Original Research Article

Isolation of thymoquinone from *Nigella sativa* L. and *Thymus vulgaris* L., and its anti-proliferative effect on HeLa cancer cell lines

Ayesha Siddique Butt[1]*, Numrah Nisar[1], Nadia Ghani[1], Imran Altaf[2], Tahira Aziz Mughal[1]

[1]*Department of Environmental Science, Lahore College for Women University, Lahore, Pakistan,* [2]*Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan*

For correspondence: **Email: aqua_feb_13@hotmail.com; **Tel:** +92-321-9489301*

Abstract

Purpose: To isolate thymoquinone (TQ) from Nigella sativa L. and Thymus vulgaris L., and investigate its anti-proliferative effect on HeLa cancer cells.

Method: Pulverized dried samples of N. sativa seed (100 g) and aerial parts of T. vulgaris (1000 g) were subjected to Soxhlet extraction using methanol and n-hexane combined in different proportions. Thymoquinone (TQ) was then isolated from the extracts using high performance liquid chromatography (HPLC). The isolated TQ was further subjected to Fourier Transform Infrared (FTIR) spectroscopy to identify its functional groups. The anti-proliferative effect of TQ on HeLa cancer cells was evaluated using 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay.

Results: Extract yield from N. sativa was significantly higher than from T. vulgaris, and also increased with increase in the proportion of methanol in the extraction solvent (p < 0.05). Methanol and n-hexane (4:1, v:v) yielded the highest amount of oil, with yields of 15.8 and 9.7 ml/25 g dry weight (d.wt.) from N. sativa and T. vulgaris, respectively. The results obtained from HPLC showed that the concentration of TQ isolated from N. sativa (388.61 µg/ml) was significantly higher than that from T. vulgaris (357.03 µg/ml, p < 0.05). The anti-proliferative effects of TQ standard and TQ isolated from N. sativa on HeLa cancer cells were dose-dependent, and was highest at the lowest concentration. The number of viable cells significantly decreased with increase in TQ concentration (p < 0.01). TQ from N. sativa significantly reduced the number of viable cells even at the lowest concentration when compared to TQ standard (p < 0.05). Cell death was significantly higher in TQ-treated groups than in untreated cancer cells.

Conclusion: The results obtained in this study show that N. sativa is a potential source of TQ, with the yield enhanced by modifying the extraction procedure or solvent used. Furthermore, TQ isolated from N. sativa exerts a dose-dependent anti-proliferative effect on HeLa cancer cells.

Keywords: Thymoquinone, Nigella sativa, Thymus vulgaris, Anti-proliferative effect

INTRODUCTION

Plants are sources of important phytochemical compounds that exhibit anticancer properties [1].

Local herbs such as *Nigella sativa* and *Thymus vulgaris* have received lots of attention in recent times due to the anti-tumor properties of their extracts. *Nigella sativa* which is native to

Mediterranean regions such as South west Asia, Southern Europe and North Africa has gained prominence due to the presence of bioactive compounds that are effective against a number of diseases [2]. Its oil possesses varied pharmacological activities such as anti-cancer, anti-hypertensive, anti-diabetic, analgesic, anti-inflammatory, anti-microbial, hepato-protective, gastrointestinal and antioxidant properties [3]. The key components of the seed include fixed oil (32 - 40 %), volatile oil, saponins, essential oils, protein and alkaloids. The fixed oils contain eicosadienoic, linoleic, oleic, palmitic, myristic, and stearic acids [4]. Volatile oils from *N. sativa* (0.40 – 0.45 %) contain carbonyl fractions, thymoquinone (TQ), dithymoquinone, carvacrol, *p*-cymene, *t*-anethole and thymohydroquinone (THQ) [5,6].

Thymoquinone (TQ, 2-isopropyl-5-methyl-benzoquinone) is also present in *Monarda didyma* L*, Monarda media Wild, Monarda menthifolia, Satureja hortensis* L*., Satureja montana* L*., Thymus pulegioides* L*., Thymus serpyllum* L. and *Thymus vulgaris* L. Studies have shown that TQ is the major bioactive component in *N. sativa* and *T. vulgaris* oils, and has been extensively studied [7]. The anti-tumor activity of TQ is determined by targeting its mode of action. Some authors have speculated that its mode of action is via the induction of apoptosis, because it promotes the expression of tumor suppressor gene p53 in a time-dependent manner. Studies involving human cervical cancer cell lines have revealed dysfunctional p53 in C33A and HT-3 cells, while in HPV-positive cells the expression of p53 is repressed by HPV-E6 oncoprotein [8]. It has been shown that the combination of selenomethionine and TQ damaged Siha cells and reduced their proliferation [9]. The aim of this study was to isolate TQ from *N. sativa* and *Thymus vulgaris,* and investigate its anti-proliferative effect on HeLa cancer cells.

EXPERIMENTAL

Materials and reagents

Thymoquinone standard (98 %), methanol (HPLC grade), n-hexane, isopropyl alcohol (HPLC grade), dimethyl sulfoxide (DMSO) were of analytical grade and were products of Sigma Aldrich, Germany. Dulbecco's Modified Eagle's medium (DMEM) and MTT assay kit were products of Trevigen, (USA), while human cervical adenocarcinoma (HeLa cell lines) were obtained from the Quality Operation Laboratory, Microbiology section, UVAS, Lahore. Grinding mill was a product of Food Mixer National

(Japan). Nikon inverted microscope was purchased from Eclipse Ts2, Nikon, Inc. (USA), while Multiskan Ex-microplate reader was a product of Thermo Electron Corporation (USA).

Plants

The seeds of *N. sativa* and *T. vulgaris* were obtained from the seed stock of Botany Department, Lahore College for Women University (LCWU), Lahore, Pakistan, and nursed in the botanical garden under controlled conditions. Both plants were identified by Prof. Tahira A. Mughal.

Extraction

Pulverized dried samples of *N. sativa* seeds (100 g) and aerial parts of *T. vulgaris* (1000 g) were subjected to Soxhlet extraction based on the method described by Ashraf *et al* with some modifications, using methanol and *n*-hexane combined in different proportions [10]. About 25 g of sample was placed in the cotton cellulose extraction thimble (25 x 80 mm) with the respective solvent combinations and extraction lasted 6 h. Extracts from both samples were collected and re-extracted thrice with 30 ml methanol in a separating funnel to achieve maximum extraction. The extracts were concentrated using a vacuum rotary evaporator at 40 °C for 5 min. In order to achieve maximum solvent decantation, the extracts were centrifuged at 4000 rpm for 30 min, and the top fatty layer was collected and stored at 4 °C. The procedure was repeated in duplicates for the other solvent combinations. The yield of extracted oil was calculated as the ratio of the quantity of oil obtained to the quantity of plant material, expressed as a percentage.

HPLC analysis

Thymoquinone standard (100 μg/ml) was prepared in methanol and used to develop the calibration curve for the quantification and identification of TQ isolated from the extracts. The analysis was carried out using Waters 600 HPLC coupled with controllers, pumps and 2996 photo diode array (PDA), and the system was controlled using Empower 3 Chromatography Data software. The column utilized for the detection and separation of TQ was a 4.6 x 250 nm ODS C-18 with particle size of 5 μm. Elution was adjusted to isocratic mode using acetonitrile (solvent A) and methanol (solvent B) (30 : 70 v:v) at a flow rate of 1.5 ml/min. The sample total run time was 25 min, and the column temperature as maintained at 25 °C. The injection volume was 20 μl and detection was made at 254 nm at a

resolution of 1.2 nm. The column was washed with acetonitrile (100 %) and equilibrated for 25 min to remove impurities before the injection of samples. The retention times of the extracted oils were compared with those of TQ standard.

Quantification of extracted TQ

A standard calibration curve of TQ was used to quantify the concentrations of TQ isolated from the plants, by extrapolation. This was further confirmed by comparing the peak areas of both samples and standard.

Purification of TQ

Different eluate fractions from the HPLC were identified by comparing their retention times with those of standard. The fractions were concentrated using a vacuum rotary evaporator at 40 °C for 5 min. The remaining extracts from both plants were re-purified using HPLC to increase the purity of the isolated TQ.

FTIR spectroscopy

FTIR spectra of TQ standard and HPLC fractions of *N. sativa* were obtained from IR Tracer - 100 Fourier Transform Infrared Spectrophotometer equipped with ATR accessory and MCT detector. The resolution was adjusted at 400 - 4000 cm^{-1} with a resolution of 4 cm^{-1} at 100 scans. The IR spectrum of the isolated TQ was compared with that of TQ standard.

MTT assay

This was performed to evaluate the anti-proliferative effect of TQ on HeLa cell lines. The cells were cultured in DMEM for 24 h to obtain a monolayer of adherent cells. Different dilutions of TQ standard (100, 50, 25, 12.5, 6.25, 3.12 & 0.78 µM) were prepared in 96-well plates. Serial dilutions of TQ isolated from *N. sativa* were made and also added to HeLa cells in DMEM for comparison with those treated with TQ standard. Dimethyl sulfoxide (0.1 %) and methanol were used to dissolve the TQ standard and isolated TQ, respectively. Negative control groups were treated with the respective solvents, while the positive control group was left untreated. After treatment, the 96-well plates were incubated for 24 h and the cells examined under a light microscope. The procedure was performed in triplicate, and the extent of cell proliferation was calculated. The wells were incubated in the dark for 2 h and absorbance of each well was measured within 20 min at 492 nm using Multiskan Ex-microplate reader. Inhibition (H) of cell proliferation was determined as in Eq 1.

$$H\ (\%) = \{(A_E - A_{NC})/(A_{PC} - A_{NC})\}100 \ .... \ (1)$$

where A_E = absorbance of experimental well; A_{NC} = absorbance of negative control well; and A_{PC} = absorbance of positive control well. The protocol used for the MTT assay is shown in Table I.

Statistical analysis

Data are expressed as mean ± SEM, and the statistical analysis was performed using SPSS (version 16.0). Multiple comparison was performed using Tukey's multiple comparison tests. Values of $p < 0.01$ were considered statistically significant.

RESULTS

Yield of extracted oil

The yield of extracts from *N. sativa* was significantly higher than that from *T. vulgaris,* and increased with increase in the proportion of methanol in the extraction solvent ($p < 0.05$; Table 2). Methanol and *n*-hexane (4 : 1) yielded the highest amount of oil. The oil yields were 15.8 ml/25 g d.wt. and 9.7 ml/25 g d.wt. in *N. sativa* and *T. vulgaris*, respectively.

Chromatograms of TQ

The results of HPLC analysis showed that TQ standard (100 µg/ml) peaked at retention time of 5.5 min, and the concentration was 383.2 µg/ml. The concentration of TQ isolated from *N. sativa* (388.61 µg/ml) was significantly higher than that of *T. vulgaris (*357.03 µg/ml). However, there was no significant difference in the concentration of TQ standard and PQ isolated from *N. sativa* ($p > 0.05$). These results are shown in Figure 1.

Table 1: Treatment protocol in the MTT assay

Group	Treatment
Experimental I (E1)	TQ standard + 0.1 % DMSO + DMEM medium + cancer cell culture
Negative control I (C1)	0.1 % DMSO + DMEM medium + cancer cell culture
Experimental 2 (E2)	TQ from *N. sativa* + 0.1 % DMSO + DMEM medium + cancer cell culture
Negative control 2 (C2)	Methanol + 0.1 % DMSO + DMEM medium + cancer cell culture
Positive control (P)	Untreated cancer cell culture

Table 2: Yield of extracted oil (g)

Sample	Solvent ratio (*n*-hexane : methanol)		
	4 : 1	**3 : 2**	**1 : 4**
N. sativa	3.23 ± 0.10	5.7 ± 0.14	12.23 ±0.10
T. vulgaris	0.3 ± 0.05*	2.9 ± 0.12*	5.82 ± 0.08*

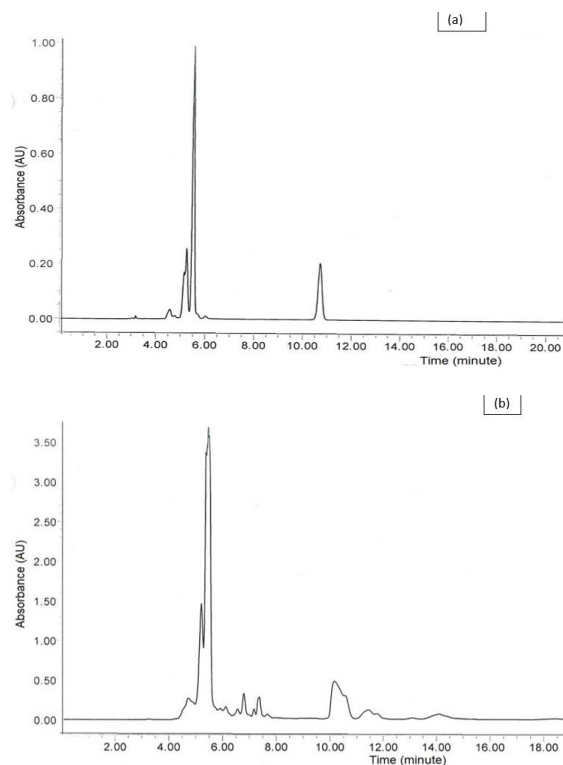*P* < 0.01, when compared to *N. sativa*

Figure 1: HPLC chromatogram showing retention time peak of (a) TQ standard and (b) TQ isolated from *N. sativa*

FTIR spectra

To establish the identity of TQ isolated from the plant extracts, functional group analysis was carried out using FTIR and the results were compared with the IR spectrum of TQ standard and existing literature. Peaks at 3498 cm^{-1} represented primary amines –NH$_2$, peaks at 3023 and 2897 cm^{-1} showed aliphatic C-H stretching (CH$_3$), peaks at 1655 cm^{-1} showed ester C=O stretching, and 1460 cm^{-1} peaks represented aliphatic C-H bending CH$_2$. Peaks at 1200 showed ester C-O stretching, and peaks from 943 to 727 cm^{-1} showed trans–CH=CH-. The overall pattern depicted clearly that the purified sample was TQ (Figure 2).

Anti-proliferative effect of TQ

The anti-proliferative effects of TQ standard and TQ isolated from *N. sativa* on HeLa cancer cells were dose-dependent, and was highest at the lowest concentration (Figure 3). The number of viable cells significantly decreased with increase in concentration of TQ (*p* < 0.01). The TQ from *N. sativa* significantly reduced the number of viable cells even at the lowest concentration, when compared to the TQ standard (Figure 3). Cell death was significantly higher in TQ-treated groups than in the untreated cancer cells (Figure 4).

DISCUSSION

The yields of seed oils depend on the extraction methods, solvents, time and temperature [12,13].

In the present study, the yield of oil from *N. sativa* was significantly higher than that from *T. vulgaris,* and increased with increase in the proportion of methanol in the extraction solvent. These results are in agreement with those previously reported [6,9].
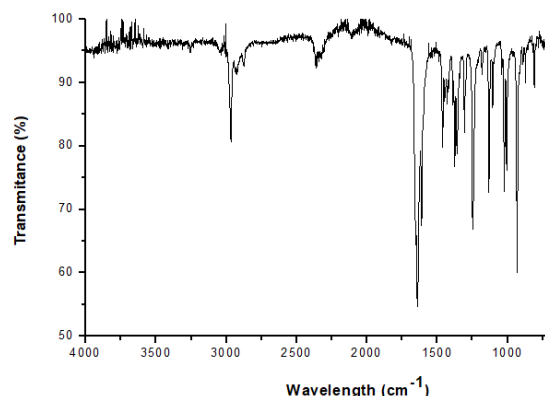

Figure 2: IR spectrum of HPLC-purified TQ isolated from *N. sativa*
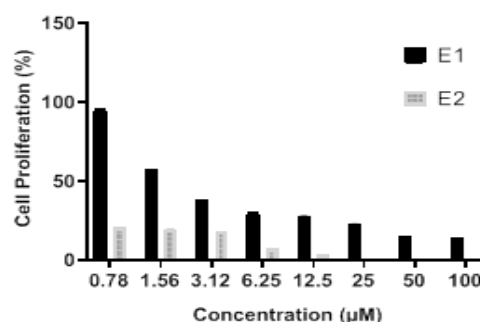

Figure 3: Anti-proliferative effect of TQ standard (E1) and TQ isolated from *N. sativa* (E2)
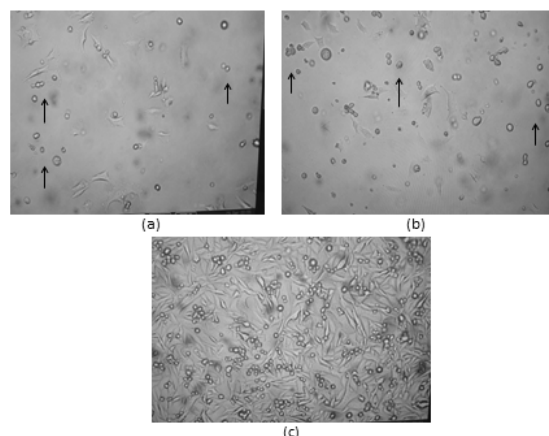
Figure 4: Microscopic images of HeLa cancer cells treated with: (a) TQ standard showing 15 % cell proliferation; (b) TQ isolated from *N. sativa* showing 1 - 2 % viable cells; and (c) untreated cell culture

Studies have shown that TQ is the major bioactive component in *N. sativa* and *T. vulgaris* [9]. In a previous study, the yield of TQ from *N. sativa* (48.9 %) was shown to be significantly higher than that from *T. vulgaris* (23.2 %) [14]. Some authors have reported that starting from 20 g of pulverized sample, the yields of TQ from *N. sativa* was between 856 and 1881 mg/g, using silica gel purification method [7, 10]. The yield of TQ obtained in this study was lower than those reported by Ashraf *et al* and Taborsky *et al.* This difference may be due to variation in geographic location, and quality of seeds or plants. Seed fat usually varies with region where they are grown and this affects the concentrations of bioactive compounds in plants. In this study, the concentration of TQ isolated from *N. sativa* was significantly higher than that from *T. vulgaris.* However, there was no significant difference in the concentration of TQ standard and TQ isolated from *N. sativa.*

Several studies have shown that TQ isolated from *N. sativa* is effective against a number of cancers [15,16]. In this study, the anti-proliferative effects of TQ standard and TQ isolated from *N. sativa* on HeLa cells were dose-dependent, and was highest at the lowest concentration. The number of viable cells was significantly decreased with increase in concentration of TQ. The TQ from *N. sativa* significantly reduced the number of viable cells even at the lowest concentration when compared to TQ standard. Cell death was significantly higher in TQ-treated groups than in the untreated cancer cells. These results suggest that TQ exerts dose-dependent anti-cancer effects against HeLa cancer cells. Studies have shown that TQ exerts anticancer effect on different types of cancers through *in vitro* and *in vivo*

mechanisms involving cell death signaling pathways, proliferation, angiogenesis and tumor-induced immunosuppression [17,18]. It has been shown that MTT assay is a standardized and recognized test for screening possible cytotoxic effects of substances on cancer cells [20].

The yield and concentration of TQ depend on the extraction method and solvents used [19]. In a previous study, Tabasi *et al* evaluated the anticancer activities of *N. sativa* and its TQ isolate against human renal cell carcinoma (ACHN) and fibroblast L929 cell lines, and reported significant increases in cancer cell death [20]. Although the complete anti-proliferation mechanism of TQ against cancer cells is still not fully elucidated, some authors have proposed TQ cytotoxicity and apoptotic induction as possible mechanisms of tumor inhibition in animal models [21,22]. These mechanisms involve anti-oxidant activity, cell cytotoxicity, immuno-modulatory action and apoptosis induction [23,24].

CONCLUSION

The results obtained in this study show that *N. sativa* is a potential source of TQ, and its yield is enhanced by modifying the extraction procedure or solvent used. Furthermore, TQ isolated from *N. sativa* exerts a dose-dependent anti-proliferative effect on HeLa cancer cells.

DECLARATIONS

Acknowledgement

Conflict of Interest

No conflict of interest associated with this work.

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