or decreasing, thereby preserving the oil's primary quality with minimal changes. However, the results of this study indicate that storing the oil at room temperature not only does not harm its quality but also increases important index components such as thymol and carvacrol. Additionally, tests showed that the antimicrobial properties of the oils stored at both room temperature and in the refrigerator were not affected. These findings may be applicable to storing essential oils with similar chemical properties, and they could benefit essential oil producers and consumers in the pharmaceutical and cosmetic industries.

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