

IN VITRO CYTOTOXIC ACTIVITY OF NIGELLA SATIVA L. ON HUMAN MALIGNANT MELANOMA CELL LINES

NIZAMI DURAN¹, GULAY GULBOL DURAN², EMRAH AY¹, DURMU ALPASLAN KAYA³, MADALINA GEORGIANA ALBU KAYA⁴, AHMET MERT³

¹Mustafa Kemal University, Medical Faculty, Department of Microbiology & Clinical Microbiology, Hatay-Turkey, nizamduran@hotmail.com

²Mustafa Kemal University, Medical Faculty, Department of Medical Biology & Genetics, Hatay-Turkey, gulaygulbol@gmail.com

³Mustafa Kemal University, Faculty of Agriculture, Department of Field Crops, Hatay-Turkey, dak1976@msn.com

⁴INCDTP -Division Leather and Footwear Research Institute (ICPI), 93 Ion Minulescu St., 031215, Bucharest, Romania, albu_mada@yahoo.com

Nigella is a medicinal plant that has various pharmacological properties. It is widely used in folk medicine to treat many diseases in the world. It was aimed to investigate the essential oils *Nigella sativa* L. on two human melanoma cells (A-375 and A-2058 cell lines). In this study, two different cancer cell lines (A-375 and A-2058 cell lines) and one normal cell culture (vero cells) were used. Firstly, the non-toxic concentrations of the essential oils of *Nigella sativa* L. were determined on normal cells (Vero cell line). And then, the cytotoxicity test were applied at these non-toxic concentrations. The cytotoxic activity of the essential oils of *Nigella sativa* L. on the cell lines was measured by MTT and Trypan blue assays. The chemical components analyses of the *Nigella sativa* L. oils were carried out by GC-MS. Medically important various components of *Nigella sativa* L. were determined in the GC-MS analysis. Some of these components are as follows; anethole (22.97%), thymoquinone (21.36%), -thujene (6.22%), longifolene (5.76%), trans-isoeugenol (3.55%), carvacrol (2.23%). A total of 24 compounds were identified. The essential oils of *Nigella sativa* L. were found to inhibit the cell proliferation of human malignant melanoma cells. The IC50 values of the essential oils of *Nigella sativa* L. It were investigated as compared with a standard drug (methotrexate). Statistically significant decrease on the cell proliferations was found in the cells treated with the essential oils of *Nigella sativa* L. It was found to be non-toxic on normal cells (on Vero cell line) at the effective concentrations of the essential oils. While the non-toxic concentrations of essential oils on Vero cell line were found to be 12.5 µg/ml, the effective concentrations for A-375 and A-2058 cell lines were as 1.56 µg/ml and 3.12 µg/ml, respectively. In conclusion, the essential oils of *Nigella sativa* L. were found to be significantly inhibited the cell proliferation on the human melanoma cells. The effect may arise from the components situated in the structure of *Nigella sativa* L. such as anethole, thymoquinone, -thujene, longifolene, trans-isoeugenol and carvacrol. These components of this plant have been found promising in the treatment for human melanomas. But further studies, especially further animal studies should be needed to explain the action mechanisms of these components.

Keywords: A-375 cell line, A-2058 cell line, melanoma, cytotoxicity, proliferation, MTT.

INTRODUCTION

Turkey has a rich flora in terms of medicinal plants. There is plenty of endemic plant species especially in the Hatay region located in the south of Turkey. *Nigella* is a useful plant that naturally grown or cultured in Hatay commonly used in the treatment of various diseases. The seeds of *Nigella sativa* L. have been reported to use in the treatment of many different diseases and disorders. In Islamic literature, it is considered as one of the major herbal medicine (Ali and Blunden, 2003).

In folk medicine, *Nigella* oil has been widely used to treat many diseases such as peritonitis, cough, bronchitis, oedema, headache, rheumatism, fever, influenza and eczema for centuries in many countries (Burits and Bucar, 2003).

Some studies were carried out about the anti-carcinogenic effects of *Nigella spp.*, although the number of studies on human malignant melanoma is quite limited (Alenzi *et al.*, 2010; Alhebshi *et al.*, 2013; El-Mahdy *et al.*, 2005; Ulasli *et al.*, 2013; Woo *et al.*, 2011).

Even, we did not find any studies that investigate the effectiveness of *Nigella sativa* L. against human melanoma cells. Melanoma is a skin malignant tumor that occurred as a result of the melanocyte transformation and one of the important cancer types for human health. Studies indicated that melanoma begin to appear more often than the other cancer types all over the world (Sun and Schuchter, 2001).

The incidence of melanoma has been reported to increase day by day. As in other cancers, there is no powerful drug choice for pharmacological treatment in melanoma chemotherapy. In addition to this, the drugs used in the treatment of human malignant melanoma have been reported to show high toxicity and low effectiveness. Hence, new drug research for the treatment of melanoma continues rapidly (Atallah and Flaherty, 2005; Goldberg *et al.*, 2007).

In this study, we aimed to investigate *in-vitro* cytotoxic and antiproliferative activities of *Nigella sativa* L. on two human malignant melanoma (A-375 and A-2058 cell lines) cells compared to normal cells (Vero cell line).

MATERIALS AND METHODS

Isolation of the Essential Oil

Black Cumin (*Nigella sativa* L.) seeds were obtained from the spice bazaar of Antakya. Essential oils were obtained from seeds after powdered. The seeds of black cumin (100 g) were extracted by hydrodistillation with 1 L distilled water for 3 h using Neo-Clevenger apparatus. The oils were dried over anhydrous sodium sulfate and then stored in dark color glass bottles, at -4 °C ready for GC-MS analysis.

Analysis of GC/MS

Analysis of the essential oils carried out by using Thermo Scientific Focus Gas Chromatograph equipped with MS, auto sampler and TR-5MS (5% Phenyl Polysilphenylenesiloxane, 0.25 mm x 30 m i.d, film thickness 0.25). The carrier gas was helium (99.9%) at a flow rate of 1 mL/min; ionization energy was 70 eV. Mass range m/z 50-650 amu. Data acquisition was scan mode. MS transfer line temperature was 250 °C, MS Ionization source temperature was 220 °C, the injection port temperature was 220 °C. The samples were injected with 250 split ratio. The injection volume was 1 µL. Oven temperature was programmed in the range of 50 to 220 °C at 3 °C /min. The structure of each compound was identified by comparison with their mass spectrum (Wiley9 library). The data were handled using Xcalibur software program. The retention indices (RIs) were calculated for all volatile constituents using a homologous series of n-alkane standard solutions C₈-C₂₀ (Fluka, product no. 04070) and C₂₁-C₄₀ (Fluka, product no. 04071).

Table 1. Essential oil components *Nigella sativa* L.

RT	RI	Compound Name	Cas #	Area %
3,48	1016	-Thujene	2867-05-2	6,22
3,65	1029	-Pinene	80-56-8	3,71
4,06	1056	Camphene	79-92-5	0,93
5,06	1107	Sabinene	3387-41-5	1,83
5,19	1114	-Pinene	127-91-3	1,46
6,56	1172	-Myrcene	123-35-3	0,43
6,88	1183	Limonene	138-86-3	4,55
7,06	1189	Eucalyptol	470-82-6	0,74

RT	RI	Compound Name	Cas #	Area %
7,12	1191	-Phellandrene	555-10-2	0,13
8,21	1230	-Terpinene	99-85-4	0,44
9,15	1260	p-Cymene	99-87-6	17,16
18,89	1524	trans-Sabinene hydrate	17699-16-0	0,31
19,36	1537	Linalyl acetate	115-95-7	0,22
19,94	1552	Endobornyl acetate	76-49-3	0,96
20,38	1563	Caryophyllene	87-44-5	0,67
20,89	1576	Terpinen-4-ol	562-74-3	0,3
25,38	1695	-Bisabolene	495-61-4	0,21
29,03	1796	Anethole	4180-23-8	22,97
29,90	1822	p-Cymenol	1197-01-9	0,86
36,65	2023	Thymoquinone	490-91-5	21,36
40,09	2151	trans-Isoeugenol	5932-68-3	3,55
41,68	2215	Carvacrol	499-75-2	2,23
43,22	2255	Longifolene	475-20-7	5,76
44,65	2290	Longifolenaldehyde	19890-84-7	1,39

Cell Culture Studies

In the present study, as cancer cell cultures two different human malignant melanoma cell lines (A375 and A-2058 cell lines) and as a normal cell culture Vero cell line were selected. Human melanomas and Vero cell cultures were obtained Mustafa Kemal University, Virology laboratory's culture collections, Hatay, Turkey. The cells were cultured in RPMI-1640 medium, supplemented with 10% (v/v) fetal bovine serum, L-glutamine and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). The incubation of the cell cultures was performed at 37°C with 5% CO₂ in a humidified incubators.

Effect of DMSO

Dimethyl sulfoxide (DMSO) was selected as a solvent to solve the essential oils of *Nigella sativa* L. In order to test the effects of DMSO (Sigma, USA), 1x10⁶ cells were inoculated into each well of micro plates containing RPMI-1640 medium (Gibco-BRL). And then, the cultures were incubated for 72 hours in the presence of decreasing amounts of DMSO (4, 2, 1.5, 1.0, 0.8, 0.4, 0.2, 0.1%). The non-toxic concentration was determined equals and lower than 2%. In the experiments, the essential oils of *Nigella sativa* L. were dissolved in 1% DMSO.

Trypan Blue Exclusion

The cell viabilities in the cultures, trypan blue dye assay method was used. For this dying, 100 µl of *Nigella sativa* L.'s oils was mixed with 800 µl of PBS (phosphate buffer saline) and 100 µl cell suspension was added. Each concentration of the essential oils was performed in triplicate. Then, the prepared mixtures were incubated at 37°C in an incubator for 30 min. Finally, 100 µl of trypan blue dye was added to this mixture. The viability of the cells was determined in a haemocytometer by a light microscope (Gupta, 2002).

MTT Test

MTT assay was performed as previously described by Mossmann (Mosmann, 1983). The Vero (normal cells), A375 (human melanoma) and A-2058 (human melanoma) cell lines were cultured in RPMI-1640 medium with 10% (w/v) fetal bovine serum. Incubation of the cells was performed above mentioned conditions. The culture cells inoculated in 96-well plates overnight. The plaques were assessed at the incubation of

96 hours. The cells in the plaques were washed at least three times with PBS after incubation, and then, 100 μ L of 0.5 mg/mL MTT were added to all wells and incubated at 37 $^{\circ}$ C. The plaques were incubated for 30 minutes at the same conditions. MTT reduction ratio was determined by measuring the difference in absorbance at 570 nm using a microplate reader. All tests were performed in triplicate.

Statistical Analysis

All measurements were expressed as mean \pm standard deviation. Statistical analyses of data were performed using Student t-test. The p value < 0.05 was considered significant. All data were obtained in the experiments repeated three times. For statistical evaluation, SPSS, a Windows based program was used to assess data.

RESULTS AND DISCUSSION

The chemical composition of the essential oils of *Nigella sativa* L. is given in Table 1. The chemical components analyses of the *Nigella sativa* L. oils were carried out by GC-MS. A total of 24 compounds of *Nigella sativa* L.'s oils were identified. In the GC-MS analysis, *Nigella sativa* L. was found to have many medically important components. Some of these these valuable components and their percentages as follows; anethole (22.97%), thymoquinone (21.36%), - thujene (6.22%), longifolene (5.76%), trans-isoeugenol (3.55%), carvacrol (2.23%). The essential oils of *Nigella sativa* L. were found to inhibit the cell proliferation of human malignant melanoma cells. The IC_{50} values of the essential oils of *Nigella sativa* L. were compared with a standard drug (Methotrexate). Statistically significant decrease on the cell proliferations was found in the cells treated with the essential oils of *Nigella sativa* L. It was found to be non-toxic on Vero cell line at these effective concentrations of the essential oils.

In the present study, while the non-toxic concentrations of essential oils on Vero cell line were found to be 12.5 μ g/ml, the effective concentrations for A-375 and A-2058 cell lines were as 1.56 μ g/ml and 3.12 μ g/ml, respectively (Figure 1).

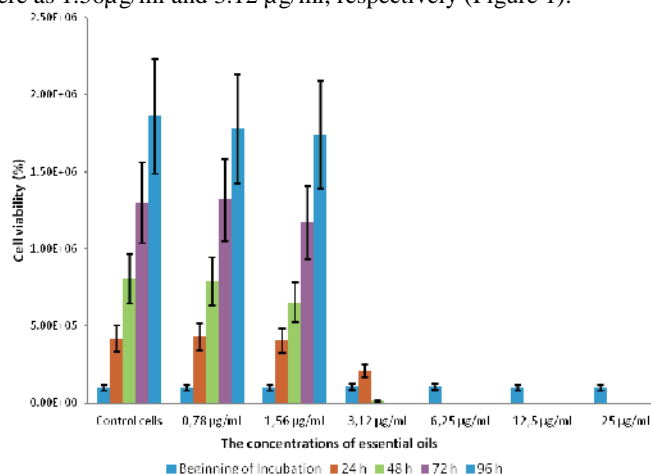


Figure 1. Cytotoxicity of the essential oils of *Nigella sativa* L. on A-375 cell line

When the essential oils of *Nigella sativa* L. compared to MTX, the efficacy of *Nigella sativa* L. found to be lower than MTX. But, cancer cell proliferation significantly inhibited by the essential oils of *Nigella sativa* L. (Figure 2).

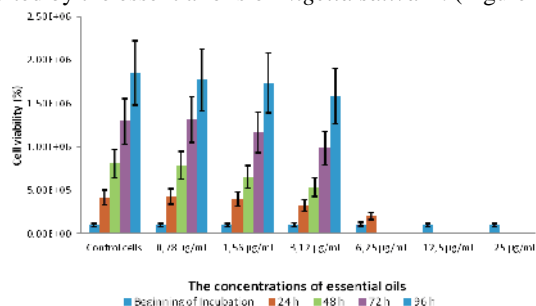


Figure 2. Cytotoxicity of the essential oils of *Nigella sativa* L. on A-2058 cell line

In the GC-MS analyses, anethole (22.97%), thymoquinone (21.36%), -thujene (6.22%), longifolene (5.76%), trans-isoeugenol (3.55%), carvacrol (2.23%) were identified as the major constituents of *Nigella sativa* L. The studies conducted previously in the literature reported that the phytochemical anethole possesses the anticancer activity against breast cancer cells (Muthukumari *et al.*, 2013). Thymoquinone is one of the most common components of the *Nigella sativa* L. Similarly, thymoquinone has reported to have a significant anticancer agent for mouse colon carcinoma (MC38) cells (Agbaria *et al.*, 2015). Before the studies, it's reported that carvacrol has an anti-proliferative and pro-apoptotic effects on human hepatocellular carcinoma cell line HepG-2 (Yin *et al.*, 2012).

The components of *Nigella sativa* L. on various cancer cell lines were studied, though, we have not found any study that performed on human melanoma cells. As we know, this study is one of the first studies carried out on human melanoma cells in the south of Turkey.

Besides, in the experiment, the inhibition concentrations of the essential oils of *Nigella sativa* L. against as cancer cell cultures in two different cell lines (A-375 and A-2058 cell lines) and a healthy cell line (Vero cell line) were evaluated. While the IC₅₀ values of the essential oils of *Nigella sativa* L. against A-375 and A-2058 cell lines were determined as 1.56 µg/ml and 3.12 µg/ml, respectively, this value for Vero cells was determined as 12.5µg/ml. Although, the IC₅₀ values for MTX against A-375 and A-2058 cell lines were determined to be lower than *Nigella sativa* L., the essential oils of *Nigella sativa* L. were found to be highly active to inhibit the proliferation of melanoma cells (Figure 3).

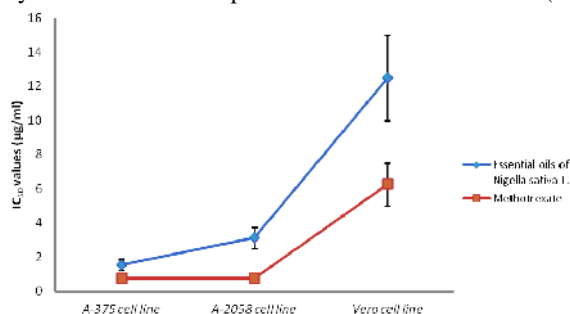


Figure 3. IC₅₀ values of the essential oils of *Nigella sativa* L. on three different cell lines

CONCLUSIONS

In conclusion, the essential oils of *Nigella sativa* L. were found to be significantly inhibited the cell proliferation on the human melanoma cell lines. The effect may arise from the components situated in the structure of *Nigella sativa* L. such as anethole, thymoquinone, -thujene, longifolene, trans-iso Eugenol, carvacrol. The components of this plant have been found promising in the treatment of human melanoma. But further studies, especially further animal studies should be needed to explain the action mechanism of action. To explain the mechanism of the effect, additional studies are required.

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