

Assessment of the effect of solvent polarity on *Nigella sativa* extracts of different origin

[Evaluación del efecto de la polaridad del disolvente en extractos de *Nigella sativa* de diferente origen]

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Abstract

Context: *Nigella sativa* (black cumin) has gained attention for its therapeutic potential in natural medicine. Among its components, thymoquinone and thymol have gained attention for their antitumor and therapeutic potential.

Aims: To identify the most effective solvent for extracting active compounds from *N. sativa* and to compare the yield of cytotoxic phytochemicals across different seed origins.

Methods: Twelve extracts were obtained using Soxhlet extraction by combining seeds from four geographic origins with three solvents of varying polarity (n-hexane, methanol, chloroform). Thymoquinone and thymol concentrations were quantified. Cytotoxic activity was assessed using the MTT assay on MCF-7 (breast cancer), HCT-116 (colon cancer), and HFF-1 (normal fibroblast) cell lines.

Results: The extraction yield followed the solvent polarity order: chloroform > methanol > n-hexane. The methanol extract from Sudanese seeds yielded the highest concentration of thymoquinone, while the hexane extract from Indian seeds exhibited the highest thymol content. All extracts, irrespective of seed origin and solvent used, reduced MCF-7 cell viability in a concentration-dependent manner. In HCT-116 cells, the methanol extract of Saudi origin demonstrated the most pronounced cytotoxic effect. Notably, cytotoxicity was also observed in the normal HFF-1 cell line.

Conclusions: The observed cytotoxic effects were not directly correlated with thymoquinone or thymol concentrations. These findings suggest that other bioactive constituents may contribute to the cytotoxicity observed. Further investigations are warranted to isolate and characterize additional phytochemicals from *N. sativa* that may play a more significant role in selective tumor cell cytotoxicity.

Keywords: cytotoxicity; extraction; *Nigella sativa*; thymol; thymoquinone.

Resumen

Contexto: *Nigella sativa* (comino negro) ha ganado atención por su potencial terapéutico en medicina natural. Entre sus componentes bioactivos, la timoquinona y el timol se han mostrado prometedores como agentes quimioterapéuticos derivados de plantas debido a sus propiedades antitumorales y otras propiedades medicinales.

Objetivos Identificar el disolvente más eficaz para extraer los compuestos activos de *N. sativa* y comparar el rendimiento de los fitoquímicos citotóxicos entre los distintos orígenes de las semillas.

Métodos: Se obtuvieron doce extractos mediante extracción Soxhlet combinando semillas de cuatro orígenes geográficos con tres disolventes de polaridad variable (n-hexano, metanol, cloroformo). Se cuantificaron las concentraciones de timoquinona y timol. La actividad citotóxica se evaluó mediante el ensayo MTT en las líneas celulares MCF-7 (cáncer de mama), HCT-116 (cáncer de colon) y HFF-1 (fibroblastos normales).

Resultados: El rendimiento de la extracción siguió el orden de polaridad de los disolventes: cloroformo > metanol > n-hexano. El extracto de metanol de semillas sudanesas produjo la mayor concentración de timoquinona, mientras que el extracto de hexano de semillas indias mostró el mayor contenido de timol. Todos los extractos, independientemente del origen de las semillas y del disolvente utilizado, redujeron la viabilidad de las células MCF-7 de forma dependiente de la concentración. En las células HCT-116, el extracto de metanol de origen saudí demostró el efecto citotóxico más pronunciado. En particular, también se observó citotoxicidad en la línea celular normal HFF-1.

Conclusiones: Los efectos citotóxicos observados no estaban directamente correlacionados con las concentraciones de timoquinona o timol. Estos resultados sugieren que otros componentes bioactivos pueden contribuir a la citotoxicidad observada. Se justifican nuevas investigaciones para aislar y caracterizar otros fitoquímicos de *N. sativa* que puedan desempeñar un papel más significativo en la citotoxicidad selectiva de las células tumorales.

Palabras Clave: cáncer; citotoxicidad; extracción; *Nigella sativa*; timol; timoquinona.

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INTRODUCTION

Nigella sativa L. (also known as black cumin or black seed) is an annual herbaceous plant in the family *Ranunculaceae* that is cultivated in several parts of the world as an essential spice, flavoring agent, and medicinal herb. Research on the therapeutic applications of *N. sativa* (NS) seeds has increased due to its widespread roles in immune stimulation (Ciesielska-Figlon et al., 2023), analgesic (Khan et al., 1999), anti-inflammatory (Al-Ghamdi, 2001), anti-allergic, anti-histaminic (Saadat et al., 2021), antioxidant (Burits and Bucar, 2000), antimicrobial (Bakathir and Abbas, 2011; Singh et al., 2005), anti-asthmatic (Boskabady et al., 2010), hypoglycemic (Hamdan et al., 2019), and anti-cancer (Mahmoud and Torchillin, 2013; Medenica et al., 1997; Salomi et al., 1989) activities.

Many active compounds have been identified and isolated from NS seeds. Some of them are alkaloids, quinones, terpenes, sterols, saponins, and many unsaturated fatty acids and proteins. Among these, thymoquinone, the most abundant quinone (30-48%) in NS seeds, and thymol, a natural monoterpene phenol found in NS seeds, have received more attention as a promising plant-derived chemotherapeutic agent for their antitumor activities in addition to other medicinal properties. Numerous *in vitro* and *in vivo* studies have been reported on the preventive action of thymoquinone in tumorigenesis and carcinogenesis and have been considered as a potential biomolecule to interfere with the hallmarks of cancer (Gupta et al., 2016; Schneider-Stock et al., 2014; Woo et al., 2012). Similarly, the other metabolites, thymol and its derivatives, are also reported to have anti-cancer properties by exerting suppression mechanisms on cell growth or inducing apoptosis (Deb et al., 2011; Islam et al., 2019).

Several methods and solvents are used for the extraction of phytochemicals from NS seeds. The choice of method and solvent depends on the type of plant, the part of the plant used for extraction, and the nature of the bioactive compounds. In general, polar solvents such as water, methanol, and ethanol are used to extract polar compounds, whereas non-polar solvents such as hexane, chloroform, or dichloromethane are used to extract non-polar compounds (Altemimi et al., 2017; Pandey and Tripathi, 2014; Sasidharan et al., 2011). Each of these models has its characteristic advantages and disadvantages. From a safety and recoverability point of view, solvents play a critical role in determining the overall efficiency of a process. Similarly, choosing an appropriate extraction method is also crucial as it, in some cases, depends on the intended use of an extract. The Soxhlet extraction

method is suitable for plant material that is partially soluble in the chosen solvent and for plant materials with insoluble impurities. This extraction method is preferred as it helps to extract large amounts of drug with smaller amounts of solvent, suitable for plant materials that are heat stable, and moreover, no filtration step is required (Abubakar and Haque, 2020; Azwanida, 2015).

It has been reported that the biological properties of herbal oils are associated with their chemical composition and are influenced by the origin of the plants (Bourgou et al., 2010; Celiktas et al., 2007). Hence, the qualitative and quantitative determination of the metabolites of the different extracts from different origins is necessary. There is a deficiency in the studies that correlate the metabolite constituents with the geographic origin of NS seeds and their potential biological implications. A metabolomic study with samples of NS seeds originating from four different countries has reported a difference in their constituents (Maulidiani et al., 2015). A recent study reports variation in terms of nutritional value, ash value, amount of active substance, and biological activities, NS seeds from three different geographical origins (Ahmed et al., 2020).

The present study aims to identify the most suitable solvent for the extraction of NS seed. This can increase the yield of specific phytochemicals in the extract that may be more effective on multiple cancer cell models.

MATERIAL AND METHODS

Solvents, chemicals, and instruments

Merck (Darmstadt, Germany). Thymoquinone, thymol standards, and MTT assay reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human breast cancer cell line MCF-7, colon cancer cell line HCT-116, and human fibroblast HFF-1 cell lines were purchased from ATCC, USA, and the reagents Dulbecco's modified Eagle medium (D-MEM), fetal bovine serum (FBS), trypsin-EDTA, Pen-strep, and PBS were purchased from Gibco (ThermoFisher Scientific, Germany). *Nigella sativa* seeds were obtained from a local market in Dammam city (Saudi Arabia). Analytical instrument Thermo Scientific™ Vanquish™ UHPLC (Thermo Scientific, Germany) with Chromeleon software (Dionex-Version 7.2.2.6890) and attached with Acquity BEH C-18 column (100 × 2.1 mm, 1.7 μm) and guard column (1.7 m VanGuard™) (Waters, Co. Wexford, Ireland) was used for HPLC analysis.

Extraction of NS seeds

The seeds of NS from different origins, namely, Saudi Arabia, Sudan, Syria, and India, were extracted using three organic solvents of varying polarity. A total of 12 extracts were obtained from the combination of the origin of NS seeds and the solvent used for extraction (Table 1). All seeds were powdered with the help of a mixer grinder, and 28 g of each sample was defatted separately with 200 mL of petroleum ether by the maceration method. The defatted powders were extracted for six hours with three solvents representing three ranges of polarity from lower to higher, that is, n-hexane (non-polar, 0.009), chloroform (semipolar, 0.259), and methanol (polar, 0.762), by the Soxhlet method. These extracts were concentrated by rotary evaporator (Buchi, R-215; Switzerland). The concentrated extracts were kept in an air-tight glass container at 5–10°C until further study. The percentage of extraction yield was calculated using the formula: Yield of extraction (%) = $(W1 \times 100)/W2$, where W1 is the weight of the extract residue obtained after solvent removal, and W2 is the weight of the ground sample taken for extraction (28 g).

Table 1. Twelve extracts were obtained after extraction procedures using different combinations of the origin of NS seeds and the extraction solvents.

Origin of NS seeds	Solvents used for extraction		
	Methanol (M)	n-Hexane (H)	Chloroform (C)
Saudi (SA)	SaM	SaH	SaC
Sudan (SU)	SuM	SuH	ScC
Syria (SY)	SyM	SyH	SyC
India (IN)	InM	InH	InC

Fourier-Transform Infrared Spectroscopy (FTIR) assay

FTIR spectra of all 12 extracts were measured using an FTIR spectrophotometer (Bruker Tensor 27 series, Germany) equipped with a diamond attenuated total reflectance (ATR). The spectra were obtained by pouring a small quantity of each homogenous sample onto the window and scanning between wavenumbers 400–4000 cm^{-1} with a resolution of 2 cm^{-1} .

Ultra-High-Pressure Liquid Chromatography-Diode Array Detector (UHPLC-DAD)

Preparation of standard and working solutions

An individual stock solution (1 mg/mL) was prepared for thymoquinone and thymol, sonicated, and filtered through a 0.2 μm membrane filter. The stand-

ards were mixed in the required amount to prepare mix standards (thymoquinone and thymol). The working solutions were prepared in the linearity range of 0.1–200 $\mu\text{g/mL}$ (0.1, 1, 5, 10, 50, 100, and 200 $\mu\text{g/mL}$) and stored in the refrigerator until the analytical procedures were completed.

Method development for UHPLC and UHPLC-DAD analysis

For UHPLC, the mobile phase consisted of water (A) and acetonitrile (B), a guard-protected Acquity BEH C-18 column (Waters) placed in an oven maintained at 40°C, automatic injector with an injection volume of 0.5 μL , and DAD at max plot (190–400) as well as selective wavelengths of 270, 272, 274, and 276 nm. For UHPLC-method development, a linearity range of 0.100–200 $\mu\text{g/mL}$ was used to develop a calibration curve with an R^2 value. Percentage accuracy, limit of detection (LOD), and limit of quantification (LOQ) were calculated from the regression equation using the formula [1] (Ahmad et al., 2021).

$$\text{Accuracy (\%)} = \frac{\text{Concentration recovered}}{\text{Initial concentration}} \times 100 \quad [1]$$

$$\text{LOD} = 3.3 \text{ (SD of intercept/slope)}$$

$$\text{LOQ} = 10 \text{ (SD of intercept/slope)}$$

Preparation of the NS seed extracts for UHPLC analysis

The NS seed extracts (10 mg/mL) were diluted to 50 $\mu\text{g/mL}$ final concentrations and injected into UHPLC for thymoquinone and thymol quantification. The samples' results were calculated (W/W) for the final amount/10 mg extract and reported.

Cell culture experiments

Cell culture setup

MCF-7, HCT-116, and HFF-1 cells were maintained in D-MEM supplemented with 1% L-glutamine, 1% penicillin-streptomycin, 10% heat-inactivated FBS, and 5% CO_2 atmosphere incubator set at 37°C. All the experiments were performed according to these conditions.

Cell viability assay using MTT

Cell viability was assessed by the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay (van Meerloo et al., 2011). Cells (15,000/well) were cultured in 96-well plates and incubated overnight. The master concentrations of extract in DMSO were diluted to the required concentrations using the media, added to the respective wells, and incubated for 24 hours. DMSO with the same extract volume in the media (restricted to not

more than 1%) was used as a control. MTT (0.5 mg/mL in PBS) was added, and after 3 hours of incubation, the formed formazan was dissolved in DMSO and quantified spectrophotometrically at 570 nm. Results are expressed as the relative percentage of the control cells (100%). All the assays were performed in triplicate in three independent experiments.

Selectivity index

To evaluate the extract's efficacy in cytotoxicity, the selectivity index (SI) for each extract was calculated by dividing the IC_{50} of the normal cell line HFF-1 by the IC_{50} of the cancer cell lines MCF-7 and HCT-116 using the formula [2]. The extract displaying SI values higher than 3 was considered to be highly selective (Machana et al., 2011).

$$\text{Selectivity Index (SI)} = \frac{IC_{50} \text{ of normal cell line}}{IC_{50} \text{ of cancer cell line}} \quad [2]$$

Statistical analysis

The data presented were performed in triplicate and are expressed as the mean $\pm$ SEM. Any significant difference was determined by one-way analysis of variance (one-way ANOVA), at $p < 0.05$, unless stated differently, using the GraphPad Prism 9 statistical package (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

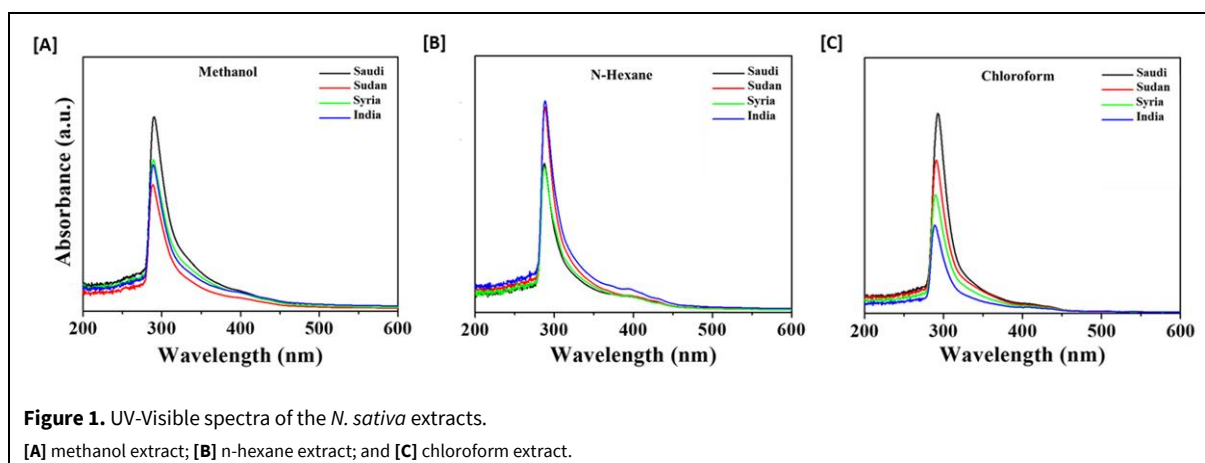
UV-absorption and FTIR analysis

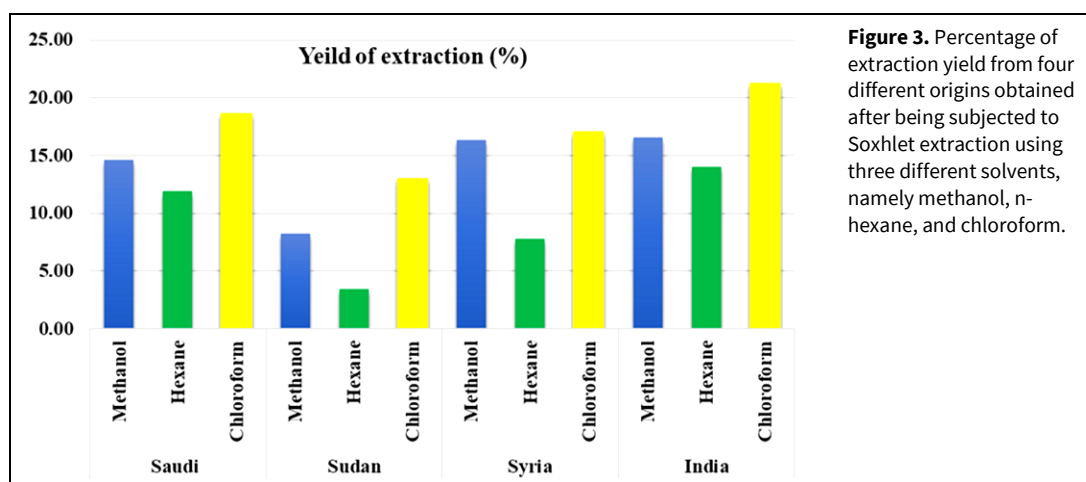
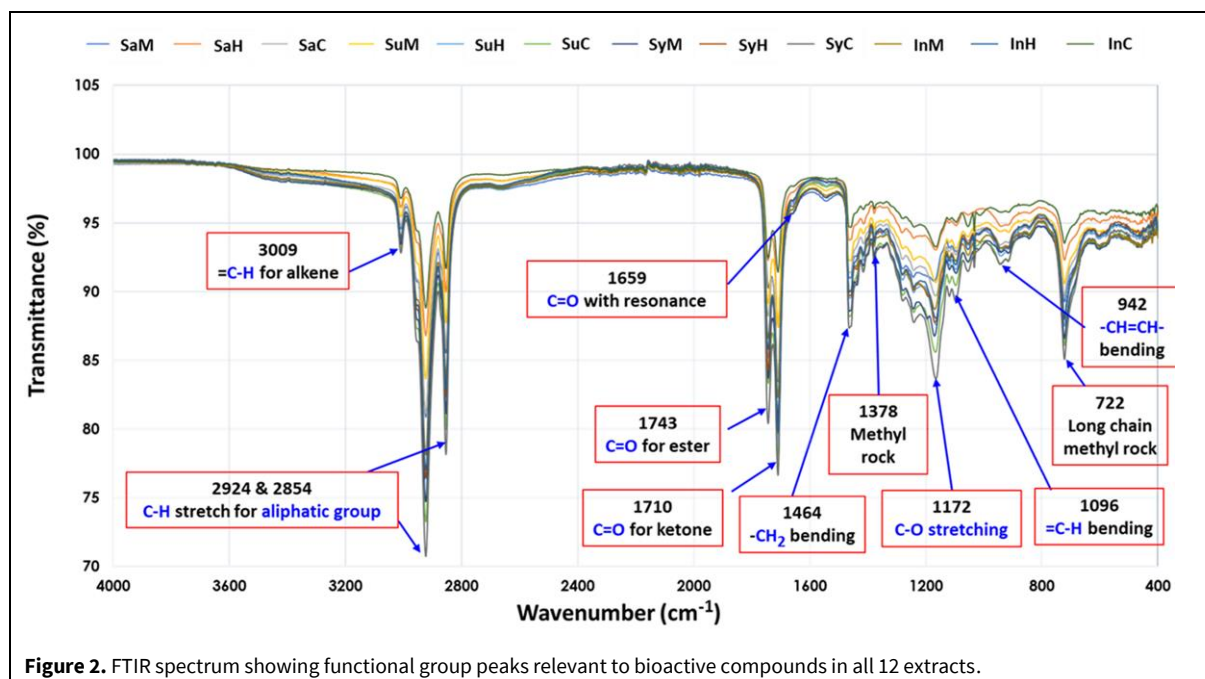
The UV-absorption spectrum of all 12 extracts was acquired, and all received within the wavelength (λ_{max}) of NS seed extract, ranging from 200 to 500 nm

(Fig. 1). The FTIR spectroscopy analysis shows the presence of various functional groups that are represented in many active compounds in NS seed extracts. Many active compounds, such as thymoquinone and thymol, with promising therapeutic potential, have been identified in different varieties of NS seed extracts, and the functional groups of these compounds describe and define the physical and chemical properties of these compounds. The FTIR data obtained for the 12 extracts are presented in Fig. 2, which shows a similar pattern of peaks in all 12 extracts. These data confirm the successful extraction of phytochemicals from NS seeds, and the FTIR peaks show the presence of similar important metabolites with various functional groups in all 12 extracts.

Effect of solvent polarity on extraction yield

The quantity of plant extracts extracted in a specific solvent is frequently an approximate assessment of the quantity of compounds the plant material contains (Zhang et al., 2018). The percentage of extraction yield from NS seeds of different origins, namely, Saudi, Sudan, Syria, and India, using the three solvents such as methanol, n-hexane, and chloroform representing the high, middle, and low polarity, respectively, were determined (Fig. 3). The order of the solvents which increased the percentage of extraction yield in all varieties was as follows: chloroform > methanol > n-hexane. The percentage of extraction yield was different in each variety of NS seeds. The order of extraction yield using chloroform was as follows: India (21.23%) > Saudi Arabia (18.57%) > Syria (17.0%) > Sudan (12.96%), and this pattern remains for the other solvents. The lowest percentage of extraction yield among all extracts was the SuH extract (3.46%).





Quantification of thymoquinone and thymol using HPLC analysis

It is reported that thymoquinone and thymol, which constitute the most abundant ingredients of NS seed extract, can independently induce many pharmacological effects (Tiwari et al., 2024). The levels of thymoquinone and thymol were quantified in all 12 extracts using HPLC analysis (Supplementary data, Table S1, Figs. S1-S2). As shown in Fig. 4, the highest level of thymoquinone concentration was present in the methanol extract of Sudan seeds. At the same time, in Syrian seeds, the high level of thymoquinone was in hexane and chloroform extracts, and in Indian seeds, it was in chloroform extract. No significant difference in the level of thymoquinone was observed in the three extracts of Saudi seeds. In the case of thymol, the highest level of thymol concentration was present in the hexane extract of Indian seed and then

in that of Sudan and Syrian seeds. Like in thymoquinone, no visible changes could be seen in the level of thymol in the extracts of Saudi seeds.

Effect of extracts on cell viability

Metabolites thymoquinone and thymol have been shown to have cytotoxic effects on several cancer cell lines. To prove whether the cytotoxic effect is due to the presence of these metabolites, the MTT assay was performed in two cancer cell lines, namely MCF-7 and HCT-116, which represent breast and colon cancer, respectively. A normal fibroblast cell line, HFF1, was also used to determine whether this cytotoxic effect is specific to only cancer cell lines. Regardless of the origin of the seeds and the solvents used for the extraction, every extract inhibited the viability of the MCF-7 cell line in a concentration-dependent manner. In HCT-116, cell viability was significantly inhibited

with methanol extracts of Saudi origin rather than those of Syrian origin. Meanwhile, n-hexane and chloroform extracts of all four origins had less or no cytotoxic effect on the HCT-116 cell line. A significant cytotoxic effect was also present in the normal cell line HFF-1. The significance was greater in methanol, n-hexane, and chloroform extracts of Saudi origin, even with the low concentrations, compared to the extracts of another origin, as shown in Fig. 5. SI,

which indicated the selective effects of the NS seed extract against cancer cells versus non-cancerous cells of all 12 extracts, was calculated as the ratio of the IC₅₀ for the normal cell line (HFF1) to the IC₅₀ for a respective cancerous cell line (MCF-7 and HCT-116) and tabulated in Table 2. The extracts with SI over 3 were present only in the methanol and n-hexane extracts of Sudan origin on the HCT-116 cell line.

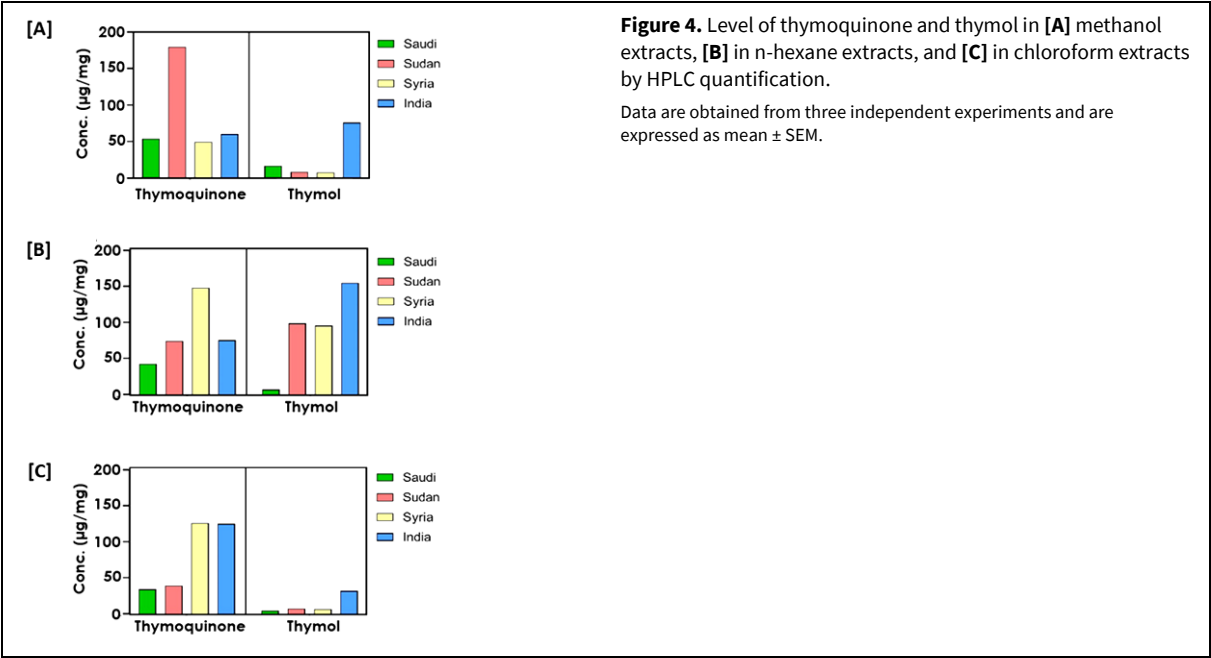


Figure 4. Level of thymoquinone and thymol in [A] methanol extracts, [B] in n-hexane extracts, and [C] in chloroform extracts by HPLC quantification. Data are obtained from three independent experiments and are expressed as mean ± SEM.

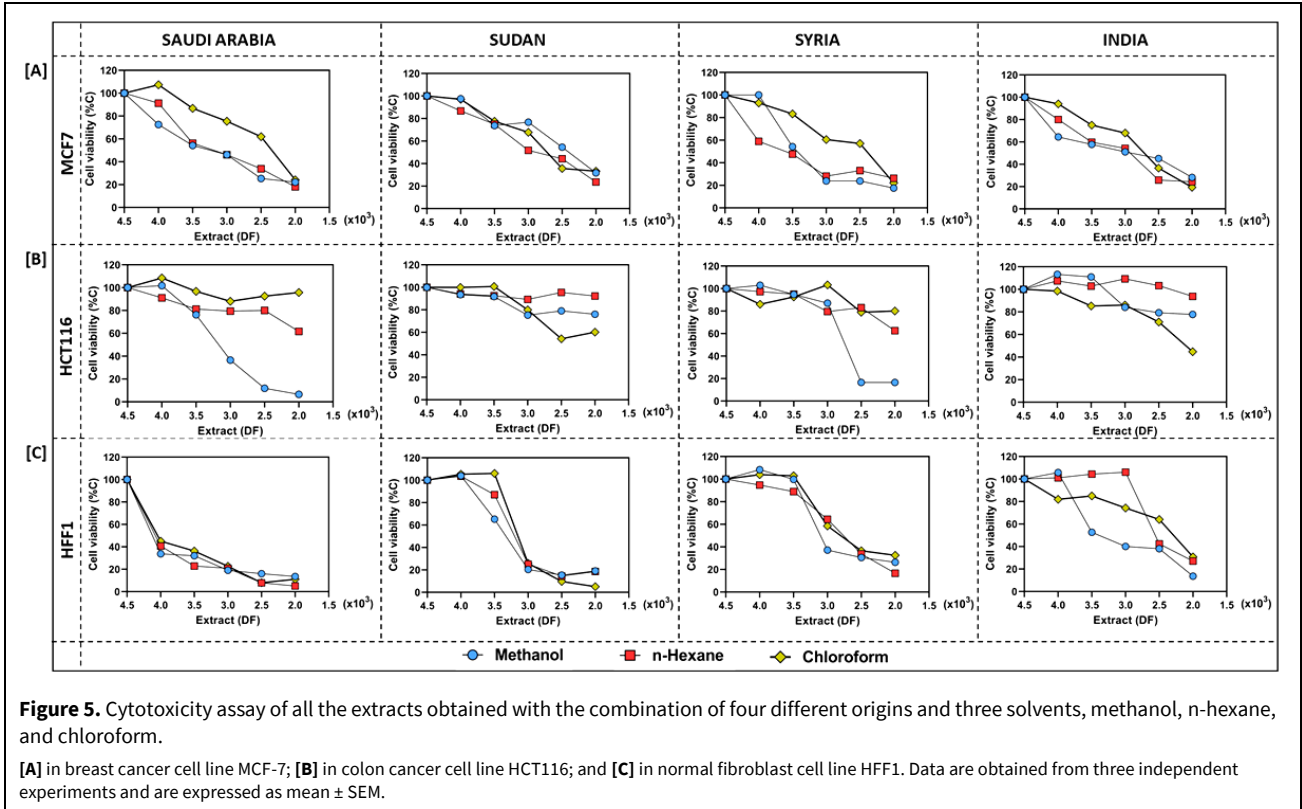


Figure 5. Cytotoxicity assay of all the extracts obtained with the combination of four different origins and three solvents, methanol, n-hexane, and chloroform. [A] in breast cancer cell line MCF-7; [B] in colon cancer cell line HCT116; and [C] in normal fibroblast cell line HFF1. Data are obtained from three independent experiments and are expressed as mean ± SEM.

Table 2. The selectivity index of *N. sativa* extracts.

Origin	Solvent extract	MCF-7	HCT-116
Saudi	Methanol	1.6429	1.3785
	n-Hexane	1.8599	2.2000
	Chloroform	1.1711	2.6700
Sudan	Methanol	1.8140	3.4286
	n-Hexane	1.7661	4.1370
	Chloroform	1.3392	1.7079
Syria	Methanol	0.9110	1.0421
	n-Hexane	0.5672	1.2324
	Chloroform	0.2874	0.4425
India	Methanol	0.2101	0.3226
	n-Hexane	0.2618	0.9804
	Chloroform	0.2203	0.2336

DISCUSSION

The polarity of the solvent used for the extraction can lead to extracts with specific constituents having diverse biochemical properties. Recent studies reveal that the solvents and their polarity can affect many characteristics, such as extraction yield, phytochemical contents, antioxidant activity, reducing properties, and free radical scavenging activities (Akinmoladun et al., 2022; Nawaz et al., 2020). It is reported that the extraction using highly polar solvents can increase yield with strong antioxidant compounds but low phenolic and flavonoid content compared to non-polar ones (Nawaz et al., 2020). A recent study reported that the extraction yield of NS seed was increased using non-polar solvents such as hexane (Shafodino et al., 2022). In the present study, the increased yield of extraction was present in chloroform extracts, followed by methanol extracts, indicating that the present extraction method may increase the yield with semipolar solvents rather than polar ones. This conflict could be due to the minor differences in the extraction method, as we included a defatting step using petroleum ether before subjecting it to the Soxhlet extraction method using different solvents. As mentioned above, the yield of extraction of a plant seed is mainly characterized by the solvents and their polarity used for the extraction, but at the same time, it is not necessary that the yield could influence the activity under investigation performed (Kchaou et al., 2013; Mohammed et al., 2019). In a previous study, the solvent ethanol extracted more active components from NS seed than the solvent hexane could, but at the same time, the extraction yield was higher in hexane than in ethanol (Shafodino et al., 2022). The other factors that could affect the extraction results are time

and temperature. In our extraction method, these two parameters were maintained constant throughout the extraction procedure of these 12 combinations, and hence, the changes in the extraction yield and their characteristics could only be associated with the respective solvents used for the extraction.

The FTIR spectrum obtained for all 12 extracts shows the presence of typical peaks representing the major functional groups of many metabolites of NS seed extracts. The peaks at wavenumber 3009 cm⁻¹ (C-H stretching for a vinyl group), 2924 and 2854 cm⁻¹ (C-H stretching for aliphatic groups indicating the existence of methyl and isopropyl substituents), 1743, 1710, and 1689 cm⁻¹ (C=O stretch for carbonyl compounds such as aldehydes, ketones, esters, and carboxylic acids), 1464 cm⁻¹ (C-H absorption scissoring and methyl rock), 1172 cm⁻¹ (C-O stretching), and 1096 cm⁻¹ (=C-H bending) are indicating the presence of many active metabolites in extracts that generally represent in the spectrum of NS seed extracts (Norziah et al., 2015). At the same time, the peak at 3350.20 cm⁻¹ attributed to the O-H functional group, reported in a previous study, was absent in our data (Shafodino et al., 2022).

All twelve extracts induced cytotoxicity in all cell models, irrespective of the solvent's characteristics or the seed's origin. No notable variations in cytotoxicity patterns were observed among extracts in all cell lines except for methanol extracts, which had an enhanced effect compared to n-hexane and chloroform extracts. The selection of the appropriate extraction method and the solvent for the extraction of medicinal plants is based on their efficiency in inducing these medicinal properties. Numerous studies have reported the antitumor and anti-angiogenic effects of NS seed ex-

tract, specifically thymoquinone and thymol, in cancer cell models or animal models (Fatfat et al., 2021; Islam et al., 2019; Vahitha et al., 2024; Woo et al., 2011). In the present study, the concentration of these metabolites is not directly associated with the cytotoxic effect of the extract. Previously, other metabolites, such as α -hederin, have also been reported to be more efficient in inhibiting the cell growth of oral cancer models than thymoquinone (Dagtas and Griffin, 2021). Saudi extracts with no significant increase in thymoquinone and thymol levels also showed anti-tumor activity in MCF-7 cells, along with the other extracts. Interestingly, Saudi methanol extract was the only one that showed cytotoxicity on HCT-116 cells. These could be due to the presence of active constituents other than thymoquinone and thymol, especially in Saudi and Syrian methanol extracts, which need to be identified. However, no significant changes in the pattern of the cytotoxic effect of any extract were observed in the normal cell line HFF-1 compared to the cancer cell lines MCF-7 and HCT-116. Among these 12 extracts, only two extracts, namely, methanol and n-hexane, showed high cytotoxic activity (with SI >3) against HCT-116 cancer cells, and their SI values were higher than 3.4. On the other hand, no solvent or origin extracts were found to be selective for MCF-7 cell lines with respect to control cells. This scenario indicates a high specificity of extracts obtained with low to medium solvent polarities for colon cancer. Previously, it has been reported that the ethyl-acetate column chromatographic fraction of NS seed extracts was non-toxic to non-cancerous human umbilical cord endothelial cells, and the methanol extract was minimally cytotoxic to normal lymphocytes (Salomi et al., 1991; Swamy and Tan, 2000). On the other hand, it has also been shown to have cytotoxicity in normal fibroblast cells (Islam et al., 2004). Whether the cytotoxic effect of NS seed extract is specific to cancer cells or not remains controversial and needs to be concluded. All twelve combinations of NS seed extracts exhibited cytotoxic effects in all three cell lines. The cytotoxicity level was not completely dependent on the concentration of thymoquinone and thymol. Moreover, the cytotoxicity of the extracts was measured based on applying the MTT assay, which can be considered a limitation of the present study. MTT assay has been used for nearly four decades as a standard tool to measure cell proliferation/viability, drug cytotoxicity, and mitochondrial/metabolic activity of cells (Stockert et al., 2018). Several studies have also revealed limitations of the MTT assay and various confounding factors, such as the type of culture media, the cell number, MTT reagent concentration, time of incubating cells with MTT, removal of the supernatant following MTT incubation, the wavelength at which optical density is measured, and these

factors can influence the assay (Ghasemi et al., 2021). Further studies with more precise methods that identify the efficacy of the extracts and the presence of other novel phytochemicals that may have more therapeutic roles in inducing tumor cell-targeted cytotoxicity are recommended.

CONCLUSION

This study showed that the cytotoxicity of 12 *N. sativa* seed extracts from different origins was independent of the extraction yield and the concentration of thymoquinone and thymol. Additional studies should be conducted to identify other cytotoxic phytochemicals.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Chathoth S	Nawaz M	Amir M	Ahmed R	Aldholmi M	Al-Mofty S	Cyrus C	Vatte C	SalahuddinM
Concepts or ideas	x								x
Design	x								x
Definition of intellectual content	x								x
Literature search	x								x
Experimental studies	x	x	x	x	x	x			x
Data acquisition	x	x		x		x			x
Data analysis	x	x	x	x	x	x	x	x	x
Statistical analysis							x	x	
Manuscript preparation	x								x
Manuscript editing	x						x	x	
Manuscript review	x	x	x	x	x	x	x	x	x

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Supplementary data

Quantification of thymoquinone and thymol using HPLC assay

The chromatographic conditions developed during UHPLC-MD were as follows: mobile phase with an isocratic flow (%) of 50 (H₂O): 50 (ACN), flow rate of 0.6 mL/min, injection volume of 5 µL, column oven temperature maintained at 40°C, and a selective UV wavelength of 274 nm. The total runtime for the method was 2.5 min, with a retention time (min) of 1.57 ± 0.10 for thymoquinone and 1.91 ± 0.10 for thymol. The Rs (peak resolution) for TQ was 3.15, i.e., >1.5.

The UHPLC-MV resulted in an accuracy of 100.17 ± 63.1% (thymoquinone) and 104.46 ± 10.42% (thymol) with LOD (7.7 and 9.1 µg/mL) and LOQ (23.4 and 27.7) for thymoquinone and thymol, respectively. The R² (coefficient of determination) values for both thymoquinone and thymol were observed to be 0.999 in the linearity range of 0.1-200 µg/mL. The SE and SD for the intercept, along with the regression equations for thymoquinone and thymol, are shown in detail in Table S1. The regression data for UHPLC-MDMV was extracted via Chromeleon software (Dionex V 7.2.2.6890). The chromatogram with retention times for thymoquinone and thymol is shown in Fig. S1, whereas the calibration curves are presented in Fig. S2.

Table S1. Characteristics of parameters validated for thymoquinone (TQ) and thymol (TY) using UHPLC-DAD MDMV.

Drug	RT	λ (nm)	Rs	SEM of intercept	SD of intercept	Accuracy (%)	Linearity range (µg/mL)	r ²	LOD (µg/mL)	LOQ (µg/mL)	Regression equation
TQ	1.57	274	3.15	0.11	0.29	100.17 ± 63.1	0.1-200	0.99	7.7	23.4	0.124x + 0.3191
TY	1.91	274	-	0.12	0.31	104.46 ± 10.42	0.1-200	0.99	9.1	27.7	0.1141x + 0.1795

RT: Retention time; Rs: Resolution; SEM: Standard error of mean; SD: Standard deviation; LOD: Limit of detection; LOQ: Limit of quantification.

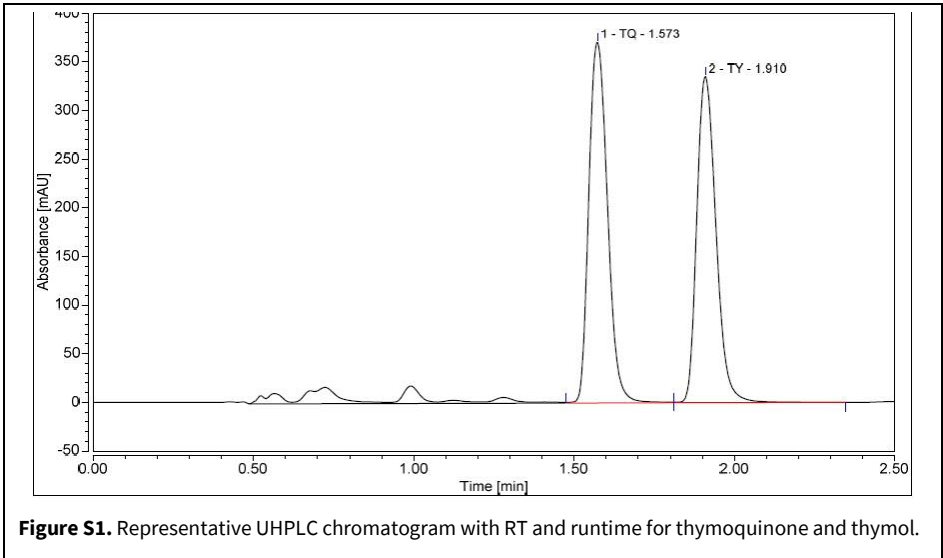


Figure S1. Representative UHPLC chromatogram with RT and runtime for thymoquinone and thymol.

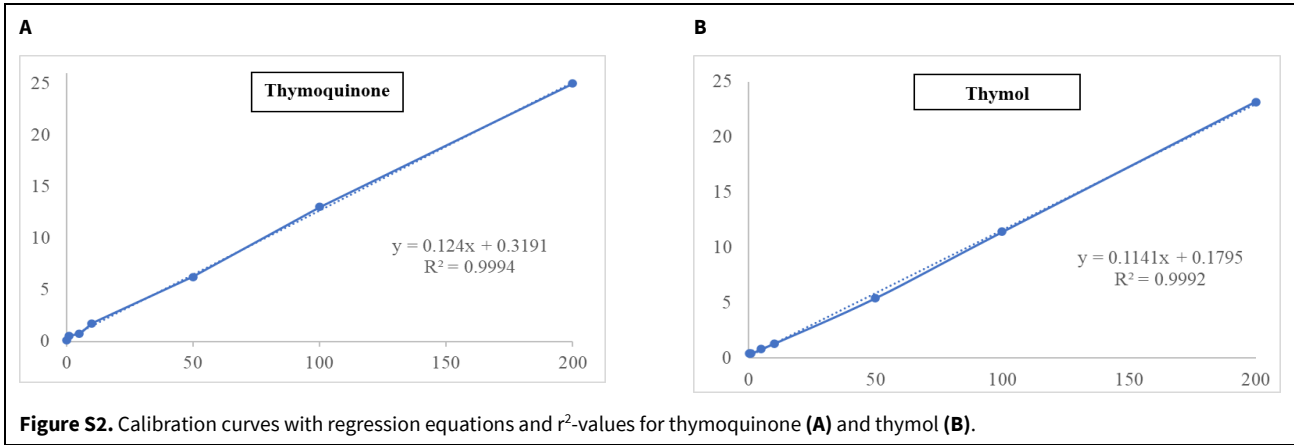


Figure S2. Calibration curves with regression equations and r²-values for thymoquinone (A) and thymol (B).