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RESEARCH ARTICLE

A comprehensive report on GC-MS profiling, FTIR analysis and HPLC quantification of pharmaceutically vital metabolite thymoquinone from Nigella seeds

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Abstract

The present investigation aimed to gain insights into the structure of bioactive metabolic compounds in *Nigella sativa* L. seed oil. Initially, spectroscopic methods viz., GC-MS and FTIR were employed to determine functional groups, substituents, and conjugated double bonds in Nigella oil. GC-MS analysis identified 11 different amalgams, with p-cymene, γ-terpinene and α-thujene being the major components. The FTIR spectrum revealed the presence of strong, sharp, and weak peaks, along with critical functional groups corresponding to C-H, O-H, C-C, C≡N, and N-O, indicating the presence of pharmaceutically active constituents of the seed oil in the wavelength range of 400 – 4000 cm^{-1}. HPLC analysis indicated that the percent composition of thymoquinone in the seed extract was reported as 0.90% at a wavelength of 254 nm. In the examined samples, thymoquinone and standard thymoquinone both showed a peak Rf value of 3.656. The study's findings revealed that thymoquinone is a potential phytochemical present in the oil. Furthermore, the identified biomolecules hold promise for use in pharmaceutical applications to enhance health standards.

Keywords

alkaloid; chemical profiling; diet; health benefits; metabolite; thymoquinone

Introduction

Phytotherapy represents an innate approach to healing or alternative therapy, involving the utilization of herbs or plants and their derivatives to address diseases and enhance overall health through various forms such as extracts, tablets, syrup, or other modes of administration (1,2). Nigella or *Kalonji* (*Nigella sativa* L.), is an annual flowering plant (Fig. 1) belonging to the Ranunculaceae family, has played a pivotal role for millennia as a vital nutritional, flavouring agent and natural health cure in traditional medicine systems. It has been employed in treating numerous disorders within ancient systems such as Ayurveda, Unani, Arabic and Chinese medicine (3). The economic importance of Nigella lies in its seeds, which encompass fixed oil, alkaloids, proteins, and saponin (4,5). The pharmacological properties of nigella seeds are predominantly attributed to their fatty and

Fig. 1. Nigella Plant.

essential oils. Thymoquinone constitutes approximately 0.0648 ± 0.0038 % of the seed oil by RP HPLC method (6). Notably, Nigella seeds maintain their position on the US-FDA's Generally Recognized as Safe (GRAS) list (7). The potent radical scavenging ability of Nigella especially against superoxide anions, significantly contributes to its antioxidant effect. Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), and natural antioxidant enzymes in the body, exhibit substantially increased transcription in the presence of thymoquinone (7). This underscores the potential of thymoquinone to influence gene expression and enhance the antioxidant mechanisms associated with Nigella seed consumption.

Phytochemistry, a branch of chemistry, focuses on the examination of chemical substances originating from plants. Its primary objectives include the documentation, isolation, and categorization of chemical compounds present in plants, with a keen emphasis on understanding their biological and pharmacological activities (8). In the realm of drug discovery and development, phytochemistry plays a pivotal role, as a multitude of contemporary pharmaceuticals trace their origins to natural sources, particularly plants (9). Plant-derived secondary metabolites have a variety of biological properties, including antioxidant capacity, immune system stimulation, antimicrobial activity, detoxification enzyme modulation, reduction in the platelet aggregation, modulation of hormone metabolism, and potential to treat cancer (10). The study of phytochemistry is instrumental in unveiling alternative sources of therapeutically significant chemicals sourced from plants. Investigating the intricacies of plant metabolites requires the use of powerful analytical techniques like GC-MS and HPLC. These techniques enable the identification and quantification of metabolites and facilitate the separation and detection of individual metabolites within complex biological samples (11). Phytochemical studies employing these analytical tools contribute significantly to the broader understanding of the chemical composition of plants and their potential applications in medicine and drug development.

Fourier Transform Infrared Spectroscopy (FTIR) emerges as a potent analytical technique for the identification and characterization of plant extracts (12). Proven as an effective method, FTIR is particularly valuable for characterizing and identifying the chemical components or functional groups (chemical bonds) present in complex plant isolates (13,14). Notably, this approach is rapid, non-destructive, and requires minimal sample preparation (15). Organic compounds are identified by examining bands in the infrared spectrum at specific frequencies that are influenced by nearby functional groups (16). Within Nigella crude extracts, thymoquinone stands out as an antioxidant/anti-inflammatory phenolic compound (17). Its efficacy in a multiplicity in *in vivo* (animal model studies) and *in vitro* (cell line studies) simulations, together with insulin resistance, dementia, and the development of cancer, has been thoroughly investigated (18-20). Previous investigations have indicated that unadulterated thymoquinone and different concentrates of nigella enhance enzyme-mediated DNA breakage (21). This suggests that thymoquinone acts as an inhibitor of topoisomerase II, which may have anti-inflammatory and anti-cancer properties like many other nutritional metabolites (22). The utilization of FTIR in these studies enhances the precision and depth of understanding regarding the chemical composition and therapeutic potential of thymoquinone in Nigella extracts.

In the given context, the present study aimed to analyze the phytochemical composition of Nigella seed oil through the application of the GC-MS technique. Furthermore, FTIR analysis was utilized to ascertain the essential functional groups within Nigella oil. Additionally, the HPLC technique was employed for the quantification of thymoquinone, a pharmacologically valuable active metabolite found in the seed oil. This comprehensive approach allows for a detailed exploration of the chemical constituents and specific bioactive compounds present in Nigella seed oil, contributing to a deeper understanding of its potential therapeutic properties.

Materials and Methods

Seed material and oil separation

Nigella seeds of the AN-20 variety were obtained from the ICAR-National Research Centre on Seed Spices, Ajmer, Rajasthan. The gathered seed samples were washed thoroughly with double-distilled water before being dried at ambient temperature. A precisely measured quantity of 50 g of seeds was then pulverized using a Malavasi mill. To prevent excessive heating, extra precautions were taken at the time of pulverisation. The subsequent particles were ranged between 250-425 µm. The finely powdered seeds were placed in a 0.5L container, strategically positioned within an ultrasonic bath. Subsequently, the container underwent ultrasonic pre-treatments employing 90 W with a constant frequency of 25 kHz for a duration of 45 min, ensuring optimal processing conditions (23). HPLC-grade

methanol was utilized for the preparation of samples for extraction. Upon completion of the pre-treatments, the extract was laid open to drying using rotary vacuum evaporator, maintaining a temperature to 40 °C in an inert environment. The resulting dried extract was then stored at low temperatures, ready for subsequent studies. This meticulous procedure ensures the preservation of the integrity of the extracted components for further detailed analyses.

GC-MS Profiling of seed oil extracted from Nigella varieties

The identification of chemical compounds within the seeds of Nigella was accomplished through Fatty Acid Methyl Esters (FAME) analysis, following the guidelines outlined in AOCS Method CE 1-62. The diluted FAME was subjected to separation on an Agilent GC-7820 A coupled with an MS-5975 system (Agilent HP-5 MS column, USA, film thickness: 0.25µm, dimensions: 30m × 0.325mm). Sample volume of 1 µl at a ratio of 20:1 was injected using an autosampler in split mode (24). Helium gas has been used as the carrier gas, and it flowed at a rate of 1.0 mL per min. The temperature of the column was programmed to ramp between 50 °C-280 °C, over 30 min, with a 3 min steady period. A temperature of 250 °C was set for the injectors. Compound identification was accomplished by comparing retention time, and confirmation was obtained by 'computer comparison of mass spectrum fragmentation patterns using the updated NIST-MS library and available mass spectra, aided by Chemstation software (Agilent Technologies, USA). This meticulous analytical approach ensures the accurate identification and confirmation of the chemical constituents present in the seeds of Nigella

Fourier Transform Infrared (FTIR) Spectroscopy analysis

Infrared spectroscopy stands out as a powerful analytical technique facilitating chemical characterization. The fundamental principle behind this approach lies in the selective absorption of infrared radiation by chemical substances. After the absorption of IR radiation, molecules undergo vibrational movements, leading to the creation of an absorption spectrum. The FTIR spectra of Nigella oil in KBr pellets (V_{max} in cm^{-1}) were obtained using a Nicolet iS10 FTIR spectrometer from Thermo Fisher Scientific. The spectra's acquired at a resolution of 4 cm^{-1} over a wavelength ranging from 4000-400 cm^{-1}. To enhance the analysis, a drop of Nigella oil was applied to the exterior of the KBr pellet. Any excess Nigella oil was carefully extracted with the help of capillary tube. Subsequently, the FTIR spectroscopic analysis was conducted on the dry pellets in accordance with the suggested protocol (25). Detailed specifications of the FTIR settings are provided in Table 1. This systematic approach ensures accurate and reliable FTIR spectroscopic analysis, contributing to the comprehensive chemical characterization of Nigella oil.

Quantification of thymoquinone by HPLC technique

The total oil accomplished after extraction using Soxhlet apparatus was employed for HPLC examination using Agilent 1200 series, equipped with a diode array detector (Agilent Technologies, USA). A Hichrom C18 reverse phase column (5 µm particle size, 250mm x 4.6mm inner diameter,) was used for chromatographic separation. The HPLC system consisted of a diode array detector (DAD/G1315D), a degasser (G13179A), and a quaternary solvent pump (G1311A). A 25 µL Hamilton manual injector was used to inject the sample and standard into the system (26).

Table 1. FTIR settings used for the measurement in the study

Model name	FT/IR-6800typeA
Serial number	Bo06661794
Accessary serial number	B124561809
Accessory	ATR PRO ONE
Light source	Standard
Incident angle	45^0
Detector	TGS
Resolution	$4cm^{-1}$
Aperture	Auto (7.1 mm)
Scanning speed	Auto (2 mm/sec)
Filter	Auto (10,000 Hz)
Gain	Auto (16)
Zero filling	On

The mobile phase consisted of an isocratic flow of methanol: water (60:40) and it was subjected for sonication for 10 minutes at 37 °C and finally filtered via 0.45 mm Millipore filter, and the final injection volume was 20 µL. The thymoquinone was found at 254 nm at 26 °C. The identification was validated by comparing it to the retention time frame while maintaining a flow rate of 1.5 mL/min. The standard thymoquinone and the isolated sample were used for validation, and quantifications were executed by means of linear calibration curves (27). This rigorous HPLC analysis ensures precise determination of thymoquinone content in the extracted oil sample.

Results

Gas Chromatography-Mass Spectrometry profiling

A GC-MS analysis of the methanolic extract of Nigella had shown the existence of numerous chromatographic peaks. The chromatogram peaks were comprehended by referring to the GC-MS library's spectrum database of recognized constituents. The data pertaining in Table 2 showed the discovered compounds, including the retention time, peak area, chemical formula, and molecular mass. The outlining disclosed 11 major compounds, with p-cymene, myristic acid and longifolene as the prime constituents in the oil. Further, 9,12-octadecadienoic acid, methyl ester occupying the 58.87 % of the peak area, it is a polyunsaturated fatty acid that possesses antioxidant, anti-inflammatory activity and reduces the risk of heart disease. Fig. 2 illustrates the GC-MS chromatogram, presenting peak areas corresponding to various chemical compounds identified in Nigella oil.

Table 2. Biochemical possessions identified by GC-MS from Nigella

Particulars	Molecular weight	Chemical formula	RT	Peak area %
p-cymene	134.22	$C_{10}H_{14}$	6.383	0.115
Longifolene	204.35	$C_{15}H_{24}$	12.816	0.055
Myristic acid	242.39	$C_{15}H_{30}O_2$	16.495	0.071
9-hexadecenoic acid, methyl ester	254.41	$C_{16}H_{30}O_2$	18.401	0.058
Hexadecanoic acid, methyl ester	270.45	$C_{17}H_{34}O_2$	18.639	10.374
9,12-octadecadienoic acid, methyl ester	280.44	$C_{18}H_{32}O_2$	20.347	58.875
11-octadecenoic acid, methyl ester	296.50	$C_{19}H_{36}O_2$	20.387	25.916
Octadecanoic acid, methyl ester	298.50	$C_{19}H_{38}O_2$	20.545	2.149
cis-11,14- eicosadienoic acid	308.50	$C_{20}H_{30}O_2$	22.041	1.982
Eicosanoic acid	312.53	$C_{20}H_{40}O_2$	22.266	0.098
9-octadecenoic acid	282.46	$C_{18}H_{34}O_2$	23.788	0.307

the characterization of compounds in the given sample. The spectrum of the sample, as illustrated in Fig 3, delineates the pertinent bonds investigated in this study. These bond exhibits a proclivity for unhindered cycling at a low energy rate, imparting considerable flexibility to the various molecular combinations. The FTIR spectra have elucidated the existence of robust, well-defined peaks, alongside more subdued features. These spectral characteristics correspond to crucial functional groups, specifically C-H, O -H, C-C, C≡N, and N-O. Their presence in the seed oil, as detailed in Table 3, signifies the existence of pharmaceutically active constituents within the sample. Furthermore, the pronounced bands observed at 2970 cm^{-1} and 2867 cm^{-1} are indicative of an aliphatic group's (C-H stretching) presence, suggesting the existence of methyl and isopropyl substituents.

HPLC quantification of thymoquinone

In this study, Nigella seed oil was utilized to assess the concentration of thymoquinone, providing valuable information for consumers. The findings of the present investigation reveal a reported percent composition of 0.90 % for

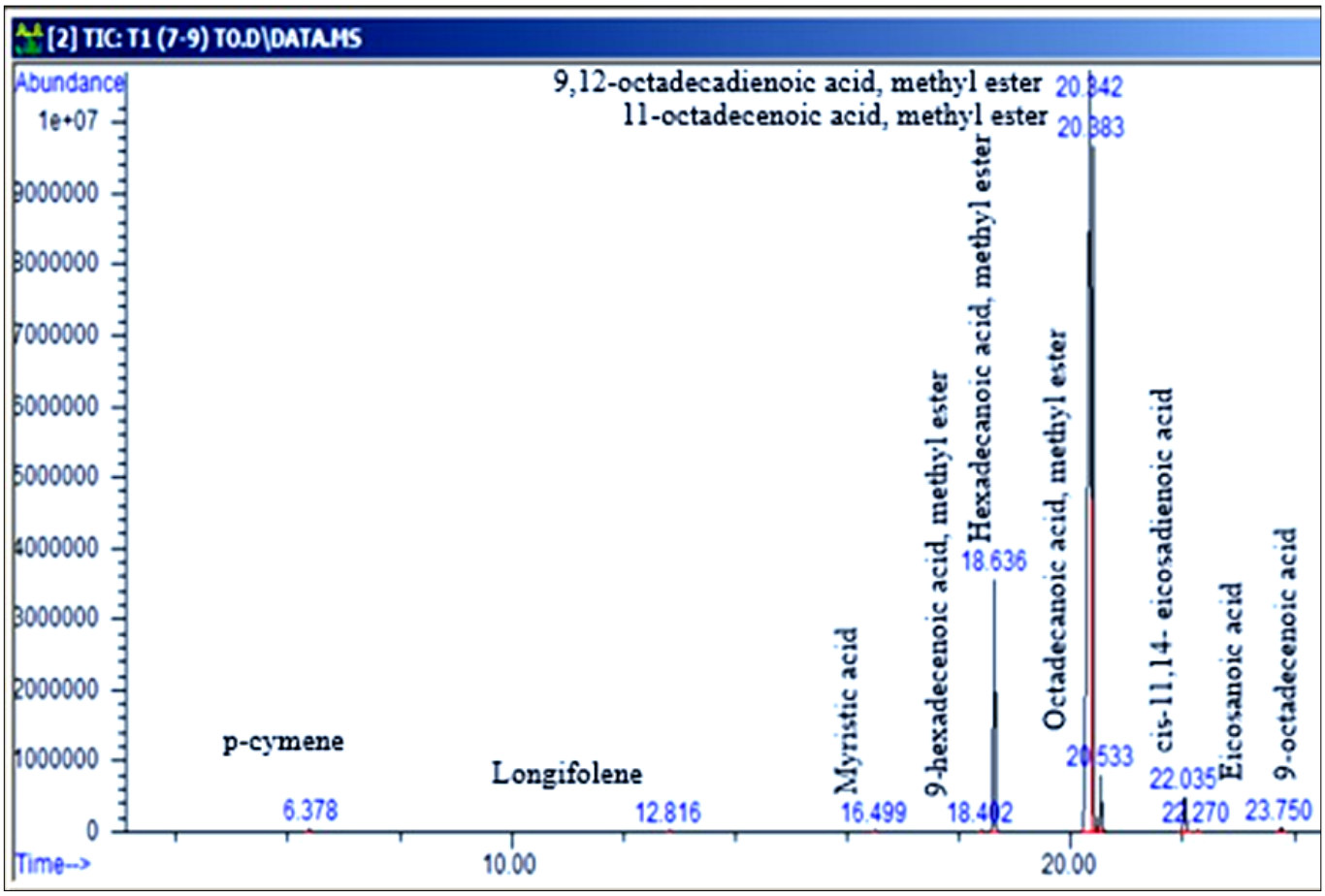

Fig. 2. GC-MS Chromatogram of Nigella.

FTIR analysis

The documentation of functional sets within the oil sample was achieved through the analysis of FTIR spectra. The seeds under investigation have been documented to contain a spectrum of theoretically significant elements in their constitution, further emphasizing the utility of FTIR as an effective and environment friendly analytical tool for

thymoquinone in the seed extract. The thymoquinone identified in Nigella oil was confirmed to match the standard reference thymoquinone at a wavelength of 254 nm. Both the standard thymoquinone (Fig. 4) and the thymoquinone from the studied samples (Fig. 5) exhibited a peak corresponding to the same Retention Factor (*Rf*) value of 3.656.

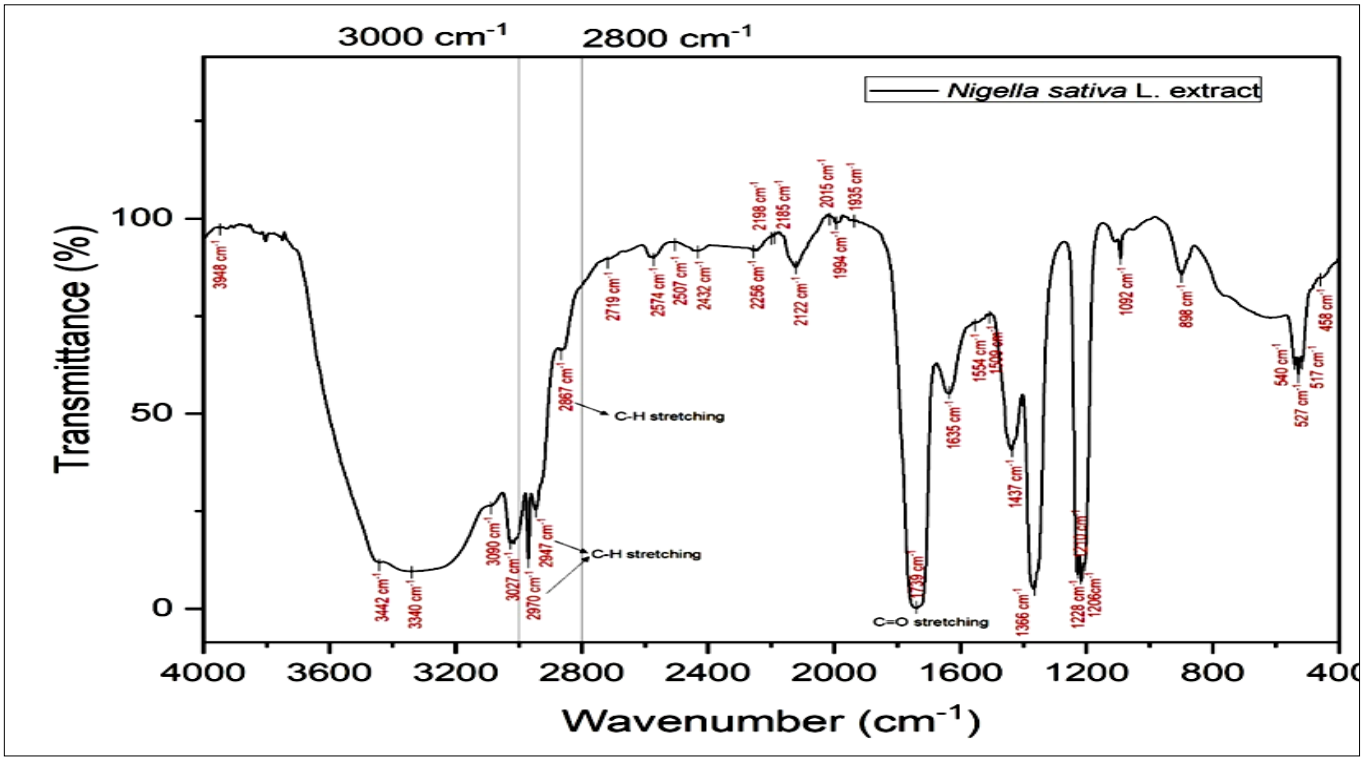

Fig. 3. FTIR image of Nigella oil.

Table 3. Range of FTIR absorbance values and respective functional group in Nigella oil

Sl. No.	Absorbance	Appearance	Functional Group	Compound type
1	3948-3970	Medium, sharp	O-H Stretching	Alcohol
2	3500-3200	Strong, broad	O-H Stretching	Alcohol
3	3200-2800	Strong, broad	N-H Stretching	Amine
4	2830-2695	Medium	C-H Stretching	Aliphatic
5	2600-2550	Weak	S-H Stretching	Thiol
6	2275-2250	Strong, broad	N=C=O Stretching	Isocyanate
7	2260-2190	Weak	C⊠N Stretching	Alkyne
8	2000-1650	Weak	C-0 Stretching	Ketone
9	1670-1300	Strong	N-O Stretching	Nitro
10	1400-1000	Medium	O-H Bending	Carboxylic acid
11	1000-650	Strong	C=C Bending	Alkene
12	600-450	Strong	N-O Stretching	Anhydrous

Discussion

Various medicinal flora and their constituent chemicals have exhibited noteworthy therapeutic potentials (28). An escalating cognizance is emerging regarding the correlation between phytochemical constituents and their associated biological activities (29, 30). Phytochemicals, characterized as secondary metabolites, typically manifest as intricate amalgamations that vary across diverse plant organs and developmental stages (31). Comprehending the composition of phytochemical ingredients is imperative for an in-depth consideration of the genuine utility the crop plant. In this present investigation, GC-MS was employed for the identification of phytochemicals, FTIR spectroscopy was utilized for the discernment of efficient chemical groups, and HPLC techniques was employed for the quantification of thymoquinone—a pharmacologically significant metabolite found in the oil of Nigella.

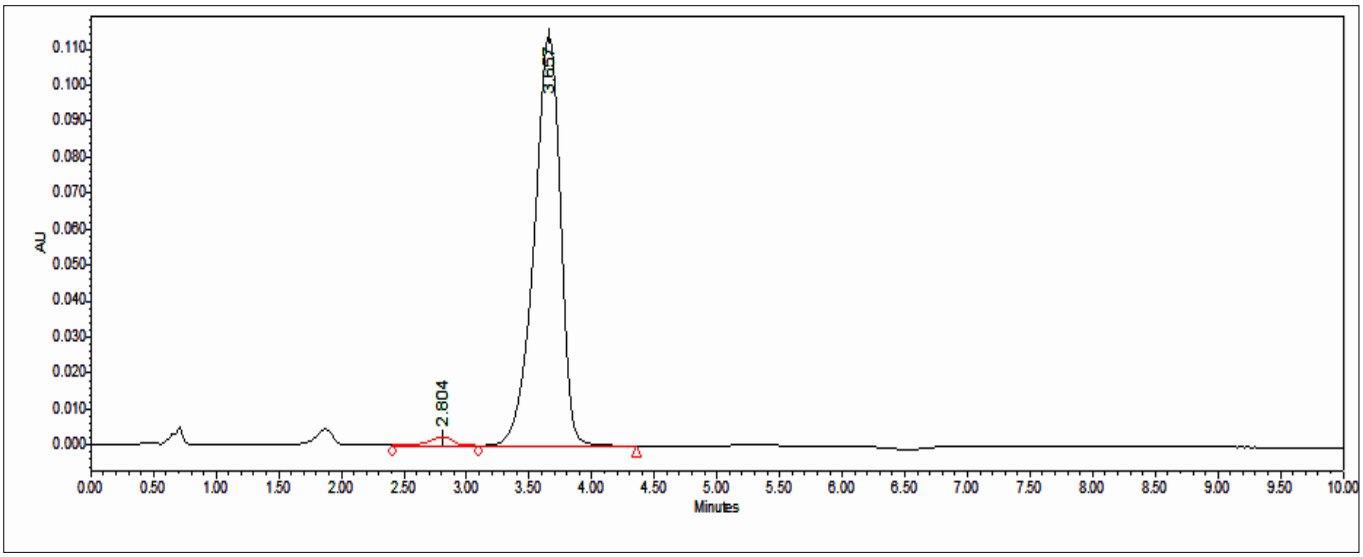

Fig. 4. HPLC chromatogram of thymoquinone standard.

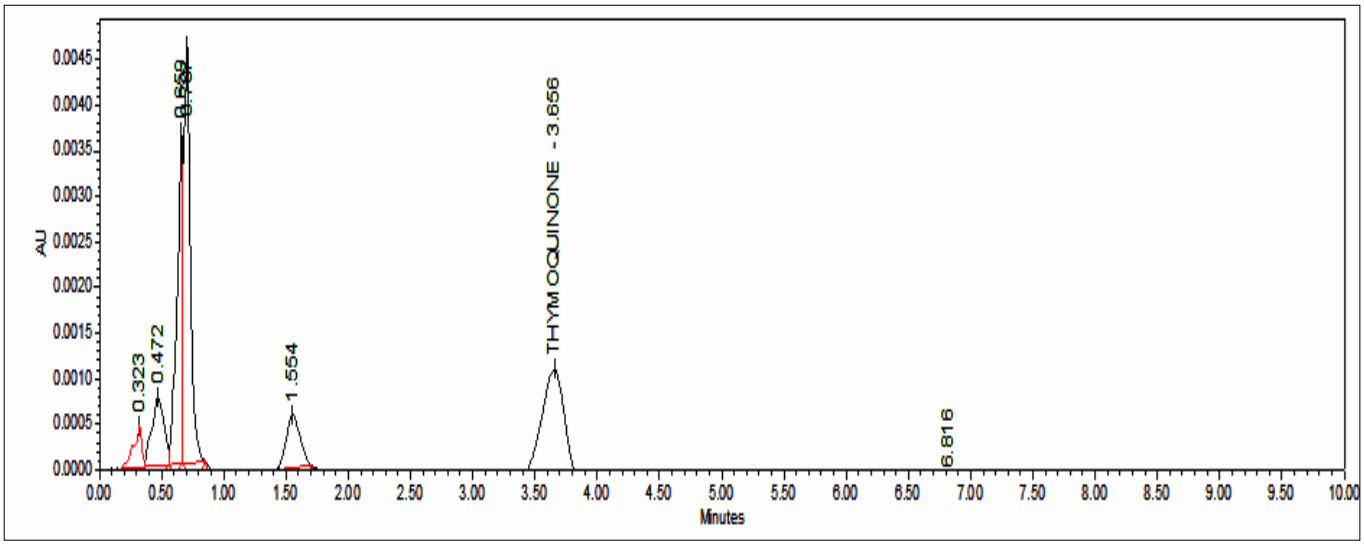

Fig. 5. HPLC chromatogram of thymoquinone from Nigella.

Gas Chromatography-Mass Spectrometry profiling

The findings of the GC-MS analysis were compared with a previous study wherein 16 unique compounds were found by GC-MS analysis, with α-thujen, p-cymene, and γ-terpinene being the initial retention time biochemical components and 9,12-octadecadienoic acid, methyl ester occupying the 58.87 % of the peak area detected in Nigella (32). P-cymene or p-isopropyltoluene is an alkyl-substituted compound naturally found in essential oils derived from aromatic and medicinal plants constitute a significant portion. Previous investigations have demonstrated that the monoterpene p-cymene possesses wide range of biological activities, like antioxidant, antidiabetic, anti-infla-mmatory, antiparasitic, antibacterial and anti-cancer properties. Furthermore, investigations have established the analgesic, neuroprotective, antinociceptive and immunomodulatory effects of p-cymene (33).

Myristic acid is another important chemical that controls vital metabolic processes in the human body through regulatory mechanisms and post-translational protein modifications (34). Furthermore, moderate myristic acid consumption has been linked to higher plasma phospholipid levels that contain long-chain omega-3 fatty acids, which may improve indicators of cardiovascular well-being in human (35).

The collective evidence suggests that Nigella oil not only exhibits potential pharmaceutical properties but also holds promise as edible oil. This dual utility underscores its significance in both medicinal and nutritional contexts. The GC-MS profiling results highlights that the qualitative composition of the oil aligns with previously published findings for fixed oils derived from nigella seeds (36). During the FAME analysis of Nigella eleven fatty acids were identified, with linoleic acid being the predominant contributor. This observation corroborates with the results of a study, which established that oleic acid, linoleic acid and palmitic acid are the primary fatty acids present in Nigella seed extract (18) . Notably, the aforementioned fatty acids are part of necessary fatty acids, which offer significant health remunerations to humans.

FTIR analysis

Fourier-Transform Infrared Spectroscopy (FTIR) is acknowledged as a "green analytical tool" owing to its expeditious, nondestructive, and straightforward methodology, rendering it more economical in terms of both chemicals and solvents compared to alternative instrumental techniques (12). The outcomes of the FTIR analysis are corroborated with previous studies (37,38) conducted on Nigella, where the seed extract was reported to contain alcohols, alkenes, aromatic amines, aliphatic amines, and alkyl halides. A prominent and robust band observed at 1739 cm^{-1} is attributed to C-O stretching, signifying the presence of a ketone group. Additionally, due to the resonance frequency impact reduction of the carbonyl group, an absorption band at 1739 cm^{-1} aligns with the C-O stretching characteristic of the metabolite of interest, namely thymoquinone. Numerous plant species use the alkanes in their cuticle and epicuticular wax to protect against moisture loss, mineral leaching, protection against microorganisms and destructive pests and insects. Alkenes, identified in the FTIR spectra, play versatile roles in various applications such as plastic production, fuel, illuminant, raw materials for alcohol and aldehyde production, fruit ripening, anaesthesia, and in the synthesis of mustard gas. Amines and amides, crucial components in protein synthesis, are also detected in the spectra. The sensitivity, adaptability, and speed of FTIR spectroscopy render it highly promising for food analysis and quality control. Operating within mid-infrared wavelengths ranging from 4000 cm^{-1} to 400 cm^{-1}, FTIR analysis in this study demonstrates its efficacy in determining simple organic molecules. Moreover, it has proven to be a reliable and perceptive method for detecting the biomolecular composition present in the analysed sample (39).

HPLC quantification of thymoquinone

High Performance Liquid Chromatography (HPLC) stands out as the most widely employed and reliable technique for the analysis of metabolites in plant extracts and the evaluation of various phytoconstituents (40). Thymoquinone, a prominent metabolite found in nigella seeds, necessitated precise calibration and formulation, prompting

the selection of HPLC as a reliable and intuitive analytical method. Ensuring the quality and optimal levels of active principles for bio-effectiveness in herbal suppository formulations demands standardization concerning raw constituent quality, manufacturing techniques, and composition (41). The percentage composition of thymoquinone in the current study closely aligns with the findings in a previous study, where the percent thymoquinone in seeds and seed oil ranged between 0.014-0.376 % and 0.142-0.619 %, respectively (42). The HPLC analytical technique, being a well-established and widely utilized technique for the separation and quantification of components in a mixture, plays a crucial role in determining phytochemicals in medicinal plants. The comprehensive quantification of these phytochemicals is essential to ensure the reliability and repeatability of pharmacological research and the quality of medicinal plant products (43). Establishing a chemical marker is imperative for quality control in medicinal plants and their derivatives. The absence of such chemical markers remains a significant challenge to maintain the quality of medicinal plants (44). In light of these considerations, the current study focused on the quantification of thymoquinone from Nigella seed extract using the HPLC technique, ensuring both the presence and accurate quantification of this active metabolite.

Conclusion

Nigella has been acknowledged and employed by millions for centuries, standing out as one of the most extensively researched and utilized natural components. The seed and its oil have garnered significant attention owing to their wide-ranging therapeutic potential for addressing various diseases. Our investigation shows that Nigella seeds contain a wide range of valuable biochemical components. The discovery of these biochemical compounds justifies the historical usage of the seeds by traditional practitioners to treat a variety of diseases. The FTIR analysis elucidates distinctive functional group stretching within the given sample, spanning wavelengths from 400 - 4000 cm^{-1}, confirming the existence of diverse functional groups in the samples. Thymoquinone, identified as an essential phytocompound, exhibits a broad spectrum of pharmacological effects. Therefore, it becomes imperative to ascertain its concentration in the seeds. Given its distribution in small amounts, the HPLC approach emerges as the most suitable technique for accurate quantification. The amalgamation of these findings underscores the multifaceted nature of Nigella and its potential as a valuable natural resource with a rich reservoir of bioactive compounds.

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Authors' contributions

All authors have significantly contributed to finalising the research RY, VIP, SNS designed the experimental set-up. AN, KKV, YD, PPR & RY analysed the data and wrote the first draft, while the final draft was read and approved by all authors. JR, DP, SJ & BSK final correction and finalisation of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Grammarly (free version) to improve the language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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