Journal of Medicinal Plants and By-Products (2024) 4: 1149– 1158
https://doi.org/10.22034/jmpb.2024.366267.1715

Original Article

Optimizing Thymoquinone Content of Iranian *Nigella sativa* Essential Oil and Challenges in Commercializing it as an Active Pharmaceutical Ingredient

Elham Erami [1]*, Mohammad Fazilati [1], Hossein Salavati [2] and Mahmood Sabahi[3]

[1]*Department of Biochemistry, Payame Noor University, Tehran, Iran,*
[2]*Department of Chemistry Payame Noor University, Tehran, Iran*
[3]*Chemical and Bimolecular Engineering Georgia institute of Technology: Atlanta, GA, US*

Article History

Received: 01 July 2024
Accepted: 06 September 2024
© 2012 Iranian Society of Medicinal Plants.
All rights reserved.

Keywords
RP-HPLC
API
Solvent extraction
Cold press

***Corresponding author**
elham.erami@gmail.com
elhamerami@isfahan.pnu.ac.ir
ORCID: 0009-0008-5125-2167

ABSTRACT

The objective of this study was to produce the essential oil of *Nigella sativa* L. (*N. sativa*) with the highest thymoquinone (TQ) content and explore the challenges associated with its commercialization as an active pharmaceutical ingredient. Commercially sourced seeds of *N. sativa* were obtained from reputable vendors. Ground seeds underwent extraction using a variety of polar and non-polar solvents. The resulting essential oil samples were subjected to analysis via reverse-phase high-performance Liquid Chromatography (RP-HPLC) to quantify TQ content, employing a method of quantification following the validation of the method. Major components of hexane extract were analyzed by GC-MS. Non-polar solvents at ambient temperature produced the essential oil with the highest TQ content. Extraction at higher temperatures has an adverse effect on TQ and is not recommended. The Cold pressed oil showed very low TQ content. However, treatment of this oil with hexane for an extended period of times released TQ from the matrix of the oil. Moreover, we refined the extraction methodology to produce essential oil with the highest TQ content. n-heptane which has a better toxicity profile than n-hexane is the best solvent for producing the essential oil with the highest TQ content. Optimization of powder/solvent ratio, temperature, and time of extraction would provide the most economically viable option. Cold press produces an oil with a different composition in which TQ is bonded to the matrix of the oil. Comparing the therapeutic effectiveness of the two oils is recommended for future studies. The challenges associated with the commercialization of *N. sativa* oil as a pharmaceutical product are also discussed.

INTRODUCTION

N. sativa, also known as black seed, belongs to the *Ranunculaceae* family and holds a prominent position in traditional medicine across numerous countries. It has been used for centuries and much is written about its therapeutic properties. The isolation of the essential oil of *N. sativa* in late 19th century marked a pivotal moment, initiating extensive research into its composition and therapeutic properties [1].

N. sativa originates from regions such as the Mediterranean, the Indian Subcontinent, and West Asia, with significant cultivation observed across various parts of Iran. Recent research conducted by Khalesro *et al*. [2] investigated the influence of farming conditions on essential oil production from *N. sativa* in the western province of Kurdistan [2]. However, while factors impacting oil yield were addressed, data regarding the TQ content within the essential oil were lacking. Additionally, several other research groups have explored the effects of factors such as fertilizers [3], planting seasons [4], irrigation methods [5], and genetic enhancement [6] on plant growth and essential oil yield in various regions of Iran. Nevertheless, none of these studies specifically addressed the TQ content of the essential oil. Commercial production of *N. sativa* is also reported by Ministry of Agriculture Jihad of Iran (Personal communication with the MAJI).

The antioxidant properties of *N. sativa* oil are correlated to the presence of phenolic compounds and specifically to TQ. Historically, cold press has been the dominant method for production of *N. sativa* oil. In search for essential oil with highest content of TQ, various extraction methods and including steam distillation [7], Soxhlet extraction [8], solvent extraction [9], and supercritical fluid extraction (SFE) [10] have been employed. The phenolic profile of *N. sativa* seeds was investigated through extraction with various alcohols, followed by assessment of the antioxidant activity of the resulting extracts [11]. The study identified hydroxybenzoic acid and caffeic acid as the predominant components, with vanillic, syringic, gallic, and p-coumaric acids also present. Notably, TQ was not mentioned in this study which highlights the significance of solvent selection in extraction of the essential oil. Recent studies have also demonstrated the degradation of TQ in aqueous media and under light [12]. Iqbal *et al*. found that hexane and benzene afford essential oils with highest content of TQ [13]. Superiority of SFE and solvent extraction was demonstrated by Kokoska *et al*. [7]. In an interesting article, Mohammed *et al*. [14], compared chemical composition of the essential oil of *N. sativa* produced by SFE and cold press, noting significant differences in the oil profiles and concluding that SFE extraction affords the highest yield and better-quality product with much higher TQ content. However, they did not offer the underlying reasons for this significant difference between the two methods of extraction.

Detailed analysis of *N. sativa* essential oil extracted with SFE method by gas chromatography-mass spectrometry (GC-MS) and ^{1}H and ^{13}C NMR spectroscopy showed about 44% quinones, 37% monoterpene hydrocarbons, 7% of oxygenated monoterpenes, 5% sesquiterpenes, 3% of oxygenated sesquiterpenes, and hydrocarbons making up the rest. TQ was the major component of the oil with about 35% [15].

Phenolic antioxidants, both natural and synthetic, have been extensively utilized in consumer products and chemical, pharmaceutical, and food industries for decades. Examples are butylated hydroxytoluene (BHT) and t-butyl hydroquinone (TBHQ) as food additives and di-tert-butyl phenol and its derivatives extensively used in consumer products Fig. 1.

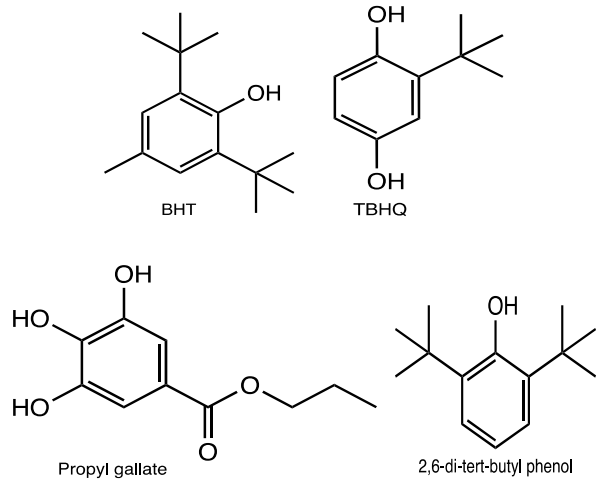

Fig. 1 Phenolic antioxidants

Phenolic compounds exhibit antioxidant properties via mechanisms such as hydrogen transfer or single electron transfer (SET). In a review article, Shahidi *et al*. [16] discussed the mechanism of action of both synthetic and naturally occurring phenolic antioxidants.

TQ is the prominent pharmaceutically active component of *N. sativa* essential oil. TQ was first isolated and identified from the essential oil of *N.sativa* in 1963 [17]. It shows anti-inflammatory, anti-oxidant, and anti-neoplastic properties both *in vitro* and *in vivo* [18]. A comprehensive review by Gali-Muhtasib *et al*. commemorating the 50th anniversary of TQ isolation delved into its chemical properties, pharmacological effects, and advancements in analog design and formulation [18], highlighting challenges such as poor bioavailability of TQ and high capacity for binding plasma proteins. To gain insights into recent advancements concerning the mechanism of action and therapeutic potential of TQ, we conducted a search in CAS-SciFinder database, yielding a wealth of 180 review articles. In 2021, Tabassum *et al*. published a comprehensive review with approximately 127 references, detailing the effects of TQ on oxidative stress, immunomodulation, and various types of cancer [19]. Following this, in 2022, Manzar Alam *et al*. provided another extensive review, supported by 280 references, summarizing the anti-cancer, antioxidant, and anti-inflammatory properties of TQ [20].

TQ, characterized as an α,β-unsaturated diketone, is the oxidized product of thymohydroquinone and does not possess inherent antioxidant properties. However, it may undergo nucleophilic 1,4-addition in the presence of compounds bearing -SH, -NH,

and -OH functional groups, generating the phenolic derivative. Khalife and Lupidi demonstrated the reduction of TQ by glutathione under nonenzymatic conditions, forming the glutathione adduct of hydrothymoquinone Fig. 2 [21].

Glutathione is a crucial antioxidant that plays a fundamental role in maintaining cellular health and protecting the body from oxidative stress. It helps neutralize free radicals, supports detoxification processes in the liver, enhances the immune system's function, and contributes to the regulation of cell growth and repair. Additionally, glutathione's addition to TQ yields a potent antioxidant in hydrothymoquinone adduct which functions as a hydrogen atom donor as well as a SET agent. This collaborative action enhances the scavenging of free radicals and mitigating oxidative damage more efficiently. Further research by Armutcu and Akyol elucidated the pivotal role of glutathione as a reducing agent in biological systems, proposing mechanisms for generation of antioxidants thymohydroquinone by SET or TQ-glutathione adduct by 1,4-addition of glutathione to TQ Fig. 3 [22].

In their review of mechanism of action of TQ, Tabassum *et al*. discuss the participation of several NADPH reductase in reducing TQ to thymohydroquinone and provide TQ based antioxidant induction pathways and potential immunomodulatory effects of TQ [19].

As indicated, a wealth of scientific research highlights the potential of essential oil extracted from *N. sativa* seeds as a potent active pharmaceutical ingredient (API). However, the development of herbal medicines comes with numerous challenges, particularly the need to ensure a consistent and safe supply of high-quality plant material. Commercial production of essential oil with high TQ content necessitates a well-defined extraction process. The available literature on producing essential oil with high TQ content from *N. sativa* seeds is inconsistent and sometimes confusing.

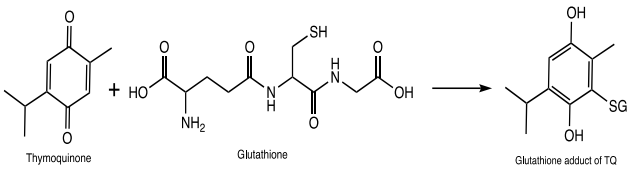

Fig. 2 Reaction of TQ with glutathione

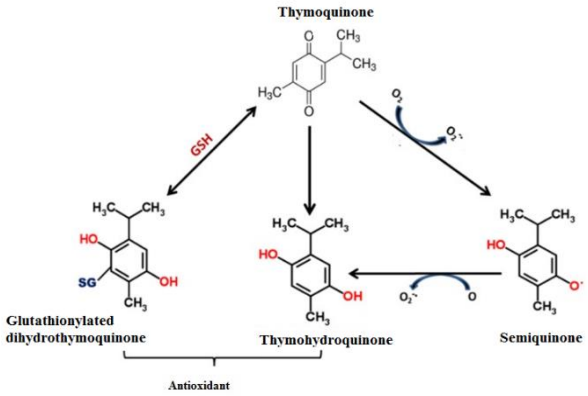

Fig. 3 Glutathione as reducing agent generating thymohydroquinone

Moreover, while a significant difference in the TQ content of essential oil produced by supercritical fluid extraction (SFE) or hydrocarbon solvents compared to cold pressing has been observed, no explanation has been provided for this discrepancy. Therefore, it is crucial to develop an economically viable extraction process that maintains optimal levels of TQ in the essential oil and to better understand the underlying reasons for the discrepancy between cold pressing and extraction methods. Our goal is to determine the optimal conditions for producing essential oil with maximum TQ content, to understand the reasons behind the discrepancies between extraction and cold press, and to address the challenges associated with its commercialization as an API.

MATERIALS AND METHODS

MATERIAL

Certified seeds of *N. sativa*, cultivated in Semirom, Iran were provided by Pakan Bazr Isfahan and seeds from Golestan province were procured from the Ministry of Agriculture. In addition, two samples of imported seeds from India and Syria were purchased from the market. TQ with certified purity of 99.0%, was purchased from Sigma-Aldrich and HPLC grade methanol, acetic acid, hexane, and heptane, were procured from Merck.

Essential oil Preparation

N. sativa powder (20 g) was suspended in the selected solvent (80 g) and stirred either at room temperature or at the reflux temperature of the selected solvent for extended time periods. Progress of TQ extraction was monitored hourly by RP-HPLC at specific time intervals as described below.

The solvent was removed under reduced pressure at 30 °C. Then a stock solution of the resulting essential oil was prepared by dissolving the essential oil (10 mg) in methanol (10 ml) and filtering it through a 0.22 µm syringe filter (MS®PTFE Syringe Filter). This solution was stored in dark at -20 °C until used for RP-HPLC analysis.

Cold pressing of the seeds was done at a local company. The selected seed (1000 g) was pressed at room temperature and the essential oil (300 g) and the cake (700 g) were collected for analysis by RP-HPLC.

GC-MS Analysis of Essential Oil

Essential oil analysis of volatile components was carried out using a Hewlett-Packard 6890/5972 system with HP-5MS capillary column (30 m x 0.25 mm; 0.5µm film thickness) and helium carrier with a flow of 1 ml/min. The split ratio was 1:10. The column temperature was programmed from 50-300 °C at 10 °C/min. Mass spectra was recorded in m/z ranges 35-350 amu at 70 eV.

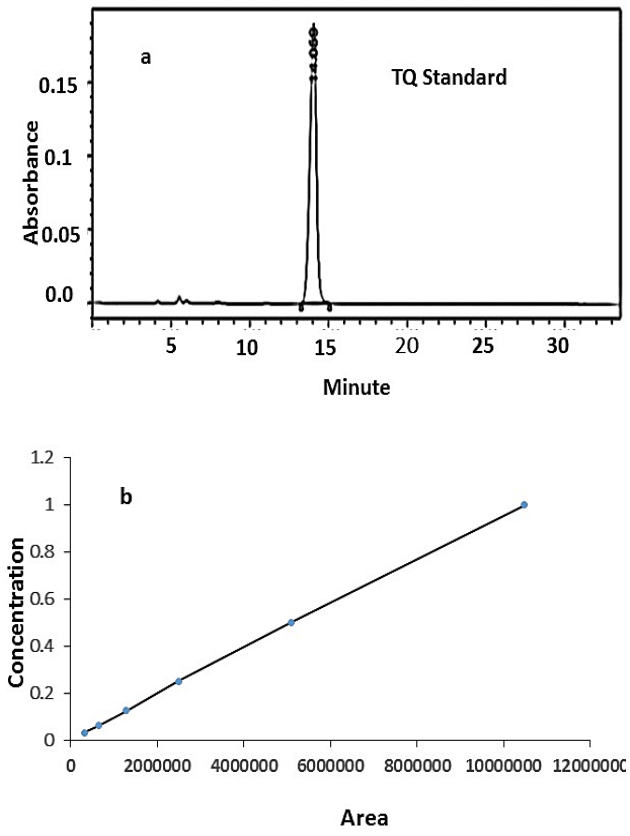

Fig. 4 (a) Chromatogram of TQ Standard and (b) calibration curve for the TQ quantification.

High Performance Liquid Chromatography

RP-HPLC analysis was conducted using a RP-C18 column, using an isocratic mobile phase of water: methanol (50:50) and 3% v/v acetic acid at 30 °C and flow rate of 0.8 ml/min [23] and UV detection at 278 nm. For quantification of TQ a linear calibration curve was generated as follows. Standard TQ solutions were prepared in methanol. The concentration range of the calibrators were 0.0625、0.125、0.25、0.5、1.00 mg/ml and a calibration graph (R^2 = 0.9998, LOD= 0.0142 mg/ml, LOQ = 0.135 mg/ml) was generated as shown in Fig. 4a and b.

Statistical Analysis

The statistical analysis was performed using SPSS 26.0 (New York, USA). Data resulting from three independent experiments was reported as mean ± SD.

RESULTS

Extractions of powdered *N. sativa* were carried out in n-hexane, n-heptane, methanol, and water under two different conditions: at room temperature and under reflux. To monitor the progress and efficacy of these extraction processes, samples were withdrawn hourly, concentrated under reduced pressure, prepared for analysis as described earlier, and analyzed by RP-HPLC. This analysis allowed us to accurately measure the yield of essential oil and the concentration of TQ at each point in time. Thymoquinone was identified by comparing the retention times of the peak of pure standard and solvent-extracted essential and cold pressed oil Typical chromatograms for solvent extracted essential and cold pressed oil are shown in Fig. 5.

The GC-MS analysis of the n-hexane extract showed a total of 15 compounds including TQ as a major constituent. thirteen significant compounds were identified by the Wiley and Adams mass spectral Libraries as observed in Table 1 and Fig. 6. The yield of essential oil increased for extractions with non-polar solvents at reflux temperature over an extended period of time, reaching a maximum of approximately 35% after 10 hours, Fig. 7a. However, the TQ content in the essential oil decreased significantly during this period, dropping from about 10% to its lowest point of 5% after 10 hours, Fig. 7b. Methanol extraction showed an increase in essential oil yield for several hours, reaching a maximum of about 10% after 10 hours. However, TQ content decreased rapidly and approached zero after 10 hours, Fig. 7b.

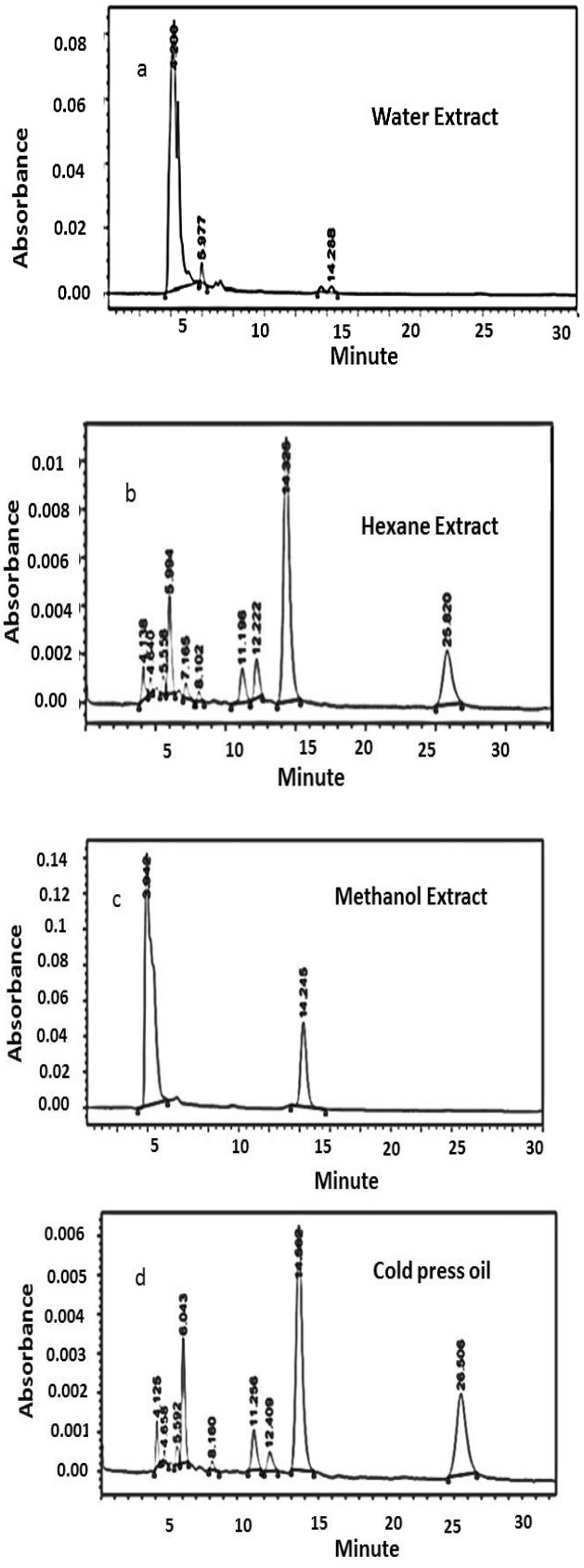

Fig. 5 Examples of HPLC chromatograms for essential oil prepared by extraction and cold press. a. water extract, b. Hexane extract, c. Methanol extract, d. cold- press oil

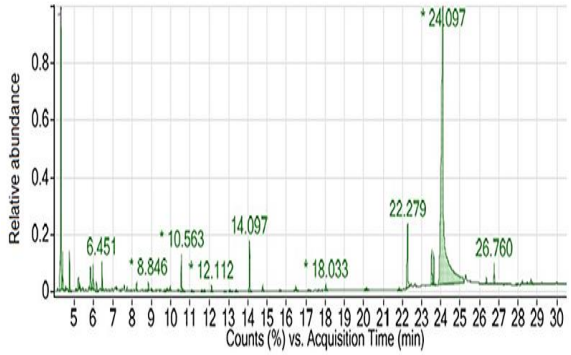

Fig. 6 GC-MS chromatography analysis of *N. sativa* oil extracted by hexane solvent.

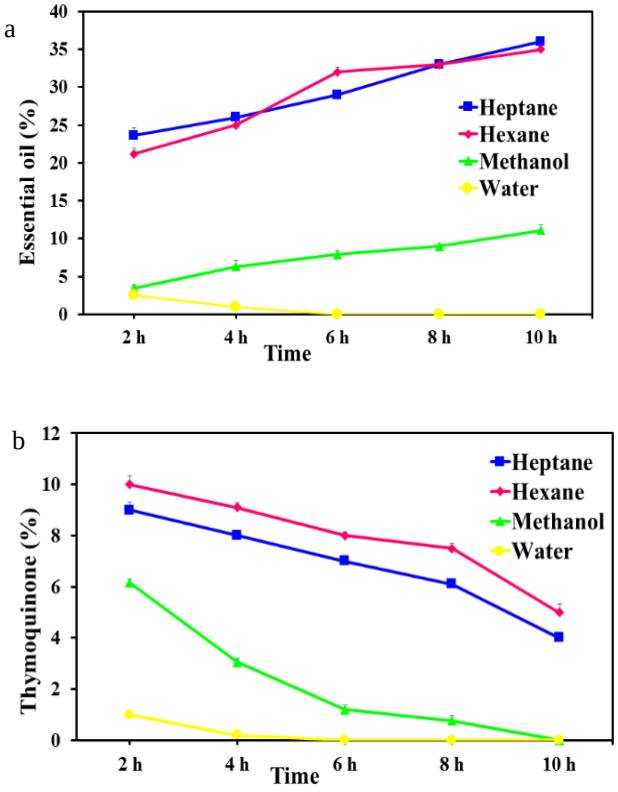

Fig. 7 Essential oils produced at reflux temperature (a) and TQ content of essential oils (b).

Extractions using the same solvents at room temperature for up to 24 hours exhibited a similar trend in essential oil production but with a significant difference in TQ content. The TQ content of the n-hexane extract increased to 18.5% after 24 hours, and similarly, the TQ content in n-heptane reached 16%, Fig. 8d. Methanol extraction at room temperature over 24 hours resulted in a higher essential oil yield of 20%, but the TQ content dropped rapidly and was not detectable after 24 hours, Fig. 8d. As expected, our results demonstrate that water is not a suitable solvent for essential oil extraction, as shown in Fig. 8d.

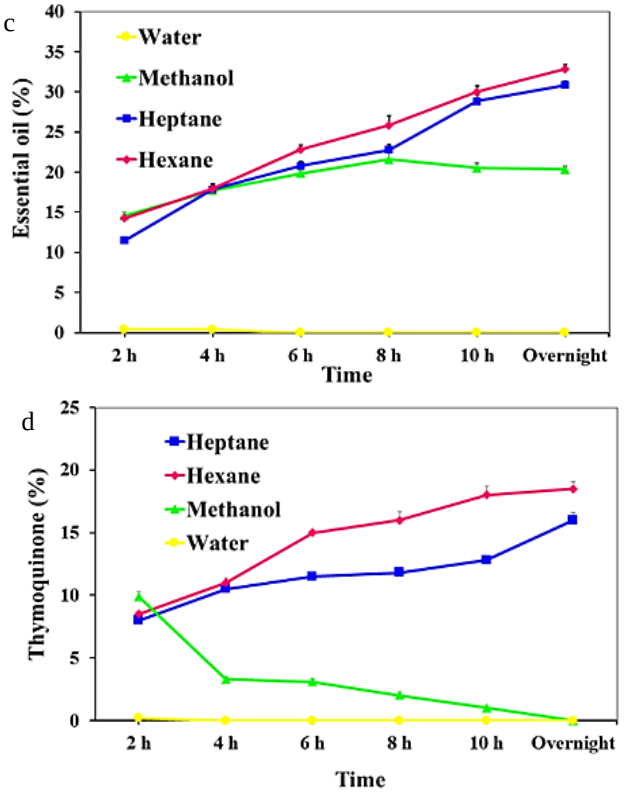

Fig. 8 Essential oil by extraction at room temperature (c) and TQ content of the essential oil (d).

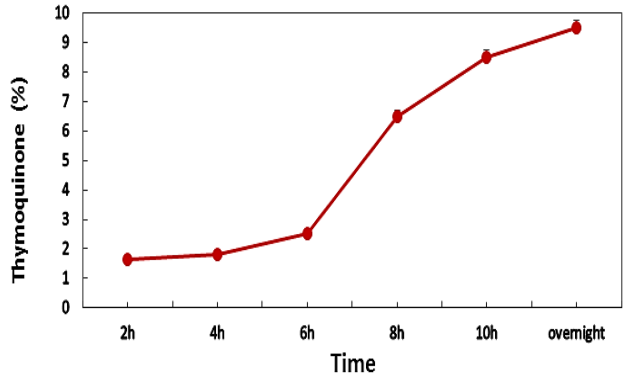

Fig. 9 TQ content of the oil released from the cake over time.

DISCUSSION

We also compared the efficiency of solvent extraction with cold pressing. Cold pressing 1000 g of seeds produced about 300 g of essential oil with a TQ content of 1.5%, which aligns well with literature reports. To understand the significant difference in TQ content between this oil and the essential oil obtained from solvent extraction with non-polar solvents, we subjected the cake and the oil from cold pressing to solvent extraction with n-hexane. Specifically, we stirred a mixture of the cake in hexane (20% w/w) for 24 hours at room temperature. The cake yielded an additional 9% essential oil with a TQ content of 2%. We also stirred a solution of the cold-pressed oil in hexane (20% w/w) at room temperature for 24 hours, which increased the overall total TQ content of the essential oil to 11% after 24 hours, Fig. 9.

TQ is a non-polar α, β-unsaturated diketone that plays a crucial role in various biochemical pathways due to its high reactivity. One of the significant reactions TQ undergoes is nucleophilic 1,4-addition, where nucleophiles add to the β-position of the conjugated system. This reaction pathway leads to the formation of thymohydroquinone, a derivative renowned for its potent antioxidant properties, as illustrated in Fig. 2. TQ's structure also includes a highly conjugated system, making it an excellent chromophore. Consequently, TQ absorbs light efficiently, which also makes it particularly sensitive to light exposure, leading to potential photodegradation or dimerization when subjected to heat or prolonged light exposure.

Table 1 Composition of the essential oil of *N. sativa* extracted by n-hexane obtained by GC-MS.

Compound	RT	Area %
α-Thujene	8.82	3.55
α-pinene	9.07	0.3
D-Limonene	10.63	0.23
O-Cymene	10.49	12.09
Tetradecane	11.04	1.3
p-cymene	11.55	8.38
Terpinen-4-ol	13.07	2.54
Thymoquinone	14	17
Carvacrol	14.76	7.38
Longifolene	16.39	1.66
α- Terpiny acetate	14.49	1.2
Ascorbic acid 2,6 dihexadecanoate	22.11	15.3
9,12 Octadecadienoic acid (z,z)	23.82	25.4

RT: retention time

Given the instability of TQ under certain conditions, our research was focused on identifying the most efficient extraction technique that would yield essential oils with the highest possible concentration of TQ. The primary objectives were twofold: firstly, to optimize the extraction parameters to enhance TQ content of the essential oil, and secondly, to compare the efficacy of different extraction methods particularly cold pressing, which is the preferred method in commercial applications, versus various solvent extraction techniques.

To achieve this, we meticulously examined the impact of several critical factors, including solvent polarity, extraction temperature, and the duration of

the extraction process, on both the yield of the essential oil and the concentration of TQ in the extracted essential oil. Specifically, we carried out extractions under two different conditions: at room temperature and under reflux. To monitor the progress and efficacy of these extraction processes, samples were withdrawn hourly, concentrated under reduced pressure, prepared for analysis, and analyzed by RP-HPLC which allowed us to accurately measure the concentration of TQ in the extracts at each time point.

Our findings revealed that non-polar solvents such as n-hexane and n-heptane are particularly effective for producing essential oil with high TQ content. These solvents, being neutral hydrocarbons, are capable of efficiently penetrating the hydrophobic organic matrix of the seeds, facilitating the release of TQ into the solvent phase without undergoing significant chemical alteration. The non-polar nature of these solvents ensures that TQ, which is non-polar itself, remains stable and intact throughout the extraction process if protected from light and excessive heat.

On the other hand, we found that polar solvents such as alcohols and water are less suitable for TQ extraction. TQ's electrophilic β-carbon in the α, β-unsaturated system is highly susceptible to nucleophilic attack by alcohols and water, which can result in unwanted side reactions, including the 1,4-addition of these nucleophiles. Such reactions lead to the formation of undesired byproducts and reduce the yield of TQ. Additionally, since TQ is thermolabile, extraction processes must be conducted at optimum conditions to avoid thermal degradation. Exposure to elevated temperatures for a long period of time can cause TQ to break down or dimerize, leading to a significant reduction in TQ content of the essential oil. So, although extended extraction times at reflux temperature increased essential oil yield but led to rapid decrease in TQ content as shown in Fig. 7a and 7b.

To further assess the thermal sensitivity of TQ, we conducted extractions with the same solvents at room temperature for up to 24 hours. Essential oil production increased to a maximum of 29.5 for non-polar solvents and 20% for methanol. The TQ content in the essential oil from non-polar solvents reached a peak of 18.5% for n-hexane and 16% for n-heptane as shown in Fig. 8c and 8d. As expected, the TQ content in the essential oil from the methanol extract rapidly decreased to zero after 24 hours. Water was confirmed to be an unsuitable solvent for essential oil extraction.

Cold pressing of *N. sativa* seeds resulted in a substantial yield of essential oil, accounting for 30 wt.% of the seed mass. However, TQ content in the oil was unexpectedly low, measured at just 1.5%. Given these results, we hypothesized that TQ might be chemically bonded to the seed matrix or associated with other components within the essential oil, limiting its extraction efficiency during cold pressing. To test this hypothesis, both the residual seed cake and the cold-pressed oil were subjected to solvent extraction using n-hexane. This approach successfully corroborated our initial suspicion. By stirring this essential oil with n-hexane at room temperature for 24 hrs. its TQ content increased to 11%. And the cake produced an additional 9% essential oil with a TQ content of 2%.

To further investigate the disparity in TQ content between the oils obtained through solvent extraction and cold pressing, we procured *N. sativa* seeds from four different sources. Essential oils were then prepared using both methods: solvent extraction with n-hexane and cold pressing at a local processing facility. The TQ content and oil yield from these extractions are summarized in table 2.

As shown in table 2, the essential oil obtained through solvent extraction consistently exhibited a higher TQ content across all seed sources compared to the oil obtained by cold pressing. These findings underscore the effectiveness of solvent extraction in maximizing TQ yield in *N. sativa* essential oil, making it a preferable method for applications where essential oil with high TQ content is desired.

Table 2 Difference in TQ content of *N. sativa* seeds assessed by Cold pressing and solvent extraction.

Region	Hexane extract	TQ	Cold press	TQ
Semirom Isfahan	25%	9.2 ± 0.18%	30%	1.5 ± 0.01%
Gorgon Golestan	24%	9.0 ± 0.18%	28%	1.3 ± 0.01%
Syria	22%	8.0 ± 0.1%	23%	0.8 ± 0.02%
India	20%	9.0 ± 0.16%	26%	1 ± 0.02%

Commercialization Challenges

Commercial production of therapeutic agents must be tightly regulated and controlled throughout the supply chain to the market. This includes the production and collection of raw materials, manufacturing the Active Pharmaceutical Ingredient (API) under Good Manufacturing Practices (GMP), and formulating and packaging the API for the market. GMP encompass a set of regulatory requirements aimed at ensuring the quality, safety, and consistency of pharmaceuticals, food, cosmetics, and medical devices. These principles include establishing a robust quality management system to govern organizational processes and resources, ensuring that personnel possess the necessary qualifications and training, maintaining suitable facilities and equipment, meticulously documenting all procedures and activities, implementing controls to monitor production processes, assessing and mitigating risks, validating critical processes and equipment, managing suppliers and materials, and adhering to guidelines to maintain product quality throughout the supply chain.

The commercialization of *N. sativa* essential oil with high TQ content as therapeutic agent begins with securing a reliable supply of the seeds. The quality and genetic variability of seeds can significantly affect the yield and TQ content of the oil. Cultivating *N. sativa* in optimal conditions, including suitable climate, soil quality, with sustainable agricultural practices, is essential for producing seeds with the highest TQ potential. The most recent organic farming regulations of Europe which came into effect on January 1, 2022, (EU) 2018/848 provides a comprehensive framework for ensuring the integrity and sustainability of organic agricultural practices. Certified organic and non-GMO seeds should be produced by organic soil management, elimination of all synthetic pesticides and herbicides, and collection and storage of seeds without any treatment with synthetic chemicals.

Our findings demonstrate that solvent extraction is the more efficient method for producing *N. sativa* essential oil with a higher TQ content. Manufacturing process development and optimization, plus economic evaluation, is the next step. We used both n-hexane and n-heptane in our extractions. The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines classified solvents according to their potential health risks [24, 25]. n-Hexane is classified as a class 2 solvent due to its neurotoxicity, while n-heptane is categorized as a class 3 solvent with lower toxicity. These classifications inform pharmaceutical manufacturers on controlling residual solvent levels in APIs to ensure compliance with regulatory standards and mitigate health risks to consumers. n-Heptane is the preferred solvent for scale-up and manufacturing. Clear specifications and impurity profiles should be prepared for the solvent and any other chemical agents involved in the process [26]. It is also imperative to establish the specifications for the final product, including composition, TQ content, solvent residue, and overall chemical profile.

In the scale-up and optimization stage, several key parameters must be investigated. For an economically viable process, the solvent-to-powder ratio, temperature and cycle time, isolation and purification, and overall process cycle time must be optimized. Recycling of the solvent and careful quality control of the reagents and product isolation are also necessary.

Life cycle assessment (LCA) is the methodology for assessing the environmental impact of products and manufacturing processes. Jimenez-Gonzalez *et al*. in pioneering papers describe Life Cycle assessment of pharmaceutical products [27-30]. Sabahi and Reichmanis demonstrated how to use Life Cycle Inventory (LCI) assessment as a sustainable chemistry and chemical engineering tool [31]. Cradle-to-gate life cycle assessment of essential oil production is the practice for understanding the energy and material intensity, plus the health and environmental impact of *N. sativa* farming, seed collection and storage, production of the essential oil, and formulation and packaging for market introduction. LCA requires the compilation and analysis of the inventory of all material inputs and environmental releases from the beginning (Cradle) to final product release to the market (Gate). ISO 14040/14044 is the core foundation for LCA methodology in the US and EU [32]. LCA helps to identify opportunities for improving the economics and health and environmental impact of the API production.

Although there is a wealth of literature and history about the therapeutic properties of the essential oil of *N. sativa*, introducing it as an API requires regulatory approval. ICH provides harmonized guidelines which are followed globally. They include

GMP requirements for manufacturing, guidelines for stability testing and shelf-life determination, impurity profile, and quality and risk management.

CONCLUSION

Essential oil of *N. sativa* and its TQ content have been extensively studied for their therapeutic properties. Various solvents and extraction methods and cold pressing have been explored for the preparation of this oil. Building on this knowledge, our study focused on identifying the optimal solvent and extraction conditions for producing essential oil with the highest TQ content while also understanding the differences between extraction and cold pressing. We identified n-heptane as the optimal solvent for producing essential oil with the highest TQ content and highlighting the limitations of cold pressing due to significantly lower TQ content. Given the heat sensitivity of TQ, extraction at temperatures below 40 °C is recommended. Further process development is necessary to refine parameters like powder particle size, solvent to powder ratio, extraction temperature and time, mixing conditions, and best isolation and purification methods.

Commercializing the essential oil with high TQ content as an Active Pharmaceutical Ingredient (API) requires navigating stringent regulatory approvals, including compliance with GMP standards and managing solvent residues. Additionally, a thorough life cycle analysis (LCA) is essential to assess the environmental impact and ensure sustainability. Addressing these challenges strategically can position the oil favorably in the health and wellness market, particularly among consumers and industries seeking enhanced therapeutic properties.

Acknowledgment

The authors appreciate the facilities and services provided by the Pasteur Institute of Iran. The authors express their gratitude to Dr. Fatemeh Taghizadeh for her support and assistance throughout this research.

Conflicts of Interest

The authors have declared that there is no conflict of interest.

REFERENCES

1. Ahmad M.F., Ahmad F.A., Ashraf S.A., Saad H.H., Wahab S., Khan M.I., Ali M., Mohan S., Hakeem K.R., Athar M.T., An updated knowledge of Black seed (*Nigella sativa* Linn.): Review of phytochemical constituents and pharmacological properties. J Herb Med. 2021; 25:100404.
2. Rahmani R., Khalesro S., Heidari G., Mokhatssi-Bidgoli A., Vermicompost and zeolite improve yield, nutrient uptake, essential and fixed oil production, and composition of *Nigella sativa* L. Front Sustain Food Syst. 2023; 7:1214691.
3. Rezaei-Chiyaneh E., Seyyedi S.M., Ebrahimian E., Moghaddam S.S., Damalas C.A., Exogenous application of gamma-aminobutyric acid (GABA) alleviates the effect of water deficit stress in black cumin (*Nigella sativa* L.). Ind Crops Prod. 2018;112: 741-748.
4. Safaei Z., Azizi M., Davarynejad G., Aroiee H., The Effect of Planting Seasons on Quantitative and Qualitative Characteristics of Black cumin (*Nigella sativa* L.). J Med Plants By-Prod, 2017. 6(1): p. 27-33.
5. Haj Seyed Hadi M.R., Darzi M.T., Riazi G., Black cumin (*Nigella sativa* L.) yield affected by irrigation and plant growth promoting bacteria. J Med Plants By-Prod. 2016; 5(2): 125-133.
6. Salehi S.,Kiran R., Integrated experimental and analytical wellbore strengthening solutions by mud plastering effects. J Energy Resour Technol. 2016; 138(3): 032904.
7. Kokoska L., Havlik J., Valterova I., Sovova H., Sajfrtova M., Jankovska I., Comparison of chemical composition and antibacterial activity of *Nigella sativa* seed essential oils obtained by different extraction methods. J Food Prot. 2008; 71(12): 2475-2480.
8. Burits M., Bucar F., Antioxidant activity of *Nigella sativa* essential oil. Phytother Res. 2000; 14(5): 323-328.
9. Ghahramanloo K.H., Kamalidehghan B., Akbari Javar H., Teguh Widodo R., Majidzadeh K., Noordin M.I., Comparative analysis of essential oil composition of Iranian and Indian *Nigella sativa* L. extracted using supercritical fluid extraction and solvent extraction. Drug Des Devel Ther. 2017; 2221-2226.
10. Salea R., Widjojokusumo E., Hartanti A.W., Veriansyah B., Tjandrawinata R.R., Supercritical fluid carbon dioxide extraction of *Nigella sativa* (black cumin) seeds using taguchi method and full factorial design. Optimization. 2013; 13(14): 16-17.
11. Benazzouz-Smail L., Achat S., Brahmi F., Bachir-Bey M., Arab R., Lorenzo J.M., Benbouriche A., Boudiab K., Hauchard D., Boulekbache L., Biological properties, phenolic profile, and botanical aspect of *Nigella sativa* L. and *Nigella damascena* L. seeds: A comparative study. Molecules. 2023; 28(2): 571.
12. Salmani J.M.M., Asghar S., Lv H., Zhou J., Aqueous solubility and degradation kinetics of the phytochemical anticancer thymoquinone; probing the effects of solvents, pH and light. Molecules. 2014; 19(5): 5925-5939.
13. Iqbal M.S., Ahmad A., Pandey B., Solvent based optimization for extraction and stability of thymoquinone from *Nigella sativa* Linn. and its quantification using RP-HPLC. Physiol Mol Biol Plants. 2018; 24: 1209-1219.

14. Mohammed N.K., Abd Manap M.Y., Tan C.P., Muhialdin B.J., Alhelli A.M., Hussin A.S.M., The effects of different extraction methods on antioxidant properties, chemical composition, and thermal behavior of black seed (*Nigella sativa* L.) oil. Evid Based Complement Alternat Med. 2016;2016.
15. Tiruppur Venkatachallam S.K., Pattekhan H., Divakar S., Kadimi U.S., Chemical composition of *Nigella sativa* L. seed extracts obtained by supercritical carbon dioxide. J Food Sci Technol. 2010; 47: 598-605.
16. Shahidi F., Ambigaipalan P., Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. J Funct Foods. 2015; 18: 820-897.
17. Gad A., El–Dakhakhny M., Hassan M., Studies on the chemical constitution of Egyptian *Nigella sativa* L. oil. Planta Medica. 1963; 11(02): 134-138.
18. Gali-Muhtasib H., Roessner A., Schneider-Stock R., Thymoquinone: a promising anti-cancer drug from natural sources. Int J Biochem Cell Biol. 2006; 38(8): 1249-1253.
19. Tabassum S., Rosli N., Ichwan S.J.A., Mishra P., Thymoquinone and its pharmacological perspective: A review. Pharmacol Res - Mod Chin Med. 2021; 1: 100020.
20. Alam M., Hasan G.M., Ansari M.M., Sharma R., Yadav D.K., Hassan M.I., Therapeutic implications and clinical manifestations of thymoquinone. Phytochemistry. 2022; 200: 113213.
21. Khalife K., Lupidi G., Nonenzymatic reduction of thymoquinone in physiological conditions. Free Radic Res. 2007; 41(2): 153-161.
22. Armutcu F., Akyol S., Akyol O., The interaction of glutathione and thymoquinone and their antioxidant properties. Electron J Gen Med. 2018; 15(4).
23. Erdoğan Ü., Yılmazer M., Erbaş S., Hydrodistillation of *Nigella sativa* seed and analysis of Thymoquinone with HPLC and GC-MS. Bilgesci. 2020; 4(1):27-30.
24. International Council for Harmonization of Technical Requirements for Pahramaceuticals for Human Use (ICH). Available from: https://www.ich.org/.
25. Available from: https://www.ema.europa.eu/en/partners-networks/international-activities/multilateral-coalitions-initiatives/international-council-harmonisation-technical-requirements-registration-pharmaceuticals-human-use-ich.
26. Q3C (R8) Impurities: Guidance for Residual Solvents Guidance for Industry. 2021.
27. Jiménez-González C., Constable D.J., Ponder C.S., Evaluating the "Greenness" of chemical processes and products in the pharmaceutical industry—a green metrics primer. Chem Soc Rev. 2012; 41(4): 1485-1498.
28. Jiménez-González C., Curzons A.D., Constable D.J., Cunningham V.L., Cradle-to-gate life cycle inventory and assessment of pharmaceutical compounds. Int J Life Cycle Assess. 2004;9: 114-121.
29. Jiménez-González C., Kim S., Overcash M.R., Methodology for developing gate-to-gate life cycle inventory information. Int J Life Cycle Assess. 2000; 5: 153-159.
30. Jiménez-González C.n., Ollech C., Pyrz W., Hughes D., Broxterman Q.B., Bhathela N., Expanding the boundaries: developing a streamlined tool for eco-footprinting of pharmaceuticals. Org Process Res Dev. 2013; 17(2): 239-246.
31. Reichmanis E., Sabahi M., Life cycle inventory assessment as a sustainable chemistry and engineering education tool. ACS Publications. 2017.
32. ISO 14044:2006(en) Environmental Management-Life Cycle Assessment-Requirements and guidelines. https://www.iso.org/Standard/38498.html.