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Enhanced Thymoquinone Recovery from Cold-Pressed Black Seeds (*Nigella sativa* L.) through Freeze-Thaw and Microwave Pretreatment Techniques

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ABSTRACT

The quality of black seed oil depends greatly on the extraction method used. Traditional methods like cold pressing are valued for producing high-quality oil that retains its natural nutrients and flavor. However, these methods often come with a trade-off, as they tend to yield less oil and lower levels of bioactive compounds, making them less efficient for large-scale production. To overcome these limitations, seed pretreatment techniques were investigated. In this study, black seeds were subjected to freeze-thaw and microwave pre-treatment before cold pressing to increase the content of thymoquinone, which is a key bioactive compound in black seeds. For the freeze-thaw treatment, black seeds were frozen at -17 °C for 24 and 48 h, followed by thawing at 50 °C for 1 h. This process was repeated for 1, 2, and 3 cycles. Microwave treatment involved subjecting seed samples to microwave at a frequency of 2450 MHz and power levels of 400 W and 640 W for durations of 1, 2, and 3 min. Subsequent oil extraction was performed by using cold pressing. HPLC analysis showed a significant increase in thymoquinone content with freeze-thawed seeds (for 48 h and 3 cycles) showing a remarkable increase like 79.93% according to untreated black seeds. Microwave-pretreated seeds at 640 W for 3 min exhibited more than double thymoquinone content compared to untreated seeds. Other quality parameters, including moisture, specific gravity, acidity, peroxide value, and iodine value, shows comparable characteristics, while significant enhancing the sensory analysis of the pretreated oil ($p < 0.05$). These findings suggest that freeze-thaw and microwave pretreatments can serve as innovative methods for enhancing thymoquinone levels in Indian black seed oil, providing a promising avenue for improving the overall quality of this valuable natural product.

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Introduction

Black seeds (*Nigella sativa* L.) are annual herbaceous plants from the Ranunculaceae family, growing 20–60 cm tall and maturing within a year. The plant produces capsule fruits containing numerous black aromatic seeds (Sarwar et al., 2024). These seeds, also known as black cumin or black caraway, have long been recognized as "seeds of blessing." Black seeds and cold-pressed black seed oil have been part of traditional diets in Asia, Africa, America, and Europe due to their beneficial fatty acid composition and bioactive compounds (Szydłowska-Czerniak et al., 2022).

Recently, black seeds and their oil have gained popularity as novel foods in Eastern and Western Europe, as recognized by the European Commission's Novel Food Catalogue (Tauferova et al., 2021). The nutritional composition of black seeds varies with geographical and environmental factors, including location, season, soil conditions, climate, and cultural practices. Known for their potent anti-inflammatory properties, black seeds have traditionally been used to treat fever, headaches, anxiety, diarrhea, asthma, and strokes. Black seeds contain 32–40% oil and 1.4% essential oil, making them a valuable source for oil extraction. Black seed oil is particularly valued for its bioactive phytochemicals, which have strong antioxidant properties and numerous health benefits. Among these, thymoquinone stands out as the primary active compound with antioxidant, anti-diabetic, anti-inflammatory, anti-cancer, antiviral, and antimicrobial effects (Abbas et al., 2024).

Over the past few decades, various extraction methods have been developed to obtain black seed oil, ranging from conventional techniques to advanced green technologies. These methods include cold pressing, supercritical fluid extraction, Soxhlet extraction, hydrodistillation, microwave-assisted extraction, ultrasound-assisted extraction, steam distillation, pulsed electric field extraction, and accelerated solvent extraction (Moghimi et al., 2018). Among these, cold pressing is widely preferred in the industry for producing high-quality oil without the

use of heat or chemicals. However, it is associated with low oil yields and residual nutritional content in the seed cake.

To address this limitation, researchers have explored pretreatment methods to improve oil yields and enhance the quality of the extracted oil. One promising technique is freeze-thaw pretreatment, which can significantly increase the release of bound bioactive compounds. Freezing and thawing alter the structural integrity of plant tissues by forming large ice crystals and changing the ionic strength and pH through freeze concentration. These changes destroy cell membranes and break down high molecular weight molecules, increasing surface hydrophobicity (Zhao et al., 2015). The repeated freeze-thaw cycles enhance cell permeability, softening the tissues and facilitating the release of oil from lipid vacuoles (Chao et al., 2022). This method is effective, inexpensive, and environmentally friendly, making it a suitable pretreatment option for black seed oil extraction.

Microwave pretreatment is another innovative approach for enhancing oil extraction. Microwaves, operating within the 300 MHz–300 GHz frequency range, generate heat by converting electromagnetic energy through ionic conduction and dipole rotation (Bakhshabadi et al., 2017; Gaber et al., 2018). This rapid heating increases intracellular pressure, ruptures cell walls, and facilitates the release of oil. Compared to traditional extraction methods, microwave pretreatment offers numerous advantages, including reduced processing time, lower energy consumption, higher oil yield, and improved oil quality (Kaseke et al., 2021; Walayat et al., 2024).

Despite the advantages of cold pressing, it yields only 30–32% oil, with the residual seed cake retaining 10–12% oil content. Moreover, the concentration of thymoquinone in cold-pressed oil ranges from 0.80 to 1.00 mg/ml when extracted from Indian black seeds (Mohammed et al., 2016). To maximize the extraction yield and thymoquinone content, pretreatments such as freeze-thaw and microwave can be applied before cold pressing. These methods can enhance oil recovery, improve bioactive compound release, and optimize the overall efficiency of the extraction process.

The goal of this study is to evaluate the effects of freeze-thaw and microwave pretreatments on the yield, physical properties, and chemical composition of cold-pressed black seed oil. By comparing these two pretreatment methods, the aim is to identify the optimal approach for achieving higher thymoquinone yields in black seed oil. This study will demonstrate that both microwave and freeze-thaw pretreatments are effective strategies for enhancing oil extraction, with significant implications for the food and nutraceutical industries.

Materials and Methods

Black seeds were obtained from Bio Extracts (Pvt) Ltd., Ratmalana, Sri Lanka, which were imported from India. Chemicals including chloroform, glacial acetic acid, iso-octane and cyclohexane were purchased from LOBA CHEMIE PVT. LTD, India. HPLC-grade thymoquinone was purchased from Sigma-Aldrich (Taufkirchen, Germany). Ethanol and iodine were purchased from VWR Chemicals (France) and Chem-Lab NV (Belgium).

Pretreatment of Black Seeds

For freeze-thaw pretreatment, black seed samples were kept in a freezer (MEZ64648231, LG Electronics, South Korea) at -17 ℃ for 24 and 48 h. Black seed samples were thawed in a 50 °C hot water bath (GEMMYCO YCW-010E, Gemmy Industrial Corporation, Taiwan) for 1 h. This process was repeated for one, two and three cycles at the same temperature and time.

For microwave pre-treatment, 200 g black seed samples were weighed and arranged in a Pyrex dish and per time and placed in a microwave oven (SMW823AY7, SINGER, Sri Lanka) at a frequency of 2450 MHz and 400 W and 640 W power levels at time of radiation 1, 2, and 3 min. After this the samples were cooled to room temperature.

Extraction of oil by Cold Pressing

1 kg of pretreated black seeds were introduced separately to a ten-headed cold-pressed oil expeller (Turkey). Oil from cold-pressed black seed samples without pre-treatment was used as a control. After 24 h the oil was manually filtered using filter papers.

Chemical and Physical Analysis of Black Seed Oil

The physical (oil yield, humidity and specific gravity) and chemical parameters (thymoquinone, acid value, peroxide value, and iodine value) of untreated and treated black seed oil samples were analyzed. The total oil yield is calculated. The humidity and specific gravity of black seed oil were measured respectively according to methods described in SLS 313:PART 3/SEC 5:2009 – ISO 662:1998E) and SLS 313:PART 1/SEC 2:2009 – UDC 665.2:613.268. The chemical parameters acid, peroxide and iodine value of the oil were analyzed respectively according to SLS standards for animal and vegetable fats and oils: 313:PART 2/SEC 6:2009 – ISO 660:2009, SLS 313:PART 3/SEC 7:2009 – ISO 3960:2007, and SLS 313:PART 2/SEC 2:2019 – ISO 3961:2018(E).

HPLC analysis of Thymoquinone

The HPLC method adopted by Salmani et al. (2014) was used to quantify the thymoquinone percent in black seed oil. Briefly, the oil sample (50 mg) was dissolved in 10 mL of methanol to extract thymoquinone from seed. Then, the sample was mixed by inverting the tube 10 times and centrifuged at 4000rpm at room temperature for 5 min. to separate the methanol layer. The upper methanol layer, which is well separated from the bottom oil, was collected and filtered using a 0.45-µm syringe filter. HPLC analysis was performed using Agilent 1260 Infinity II equipment (Agilent Technologies, Santa Clara, CA, USA). A mixture of acetonitrile and water in the ratio of 60:40 was used as the mobile phase at a flow rate of 1 mL/min using an Agilent 300SB-C18 (4.6 mm × 250 mm, 5 µm) analytical column. The volume of sample for injection was 15 µL and detection of the analyte was performed at an ultraviolet wavelength using a diode array detector at 254 nm having a column oven temperature of 28 ◦C and sample cooler temperature of 8 ◦C ± 0.3 ◦C. The determination of the analyte was done by comparing the retention time (3.783 min) of the pure standard with that of the real samples.

Sensory Evaluation of Black Seed Oil

The treated and untreated black seed oil samples were submitted to a sensory evaluation panel to determine the color, taste, odor and overall acceptability. Thirty untrained members in the Bio Extract (Pvt) Ltd, Ratmalana, Sri Lanka participated in the sensory evaluation by filling ballot paper on a five-point hedonic scale: 1 = strongly dislike, 5 = strongly like. All black seed oil samples were coded with three-digit numbers and served in random order.

Statistical Analysis

Statistical analyses were carried out using SAS 9.4 to determine the significance of differences. One-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used to compare and identify significant differences ($p < 0.05$) between the group means. The results were reported as the mean values of three repeated experimental trials. Statistical analysis of the sensory evaluation was carried out for all experiments using Friedman's test with the R software.

Results and Discussion

Thymoquinone Content of Black Seeds Oil

The results of this research indicated that the thymoquinone percentage of black seed oil pretreated for 24 h increased the freeze-thaw cycles by 1, 2 and 3 cycles compared to the untreated black seed oil sample (8.94%, 22.01% and 74.54%, respectively (Figure 1a). Furthermore, after 48 h of 1, 2 and 3 freeze–thaw cycles, the extracted thymoquinone content increased by 40.60%, 61.71% and 79.93%, respectively, compared with untreated black seed oil (Figure 1b). The thymoquinone percentage of freeze-thaw pretreated oil samples was significantly different from untreated oil samples ($p < 0.05$). It can be reported that freeze-thaw pretreatment can be affected on rupture of cell membranes and cell walls of plant tissues and release the bound bioactive compounds from vacuoles and travels through loosened plant tissues (Lee et al., 2020).

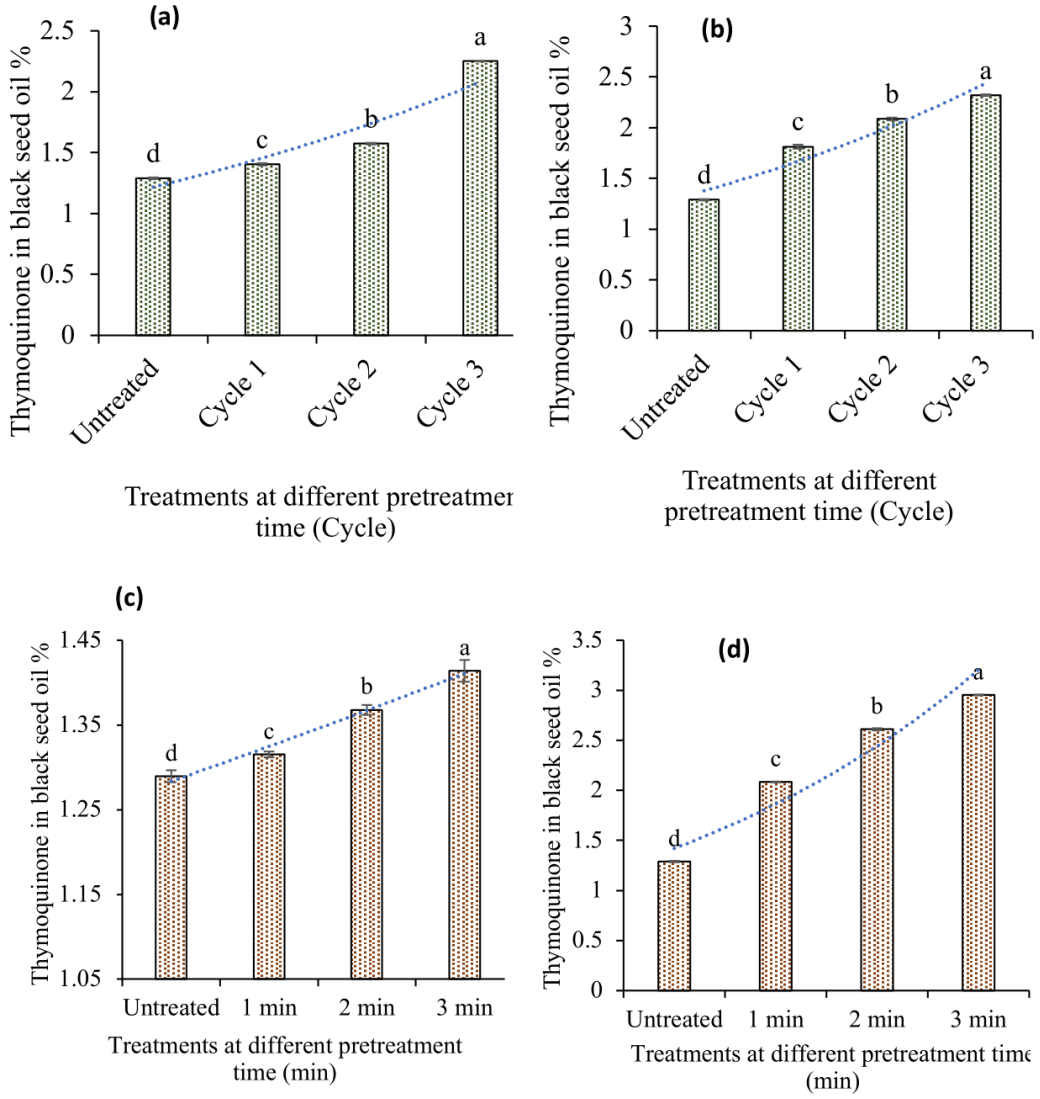

Figure 1. Thymoquinone percentage of oils extracted from (a) 24 h freeze-thaw pretreated and untreated black seeds, (b) 48 h freeze-thaw pretreated and untreated black seeds, (c) 400 W microwave pretreated and untreated black seeds, (d) 640 W microwave pretreated and untreated black seeds.

Table 1. Physical and chemical parameters of 24h and 48 h freeze-thaw pretreated and untreated black seed oil samples.

	Untreated	1 Cycle	2 Cycle	3 Cycle
Frozen time: 24 h				
Physical parameters				
Oil yield (g)	344.10± 2.30^{b}	351.35 ± 1.62^{b}	361.28 ± 1.62^{a}	366.55 ± 2.83^{a}
Moisture (%)	1.56 ± 0.01^{c}	1.67 ± 0.01^{a}	1.61 ± 0.01^{b}	1.46 ± 0.02^{d}
Specific Gravity	0.92 ± 0.00^{ab}	0.92 ± 0.00^{a}	0.92 ± 0.00^{a}	0.92 ± 0.00^{b}
Chemical parameters				
Acid value (mg KOH/g)	2.79 ± 0.04^{c}	3.27 ± 0.01^{a}	3.05 ± 0.03^{b}	2.31 ± 0.07^{d}
Peroxide value (meq/kg)	40.32 ± 0.05^{a}	34.20 ± 0.09^{d}	35.26 ± 0.02^{c}	36.18 ± 0.06^{b}
Iodine value (g/100g of fat)	116.91 ± 0.12^{a}	100.08 ± 0.04^{b}	100.42 ± 0.12^{b}	116.23 ± 0.58^{a}
Frozen time: 48 h				
Physical parameters				
Oil yield (g)	344.10 ± 2.30^{c}	356.53 ± 1.56^{b}	365.68 ± 4.24^{ab}	374.78 ± 2.55^{a}
Moisture (%)	1.56 ± 0.01^{d}	1.70 ± 0.01^{a}	1.62 ± 0.01^{b}	1.58 ± 0.00^{c}
Specific Gravity	0.92 ± 0.00^{b}	0.92 ± 0.00^{a}	0.92 ± 0.00^{b}	0.91 ± 0.00^{c}
Chemical parameters				
Acid value (mg KOH/g)	2.79 ± 0.04^{b}	2.90 ± 0.01^{a}	2.7 ± 0.01^{c}	2.31 ± 0.03^{d}
Peroxide value (meq/kg)	40.32 ± 0.05^{a}	35.83 ± 0.04^{d}	36.09 ± 0.02^{c}	37.44 ± 0.04^{b}
Iodine value (g/100g of fat)	116.91 ± 0.12^{c}	119.40 ± 0.75^{b}	122.77 ± 0.23^{a}	123.40 ± 0.36^{a}

Note: Values are means of three replicates. *a-d* Mean ± SD in same row with different superscript indicates that there are significant different ($p < 0.05$).

The thymoquinone percentage of 400 W microwave pretreated black seed oil increased within the microwave radiation times of 1, 2 and 3 min. compared to the untreated black seed oil sample at 2.00%, 6.07% and 9.65%, respectively (Figure 1c). In addition 640 W microwave pretreated black seed oil increased within the microwave radiation times of 1, 2 and 3 min. compared to the untreated black seed oil sample. It was 61.62%, 102.50% and 129.00% higher than that of the untreated samples, respectively (Figure 1d). With a power of 640 W and a duration of 3 minutes, the yield of the active compound was doubled compared to the untreated sample.

The thymoquinone percentage of microwave-pretreated oil samples was significantly increased with pretreatment time compared to untreated oil sample, showing a positive correlation between pretreatment time and thymoquinone content ($r = 0.94$ and 0.99) ($p < 0.05$). Microwave assisted extraction of essential oils works by selectively targeting polar water molecules in the glands, trichomes or vascular tissues. Localized heating, which occurs at or above the boiling point of water, causes the cell walls to expand and rupture by releasing essential oils. One of the main advantage of microwave pretreatment is; it takes a very short time to preserve thermolabile compounds, such as thymoquinone. Therefore, it is an effective method for extracting bioactive compounds from different plants, which avoids the degradation of compounds (Destandau et al., 2015).

The results indicated that the oil yield of black seeds increased with the exposure time for pretreatment and the number of freeze-thaw cycles applied (Table 1). Comparing all treatments, 48 h and 3 cycle freeze-thaw pretreatment for black seeds showed the highest oil yield extracted from black seeds. According to Phothiset et al. (2014), before oil extraction, frozen seeds may be repeatedly thawed and refrozen but this may damage texture. Treated oil has higher humidity content than the untreated sample but applying freeze-thaw cycles decreased the humidity significantly ($p < 0.05$). During the freezing it forms large ice crystals and inside water cells diffuse outward. It increases the moisture of extracted oil but the repeated thawing process may decrease the moisture. There was not considerable difference reported with specific gravity. With 3 cycles of freeze-thaw pretreatment, acidity was decreased. When presenting natural antioxidants can also reduce the free fatty acid content of seed oil. Black seed oil has high tocopherol content (Matthaus et al., 2011). It may reduce the acidity in freeze-thaw pretreated samples. Peroxide value (PV) is another important parameter for assessing the quality of edible oils. The PV was decreased significantly by applying freeze-thaw pretreatment than untreated ($p < 0.05$), could be due to reducing the oxidation of oil at very temperature. Normally black seed oil had reported high PV than other edible oils (Ashrafi et al., 2023). Decreasing PV is an indication for good shelf life of oil. However, with increasing the number of cycles, this value has been increased again. This can be attributed to the disruption of the cellular structure caused by the freeze-thaw (FT) treatment, which facilitated increased interaction between the oil and intrinsic enzymes such as lipase and lipoxygenase, leading to enhanced hydrolysis and oxidation (Zhang et al., 2024). Iodine value was increased with the freeze-thaw cycles (. If the oil resulted with the presence of more iodine, it may more reactive, less stable and more susceptible to oxidation (Table 1 & 2).

The oil yield of black seeds was increased within the microwave power level and the radiation time. Comparing all treatments, 640 W power level and 3 min. microwave pretreatment had shown the highest oil yield extracted from black seeds (Table 2). The generation of heat energy causes the increment of the temperature of seeds and it creates intracellular pressure and influences the rupturing of oil seeds' cell walls and cell membranes. It facilitates the release of oil (Kaseke et al., 2021). With the microwave radiation time (1-3 min), the moisture of black seed oil was decreased significantly ($p < 0.05$).

Table 2. Physical and chemical parameters of 400 W and 640 W power microwave pretreated and untreated black seed oil samples

	Untreated	1 min	2 min	3 min
Microwave power: 400 W				
Physical parameters				
Oil yield (g)	344.10 ± 2.30^{d}	352.22 ± 2.43^{c}	358.37 ± 1.07^{b}	365.54 ± 1.09^{a}
Moisture (%)	1.56 ± 0.01^{a}	1.54 ± 0.02^{a}	1.47 ± 0.02^{b}	1.40 ± 0.00^{c}
Specific Gravity	0.92 ± 0.00^{ab}	0.92 ± 0.00^{b}	0.92 ± 0.00^{ab}	0.92 ± 0.00^{a}
Chemical parameters				
Acid value (mg KOH/g)	2.79 ± 0.04^{a}	2.37 ± 0.06^{b}	2.33 ± 0.04^{b}	2.28 ± 0.03^{b}
PV (meq/kg)	40.32 ± 0.05^{c}	42.02 ± 0.59^{b}	42.37 ± 0.37^{b}	43.69 ± 0.54^{a}
Iodine value (g/100g of fat)	116.91 ± 0.12^{a}	116.90 ± 0.84^{a}	109.77 ± 1.10^{b}	104.04 ± 0.82^{c}
Microwave power: 640 W				
Physical parameters				
Oil yield (g)	344.10 ± 2.30^{c}	376.24 ± 1.52^{b}	381.90 ± 1.99^{ab}	385.59 ± 2.30^{a}
Moisture (%)	1.56 ± 0.01^{a}	1.49 ± 0.00^{b}	1.31 ± 0.02^{c}	1.24 ± 0.01^{d}
Specific Gravity	0.92 ± 0.00^{b}	0.91 ± 0.00^{c}	0.92 ± 0.00^{a}	0.92 ± 0.00^{a}
Chemical parameters				
Thymoquinone (%)	0.84 ± 0.01^{d}	1.35 ± 0.00^{c}	1.69 ± 0.00^{b}	1.92 ± 0.00^{a}
Acid value (mg KOH/g)	2.79 ± 0.04^{a}	2.33 ± 0.02^{b}	2.32 ± 0.03^{b}	2.25 ± 0.05^{b}
PV (meq/kg)	40.32 ± 0.05^{d}	42.97 ± 0.08^{c}	43.79 ± 0.02^{b}	45.48 ± 0.08^{a}
Iodine value (g/100g of fat)	116.91 ± 0.12^{a}	115.69 ± 0.40^{b}	106.46 ± 0.23^{c}	99.19 ± 0.47^{d}

Note: Values are means of three replicates. *a-d* Mean ± SD in same row with different superscript indicates that there are significant different ($p < 0.05$)

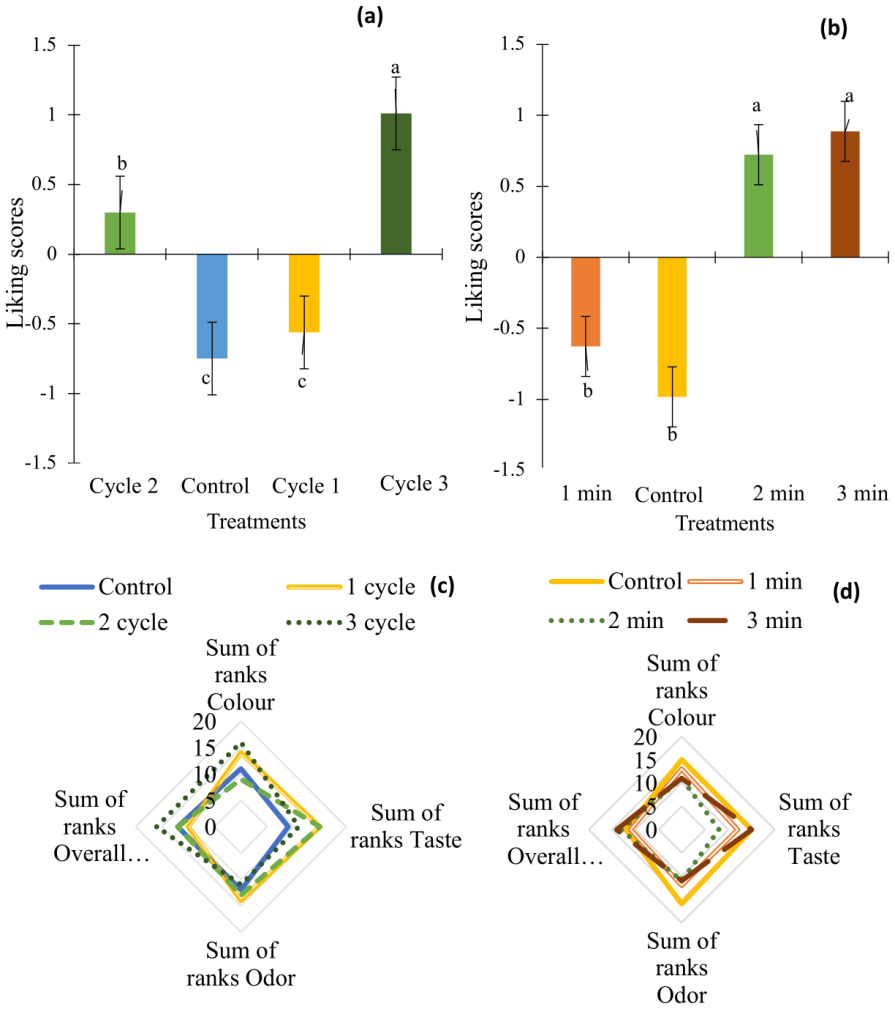

Figure 2. LS Means of Liking Scores of overall acceptability for different treatments (a, b) and Radar chart of sensory analysis for colour, taste, odor and overall acceptability (c, d). (a) & (c); 48 h freeze-thaw pretreated and untreated, (b) & (d); 640 W power microwave pretreated and untreated.

Increasing the exposure time to higher temperatures, it causes low moisture content due to the loss of cells' water content through evaporation. Even though no considerable difference was reported with specific gravity.

With 3 cycles of microwave pretreatment, acidity and iodine values were decreased. Peroxide value was increased significantly by applying microwave pretreatment than untreated ($p < 0.05$). As a result, applying microwave pretreatment elevates the tocopherol and other antioxidant compounds. It may affect to reduction of the acid value with the increasing temperature. The reduction in iodine value after applying microwave pretreatment may be caused by a decrease in the number of unsaturation sites as a result of the breakage of the long-chain fatty acids (Kittiphoom and Sutasinee, 2015). Peroxide value was increased with the increasing temperature because of lipid oxidation (Kaleem et al., 2015).

Sensory Analysis of Black Seed Oil

Sensory evaluation of black seed oil involves assessing its characteristics such as colour, flavour aroma, and texture so that it determines customer satisfaction and market value. Therefore, it is important to decide on the sensory attributes of cold-pressed extracted black seed oil samples by applying pretreatments against the untreated samples.

The results indicate that significant distinguishable variance was found in sensory evaluation of oil samples from 24 h freeze-thaw pretreated and 48 h freeze-thaw pretreated with untreated oil samples respectively. It can be stated that freeze-thaw pretreatment apply before oil extraction may be affected by the sensory attributes of black seed oil.

Similarly, significantly higher acceptability was shown in the 3-cycle freeze-thaw pretreated oil and the 2 and 3 minute microwave-treated oil than in other samples (Figure 2). It can be stated that microwave and freeze-thaw pretreatments may have been affected for making positive sensorial changes in cold-pressed extracted black seed oil even at the higher phenolic level or higher thymoquinone content. The phenolic compounds in black seed oil primarily contribute to development of its astringent and pungent taste (Alkhatib et al., 2024). Therefore, innovative ways such as incorporating oil into the diet and encapsulation can be used to mask the unliked taste of black seed oil.

Conclusion

This research demonstrates that freeze-thaw pretreatment and microwave pretreatment are promising novel methods for enhancing the extraction of thymoquinone from Indian black seed oil. Both techniques significantly improved the qualitative and quantitative properties of the oil. The optimal conditions identified in this study were 48 hours with three freeze-thaw cycles for the freeze-thaw pretreatment and 640 W for 3 minutes for the microwave pretreatment. Our findings revealed that both pretreatments not only increased the oil yield but also improved the physicochemical properties of the black seed oil. Additionally, both methods had a positive impact on the sensory properties of the oil, enhancing attributes such as aroma, flavor, and overall quality. These improvements suggest the potential of freeze-thaw and microwave pretreatments to optimize the production of black seed oil for commercial applications. Further studies are recommended to confirm these results, focusing on the long-term stability of the oil, scalability of the pretreatment processes, and the potential influence of these methods on other bioactive compounds in black seed oil.

Declarations

Author Contribution Statement

H.B.: Data collection, investigation, formal analysis, and writing the original draft

N.S.: Data collection and investigation

S.R.: Project administration, supervision, conceptualization, methodology, review and editing

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Conflict of Interest

The authors declare no conflict of interest.

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