



The influence of *Nigella sativa* essential oil on proliferation, activation, and apoptosis of human T lymphocytes in vitro

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ARTICLE INFO

Keywords:

Nigella sativa
Essential oil
Thymoquinone
T cells
Proliferation
Apoptosis

ABSTRACT

In previous work, we tested the immunomodulatory effect of *Nigella sativa* (NS) fatty oil. Our results demonstrated that unrefined, obtained by cold pressing black cumin seed oil inhibited lymphocytes' proliferation and induced their apoptosis in a dose-dependent manner. In this study, we examined the immunomodulatory properties of essential oil (EO) obtained from the NS seeds by hydrodistillation and its two main constituents: thymoquinone (TQ) and p-cymene. We analyzed the proliferation, activation phenotype, and apoptosis rates of human T lymphocytes stimulated with an immobilized monoclonal anti-CD3 antibody in the presence of serial ethanol dilutions of tested oil or serial distilled water dilutions of tested compounds with flow cytometry. Our results showed that NSEO significantly inhibited the proliferation of CD4⁺ and CD8⁺ T lymphocytes, induced cell death in a dose-dependent manner, and reduced the expression of CD28 and CD25 antigens essential for lymphocyte activation. TQ inhibited the proliferation of T lymphocytes and induced cell death, particularly in high concentrations. Meanwhile, p-cymene did not influence lymphocyte proliferation. However, its high concentration induced cell necrosis. These results show that the essential oil from *Nigella sativa* has powerful immunomodulatory properties, which, at least partially, are related to the TQ component.

1. Introduction

The *Nigella sativa* (NS) seeds are sourced from an annual herbaceous plant belonging to the Ranunculaceae family. The black and redolent seeds have been traditionally used in the Middle East, Egypt, and India as an adjunct to many dishes (meat, bread). In addition, black cumin seeds are substituted for pepper due to their gentle activity on the digestive system [1]. Moreover, they were used to treat an affliction of the respiratory or digestive system, headache, fever, and rheumatism [2].

NS seeds contain 30–40% fixed oil, 20–30% protein, 3,7–4,7% ash, and 25–40% total carbohydrates with antioxidants lignans such as saponin, or melantin [3]. That percentage content depends on time, location, and method of harvesting. The oil fraction contains a fatty oil rich in unsaturated fatty acids, mainly linoleic acid (50–60%), oleic acid (20%), eicodadienoic acid (3%), and dihomolinoleic acid (10%). Saturated fatty acids (palmitic, stearic acid) amount to about 30% [4].

Additionally, the oil contains alkaloids, phytosterols, tocopherols, saponins, flavonoids, and finally, essential oil (EO) (0,4–2,5%) [5]. Numerous active compounds from the NSEO have been isolated, identified, and reported so far in many experiments. The proportion of active compounds is different, but the most important active compounds are thymoquinone (TQ), p-cymene, carvacrol, sesquiterpene longifolene, trans-anethole, 4-terpineol, thymol, and α -pinene. Studies show that some of these substances may significantly affect human health [6].

NS has been shown to possess antibacterial [7–9], antioxidant activity [10–14], and antitumor [15] properties. However, in recent years, some articles have been published showing that NS oil or extract can modulate the immune response in various diseases associated with hypersensitivity reactions, including asthma, allergic rhinitis, or rheumatoid arthritis. For example, one of the clinical trials showed that boiled extract of NS administered to asthma patients caused a reduction in disease symptoms, like the frequency of attacks and wheezing at subsequent visits [16]. In another study, a one-month supplementation with

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<https://doi.org/10.1016/j.bioph.2022.113349>

Received 24 May 2022; Received in revised form 24 June 2022; Accepted 24 June 2022

Available online 29 June 2022

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NS seeds increased the percentage of cytotoxic (CD8⁺) T cells and the activity of polymorphonuclear leukocytes in patients with allergic rhinitis [17]. Finally, Kheirouri et al. [18] demonstrated that treatment with black seed oil reduced rheumatoid arthritis (RA) symptoms. In this study, the treatment also decreased the percentage of CD8⁺ T lymphocytes and increased CD4⁺CD25⁺ T cells.

To explain some of these results, in our previous work, we examined how cold-pressed NS oil influences human lymphocytes in vitro [19]. Our results demonstrated that the lowest ethanol dilutions of NS oil had a strong antiproliferative and proapoptotic effect on human lymphocytes; only 10% of lymphocytes were proliferating in the presence of 1:1 or 1:10 oil dilutions, and there was a significant increase in the percentage of cells in the early and late apoptosis phases. Reduced proliferation capacity was associated with a decreased expression of CD4 and CD28 antigens in the presence of NS oil.

Of the many components of NSEO, the active one is primarily TQ, which was shown to have antioxidant [11,12,14,20,21], and some antitumor effects [22]. Studies have shown that the antioxidant activity of TQ manifests itself in particular under the conditions of ionizing radiation in the rat model. Moreover, different authors observed a decrease in both reactive oxygen species (ROS) [11,12,14] and reactive nitrogen species (RNS) [20] in the presence of TQ under experimental conditions. A study by Salim et al. [22] showed that TQ induced mitochondria-mediated apoptosis in an acute lymphoblastic leukemia cell line in vitro. Recently, Diab-Assaf et al. [23] demonstrated that TQ inhibited proliferation and induced apoptosis of adult T-cell leukemia in a dose-dependent manner. In another study, TQ induced apoptosis in malignant T-cells by decreasing glutathione and increasing reactive oxygen species (ROS) [24].

Both TQ and p-cymene were shown to have also anti-inflammatory activity. For example, Xie et al. [25] demonstrated that p-cymene attenuates acute lung injury induced by lipopolysaccharide (LPS) by reducing inflammatory cell infiltration and pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) in the mouse model. In another study, p-cymene reduced the production of TNF- α and IL-1 β and increased IL-10 in LPS-challenged mice and murine macrophage-like cell line RAW 264.7 [26]. In addition, TQ was shown to reduce inflammatory cell infiltration and reduced cyclooxygenase-2 (COX-2) expression and prostaglandin D2 (PGD2) production in a mouse model of allergic airway inflammation [27].

In connection with reports of the immunomodulatory effects of *Nigella sativa*, the present study investigates the influence of NSEO and its two main constituents, thymoquinone and p-cymene, on human T cells, especially their phenotype, proliferation capacity, and susceptibility to apoptosis.

2. Material and methods

2.1. Plant material

The seeds of black caraway (*Nigella sativa* L.) were obtained from Makar Bakalie Sp. z o. o. Sp. k., Katowice, Poland (country of origin: India; harvest date/batch number: 02.2019/3505 0912). The material was stored in the dark in a hermetically sealed container. The moisture content of seeds was 5,74% (corrected to NSEO content), as determined by convection drying (105 °C, for 4 h, Binder FD oven, Tuttlingen, Germany). The NSEO samples were obtained within three months since the material was acquired. NSEO isolation and GC analysis were performed in the Department of Pharmacognosy of the Medical University of Gdańsk and the Department of Analytical Chemistry of the Gdańsk University of Technology.

2.2. Essential oil isolation

For NSEO isolation, a 100-g sample of seeds was frozen in liquid nitrogen and immediately ground to 0.25–0.8 mm size (15,000 rpm, 10

s; SM-450 grinder, Envisense, Lublin, Poland). The still-frozen material was moved into a 1000 ml round-bottom flask, added 400 ml water with a 0.05% (w/v) SE-15 antifoam agent (Sigma Aldrich Inc., USA), and connected to etheric oils distillation apparatus (Carl-Roth, Karlsruhe, Germany). The apparatus specification was according to European Pharmacopoeia, as presented by Bicchi [28]. The heating was provided by stirred electromantle (EMEA3, 750 rpm; Cole-Parmer, UK) and adjusted to yield a condensate flow of 3 ml/min. The hydrodistillation time was 3.0 h. Afterward, the apparatus was left to cool down for 60 min. NSEO volume was measured, and its content was expressed as % v/w. The NSEO sample was collected through the venting port of the distillation apparatus, added 100 mg anhydrous sodium sulfate, and stored in a sealed vial for 24 h at 8°C. The dehydrated sample was collected from the above sodium sulfate layer, moved to a clean vial, and stored at 8°C.

2.3. GC analysis

The qualitative GC/MS analyses of NSEO samples were conducted using a 7890 A gas chromatography coupled with a 5977 A mass selective detector, and quantitative GC/FID analyses were conducted using a 5977 A gas chromatograph with a flame ionization detector (Agilent Technologies, USA). For GC/MS analysis, 10.0 μ l of the sample was diluted with ethyl acetate (1:80 v/v) and injected (model 7693, Agilent) into the DB-5 ms 30 m x 0.25 mm x 0.25 μ m capillary column (Agilent J&W) at 250°C, at a split ratio of 1:10. The carrier gas (helium) flow was 1.1 ml/min. The oven temperature increased from 50°C to 280°C at a 7°C per minute and was held at 280°C for 20 min. The total run time was 53 min. The GC/FID analyses were conducted using the DB-5 30 m x 0.32 mm x 0.25 μ m column with the same temperature program. The flow of carrier gas (helium) was 1.5 ml/min. The obtained data were compared with retention indices and spectra from NIST Library 11.0. The main constituents of NSEO are listed in Table 1.

Table 1

Chemical composition of essential oil obtained from *Nigella sativa* seeds, determined by GC-FID. The percentage values represent means \pm SD (n = 6).

Compound	t _R (min)	RI _{exp}	RI _{lit}	Content (%)
α -thujene	5251	920	928	14,38 \pm 1,29
α -pinene	5378	928	931	3,09 \pm 0,28
camphene	5679	945	943	0,05 \pm 0,01
β -phellandrene/sabinene	6180	973	975	2,43 \pm 0,86
β -pinene	6250	979	978	2,85 \pm 0,89
n-decane	6556	1000	1000	0,07 \pm 0,02
α -terpinene	7129	1019	1008	0,87 \pm 0,02
p-cymene	7372	1029	1013	49,27 \pm 0,50
D-limonene	7431	1032	1020	2,42 \pm 0,09
eucalyptol	7497	1035	1054	0,06 \pm 0,03
γ -terpinene	8136	1061	1047	3,36 \pm 0,20
cis- β -terpineol	8360	1070	1125	0,03 \pm 0,01
p-cymenene	8879	1091	1073	0,13 \pm 0,01
cis-4-methoxy-thujane	9067	1100	1040 _{est}	0,99 \pm 0,04
n-undecane	9180	1100	1100	0,04 \pm 0,01
trans-4-methoxy-thujane	9641	1123	1040 _{est}	5,83 \pm 0,28
terpinen-4-ol	11,145	1181	1161	0,73 \pm 0,11
n-dodecane	11,386	1200	1200	0,02 \pm 0,02
β -cyclocitral	11,837	1207	1196	1,24 \pm 0,10
thymoquinone	13,007	1255	1216	2,26 \pm 0,22
bornyl acetate	13,949	1293	1269	0,17 \pm 0,01
thymol	14,167	1304	1262	0,09 \pm 0,03
carvacrol	14,396	1305	1278	1,57 \pm 0,37
α -longipinene	15,545	1361	1358	0,81 \pm 0,06
longifolene	16,911	1417	1402	3,69 \pm 0,25
caryophyllene	17,251	1430	1424	0,06 \pm 0,01
β -bisabolene	19,365	1514	1496	0,06 \pm 0,01
4-methoxy-2,3,5-trimethylphenol	20,531	1555	1430	0,50 \pm 0,13
Total (%)				97,06 \pm 0,57

RI_{exp}, experimental retention index; RI_{lit}, literature retention index (according to NIST database); t_R, retention time.

2.4. Blood samples

The study groups comprised 18 prescreened, healthy people with a mean age of $28 \pm 5,7$. None of them took in any medicinal products that influence the immune system. All participants were informed about the purpose of the tests and gave their written informed consent. The Bioethical Committee for Scientific Research at the Medical University of Gdansk approved the study. We performed all the experiments following the relevant guidelines and regulations.

We collected 25 ml of peripheral venous blood from each volunteer in tubes containing EDTA as the anticoagulant after overnight fasting.

2.5. PBMC isolation and stimulation

We isolated peripheral blood mononuclear cells (PBMCs) by centrifugation on Histopaque®– 1077 gradient (Sigma Aldrich Inc., USA). PBMCs were stained with Violet Proliferation Dye 450 (VPD450) (Becton Dickinson, USA) for 10–15 min in the dark at 37 °C according to Witkowski's protocol [29] and resuspended in a complete culture medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin). Then cells (1,5 million on each well plate) were incubated with an immobilized monoclonal anti-CD3 antibody with the addition of 1:10, 1:50, 1:100, 1:500, or 1:1000 ethanol (EtOH) dilution of NSEO in standard culture conditions (5% CO₂, 100% humidity at 37°C) for 5 days. Additionally, cells were also incubated with anti-CD3 antibody alone or with ethanol as a control.

Cells were also incubated with TQ and p-cymene (Sigma Aldrich Inc., USA). TQ was $\geq 98\%$ pure, and the stock solution was prepared with EtOH at a 1 mg/ml concentration. This stock was stored at -80°C . P-cymene was 99% pure and was stored at 4°C in a dark glass bottle. Before every experiment, the used dilutions of with were prepared with sterile distilled water. P-cymene was diluted with EtOH, and the rest of the solutions were diluted with sterile distilled water. Cells were incubated with an immobilized monoclonal anti-CD3 antibody with the addition of 200, 100, 20, 10 µg/ml TQ, 50, 5, 0,5, 0,05% p-cymene or their different combinations in standard culture conditions (5% CO₂, 100% humidity at 37°C) for 5 days.

Stimulated PBMCs were collected after 72 and 120 h and stained with the following antibodies: anti-CD4 conjugated with peridinin-chlorophyll-protein (PerCP), anti-CD8 conjugated with allophycocyanin-hilite7 (APC-H7), anti-CD28 conjugated with phycoerythrin (PE), and anti-CD25 conjugated with fluorescein-5-isothiocyanate (FITC) (all from BD Pharmingen, USA). In addition, cells also were stained with PE-conjugated annexin V and 7-aminoactinomycin D (7-AAD) according to the manufacturer protocol (BD Pharmingen, USA). Finally, stained cells were analyzed with flow cytometry using the FACSVerse instrument (Becton Dickinson, USA).

2.6. Analysis and statistics

Thirty thousand events corresponding to lymphocytes' light scatter characteristics were acquired from each sample to analyze proliferation capacity, cell susceptibility to apoptosis and necrosis, and antigen expression (Suppl. Fig. 1). We used FCSalyzer (copyright (C) 2012–2019 Sven Mostböck) to perform cytometric analysis. First, the lymphocytes were selected based on forward and side scatter characteristics (FSC and SSC) and their positivity for surface antigens (CD4, CD8, CD28, and CD25). Then, the dividing cell tracking (DCT) method was used to determine percentages of dividing cells after different simulation combinations. DCT uses VPD450, which passively diffuses across cell membranes and is cleaved by esterase activity within viable cells. The cleaved dye becomes fluorescent and covalently binds to proteins within the cells. As viable cells divide, the VPD450 dye is distributed uniformly between daughter cells so that each daughter cell retains approximately half of the VPD450 fluorescence intensity of its parent cell. In

Supplementary Figure 1, non-dividing cells are indicated with marker 1 (M1), while proliferating cells are indicated as M2. Finally, based on annexin V and 7-AAD staining, we identified cells as alive – cells negative for annexin V and 7-AAD, in early apoptosis – annexin V-positive 7-AAD-negative cells, in late apoptosis – cells positive for both annexin V and 7-AAD, and necrotic – cells only 7-AAD-positive (Suppl. Fig. 1).

Statistical data analysis was done using the GraphPad Prism program, version 9 (GraphPad Software, USA). Shapiro-Wilk and Kolmogorov-Smirnov tests were used to test for normal distribution. Since data did not pass the normality tests, an appropriate nonparametric test for repeated measures (indicated in the Results and Figure legends) was chosen with a significance level of $p < 0.05$.

3. Results

3.1. Hydrodistillation and GC analysis of NSEO

The EO content of NS seeds was $0,260 \pm 0,017\%$ v/w ($n = 6$) as determined by hydrodistillation, and the density of the volatile fraction was 0,86 g/ml. The composition of NSEO was determined by GC/MS, and the volatiles were quantified using GC/FID. The significant component of NSEO was p-cymene, which constituted roughly half of the volatile fraction (49,27%). The detailed composition of NSEO is presented in Table 1.

3.2. Influence of NSEO on T cell proliferation and antigen expression

The decrease of proliferating cells in the presence of NSEO dilutions was significant compared with cells incubated only with an immobilized antibody alone (control) or combined with 1:1000 dilution. Fig. 1A presents the percentage of proliferating CD4⁺ cells after 72 h of incubation with NSEO. There was a significant difference between cells incubated with immobilized antibody alone and combined with 1:10, 1:50 NSEO dilution variants, cells incubated with 1:10 and 1:500 NSEO dilutions, and between 1:10, 1:50, 1:100 and 1:1000 dilution variants.

After 120 h (Fig. 1B), the statistical significance existed between the percentage of cells incubated with immobilized antibody and 1:10, 1:50, 1:100 NSEO variant dilutions, between 1:10, 1:50, 1:100, and 1:1000 dilutions. Fig. 1C demonstrates the percentage of proliferating CD8⁺ cells after 72 h. There was a significant difference in the percentage of proliferating cells between incubated with immobilized antibody alone and combined with 1:10 and 1:50 NSEO dilutions, between 1:10, 1:50, and 1:1000 NSEO dilutions. Finally, Fig. 1D presents the percentage of proliferating CD8⁺ cells after 120 h. The statistical significance was seen between cells stimulated with anti-CD3 antibody alone and combined with 1:10, 1:50, 1:100 NSEO dilutions, and between 1:10, 1:50, 1:100, and 1:1000 variants.

The changes were also observed in the expression of main T cell antigens: CD4, which identifies helper T lymphocytes, CD28 – major costimulatory antigen of T lymphocytes (Suppl. Fig. 2), and CD25 (interleukin-2 receptor alpha chain) (Suppl. Fig. 3), which is a type I of transmembrane protein present on activated T cells. As seen in representative dot-plots, in the presence of 1:10, 1:50, 1:100, and 1:500 NSEO dilution, cells were characterized by reduced expression of CD4, CD28, and CD25 antigens. In addition, CD8⁺ cells (here seen on the dot-plots as CD4-negative cells) showed similar changes in CD28 and CD25.

3.3. Influence of NSEO on cell death

Supplementary Figure 4 demonstrates how NSEO changes the proportions of living and dying cells. We used annexin V and 7-AAD staining to distinguish between alive cells (annexin V and 7-AAD negative), cells in early apoptosis (annexin V positive and 7-AAD negative), in late apoptosis (positive for both annexin V and 7-AAD), and necrotic cells (cells that are only 7-AAD positive). Representative dot-plots show the

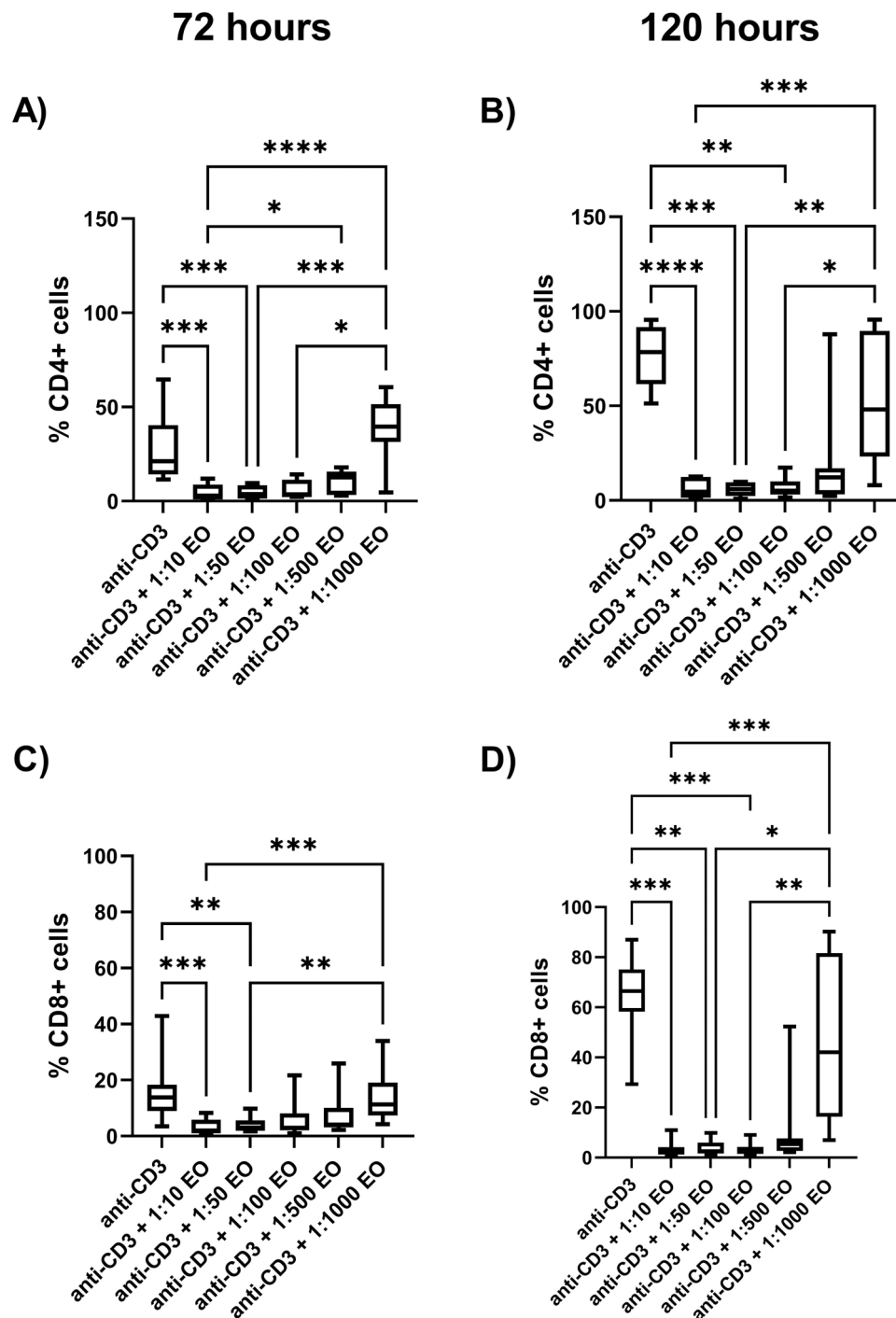


Fig. 1. Comparison of percentage of proliferating CD4⁺ (A, B) and CD8⁺ cells (C, D) stimulated with an anti-CD3 antibody with different dilutions of NSEO for 72 (A, C) and 120 (B, D) hours. Graphs show median, percentiles with the maximum and minimum value of ten independent experiments, ANOVA Friedman with Dunn's post hoc test, * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$, **** $p < 0,0001$.

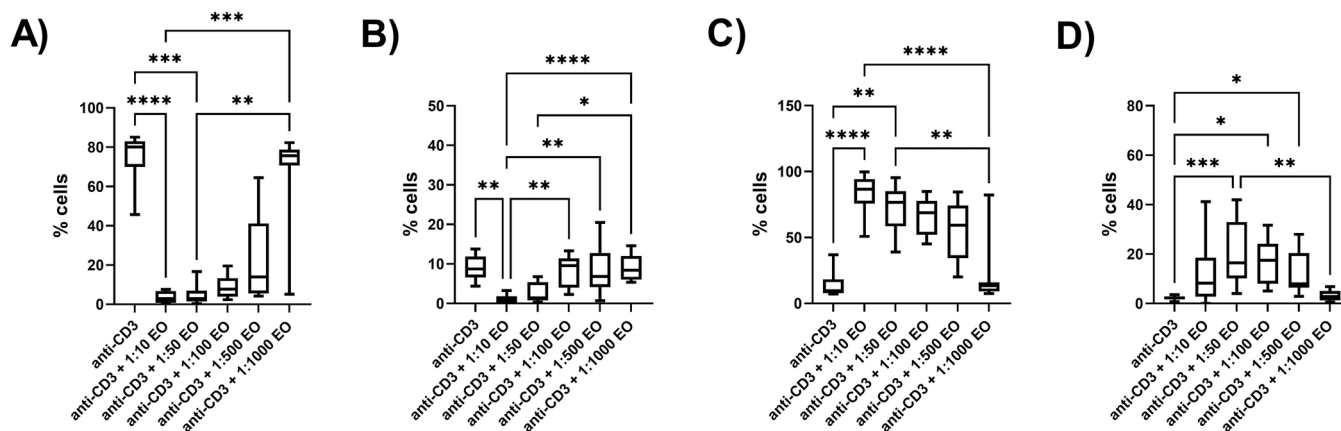
percentage of alive cells after incubation with an immobilized CD3 antibody alone (Suppl. Fig. 4A) or combined with 1:1000 NSEO dilution (Suppl. Fig. 4F) exceeding 80%. In 1:10, 1:50, and 1:100 NSEO dilution variants, most cells are in late apoptosis (Suppl. Fig. 4B-D). When cells are incubated in the presence of 1:500 NSEO dilution, approximately 14% of cells are alive, 20% of cells are in early apoptosis, less than 60% are in late apoptosis, and over 6% cells are necrotic.

The decrease of living cells and increase of cells in late apoptosis in the presence of 1:10, 1:50, or 1:100 NSEO dilutions was significant compared with cells incubated only with an immobilized anti-CD3

antibody alone or a combination with 1:1000 NSEO dilution after 72 (Fig. 2A and C) and 120 (Fig. 2E and G) hours of stimulation. In addition, there was a significant decrease of cells in the stage of early apoptosis in the presence of 1:10 and 1:50 NSEO dilutions (Fig. 2B and F). Also, the percentage of necrotic cells was significantly increased when cells were incubated with 1:10, 1:50, or 1:100 NSEO dilution variants but only after 72 h of stimulation (Fig. 2D).



72 hours



120 hours

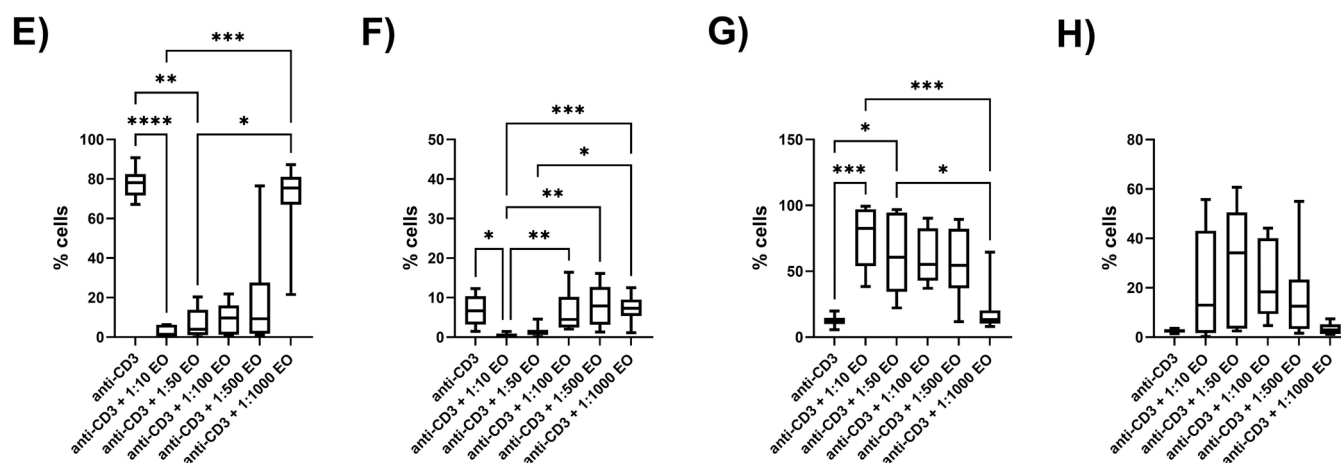


Fig. 2. Comparison of percentage of living, apoptotic, and necrotic lymphocytes stimulated for 72 (A-D) and 120 (E-H) hours with an anti-CD3 antibody with different dilutions of NSEO. Graphs show median, percentiles with the maximum and minimum value of nine independent experiments, ANOVA Friedman with Dunn's post hoc test, * $p < 0,05$, ** $p < 0,01$, *** $p < 0001$, **** $p < 0,0001$.

3.4. Influence of thymoquinone and p-cymene on T cell proliferation and apoptosis

Next, we examined the influence of TQ and p-cymene on the proliferation and apoptosis of lymphocytes. Fig. 3 shows the percentage of proliferating $CD4^+$ and $CD8^+$ cells stimulated with an immobilized anti-CD3 antibody and different TQ and p-cymene concentrations added separately (Fig. 3A-D). Fig. 3A and B present the percentage of proliferating $CD4^+$ cells after 72 and 120 h of incubation, respectively. There was a significant decrease in the percentage of proliferating cells in the presence of TQ highest concentrations (200 and 100 $\mu\text{g}/\text{ml}$) compared to cells incubated with anti-CD3 antibody alone or combined with p-cymene. A similar effect was observed for $CD8^+$ cells (Fig. 3C and D). On the other hand, p-cymene combined with an anti-CD3 antibody did not affect the proliferation of $CD4^+$ or $CD8^+$ cells (Fig. 3A-D).

Fig. 3E-H shows the percentage of proliferating $CD4^+$ and $CD8^+$ cells stimulated with an immobilized anti-CD3 antibody with TQ and p-cymene mixed up in different combinations. There was a significant decrease in the percentage of proliferating $CD4^+$ (Fig. 3E) and $CD8^+$ (Fig. 3G) cells in the presence of 200 $\mu\text{g}/\text{ml}$ TQ combined with 50% or 0,05% p-cymene after 72 h of incubation. However, no difference was

seen in the percentage of proliferating $CD4^+$ or $CD8^+$ cells in the presence of 100 $\mu\text{g}/\text{ml}$ TQ and 5% or 0,5% p-cymene (Fig. 3E-H). After 120 h, a considerable reduction in the proliferating $CD4^+$ (Fig. 3F) and $CD8^+$ (Fig. 3H) cells was observed in the presence of 200 $\mu\text{g}/\text{ml}$ TQ and 50% p-cymene. Combinations of lower concentrations of TQ combined with different concentrations of p-cymene did not affect the proliferation capacity of lymphocytes.

Fig. 4 compares the percentage of living, apoptotic, and necrotic lymphocytes stimulated with an anti-CD3 antibody with different concentrations of TQ or p-cymene for 72 h and 120 h. In the presence of 200 $\mu\text{g}/\text{ml}$ TQ, there was a significant decrease of alive cells after 72 (Fig. 4A) and 120 (Fig. 4E) hours of stimulation. At the same time, the percentage of cells in late apoptosis (Fig. 4C and G) or necrosis (Fig. 4D and H) was increased in the presence of 200 $\mu\text{g}/\text{ml}$ TQ. No difference was seen in the percentage of cells in early apoptosis (Fig. 3B and F). In general, p-cymene alone did not affect the percentage of living or apoptotic cells. However, in the presence of 50% p-cymene, there was a significant increase in the percentage of necrotic cells after 72 (Fig. 4D) and 120 (Fig. 4H) hours.

Fig. 5 shows the comparison of the percentage of living, apoptotic, and necrotic lymphocytes stimulated with an anti-CD3 antibody with TQ

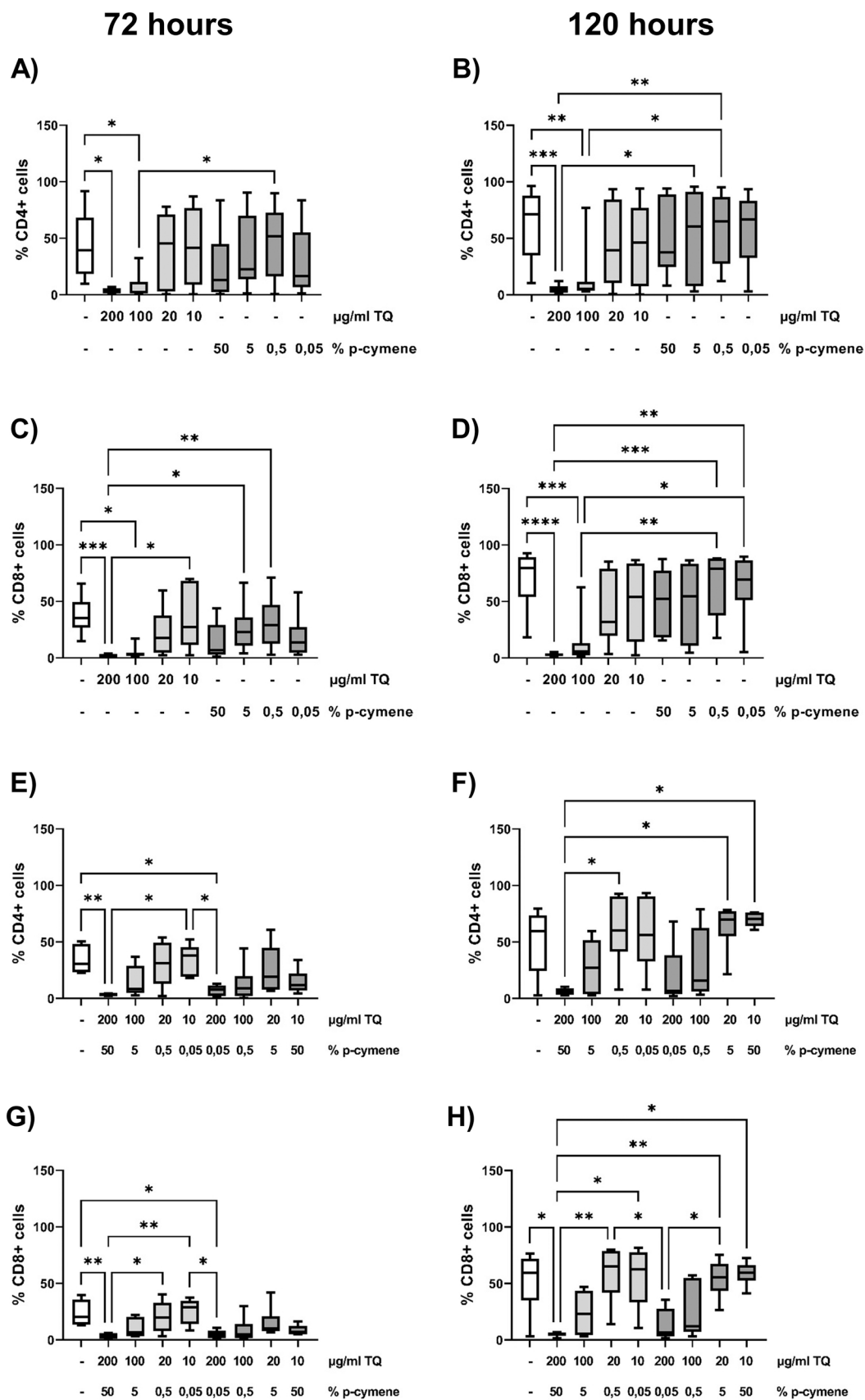


Fig. 3. Comparison of percentage of proliferating CD4⁺ and CD8⁺ cells stimulated with an anti-CD3 antibody alone (-) and with different dilutions of TQ and p-cymene and their combinations for 72 (A, C, E, G) and 120 (B, D, F, H) hours. Graphs show median, percentiles with the maximum and minimum value of seven independent experiments, ANOVA Friedman with Dunn's post hoc test, * p < 0,05, ** p < 0,01, *** p < 0001, **** p < 0,0001.

72 hours

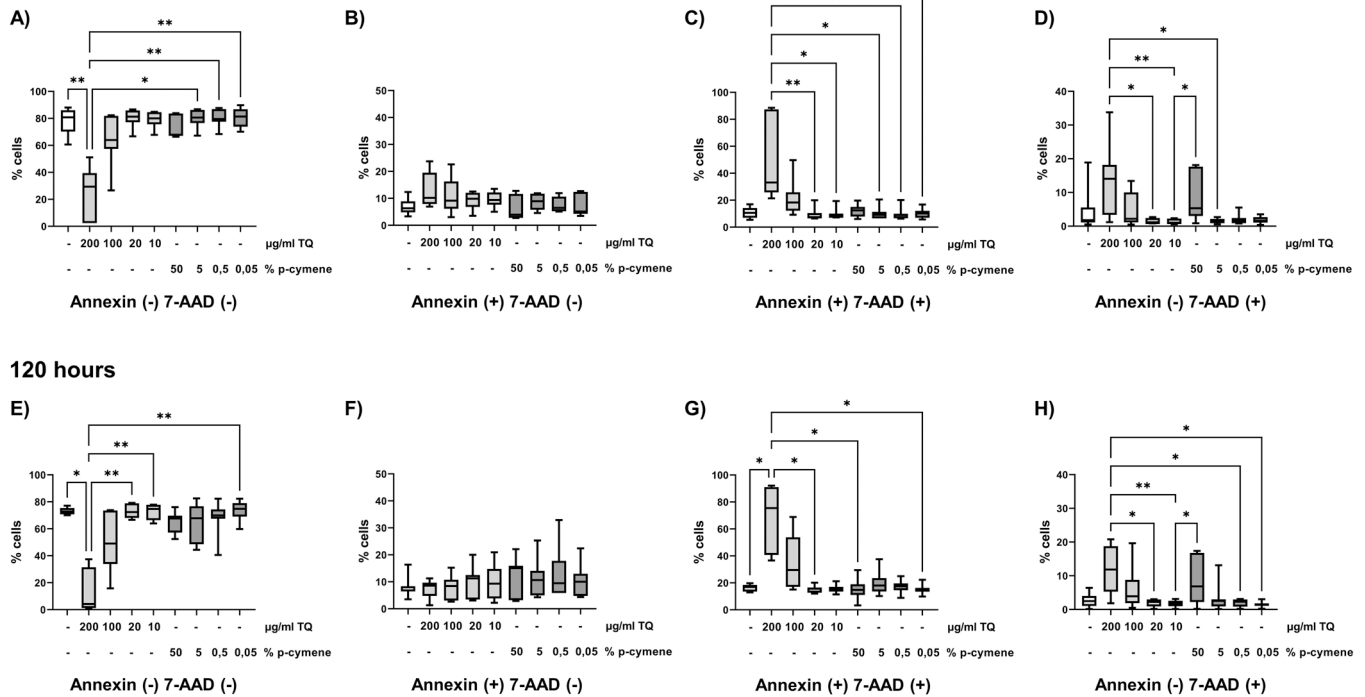


Fig. 4. Comparison of the percentage of living, apoptotic, and necrotic lymphocytes stimulated for with an anti-CD3 antibody alone (-) and with different TQ and p-cymene dilutions for 72 (A-D) and 120 (E-H) hours. Graphs show median, percentiles with the maximum and minimum value of seven independent experiments, ANOVA Friedman with Dunn's post hoc test, * $p < 0,05$, ** $p < 0,01$.

72 hours

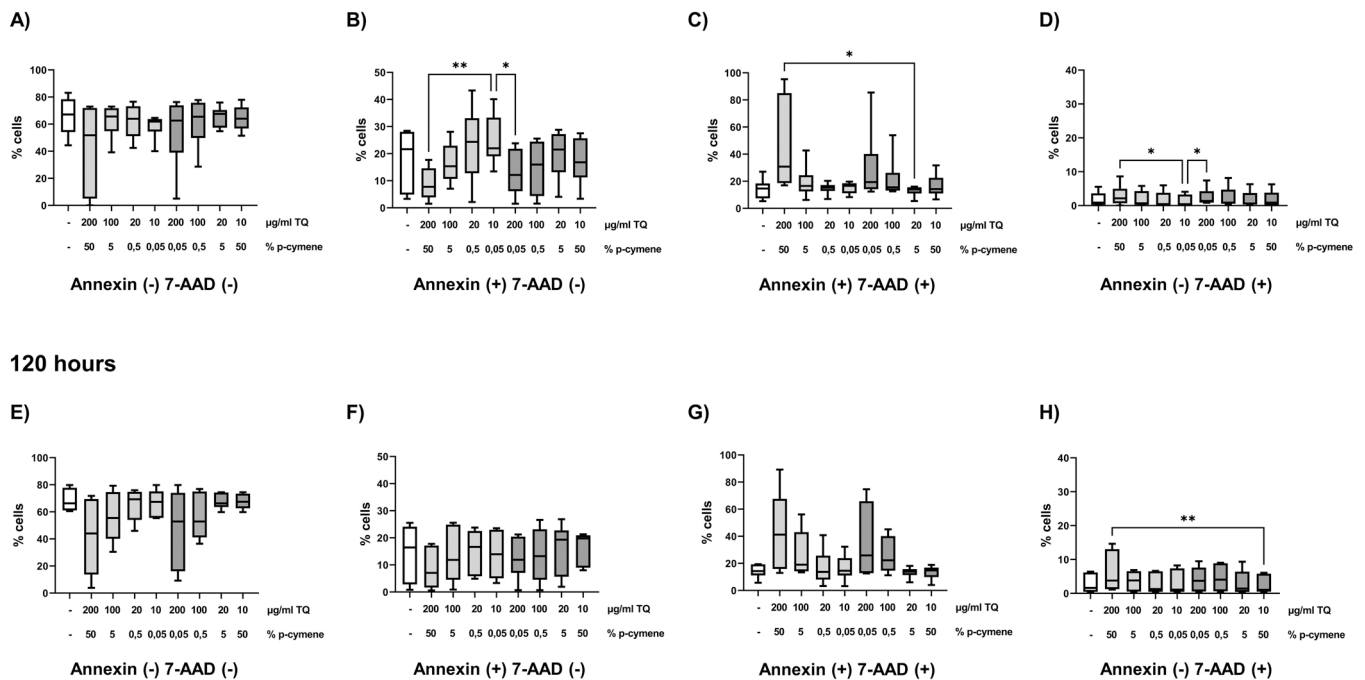


Fig. 5. Comparison of percentage of living, apoptotic, and necrotic lymphocytes stimulated for 72 h with an anti-CD3 antibody alone (-) and with different combinations of dilutions of TQ and p-cymene for 72 (A-D) and 120 (E-H) hours. Graphs show median, percentiles with the maximum and minimum value of six independent experiments, ANOVA Friedman with Dunn's post hoc test, * $p < 0,05$, ** $p < 0,01$, (n = 6).

and p-cymene mixed up in different combinations. There was no significant difference in the percentage of living cells after 72 (Fig. 5A) and 120 (Fig. 5E). There was a significant difference in the percentage of

cells in early apoptosis in the presence of 200 µg/ml TQ combined with 50% or 0,05% p-cymene but only after 72 h of incubation (Fig. 5B). The percentage of cells in late apoptosis was only increased in the presence

of 200 µg/ml TQ combined with 50% p-cymene but only compared to cells incubated in the presence of 20 µg/ml TQ combined with 5% p-cymene for 72 h (Fig. 5C). No difference was seen after 120 h (Fig. 5G). There was a significant increase of necrotic cells but only in the presence of 200 µg/ml TQ combined with 50% or 0,05% p-cymene but only compared to a combination of 10 µg/ml TQ combined with 0,05% p-cymene for 72 h (Fig. 5D). After 120 h, the difference in the percentage of necrotic cells was observed only between a combination of 200 µg/ml TQ with 50% p-cymene and 10 µg/ml TQ with 50% p-cymene (Fig. 5H).

4. Discussion

As shown in the Results, the EO content of *Nigella sativa* seeds was less than 0,5. According to previous papers concerning NS volatiles, the reported yield falls within a wide range of 0,08–1,7% [30,31]. As revealed by GC analysis, the significant component of NSEO was p-cymene, which constituted roughly half of the volatile fraction (49, 27%). This observation agrees with several previous studies that showed p-cymene as a dominant constituent of NSEO [32,33].

Recently, we have demonstrated that cold-pressed NS oil influences human lymphocytes in vitro [15]. It had a solid antiproliferative and proapoptotic effect on T cells accompanied by reduced expression of CD4 and CD28 antigens. Therefore, we decided to explore how essential oil from NS seeds influences human T cells in vitro. In the presented model, the human PBMCs were stimulated with an immobilized monoclonal anti-CD3 antibody in the presence of different NSEO dilution variants. Thus, T cell activation was mediated through the TCR/CD3 complex, which in vivo is associated with recognizing antigens. Next, we examined the proliferation capacity of helper (CD4⁺) and cytotoxic (CD8⁺) T cells and cell susceptibility to apoptosis and necrosis.

Our findings demonstrate that the lowest (1:10, 1:50, and 1:100) NSEO dilutions significantly inhibited the proliferation of CD4⁺ and CD8⁺ T cells, reducing the percentage of dividing cells to 2–3%. Additionally, NSEO had powerful proapoptotic effects, especially in the presence of the lowest (1:10 and 1:50) dilutions. The presented results showed an increase in the percentage of cells, mainly in the late apoptosis phase. The action of NSEO is much stronger than that of cold-pressed oil; oil also induced cell apoptosis, but there was a marked increase in the percentage of cells, especially in its early phase [15]. Also, after the first three days of incubation with NSEO, necrosis symptoms appear in the cells incubated with the lowest dilutions. Reduced proliferation capacity and susceptibility to apoptosis were accompanied by changes in two crucial antigens, CD28 and CD25, which are essential for activating T cells.

Our results indicate that alleviation of symptoms after consuming NS oil, seeds, or extract seen in patients with asthma, allergic rhinitis, or RA maybe be related to NSEO content. Both allergy and autoimmune diseases are linked with abnormal function of CD4⁺ T cells, which can recognize allergens or autoantigens and trigger a response of CD8⁺ T cells and induce humoral response dependent on B cell activity. The EO content in NS seeds is relatively low (less than 0,5%), but our in vitro studies show it exhibits powerful antiproliferative, proapoptotic, and even pronecrotic properties. The obtained results indicate that while the consumption of cold-pressed NS oil would not be harmful, the direct effect of NSEO may be associated with a robust cellular response. Recently, Gaudin et al. [34] have reported the case of three people who consulted a doctor because of acute contact dermatitis after applying NS oil directly to the skin. They presented polymorphic skin lesions spreading beyond the area of oil application, and the skin biopsy revealed keratinocyte apoptosis with a moderate perivascular infiltrate of lymphocytes in the dermis.

To identify the compound responsible for observed NSEO properties, we repeated experiments using TQ and p-cymene. P-cymene is a dominant constituent of NSEO. Meanwhile, TQ is described in the literature as a compound responsible for antioxidant and anti-inflammatory effects. Our findings demonstrate that TQ in the highest concentrations

(200, 100 µg/ml) significantly inhibited the proliferation of CD4⁺ and CD8⁺ cells, while p-cymene did not affect lymphocyte proliferation. Moreover, it seemed to protect cells from the antiproliferative activity of TQ, but only when the TQ was at the dose of 100 µg/ml. Also, in its highest concentration of 200 µg/ml, TQ significantly reduced the percentage of living cells and increased the percentage of cells in late apoptosis or necrotic cells. Interestingly, even though 50% p-cymene alone showed a pronecrotic effect at such a concentration, the percentage of necrotic cells was reduced when both 200 µg/ml TQ and 50% p-cymene were present in cell culture. Also, the percentage of living cells was higher in the presence of 200 µg/ml TQ and 50% p-cymene.

It can be concluded that TQ has significant antiproliferative and cytotoxic properties, which is in line with the observations of other authors, at least with regard to the effect on the cells of the immune system. Diab-Assaf et al. [23] demonstrated that TQ inhibited proliferation and induced apoptosis of T cells responsible for adult T-cell leukemia by an up-regulation of p53, and p21 and a down-regulation of Bcl-2, while Dergarabetian et al. [24] showed that it induced apoptosis in malignant T cells by increasing ROS production. Furthermore, in the most recent study by Glamoclija et al. [35], TQ alone or combined with metformin has been shown to induce apoptosis and inhibit the proliferation of different leukemia cell lines, including cells resistant to imatinib. In our experimental model, p-cymene, at least partially, counteracted the effects of TQ. P-cymene is mainly known for its anti-inflammatory properties, resulting from reducing inflammatory cell infiltration and pro-inflammatory cytokine production in various animal models [25,36], which could explain how it protects cells from the effects of TQ in vitro.

5. Conclusions

Our studies demonstrate that essential oil sourced from *Nigella sativa* seeds has a potential proapoptotic and antiproliferative effect on human T lymphocytes in vitro. TQ, one of the NSEO components, may be responsible for these properties. However, it should be emphasized that there is only a few percent of the TQ in NSEO. So TQ is probably not the only one responsible for such strong immunomodulatory properties of NSEO. Meanwhile, even though p-cymene seems to have some pronecrotic properties, at the same time, it at least partially counteracts some of the effects of TQ. Our results could explain the immunosuppressive effect of NS seeds, extracts, or oils in patients suffering from diseases resulting from hypersensitivity reactions, like asthma or rheumatoid arthritis, but adverse skin reactions in other patients after applying the oil. Therefore, additional studies are necessary to determine in what form NS can be used safely in patients suffering from allergic or autoimmune diseases.

Funding

This work was supported by the statutory funds of the Medical University of Gdańsk: 02–0058/07 (granted to J.M.W.) and Young Researcher's grant 01–0422/08 (granted to K.C.-F.).

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

All cytometric analyses were performed using the instruments acquired for the Cellular Function and Pathology Imaging Network of the Medical University of Gdańsk and the University of Gdańsk.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at [doi:10.1016/j.biopha.2022.113349](https://doi.org/10.1016/j.biopha.2022.113349).

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