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Wpływ olejku eterycznego z nasion czarnuszki siewnej (*Nigella sativa*) na aktywację, proliferację i apoptozę limfocytów T

ROZPRAWA NA STOPIEŃ DOKTORA W DZIEDZINIE NAUK MEDYCZNYCH

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Niniejszą pracę dedykuję synowi Cezaremu, pamiętaj, że w życiu nie ma rzeczy niemożliwych.

SPIS TREŚCI

SŁOWA KLUCZOWE / KEYWORDS

Słowa kluczowe:

Czarnuszka siewna,
Olejek eteryczny,
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Nigella sativa,
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T Lymphocytes,
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Inhibition of proliferation,
Proapoptotic activity

WYKAZ SKRÓTÓW / LIST OF ABBREVIATIONS

7-AAD – 7-aminoaktynomycyna D / *7-aminoactinomycin D*

CTLA-4 – antygen-4-cytotoksycznych limfocytów T / *cytotoxic T-lymphocyte-associated antigen-4*

DCT – śledzenie komórek dzielących się / *dividing cell tracking*

EDTA – kwas etylenodiaminotetraoctowy / *ethylenediaminetetraacetic acid*

EO – olejek eteryczny / *essential oil*

EtOH – etanol / *ethanol*

GC – chromatografia gazowa / *gas chromatography*

GC/MS – chromatografia gazowa spektrometria masowa / *gas chromatography mass spectrometry*

HLA – ludzki antygen leukocytarny / *human leukocyte antigen*

HT – zapalenie tarczycy Hashimoto / *Hashimoto's thyroiditis*

IFN-γ - interferon gamma / *interferon gamma*

IL – interleukina / *interleukin*

NO – tlenek azotu / *nitric oxide*

NS – czarnuszka siewna / *Nigella sativa*

NSEO – olejek eteryczny z nasion czarnuszki siewnej / *Nigella sativa essential oil*

PBS – sól fizjologiczna buforowana fosforanami / *phosphate-buffered saline*

PMBC – jednojądrzaste komórki krwi obwodowej / *peripheral blood mononuclear cells*

RZS – reumatoidalne zapalenie stawów / *rheumatoid arthritis*

TG – tyreoglobulina / *thyroglobulin*

TGF-β – transformujący czynnik wzrostu beta / *transforming growth factor beta*

TNF – czynnik nekrozy nowotworu / *tumor necrosis factor*

TPO – peroksydaza przeciwtarczycowa / *thyroid peroxidase*

TQ – tymochinon / *thymoquinone*

TSH – hormon stymulujący tarczycę / *thyroid-stimulating hormone*

T3 – trójjodotyronina / *triiodothyronine*

VPD450 – fioletowy barwnik proliferacyjny 450 / *Violet Proliferation Dye 450*

WBC – krwinki białe / *white blood cells*

WYKAZ PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ / LIST OF PUBLICATIONS INCLUDED IN THE DOCTORAL DISSERTATION

1. Praca oryginalna

   Ciesielska-Figlon K, Daca A, Kokotkiewicz A, Łuczkiewicz A, Zabiegała B, Witkowski J, Lisowska K.

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

   Biomedicine & Pharmacotherapy 2022; vol. 153: 113349.

   Impact Factor – 7.5; Punktacja MNiSW – 100; Q1

2. Praca oryginalna

   Ciesielska-Figlon K, Wojciechowicz K, Daca A, Kokotkiewicz A, Łuczkiewicz A, Witkowski J, Lisowska K.

   The impact of Nigella sativa essential oil on T cells in women with Hashimoto's thyroiditis.

   Antioxidants 2023; vol. 12: 1246.

   Impact Factor – 7.0; Punktacja MNiSW – 100; Q1

3. Praca przeglądowa

   Ciesielska-Figlon K, Wojciechowicz K, Wardowska A, Lisowska K.

   The immunomodulatory effect of Nigella sativa.

   Antioxidants 2023; vol. 12: 1340.

   Impact Factor – 7.0; Punktacja MNiSW – 100; Q1

   Łączny współczynnik Impact Factor: 21.5

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STRESZCZENIE

Substancje pochodzenia roślinnego cieszą się niezmiennie dużym zainteresowaniem naukowców, a wśród roślin wymienianych w literaturze, ważne miejsce zajmuje czarnuszka siewna (*Nigella sativa*). Roślina należy do rodziny Jaskrowatych. Jej owoce zawierają drobne i aromatyczne nasiona, z których pozyskuje się olej, zawierający frakcję tłustą bogatą w nienasycone kwasy tłuszczowe, frakcję eteryczną oraz substancje pochodzenia roślinnego takie jak: alkaloidy, tokoferole, fitosterole, saponiny i flawonoidy. Uznaje się, że frakcja eteryczna ma największy potencjał immunomodulacyjny, ze względu na zawarte w niej substancje aktywne, takie jak tymochinon, p-cymen, alfa-tujen czy karwakrol. Pozyskiwany w procesie tłoczenia na zimno olej, w starożytności nazywany „złotem Faraonów", był uważany za panaceum na wiele różnych chorób, zwłaszcza układu oddechowego, pokarmowego i sercowo-naczyniowego. Obserwacje kliniczne wskazują, że nasiona, ekstrakty i olej z czarnuszki siewnej mogą łagodzić objawy chorób o podłożu immunologicznym takich jak astma, alergiczny nieżyt nosa oraz reumatoidalne zapalenie stawów. Z kolei tymochinon ma właściwości antyoksydacyjne i przeciwnowotworowe, a wraz z p-cymenem wykazuje właściwości przeciwzapalne. Dokładny mechanizm działania czarnuszki siewnej na układ odpornościowy nie jest jednak w pełni poznany.

W niniejszej rozprawie badano właściwości immunomodulacyjne olejku eterycznego otrzymanego z nasion czarnuszki siewnej oraz substancji chemicznych zidentyfikowanych w olejku eterycznym: tymochinonu oraz p-cymenu. Właściwości te oceniano u osób zdrowych oraz kobiet ze zdiagnozowanym zapaleniem tarczycy Hashimoto. Za pomocą cytometrii przepływowej analizowano fenotyp powierzchniowy, aktywność proliferacyjną oraz podatność na apoptozę ludzkich limfocytów T, stymulowanych monoklonalnym przeciwciałem anty-CD3, w obecności seryjnych, etanolowych rozcieńczeń badanego olejku oraz tymochinonu i p-cymenu. Dodatkowo oceniono zmiany profilu cytokinowego w nadsączach pohodowlanych. Uzyskane wyniki wykazały, że olej eteryczny z nasion czarnuszki siewnej znacząco hamuje proliferację limfocytów T $CD4^+$ i $CD8^+$ oraz indukuje śmierć komórek w sposób zależny od dawki, zarówno u osób zdrowych jak i pacjentek z zapaleniem tarczycy Hashimoto. Ponadto, olej eteryczny obniża ekspresję antygenów CD28 i CD25 istotnych dla aktywacji limfocytów. Antyproliferacyjny i proapoptotyczny efekt olejku eterycznego był związany z aktywnością tymochinonu. Z kolei p-cymen nie wpływał na proliferację ludzkich limfocytów T, ale w najwyższym stężeniu indukował ich

nekrozę, a także częściowo przeciwdziałał niektórym skutkom obecności tymochinonu. Olej eteryczny znacząco obniżał stężenie TNF, IL-17A i IL-10 w nadsączach pohodowlanych. Z kolei poziom IL-4 i IL-2 istotnie wzrastał w obecności olejku. Olejek nie wpływał na stężenie IL-6 i IFN-γ.

Uzyskane wyniki mają wysokie znaczenie poznawcze, ponieważ wyjaśniają immunosupresyjne działanie nasion, ekstraktów oraz olejków pozyskanych z nasion czarnuszki siewnej u pacjentów z chorobami wynikającymi z reakcji nadwrażliwości, takimi jak astma czy reumatoidalne zapalenie stawów. Ponadto mogą również stanowić podstawę do wdrażania terapii z wykorzystaniem substancji pochodzenia roślinnego. W tym celu konieczne są dodatkowe badania, by ustalić, jaką postać surowca roślinnego można bezpiecznie stosować u pacjentów.

STRESZCZENIE W JĘZYKU ANGIELSKIM / SUMMARY

Substances of plant origin are of continuous interest to scientists, and among the plants mentioned in the literature, black cumin (*Nigella sativa*) occupies an important place. The plant belongs to the *Ranunculaceae* family. Its fruits contain small and aromatic seeds from which oil is extracted. It contains an oily fraction rich in unsaturated fatty acids, essential oil, and substances such as alkaloids, tocopherols, phytosterols, saponins, and flavonoids. The essential fraction has the greatest immunomodulatory potential due to its active substances, such as thymoquinone, p-cymene, alpha-thujene, and carvacrol. Obtained by cold-pressing, the oil was called" "the gold of the Pharaohs" in ancient times and was considered a panacea for a wide variety of diseases, especially those of the respiratory, digestive, and cardiovascular systems. Clinical observations indicate that seeds, extracts, and oil from black cumin can alleviate the symptoms of immune diseases such as asthma, allergic rhinitis, and rheumatoid arthritis. On the other hand, thymoquinone shows antioxidant and anticancer properties, and together with p-cymene, they show anti-inflammatory properties. However, the exact mechanism of action of black cumin on the immune system is not fully understood.

In this dissertation, we report the immunomodulatory properties of the essential oil obtained from the seeds of black cumin and the chemicals identified in the essential oil, namely thymoquinone and p-cymene. These properties were evaluated in healthy people and women diagnosed with Hashimoto's thyroiditis. Using flow cytometry, the surface phenotype, proliferative activity, and susceptibility to apoptosis of human T lymphocytes stimulated with an anti-CD3 monoclonal antibody in the presence of serial ethanolic dilutions of the tested oil, thymoquinone, and p-cymene were analyzed. Additionally, changes in the cytokine profile in the cell culture supernatants were determined. The results showed that black cumin seed essential oil significantly inhibits the proliferation of $CD4^+$ and $CD8^+$ T cells and induces cell death in a dose-dependent manner both in healthy people and patients with Hashimoto's thyroiditis. The essential oil also reduces the expression of CD28 and CD25 antigens essential for lymphocyte activation. The anti-proliferative and proapoptotic effect of the oil was related to the activity of thymoquinone. In contrast, p-cymene did not affect the proliferation of human T lymphocytes but induced their necrosis at the highest concentration and partially counteracted some of the effects of thymoquinone. The essential oil significantly reduced the concentration of TNF, IL-17A, and IL-10 in cell

culture supernatants. In turn, the level of IL-4 and IL-2 significantly increased in the presence of oil. The oil did not affect the concentration of IL-6 and IFN-γ.

The results are of high cognitive significance, as they may explain the immunosuppressive effects of seeds, extracts, and oils extracted from black cumin seeds in patients with hypersensitivity reactions, such as asthma and rheumatoid arthritis. Moreover, they provide a basis for implementing therapies using plant-derived substances. On the other hand, additional research is needed to determine which form of plant material can be safely used in patients.

WPROWADZENIE

Substancje pochodzenia roślinnego, posiadające potencjał terapeutyczny niezmiennie, cieszą się znacznym zainteresowaniem wśród naukowców z dziedziny biomedycznej i farmakologicznej. Wśród roślin, wymienianych w literaturze, ważne miejsce zajmuje **czarnuszka siewna** (*Nigella sativa*). Jest to jednoroczna roślina, pochodząca z krajów Europy Wschodniej i zachodniej części Azji, która ze względu na swoje charakterystyczne cechy botaniczne, została zakwalifikowana do rodziny Jaskrowatych [1]. Jej kwitnienie przypada na okres od maja do września, a następnie kwiatostany przekształcają się w owoce, które mają postać zrośniętych mieszków. Pojedynczy mieszek zawiera drobne, czarne i aromatyczne nasiona. Wykorzystując techniki destylacji, z nasion pozyskuje się olej, w którym wyróżnia się **frakcję tłustą,** bogatą w nienasycone kwasy tłuszczowe (głównie z grupy omega-6), **frakcję eteryczną** oraz **substancje chemiczne pochodzenia roślinnego** takie jak: tokoferole, fitosterole, saponiny i flawonoidy [2]. Powszechnie uznaje się, że frakcja eteryczna ma największy potencjał immunomodulacyjny, ze względu na zawarte w niej **substancje czynne** takie jak tymochinon (ang. *thymoquinone*, TQ), p-cymen, alfa-tujen czy karwakrol [3].

Czarnuszka, zwana również czarnym kminem, to znana i szeroko stosowana od stuleci roślina. Olej pozyskiwany z jej nasion już w starożytności nazywany był „złotem Faraonów" i uważano go za panaceum dla wielu schorzeń, zwłaszcza układu oddechowego [4], pokarmowego [5] i sercowo-naczyniowego [6]. Ponadto, wiele starożytnych kultur, zwłaszcza krajów arabskich i afrykańskich, stosowało olej z nasion czarnuszki w leczeniu schorzeń alergicznych [7]. Opisywane w literaturze obserwacje kliniczne wskazują, że nasiona, ekstrakty oraz olej mogą łagodzić objawy takich chorób jak astma [8] czy reumatoidalne zapalenie stawów [9]. Nie można również zapomnieć o powszechnym zastosowaniu czarnuszki w kuchni, ze względu na wyrazisty, korzenny aromat oraz wysoko oceniane walory smakowe. W Polsce nasiona czarnuszki są popularnym dodatkiem do pieczywa i mięs, a ich sproszkowana forma jest korzystną alternatywą dla ostrych przypraw dla osób z wrażliwym układem pokarmowym [10].

Według licznych oryginalnych prac badawczych, nasiona czarnuszki siewnej, a także pozyskane z nich ekstrakty, oraz olej wykazują właściwości przeciwbakteryjne, przeciwgrzybicze, przeciwutleniające i przeciwzapalne [11]. Z perspektywy tej pracy najważniejsze są jednak doniesienia o możliwym działaniu immunomodulującym. Dotychczas, większość tego typu badań, obejmujących analizę wpływu suplementacji

substratami roślinnymi, przeprowadzono na modelach zwierzęcych, głównie na myszach i szczurach, a w pojedynczych publikacjach na innych zwierzętach takich jak ryby [12] świnki morskie [13] kurczaki [14] i owce [15]. Badania przeprowadzone na świnkach morskich immunizowanych owoalbuminą (model eksperymentalny astmy alergicznej) wykazały, że wodno-etanolowy ekstrakt z nasion czarnuszki podnosi w surowicy stężenie interleukiny 4 (ang. *interleukin* 4, IL-4) i interferonu γ (ang. *interferon gamma*, IFN-γ). Jednocześnie obserwowano redukcję zmian zapalnych w płucach zwierząt, w postaci zmniejszonej infiltracji eozynofilii i limfocytów oraz miejscowej martwicy nabłonka płuc [13]. Z kolei badania na tilapii nilowej wykazały, że dodatek nasion czarnuszki siewnej w diecie znacząco podnosi poziom globulin w surowicy, liczbę białych krwinek (ang. *white blood cell*, WBC) i aktywność fagocytarną komórek oraz obniża śmiertelność ryb w porównaniu z dietą standardową [12].

Jedne z nielicznych badań klinicznych, w których wyciąg z *N. sativa* (NS) podawano pacjentom chorującym na astmę, wykazały że jej objawy, takie jak częstotliwość napadów i świszczący oddech, znacznie się zmniejszają, u osób spożywających regularnie wyciąg z nasion [8]. Praca, opublikowana w 2016 roku, wskazuje na możliwy immunosupresyjny efekt oleju z czarnuszki wobec limfocytów B i T u chorych na reumatoidalne zapalenie stawów (RZS) [9]. Doustne podawanie oleju z NS nie miało wpływu na odsetek komórek T $CD4^+$, ale zmniejszyło odsetek komórek T $CD8^+$, zwiększyło odsetek komórek T $CD4^+CD25^+$ oraz stosunek $CD4^+/CD8^+$ w porównaniu z pacjentami z RZS otrzymującymi placebo i wynikami wyjściowymi. Praca ta nie podejmuje jednak próby wyjaśnienia mechanizmu obserwowanego efektu na poziomie komórkowym. Przeprowadzone, w ramach pracy magisterskiej, badania *in vitro* potwierdzają, że olej tłusty z nasion czarnuszki hamuje proliferację ludzkich limfocytów B i T oraz nasila ich apoptozę [16].

Za obserwowane efekty odpowiedzialne mogą być zawarte w oleju substancje czynne. Jedną z nich jest **tymochinon**, którego działanie proapoptotyczne i antyproliferacyjne obserwowano względem komórek nowotworowych, w tym białaczkowych [17,18]. Tymochinon wykazuje również właściwości antyoksydacyjne. Badania wykazują, że działanie przeciwutleniające dotyczy zwłaszcza ochronnego działania względem promieniowania jonizującego. W badaniach z wykorzystaniem modelu szczurzego podanie tymochinonu przed ekspozycją na promieniowanie gamma powodowało powstawanie znacznie mniejszych ilości reaktywnych form tlenu i azotu niż u zwierząt, którym tymochinonu nie podano [19,20,21]. **P-cymen** według doniesień literaturowych również wykazuje właściwości przeciwzapalne. W badaniach przedstawiono, że p-cymen łagodzi

ostre uszkodzenie płuc indukowane lipopolisacharydem poprzez zmniejszenie infiltracji komórek zapalnych i cytokin prozapalnych, takich jak TNF-α (ang. *tumor necrosis factor alpha*), IL-1β, IL-6 w modelu mysim [22].

Zapalenie tarczycy Hashimoto (ang. *Hashimoto's thyroiditis*, HT) ma podłoże autoimmunologiczne, które jest związane z nieprawidłową aktywnością limfocytów T i B, a także produkcją przeciwciał skierowanych przeciw antygenom tarczycy: tyreoglobulinie (ang. *thyroglobulin*, Tg) oraz tarczycowej peroksydazie (ang. *thyroperoxidase*, TPO). U podstaw patogenezy tej choroby leży zaburzenie pracy układu immunologicznego, objawiające się nagromadzeniem autoreaktywnych limfocytów z jednoczesną utratą tolerancji immunologicznej na własne tkanki, co w konsekwencji prowadzi do zniszczenia tarczycy i powstania niedoczynności [23]. Ważną rolę w procesach rozwoju choroby odgrywa apoptoza tyreocytów [24]. W chorobie Hashimoto przeważa odpowiedź immunologiczna limfocytów Th1, które wraz z makrofagami wytwarzają prozapalne cytokiny IFN-γ, TNF-α i IL-1β. To aktywuje zewnątrzkomórkową ścieżkę apoptozy komórek tarczycy wykorzystującą interakcję receptora Fas (CD95) na powierzchni limfocytów i jego ligandu, FasL (CD95L) na powierzchni tyreocytów [25,26,27,28].

Częstotliwość występowania zapalenia tarczycy Hashimoto w dużym stopniu zależy od czynników środowiskowych (m.in. stresu, obniżonego poziomu selenu, nadmiernej podaży jodu, przyjmowania niektórych leków np. soli litu). Jednak głównymi czynnikami predysponującym do rozwoju choroby są czynniki genetyczne, które powodują utratę tolerancji immunologicznej [29]. Do tej pory zidentyfikowano kilka wariantów genów odpowiedzialnych za rozwój choroby Hashimoto, w tym geny odpowiedzialne za ekspresję antygenów zgodności tkankowej (ang. *human leukocyte antigen*, HLA) zlokalizowane na chromosomie 6, czy gen kodujący antygen 4 związany z cytotoksycznymi limfocytami T (ang. *cytotoxic T-lymphocyte-associated antigen-4*, CTLA-4) na chromosomie 2 [30]. Choroba częściej rozwija się u kobiet; stosunek kobiet do mężczyzn wynosi co najmniej 10:1. Większość kobiet jest diagnozowana między 30 a 50 rokiem życia [31].

Według badań opublikowanych w 2016 roku spożycie kilku gramów czarnuszki, może wywierać korzystny wpływ na przebieg autoimmunologicznych chorób tarczycy, w tym zapalenia tarczycy Hashimoto. W badaniu przeprowadzonym przez Farhangi i wsp. [32] pacjenci z HT otrzymywali 2 g sproszkowanych nasion czarnuszki w kapsułkach. Czas trwania interwencji wynosił 8 tygodni. Po tym czasie w grupie otrzymującej sproszkowane nasiona NS, w porównaniu z grupą placebo, zaobserwowano istotne statystycznie obniżenie wskaźników antropometrycznych, takich jak waga, BMI oraz obwód talii. W grupie badanej

odnotowano znaczne zmniejszenie poziomu hormonu TSH (ang. *thyroid-stimulating hormone*) i przeciwciał anty-TPO. W grupie przyjmującej NS wykazano zwiększenie stężenia T3, natomiast stężenie T4 nie zmieniło się. W grupie otrzymującej placebo nie obserwowano podobnych efektów.

Przytoczone doniesienia literaturowe popierają hipotezę dotyczącą immunomodulujących właściwości czarnuszki siewnej. Co więcej, wydaje się, że suplementacja wybranych składników czarnuszki może modulować przebieg chorób, w tym tych o podłożu autoimmunologicznym. Jednak brakuje prac tłumaczących mechanizm immunomodulacyjnego działania NS. Zatem w opisywanej pracy podjęto próbę sprawdzenia czy olejek eteryczny pozyskany z nasion czarnuszki siewnej ma wpływ na funkcjonowanie limfocytów u osób zdrowych oraz kobiet z zapaleniem tarczycy Hashimoto.

CELE PRACY

Celem nadrzędnym przeprowadzonych badań była ocena wpływu olejku eterycznego pozyskanego z nasion czarnuszki siewnej (*Nigella sativa*) na ludzkie limfocyty T, z uwzględnieniem ich fenotypu powierzchniowego, zdolności do proliferacji oraz produkcji cytokin, a także podatności na apoptozę. Cel ten osiągnięto realizując następujące cele szczegółowe:

1. Pozyskanie olejku eterycznego metodą hydrodestylacji z nasion czarnuszki siewnej i przeanalizowanie jego chemicznej kompozycji;
2. Analizowanie wpływu wybranych **etanolowych rozcieńczeń olejku eterycznego** na limfocyty T $CD4^+$ i $CD8^+$ w grupie **osób zdrowych i kobiet chorych na zapalenie tarczycy Hashimoto** z uwzględnieniem ekspresji antygenów powierzchniowych
   w tym CD25 i CD28, ich zdolności do proliferacji, produkcji cytokin oraz podatności na apoptozę;
3. Analizowanie wpływu wybranych stężeń **tymochinonu** i rozcieńczeń **p-cymenu** na limfocyty T $CD4^+$ i $CD8^+$ **w grupie osób zdrowych** z uwzględnieniem ich zdolności do proliferacji oraz podatności na apoptozę.

Cele rozprawy doktorskiej zostały zrealizowane w cyklu dwóch spójnych tematycznie artykułów oryginalnych. Trzeci artykuł jest pracą przeglądową podsumowującą obecną wiedzę na temat immunomodulacyjnego wpływu czarnuszki siewnej.

MATERIAŁY I METODY

Nasiona czarnuszki pozyskano z firmy Makar Bakalie Sp. z o. o. Katowice, Polska (kraj pochodzenia: Indie; data zbioru/numer partii: 02.2019/3505 0912). Próbki olejku eterycznego z *N. sativa* (ang. *Nigella sativa essential oil*, NSEO) uzyskano metodą hydrodestylacji w ciągu trzech miesięcy od pozyskania materiału. Izolację NSEO przeprowadzono w Katedrze i Zakładzie Farmakognozji Gdańskiego Uniwersytetu Medycznego.

Badania doświadczalne prowadzone były w grupie kontrolnej, która obejmowała 18 osób zdrowych (9 kobiet oraz 9-ciu mężczyzn) o średniej wieku 28 lat, oraz w grupie badanej 9 kobiet z zapaleniem tarczycy Hashimoto o średniej wieku 36 lat. Do badania wykorzystano 25 ml krwi obwodowej pobieranej do probówek morfologicznych z EDTA (ang. *ethylenediaminetetraacetic acid*, kwas etylenodiaminotetraoctowy). Z krwi obwodowej wyizolowano komórki jednojądrzaste (ang. *peripheral blood mononuclear cells*, PBMC) metodą wirowania w gradiencie gęstości fikolu.

PBMC, zabarwione VPD450 (ang. *Violet Proliferation Dye 450*), znacznikiem służącym do śledzenia proliferacji komórek, przeniesione zostały na 24-dołkową płytkę, uprzednio opłaszczoną monoklonalnym przeciwciałem anty-CD3 w stężeniu 1µg/ml na dołek (250 ng/250µl/1,5 miliona komórek). Następnie do dołków dodane zostały seryjne rozcieńczenia etanolowe (EtOH) olejku eterycznego z NS (1:10, 1:50, 1:100, 1:500 i 1:1000). Komórki były inkubowane w standardowych warunkach hodowlanych: 5% CO_2, 100% wilgotności w 37ºC przez 5 dni.

Zastosowano dwa modele hodowli komórkowych:

a) hodowlę standardową, gdzie **olejek dodawany był w punkcie 0** celem sprawdzenia, jak działa na proces aktywacji limfocytów (schemat stosowano dla PBMC izolowanych od osób z grupy kontrolnej – **publikacja nr 1**),
b) hodowlę preaktywowaną, gdzie **olejek dodawany był 24 godziny** po wcześniejszej aktywacji limfocytów za pomocą przeciwciała anty-CD3 celem sprawdzenia, czy olejek wpływa na już aktywne limfocyty (schemat stosowano dla PBMC izolowanych
od kobiet z grupy kontrolnej oraz pacjentek z HT – **publikacja nr 2**).

Następnie metodą cytometrii przepływowej, z użyciem przeciwciał monoklonalnych sprzężonych z barwnikami fluorescencyjnymi, w dwóch punktach czasowych, po 72 i 120 godzinach hodowli oceniano:

- ekspresję wybranych antygenów powierzchniowych (CD28, CD25) limfocytów $CD4^+$ i $CD8^+$,
- zdolność limfocytów $CD4^+$ i $CD8^+$ do proliferacji metodą DCT (ang. *dividing cell tracking*),
- odsetek komórek apoptotycznych i nekrotycznych w populacji limfocytów z użyciem aneksyny V i 7-AAD (ang. *7-aminoactinomycin D*).

Po zgromadzeniu wszystkich próbek w postaci nadsączy z hodowli komórkowej oceniono również stężenie cytokin IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, IL-17A, wykorzystując zestaw Human Th1/Th2/Th17 CBA Kit (BD Biosciences, USA). Do oceny ww. parametrów wykorzystano cytometry przepływowe FACSVerse oraz FACSAria III (Becton Dickinson, USA) znajdujące się w Katedrze i Zakładzie Fizjopatologii GUMed.

Dane cytometryczne zanalizowano za pomocą programu FCSalyzer (Sven Mostböck, Austria). Ostateczne dane liczbowe zanalizowano w programie GraphPad Prism, wersja 9 (GraphPad Software, USA). Testy Shapiro-Wilka i Kołmogorowa-Smirnowa zostały wykorzystane do przetestowania normalności rozkładu. Ponieważ dane nie cechowały się rozkładem normalnym, wybrano odpowiedni test nieparametryczny dla powtarzanych miar – ANOVA Friedmana wraz z testem post-hoc Dunna. Na potrzeby analizy statystycznej przyjęto poziom istotności $p < 0,05$.

OMÓWIENIE PUBLIKACJI 1

Ciesielska-Figlon K, Daca A, Kokotkiewicz A, Łuczkiewicz A, Zabiegała B, Witkowski J, Lisowska K. ***The influence of Nigella sativa essential oil on proliferation, activation, and apoptosis of human T lymphocytes in vitro.*** Biomedicine & Pharmacotherapy 2022; vol. 153: 113349.

W pierwszej publikacji opisano sposób pozyskania olejku eterycznego z nasion czarnuszki siewnej, analizy jego kompozycji chemicznej oraz wyniki doświadczeń, których celem było sprawdzenie, jak wybrane rozcieńczenia olejku eterycznego, a także wybrane substancje aktywne – tymochinon i p-cymen – wpływają na fenotyp oraz aktywność limfocytów T $CD4^+$ i $CD8^+$ w grupie zdrowych ochotników obu płci.

Dzięki współpracy z Katedrą i Zakładem Farmakognozji Wydziału Farmaceutycznego GUMed, z zakupionych nasion czarnuszki siewnej, otrzymano frakcję eteryczną. Wydzielenie olejku eterycznego nastąpiło w procesie technologicznym hydrodestylacji. Następnie pozyskane próbki poddano analizie jakościowej, techniką chromatografii gazowej sprzężonej ze spektrometrią mas (ang. *gas chromatography mass spectrometry*, GC/MS), umożliwiającą identyfikację substancji w próbce. Badania analityczne zostały wykonane w ramach współpracy z Katedrą Chemii Analitycznej Politechniki Gdańskiej. Analizując próbkę olejku eterycznego z sukcesem zidentyfikowano około 95% materii i wyróżniono 28 związków organicznych. Zawartość olejku eterycznego w nasionach wynosiła 0,26%. Istotnym składnikiem olejku był p-cymen stanowiący około połowę frakcji lotnej (49,27%) z kolei tymochinon stanowił 2,26% olejku.

Do badania wpływu NSEO włączono 18 osób, z równym podziałem na płeć i średnią wieku 28 lat. Osoby, które zostały zakwalifikowane do grupy badanej musiały być w ogólnie dobrym stanie zdrowia oraz nie mogły przyjmować żadnych leków. Bezpośredni materiał do badań stanowiły PBMC. Komórki były barwione VPD450, co w kolejnym etapie doświadczeń umożliwiło analizę ich proliferacji metodą DCT. Komórki były hodowane na płytce opłaszczonej monoklonalnym przeciwciałem anty-CD3 z różnymi rozcieńczeniami olejku eterycznego lub z różnymi stężeniami tymochinonu i/lub p-cymenu. Kontrolę stanowiły komórki stymulowane samym przeciwciałem anty-CD3. Poniżej znajduje się lista wariantów użytych do stymulacji komórek:

- etanolowe rozcieńczenia olejku eterycznego: 1:10, 1:50, 1:100, 1:500, 1:1000,

- stężenia tymochinonu: 200, 100, 20, 10 μg/ml,
- stężenia p-cymenu: 50, 5, 0,5, 0,05% p-cymenu,
- kombinacje wybranych stężeń tymochinonu i p-cymenu.

W tym modelu doświadczalnym olejek eteryczny, tymochinon i p-cymen były dodawane w momencie zakładania hodowli komórkowej. Inkubacja komórek trwała 5 dni i odbywała się w standardowych warunkach hodowlanych. Po 72 i 120 godzinach komórki były zbierane z dołków i znakowane określonymi w protokole przeciwciałami. Na podstawie wykonanych barwień oceniono:

- zmiany w proliferacji limfocytów $CD4^+$ i $CD8^+$,
- ekspresję antygenów powierzchniowych CD25 i CD28,
- zmiany odsetka komórek żywych, apoptotycznych i nekrotycznych.

Obecność olejku eterycznego w hodowli PBMC powodowała spadek odsetka komórek proliferujących w obecności NSEO w rozcieńczeniach 1:10, 1:50 i 1:100 w porównaniu do wariantu kontrolnego oraz rozcieńczenia 1:1000. Różnice te osiągnęły istotność statystyczną zarówno po 72 i 120 godzinach hodowli. Olejek w rozcieńczeniach 1:10, 1:50, 1:100 i 1:500 powodował obniżenie ekspresji antygenów CD28 i CD25 na limfocytach $CD4^+$ i $CD8^+$. Obecność olejku eterycznego zmieniała proporcje żywych, apoptotycznych i nekrotycznych komórek. Spadek liczby żywych komórek i wzrost liczby komórek w późnej apoptozie w obecności rozcieńczeń 1:10 i 1:50 był istotny statystycznie w porównaniu z wariantem kontrolnym i rozcieńczeniem 1:1000 po 72 i 120 godzinach stymulacji. Nastąpiło również istotne obniżenie liczby komórek w stadium wczesnej apoptozy w obecności olejku w rozcieńczeniach 1:10 i 1:50. Ponadto, po 72 godzinach stymulacji, odsetek komórek nekrotycznych był istotnie wyższy, gdy komórki inkubowano z NSEO w rozcieńczeniach 1:10, 1:50 lub 1:100.

Biorąc pod uwagę wpływ substancji czynnych zawartych w olejku eterycznym – tymochinonu i p-cymenu – stwierdzono istotny spadek odsetka proliferujących limfocytów $CD4^+$ i $CD8^+$ w obecności tymochinonu o najwyższych stężeniach (200 i 100 μg/ml) w porównaniu z kontrolą lub komórkami hodowanymi z p-cymenem. W obecności p-cymenu, tymochinon hamował proliferację limfocytów jedynie w najwyższym stężeniu 200 μg/ml. Natomiast sam p-cymen nie wpływał na proliferację ani limfocytów $CD4^+$, ani $CD8^+$. W obecności najwyższego stężenia TQ nastąpił istotny spadek liczby żywych komórek. Jednocześnie zwiększał się odsetek komórek w późnej apoptozie lub nekrozie. Tymczasem sam p-cymen nie wpływał na odsetek żywych lub apoptotycznych komórek.

Aczkolwiek w obecności 50% p-cymenu nastąpił istotny wzrost odsetka komórek nekrotycznych po stymulacji.

Na podstawie tej pracy sformułowano następujące wnioski:

1. Olejek eteryczny pozyskiwany z nasion *Nigella sativa* ma potencjalne działanie immunomodulujące, ponieważ hamuje aktywację ludzkich limfocytów T poprzez zahamowanie proliferacji oraz indukcję apoptozy w warunkach *in vitro,* głównie w najniższych rozcieńczeniach, 1:10 oraz 1:50.
2. Za opisane właściwości może być odpowiedzialny tymochinon, który znacząco hamuje proliferację limfocytów T i indukuje ich apoptozę w najwyższych stężeniu. Jednak mając na uwadze fakt, że w olejku eterycznym znajduje się tylko kilka procent tej substancji, postawiono hipotezę, że prawdopodobnie nie jest to jedyny związek odpowiedzialny za tak silne właściwości immunomodulujące olejku eterycznego.
3. P-cymen, mimo, że stanowi większość frakcji eterycznej, nie wpływa na proliferację oraz apoptozę limfocytów. Co więcej, częściowo przeciwdziała efektom wywoływanym przez tymochinon.

OMÓWIENIE PUBLIKACJI 2

Ciesielska-Figlon K, Wojciechowicz K, Daca A, Kokotkiewicz A, Łuczkiewicz A, Witkowski J, Lisowska K. ***The impact of Nigella sativa essential oil on T cells in women with Hashimoto's thyroiditis.*** Antioxidants 2023; vol. 12: 1246.

W drugiej pracy oryginalnej sprawdzono, jak wybrane rozcieńczenia olejku eterycznego wpływają na funkcjonowanie limfocytów T $CD4^+$ i $CD8^+$ w grupie kobiet chorych na zapalenie tarczycy Hashimoto.

Do badania wpływu olejku włączono grupę 9 kobiet ze zdiagnozowanym zapaleniem tarczycy Hashimoto (średnia wieku 39 lat) oraz 9-ciu zdrowych kobiet, to znaczy, nieposiadających zdiagnozowanych chorób nowotworowych, autoimmunologicznych oraz nie przyjmujących leków na stałe, stanowiących grupę kontrolną (średnia wieku 36 lat).

Bezpośredni materiał do badań ponownie stanowiły PBMC. Komórki były barwione VPD450 zgodnie z przyjętym protokołem, co w kolejnym etapie doświadczeń umożliwiło analizę proliferacji. W tym wariancie hodowli, komórki znajdowały się przez pierwsze 24 godziny hodowane na płytce opłaszczonej przeciwciałem anty-CD3. Po 24 godzinach do komórek dodane zostały wybrane rozcieńczenia NSEO (1:10, 1:50, 1:100, 1:500 i 1:1000). Kontrolę stanowiły komórki stymulowane samym przeciwciałem anty-CD3 i/lub komórki niestymulowane. Całkowity czas inkubacji komórek wynosił 5 dni, z zachowaniem standardowych warunków hodowlanych. Po 72 i 120 godzinach komórki były zbierane z dołków i znakowane określonymi w protokole przeciwciałami sprzężonymi ze znacznikami fluorescencyjnymi. Dzięki temu możliwe było określenie zmian w proliferacji limfocytów $CD4^+$ i $CD8^+$. Ponownie oceniono zmiany odsetka komórek żywych, apoptotycznych i nekrotycznych. Ponadto, zebrano nadsącza pohodowlane, aby określić w nich stężenie wydzielanych cytokin: IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, IL-17A przy użyciu Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokines Kit.

Zarówno u zdrowych kobiet i pacjentek z HT zaobserwowano spadek liczby podziałów komórkowych limfocytów $CD4^+$ i $CD8^+$, głównie w obecności NSEO w rozcieńczeniach 1:10. Jednocześnie obserwowano spadek odsetka komórek proliferujących. Ocena żywotności komórek pokazała, że olejek eteryczny w rozcieńczeniach 1:10 i 1:50 powoduje śmierć komórek; spadł odsetek komórek żywych, komórek we wczesnej apoptozie, a jednocześnie wzrósł odsetek komórek w późnej apoptozie i nekrozie w porównaniu

do komórek kontrolnych lub wyższych rozcieńczeń olejku eterycznego. Zmiany te dotyczyły komórek od zdrowych kobiet i pacjentek z HT.

Porównując stężenia wymienionych wcześniej cytokin zaobserwowano, że zarówno u zdrowych kobiet, jak i u pacjentek z HT poziom IL-2 był obniżony po stymulacji samym przeciwciałem anty-CD3 w porównaniu do komórek niestymulowanych, podczas gdy poziom IL-4 w ogóle się nie zmienił. W grupie kontrolnej poziom IL-2 oraz IL-4 znacząco wzrastał w obecności NSEO w rozcieńczeniach 1:10 i 1:50. U pacjentek z HT stężenie IL-4 wzrosło jedynie w obecności NSEO w rozcieńczeniu 1:50. Jeśli chodzi o zmiany w stężeniach IFN-γ, TNF, IL-17A, IL-6 i IL-10, były one znacząco podwyższone po stymulacji samym przeciwciałem anty-CD3 w obu grupach badanych w porównaniu z komórkami niestymulowanymi. U pacjentek z HT, obecność NSEO, głównie w rozcieńczeniu 1:10, znacząco obniżało stężenie TNF oraz IL-17A. U zdrowych kobiet, olejek w rozcieńczeniu 1:10 i 1:50 obniżył poziom IL-17A i IL-10. Olejek eteryczny nie wpłynął znacząco na zmiany w stężeniu IFN-γ oraz IL-6 w żadnej z badanych grup.

Na podstawie tej pracy sformułowano następujące wnioski:

1. Olejek eteryczny pozyskiwany z nasion czarnuszki siewnej działa proapoptotycznie i antyproliferacyjnie na preaktywowane komórki T u pacjentek z zapaleniem tarczycy Hashimoto oraz zdrowych kobiet w warunkach *in vitro*.
2. Olejek eteryczny wpływa również na produkcję cytokin poprzez obniżenie stężenia niektórych cytokin ważnych dla regulacji procesów zapalnych, takich jak TNF, IL-17A oraz IL-10, przy jednoczesnym podwyższeniu stężeń IL-2 czy IL-4. Warto jednak zaznaczyć, że nie wpływał na produkcję IL-6 czy IFN-γ.
3. Wrażliwość limfocytów na NSEO zależy również od stanu zdrowia osób badanych. Przykładowo, olejek eteryczny nie wpływał istotnie na produkcję TNF u zdrowych kobiet, podczas gdy u pacjentek z HT w najniższych rozcieńczeniach istotnie ją zmniejszał.

OMÓWIENIE PUBLIKACJI 3

Ciesielska-Figlon K, Wojciechowicz K, Wardowska A, Lisowska K. ***The immunomodulatory effect of Nigella sativa.*** Antioxidants 2023; vol. 12: 1340.

Ostatni artykuł, zamykający cykl prac wchodzących w skład rozprawy doktorskiej, jest pracą przeglądową. Artykuł ten podsumowuje obecną wiedzę na temat immunomodulacyjnego wpływu *Nigella sativa*. W niniejszym artykule uwzględniono badania eksperymentalne dotyczące zdolności czarnuszki siewnej do modulowania stanu zapalnego i odpowiedzi immunologicznej oraz aktywności cytotoksycznej komórek układu odpornościowego. Przegląd opiera się na artykułach, książkach i referatach konferencyjnych wydrukowanych do września 2022 r., znalezionych w bazach danych Web of Science, PubMed, Wiley Online Library i Google Scholar.

Praca rozpoczyna się od ogólnej charakterystyki rośliny oraz jej nasion i pozyskanych z nich produktów (olej tłusty, olej eteryczny, ekstrakty), z uwzględnieniem najpowszechniejszych metod ich pozyskania oraz właściwości antyoksydacyjnych. Druga część pracy jest rozwinięciem głównej tematyki artykułu i przeglądem publikacji opisujących właściwości immunomodulacyjne czarnuszki siewnej z podziałem na eksperymenty z wykorzystaniem ekstraktów, oleju tłustego i olejku eterycznego uwzględniając wariant badań *in vitro* i *in vivo*.

Podsumowując najważniejsze informacje uzyskane podczas przygotowywania tego artykułu należy podkreślić fakt, że właściwości terapeutyczne *Nigella sativa* są ciągle przedmiotem badań. Jej właściwości przeciwbakteryjne, przeciwwirusowe i przeciwgrzybicze są dobrze znane i szeroko opisane, ale wiedza o jej wpływie na układ odpornościowy jest uboga. Wyniki badań opisane w tym przeglądzie pokazują, że czarnuszka wpływa na komórki odpornościowe poprzez modulowanie ich funkcji, takich jak zdolność do wytwarzania cytokin lub tlenku azotu, aktywności fagocytarnej i cytotoksycznej, proliferacji splenocytów i limfocytów T oraz podatności na apoptozę i nekrozę zarówno w warunkach *in vivo* i *in vitro*. W związku z tym *Nigella sativa* jest obiecującym źródłem składników aktywnych, może w przyszłości być stosowana w różnych warunkach klinicznych. Konieczne są jednak dalsze badania w celu zbadania dokładnych mechanizmów działania oleju tłustego, olejku eterycznego i ich najważniejszych komponentów, szczególnie u ludzi na poziomie komórkowym i molekularnym.

Niniejszy przegląd można uznać za podstawę dla nadchodzących badań i przyszłego rozwoju środków terapeutycznych, które pomogą zmniejszyć objawy chorób związanych z układem odpornościowym.

BIBLIOGRAFIA

1. Zohary D, Hopf M, Weiss E, Domestication of Plants in the Old World: The Origin and Spread of Domesticated Plants in Southwest Asia, Europe, and the Mediterranean Basin. Oxford University Press, 2012.
2. Ghahramanloo K, Kamalidehghan B, Akbari Javar H, Teguh Widodo R, Majidzadeh K, and Noordin MI, Comparative analysis of essential oil composition of Iranian and Indian Nigella sativa L. extracted using supercritical fluid extraction and solvent extraction. Drug Des. Devel. Ther., 2017; 11:2221–2226.
3. Tembhurne SV, Feroz S, Sakarkar DM. A review on therapeutic potential of Nigella sativa (kalonji) seeds. J Med Plants Res. 2014;8:166–167.
4. Gholamnezhad Z, Keyhanmanesh R, Boskabady MH. Anti-inflammatory, antioxidant, and immunomodulatory aspects of Nigella sativa for its preventive and bronchodilatory effects on obstructive respiratory diseases: A review of basic and clinical evidence. J. Funct. Foods. 2015; 17:910–927.
5. Muthu C, Ayyanar M, Raja N, Ignacimuthu S. Medicinal plants used by traditional healers in Kancheepuram district of Tamil Nadu, India. J Ethnobiol Ethnomed. 2006 Oct 7;2:43.
6. Saad B, Azaizeh H, Said O. Tradition and perspectives of arab herbal medicine: a review. Evid Based Complement Alternat Med. 2005 Dec;2(4):475-9.
7. Kalus U, Pruss A, Bystron J, Jurecka M, Smekalova A, Lichius JJ, Kiesewetter H. Effect of Nigella sativa (black seed) on subjective feeling in patients with allergic diseases. Phytother Res. 2003 Dec;17(10):1209-14.
8. Boskabady MH, Javan H, Sajady M, Rakhshandeh H. The possible prophylactic effect of Nigella sativa seed extract in asthmatic patients. Fundam Clin Pharmacol. 2007 Oct;21(5):559-66.
9. Kheirouri S, Hadi V, Alizadeh M. Immunomodulatory Effect of Nigella sativa Oil on T Lymphocytes in Patients with Rheumatoid Arthritis. Immunol Invest. 2016 May;45(4):271-83.
10. Yimer EM, Tuem KB, Karim A, Ur-Rehman N, Anwar F. Nigella sativa L. (Black Cumin): A Promising Natural Remedy for Wide Range of Illnesses. Evid Based Complement Alternat Med. 2019 May 12;2019:1528635.
11. Ahmad A, Husain A, Mujeeb M, Khan SA, Najmi AK, Siddique NA, Damanhouri ZA, Anwar F. A review on therapeutic potential of Nigella sativa: A miracle herb. Asian Pac J Trop Biomed. 2013 May;3(5):337-52.
12. Elkamel AA Mossad GM. Immunomodulation of Nile Tilapia, Oreochromis niloticus, by Nigella sativa and Bacillus subtilis. J. Aquac. Res. Dev. 2012; 3-6.
13. Boskabady MH, Keyhanmanesh R, Khamneh S, Ebrahimi MA. The effect of Nigella sativa extract on tracheal responsiveness and lung inflammation in ovalbumin-sensitized guinea pigs. Clinics (Sao Paulo). 2011;66(5):879-87.
14. Singh Y, Prakashbhai, PN Saxenam, B Ramakrishnan S. Immunomodulatory Effects of Nigella sativa Extract on Chicken Peripheral Blood Mononuclear Cells: In-vitro. J Anim Res. 2021 Apr;11(4): 579-586.
15. Elmowalid G, Amar AM, Ahmad AA. Nigella sativa seed extract: 1. Enhancement of sheep macrophage immune functions in vitro. Res Vet Sci. 2013 Oct;95(2):437-43.
16. Ciesielska-Figlon K, Lisowska KA, Mikosik-Roczynska A, Witkowski JM. Nigella sativa oil inhibits proliferation and stimulates apoptosis of human lymphocytes in vitro. Hum Immunol. 2021; 82(8):608-614.
17. Salim LZ, Mohan S, Othman R, Abdelwahab SI, Kamalidehghan B, Sheikh BY, Ibrahim MY. Thymoquinone induces mitochondria-mediated apoptosis in acute lymphoblastic leukaemia in vitro. Molecules. 2013 Sep 12;18(9):11219-40.
18. Diab-Assaf M, Semaan J, El-Sabban M, Al Jaouni SK, Azar R, Kamal MA, Harakeh S. Inhibition of Proliferation and Induction of Apoptosis by Thymoquinone via Modulation of TGF Family, p53, p21 and Bcl-2α in Leukemic Cells. Anticancer Agents Med Chem. 2018;18(2):210-215.

19. Demir E, Taysi S, Ulusal H, Kaplan DS, Cinar K, Tarakcioglu M. Nigella sativa oil and thymoquinone reduce oxidative stress in the brain tissue of rats exposed to total head irradiation. Int J Radiat Biol. 2020 Feb;96(2):228-235.
20. Cikman O, Ozkan A, Aras AB, Soylemez O, Alkis H, Taysi S, Karaayvaz M. Radioprotective effects of Nigella sativa oil against oxidative stress in liver tissue of rats exposed to total head irradiation. J Invest Surg. 2014 Oct;27(5):262-6.
21. Ahlatci A, Kuzhan A, Taysi S, Demirtas OC, Alkis HE, Tarakcioglu M, Demirci A, Caglayan D, Saricicek E, Cinar K. Radiation-modifying abilities of Nigella sativa and thymoquinone on radiation-induced nitrosative stress in the brain tissue. Phytomedicine. 2014 Apr 15;21(5):740-4.
22. Xie G, Chen N, Soromou LW, Liu F, Xiong Y, Wu Q, Li H, Feng H, Liu G. p-Cymene protects mice against lipopolysaccharide-induced acute lung injury by inhibiting inflammatory cell activation. Molecules. 2012 Jul 6;17(7):8159-73.
23. Dayan CM, Daniels GH. Chronic autoimmune thyroiditis. N Engl J Med. 1996 Jul 11;335(2):99-107.
24. Bossowski A, Stasiak-Barmuta A, Czarnocka B, Urban M, Łyczkowska A, Niedziela M, Bardadin K, Czerwińska J, Dadan J, Baltaziak M. Cytofluorymetryczna analiza wybranych markerów apoptozy Fas/FasL (CD95/CD95L) w tkance gruczołu tarczowego u młodocianych pacjentów z choroba Gravesa-Basedowa oraz zapaleniem tarczycy typu Hashimoto [Cytofluorometric analysis of chosen markers of apoptosis CD95/CD95L (Fas/FasL) in thyroid tissues from young patients with Graves' disease and Hashimoto's thyroiditis]. Endokrynol Diabetol Chor Przemiany Materii Wieku Rozw. 2006;12(2):83-90.
25. Wang SH, Baker JR. The role of apoptosis in thyroid autoimmunity. Thyroid. 2007 Oct;17(10):975-9.
26. Salmaso C, Bagnasco M, Pesce G, Montagna P, Brizzolara R, Altrinetti V, Richiusa P, Galluzzo A, Giordano C. Regulation of apoptosis in endocrine autoimmunity: insights from Hashimoto's thyroiditis and Graves' disease. Ann N Y Acad Sci. 2002 Jun;966:496-501.
27. Bretz JD, Baker JR Jr. Apoptosis and autoimmune thyroid disease: following a TRAIL to thyroid destruction? Clin Endocrinol (Oxf). 2001 Jul;55(1):1-11.
28. Giordano C, Richiusa P, Bagnasco M, Pizzolanti G, Di Blasi F, Sbriglia MS, Mattina A, Pesce G, Montagna P, Capone F, Misiano G, Scorsone A, Pugliese A, Galluzzo A. Differential regulation of Fas-mediated apoptosis in both thyrocyte and lymphocyte cellular compartments correlates with opposite phenotypic manifestations of autoimmune thyroid disease. Thyroid. 2001 Mar;11(3):233-44.
29. Ragusa F, Fallahi P, Elia G, Gonnella D, Paparo SR, Giusti C, Churilov LP, Ferrari SM, Antonelli A. Hashimotos' thyroiditis: Epidemiology, pathogenesis, clinic and therapy. Best Pract Res Clin Endocrinol Metab. 2019 Dec;33(6):101367.
30. Bossowski A. Wybrane aspekty diagnostyczne i terapeutyczne w schorzeniach autoimmunizacyjnych tarczycy wieku rozwojowego. Endokrynol Pediatr. 2012; 11 (3) 40.
31. Ahmed R, Al-Shaikh S, Akhtar M. Hashimoto thyroiditis: a century later. Adv Anat Pathol. 2012 May;19(3):181-6.
32. Farhangi MA, Dehghan P, Tajmiri S, Abbasi MM. The effects of Nigella sativa on thyroid function, serum Vascular Endothelial Growth Factor (VEGF) - 1, Nesfatin-1 and anthropometric features in patients with Hashimoto's thyroiditis: a randomized controlled trial. BMC Complement Altern Med. 2016 Nov 16;16(1):471.

PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY

Publikacja 1

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

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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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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-1β, IL-6) in the mouse model. In another study, p-cymene reduced the production of TNF-α and IL-1β 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 8ºC. The dehydrated sample was collected from the above sodium sulfate layer, moved to a clean vial, and stored at 8ºC.

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 µl 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× 0.25 µm capillary column (Agilent J&W) at 250ºC, at a split ratio of 1:10. The carrier gas (helium) flow was 1.1 ml/min. The oven temperature increased from 50ºC to 280ºC at a 7ºC per minute and was held at 280ºC 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× 0.25 µm 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 ± SD (n = 6).

Compound	t_R (min)	RI_{exp}	RI_{lit}	Content (%)
α-thujene	5251	920	928	14,38 ± 1,29
α-pinene	5378	928	931	3,09 ± 0,28
camphene	5679	945	943	0,05 ± 0,01
β-phellandrene/sabinene	6180	973	975	2,43 ± 0,86
β-pinene	6250	979	978	2,85 ± 0,89
n-decane	6556	1000	1000	0,07 ± 0,02
α-terpinene	7129	1019	1008	0,87 ± 0,02
p-cymene	7372	1029	1013	49,27 ± 0,50
D-limonene	7431	1032	1020	2,42 ± 0.09
eucalyptol	7497	1035	1054	0,06 ± 0,03
γ-terpinene	8136	1061	1047	3,36 ± 0,20
cis-β-terpineol	8360	1070	1125	0,03 ± 0,01
p-cymenene	8879	1091	1073	0,13 ± 0,01
cis-4-methoxy-thujane	9067	1100	1040_{est}	0,99 ± 0,04
n-undecane	9180	1100	1100	0,04 ± 0,01
trans-4-methoxy-thujane	9641	1123	1040_{est}	5,83 ± 0,28
terpinen-4-ol	11,145	1181	1161	0,73 ± 0,11
n-dodecane	11,386	1200	1200	0,02 ± 0,02
β-cyclocitral	11,837	1207	1196	1,24 ± 0,10
thymoquinone	13,007	1255	1216	2,26 ± 0,22
bornyl acetate	13,949	1293	1269	0,17 ± 0,01
thymol	14,167	1304	1262	0,09 ± 0,03
carvacrol	14,396	1305	1278	1,57 ± 0,37
α-longipinene	15,545	1361	1358	0,81 ± 0,06
longifolene	16,911	1417	1402	3,69 ± 0,25
caryophyllene	17,251	1430	1424	0,06 ± 0,01
β-bisabolene	19,365	1514	1496	0,06 ± 0,01
4-methoxy-2,3,5-trimethylophenol	20,531	1555	1430	0,50 ± 0,13
Total (%)				**97,06 ± 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 ± 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% CO_2, 100% humidity at 37ºC) 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 ≥ 98% pure, and the stock solution was prepared with EtOH at a 1 mg/ml concentration. This stock was stored at − 80ºC. P-cymene was 99% pure and was stored at 4ºC 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% CO_2, 100% humidity at 37ºC) 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 ± 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 co-stimulatory 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

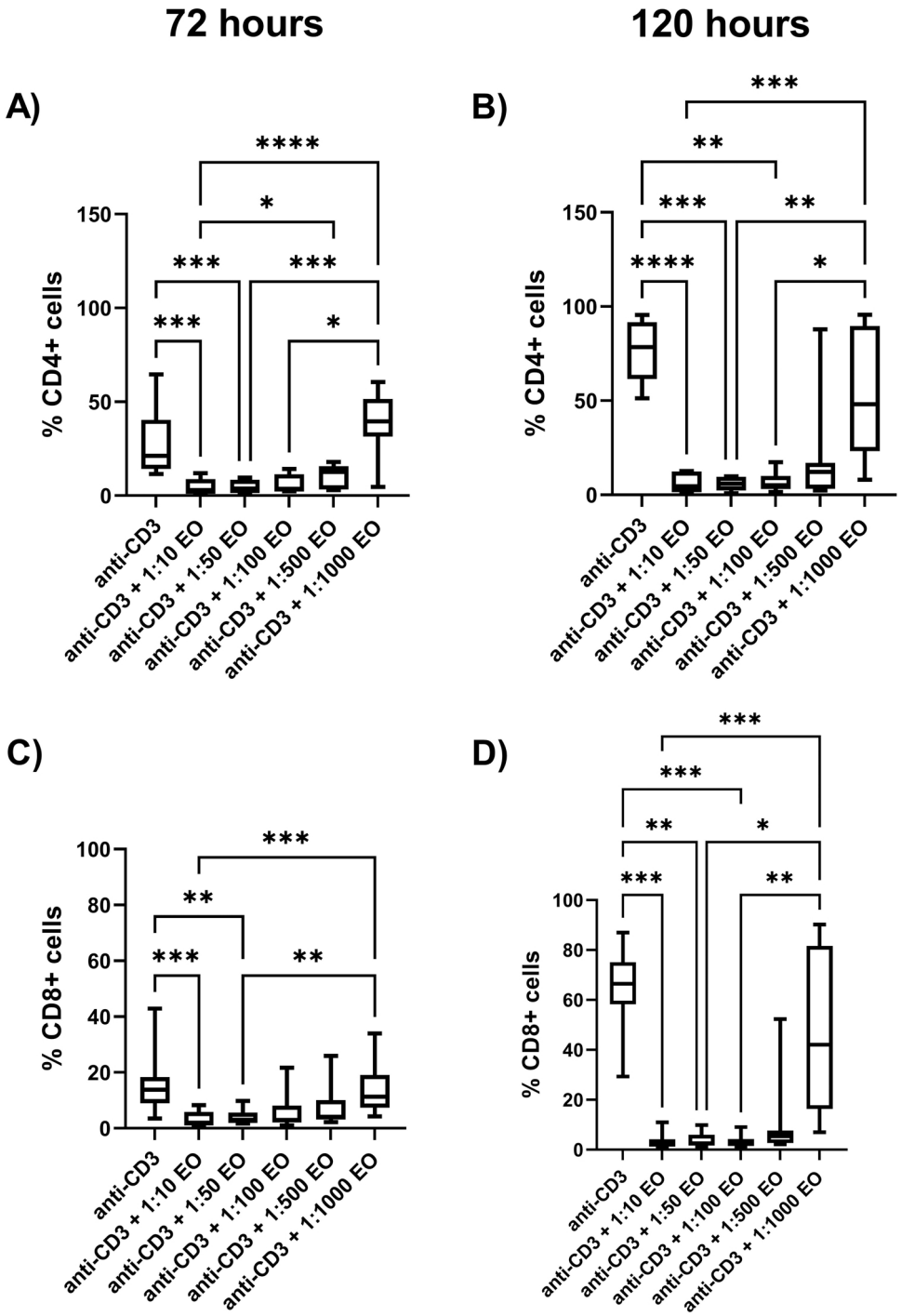

Fig. 1. Comparison of percentage of proliferating $CD4^+$ (A, B) and $CD8^+$ cells (C, D) stimulated with an anti-CD3 antibody with different dilutions of NSEO for 72 (A, C) and 120 (B, D) hours. Graphs show median, percentiles with the maximum and minimum value of ten independent experiments, ANOVA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

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).

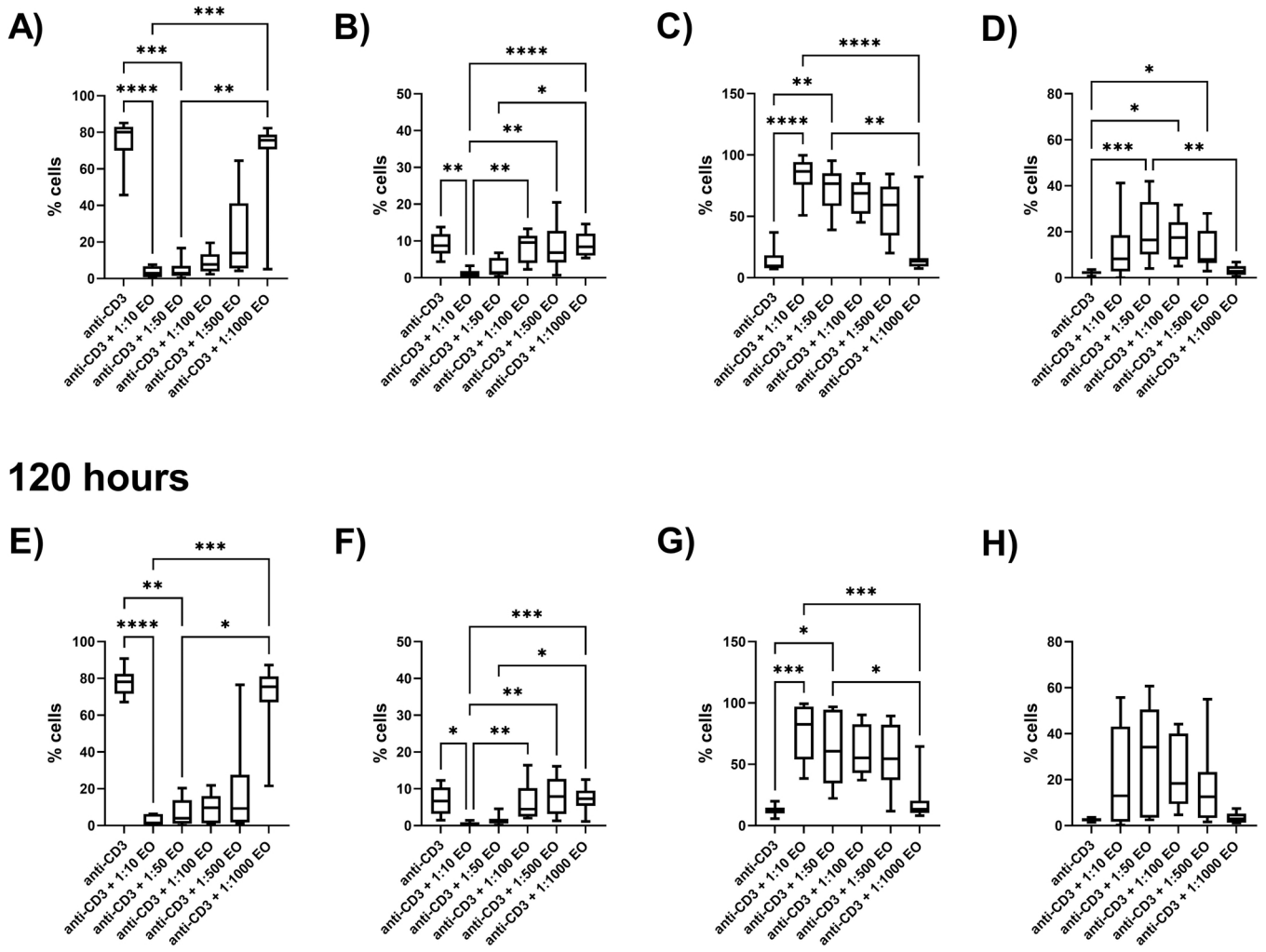

Fig. 2. Comparison of percentage of living, apoptotic, and necrotic lymphocytes stimulated for 72 (A-D) and 120 (E-H) hours with an anti-CD3 antibody with different dilutions of NSEO. Graphs show median, percentiles with the maximum and minimum value of nine independent experiments, ANOVA Friedman with 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 μg/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 μg/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 μg/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 μg/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 μg/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 μg/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

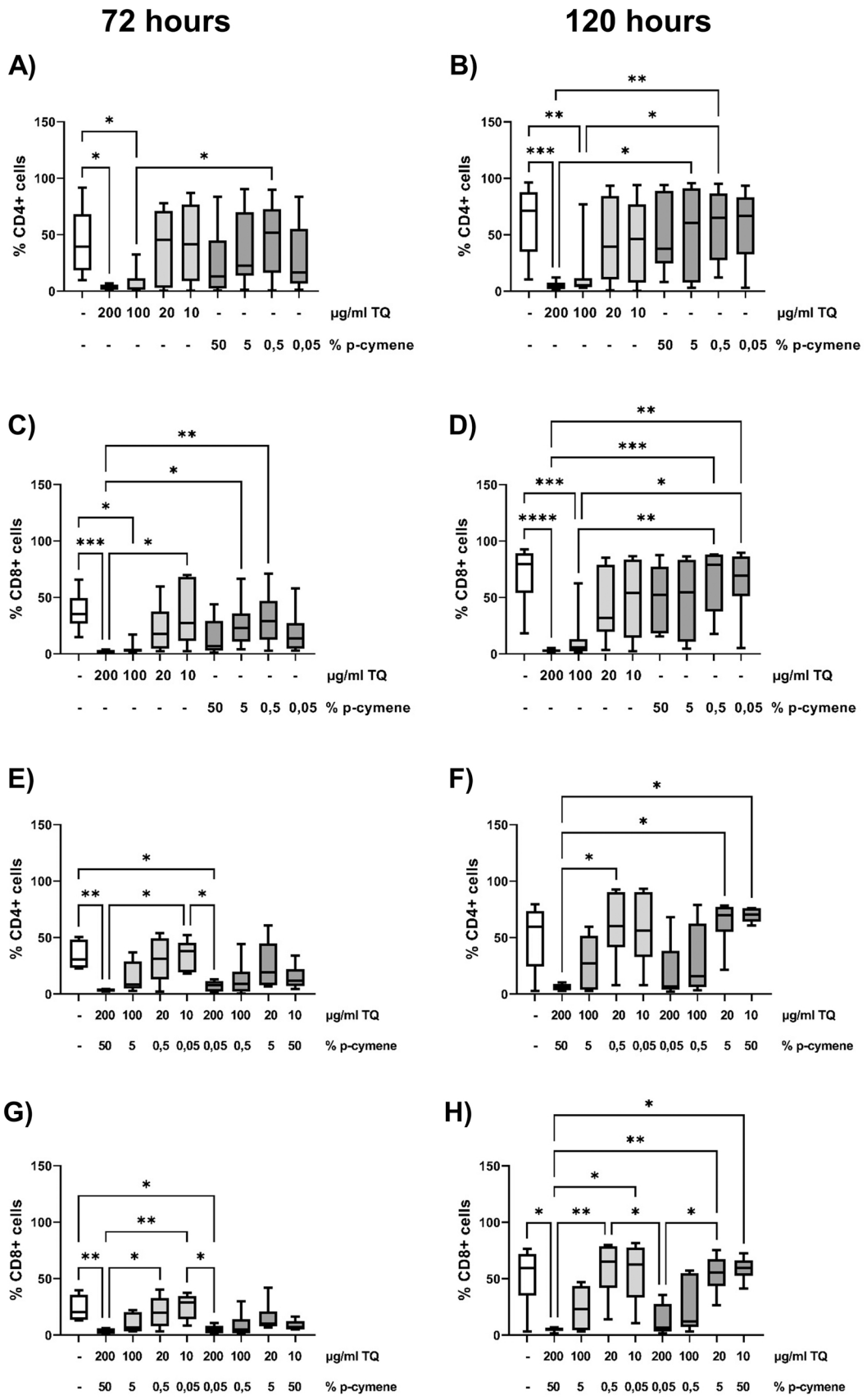

Fig. 3. Comparison of percentage of proliferating $CD4^+$ and $CD8^+$ cells stimulated with an anti-CD3 antibody alone (-) and with different dilutions of TQ and p-cymene and their combinations for 72 (A, C, E, G) and 120 (B, D, F, H) hours. Graphs show median, percentiles with the maximum and minimum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

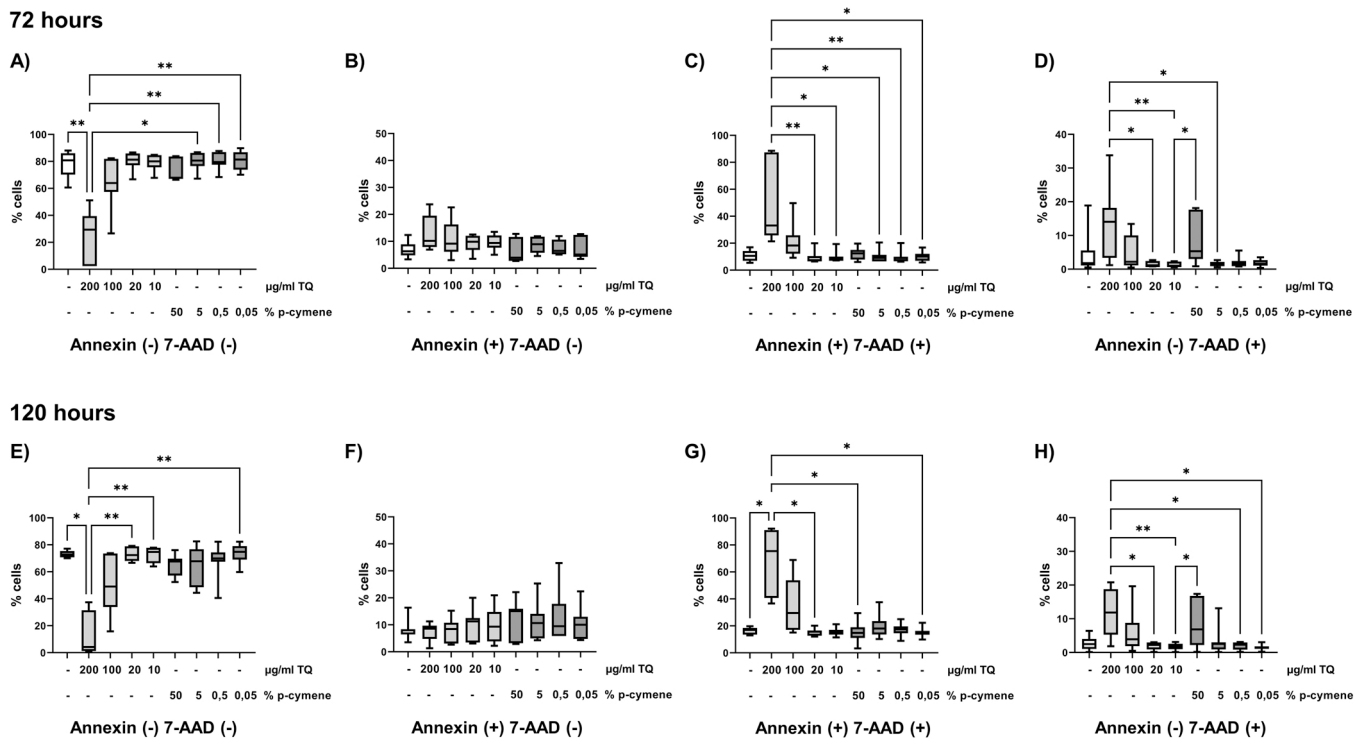

Fig. 4. Comparison of the percentage of living, apoptotic, and necrotic lymphocytes stimulated with an anti-CD3 antibody alone (-) and with different TQ and p-cymene dilutions for 72 (A-D) and 120 (E-H) hours. Graphs show median, percentiles with the maximum and minimum value of seven independent experiments,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

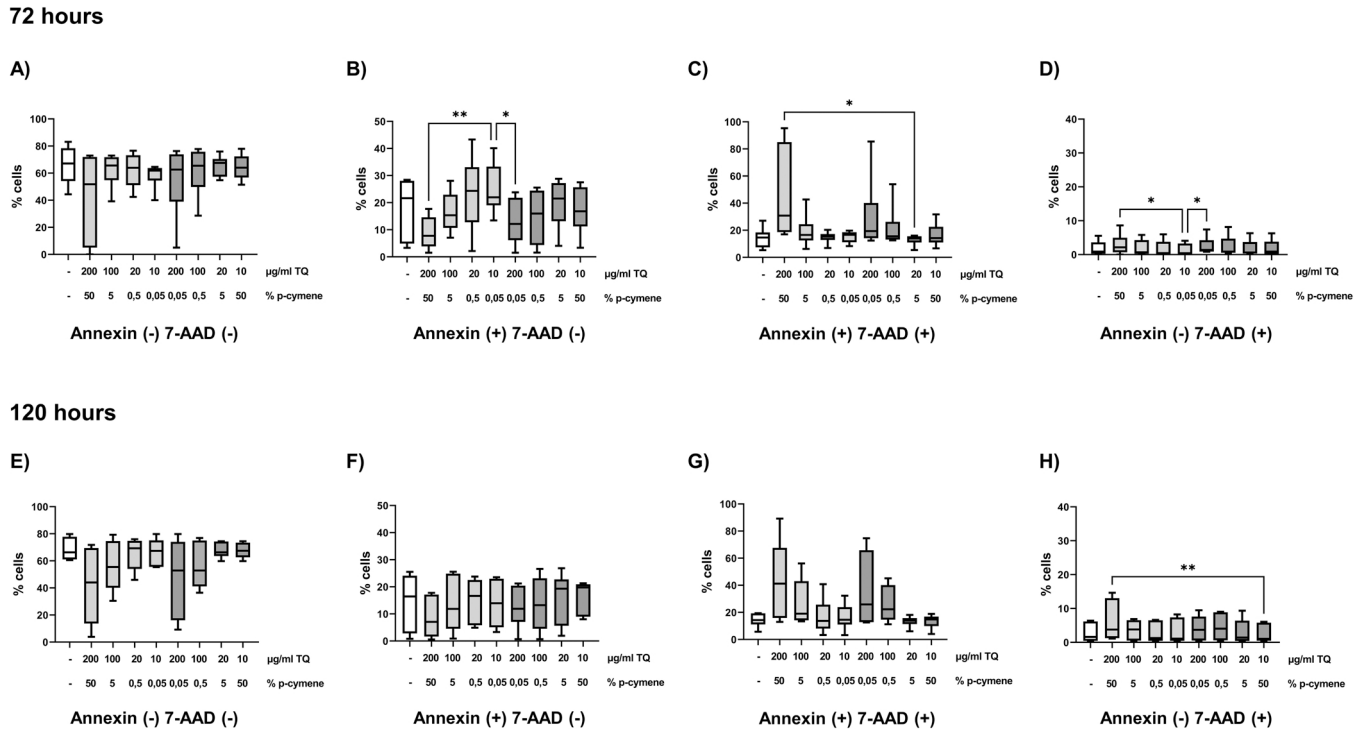

Fig. 5. Comparison of percentage of living, apoptotic, and necrotic lymphocytes stimulated with an anti-CD3 antibody alone (-) and with different combinations of dilutions of TQ and p-cymene for 72 (A-D) and 120 (E-H) hours. Graphs show median, percentiles with the maximum and minimum value of six independent 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.

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Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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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.

References

[1] F.J. Aboutabl, E.A. El-Azzouny, A.A. Hammerschmidt, Aroma volatiles of Nigella sativa L. seeds, in: E.-J. Brunke (Ed.), Progress in Essential Oil Research, De Gruyter, 1986, pp. 49–56.

[2] S. Lautenbacher, Schwarzkümmelöl, Dtsch. Apoth. Ztg. 137 (1997) 68–69.

[3] F.M. Al-Jasass, M.S. Al-Jasser, Chemical composition and fatty acid content of some spices and herbs under Saudi Arabia conditions, Sci. World J. 2012 (2012) 1–5, https://doi.org/10.1100/2012/859892.

[4] A. Ahmad, A. Husain, M. Mujeeb, S.A. Khan, A.K. Najmi, N.A. Siddique, Z. A. Damanhouri, F. Anwar, A review on therapeutic potential of Nigella sativa: a miracle herb, Asian Pac. J. Trop. Biomed. 3 (2013) 337–352.

[5] K. Ghahramanloo, B. Kamalidehghan, H. Akbari Javar, R. Teguh Widodo, K. Majidzadeh, M.I. Noordin, Comparative analysis of essential oil composition of Iranian and Indian Nigella sativa L. extracted using supercritical fluid extraction and solvent extraction, Drug Des. Devel. Ther. 11 (2017) 2221–2226, https://doi.org/10.2147/dddt.s87251.

[6] M.B. Atta, Some characteristics of nigella (Nigella sativa L.) seed cultivated in Egypt and its lipid profile, Food Chem. 83 (2003) 63–68, https://doi.org/10.1016/S0308-8146(03)00038-4.

[7] M.M. Hosseinzadeh, H. Fazly Bazzaz, B.S. Haghi, Antibacterial activity of total extracts and essential oil of Nigella sativa L. seeds in mice, Pharmacologyonline 2 (2007) 429–435.

[8] A. Mouwakeh, A. Kincses, M. Nové, T. Mosolygó, C. Mohácsi-Farkas, G. Kiskó, G. Spengler, Nigella sativa essential oil and its bioactive compounds as resistance modifiers against Staphylococcus aureus, Phyther. Res. 33 (2019) 1010–1018, https://doi.org/10.1002/ptr.6294.

[9] H. Mahmoudvand, E.S. Dezaki, F. Kheirandish, B. Ezatpour, S. Jahanbakhsh, M. F. Harandi, Scolicidal effects of black cumin seed (nigella sativa) essential oil on hydatid cysts, Korean J. Parasitol. 52 (2014) 653–659, https://doi.org/10.3347/kjp.2014.52.6.653.

[10] M. Burits, F. Bucar, Antioxidant activity of Nigella sativa essential oil, Phyther. Res. 14 (200) 323–328. ⟨https://doi.org/10.1002/1099–1573(200008)14:5%3C323::aid-ptr621%3E3.0.co;2-q⟩.

[11] O. Cikman, A. Ozkan, A.B. Aras, O. Soylemez, H. Alkis, S. Taysi, M. Karaayvaz, Radioprotective effects of Nigella sativa oil against oxidative stress in liver tissue of rats exposed to total head irradiation, J. Invest. Surg. 27 (2014) 262–266, https://doi.org/10.3109/08941939.2014.898811.

[12] E. Demir, S. Taysi, H. Ulusal, D.S. Kaplan, K. Cinar, M. Tarakcioglu, Nigella sativa oil and thymoquinone reduce oxidative stress in the brain tissue of rats exposed to total head irradiation, Int. J. Radiat. Biol. 96 (2020) 228–235, https://doi.org/10.1080/09553002.2020.1683636.

[13] M. Kaplan, E. Demir, F. Yavuz, G.I. Kaplan, M.R. Taysi, S. Taysi, M.M. Sucu, Radioprotective effect of nigella sativa oil on heart tissues of rats exposed to irradition, Int. J. Cardiovasc. Sci. 35 (2021) 214–219, https://doi.org/10.36660/ijcs.20210055.

[14] H. Alkis, E. Demir, M.R. Taysi, S. Sagir, S. Taysi, Effects of Nigella sativa oil and thymoquinone on radiation-induced oxidative stress in kidney tissue of rats, Biomed. Pharmacother. 139 (2021), 111540, https://doi.org/10.1016/j.biopha.2021.111540.

[15] V.S. Periasamy, J. Athinarayanan, A.A. Alshatwi, Anticancer activity of an ultrasonic nanoemulsion formulation of Nigella sativa L. essential oil on human breast cancer cells, Ultrason. Sonochem. 31 (2016) 449–455, https://doi.org/10.1016/j.ultsonch.2016.01.035.

[16] M.H. Boskabady, H. Javan, M. Sajady, H. Rakhshandeh, The possible prophylactic effect of Nigella sativa seed extract in asthmatic patients, Fundam. Clin. Pharmacol. 21 (2007) 559–566, https://doi.org/10.1111/j.1472-8206.2007.00509.x.

[17] H. Işık, A. Cevikbaş, U.S. Gürer, B. Kiran, Y. Uresin, P. Rayaman, E. Rayaman, B. Gürbüz, S. Büyüköztürk, Potential adjuvant effects of nigella sativa seeds to improve specific immunotherapy in allergic rhinitis patients, Med. Princ. Pract. 19 (2010) 206–211, https://doi.org/10.1159/000285289.

[18] S. Kheirouri, V. Hadi, M. Alizadeh, Immunomodulatory effect of nigella sativa oil on T lymphocytes in patients with rheumatoid arthritis, Immunol. Invest. 45 (2016) 271–283, https://doi.org/10.3109/08820139.2016.1153649.

[19] K. Ciesielska-Figlon, K.A. Lisowska, A. Mikosik-Roczynska, J.M. Witkowski, Nigella sativa oil inhibits proliferation and stimulates apoptosis of human lymphocytes in vitro, Hum. Immunol. 82 (2021) 608–614, https://doi.org/10.1016/j.humimm.2021.04.010.

[20] A. Ahlatci, A. Kuzhan, S. Taysi, O.C. Demirtas, H.E. Alkis, M. Tarakcioglu, A. Demirci, D. Caglayan, E. Saricicek, K. Cinar, Radiation-modifying abilities of Nigella sativa and thymoquinone on radiation-induced nitrosative stress in the brain tissue, Phytomedicine 21 (2014) 740–744, https://doi.org/10.1016/j.phymed.2013.10.023.

[21] S. Taysi, F.S. Algburi, Z. Mohammed, O.A. Ali, M.E. Taysi, Thymoquinone: a review of pharmacological importance, oxidative stress, COVID-19, and radiotherapy, Mini-Rev. Med. Chem. 22 (2022), https://doi.org/10.2174/1389557522666220104151225.

[22] L. Salim, S. Mohan, R. Othman, S.I. Abdelwahab, B. Kamalidehghan, B.Y. Sheikh, M.Y. Ibrahim, Thymoquinone induces mitochondria-mediated apoptosis in acute lymphoblastic leukaemia in vitro, Molecules 18 (2013) 11219–11240, https://doi.org/10.3390/molecules180911219.

[23] M. Diab-Assaf, J. Semaan, M. El-Sabban, S.K. Al Jaouni, R. Azar, M.A. Kamal, S. Harakeh, Inhibition of proliferation and induction of apoptosis by thymoquinone via modulation of tgf family, p53, p21 and Bcl-2α in leukemic cells, Anticancer. Agents Med. Chem. 18 (2018) 210–215, https://doi.org/10.2174/1871520617666170912133054.

[24] E.M. Dergarabetian, K.I. Ghattass, S.B. El-Sitt, R.M. Al-Mismar, C.O. El-Baba, W. S. Itani, N.M. Melhem, H.A. El-Hajj, A.A. Bazarbachi, R. Schneider-Stock, H. U. Gali-Muhtasib, Thymoquinone induces apoptosis in malignant T-cells via generation of ROS, Front. Biosci. 5 (2013) 706–719, https://doi.org/10.2741/e651.

[25] G. Xie, N. Chen, L. Soromou, F. Liu, Y. Xiong, Q. Wu, H. Li, H. Feng, G. Liu, P-cymene protects mice against lipopolysaccharide-induced acute lung injury by inhibiting inflammatory cell activation, Molecules 17 (2012) 8159–8173, https://doi.org/10.3390/molecules17078159.

[26] W. Zhong, G. Chi, L. Jiang, L. Soromou, N. Chen, M. Hou, W. Guo, X. Deng, H. Feng, P-cymene modulates in vitro and in vivo cytokine production by inhibiting MAPK and NF-κB activation, Inflammation 36 (2013) 529–537, https://doi.org/10.1007/s10753-012-9574-y.

[27] R. El Mezayen, M. El Gazzar, M.R. Nicolls, J.C. Marecki, S.C. Dreskin, H. Nomiyama, Effect of thymoquinone on cyclooxygenase expression and prostaglandin production in a mouse model of allergic airway inflammation, Immunol. Lett. 106 (2006) 72–81, https://doi.org/10.1016/j.imlet.2006.04.012.

[28] C. Bicchi, Essential oils. Gas Chromatography, in: I. Wilson (Ed.), Encyclopedia of Separation Science, Academic Press, 2000, pp. 2744–2755.

[29] J.M. Witkowski, Advanced application of CFSE for cellular tracking, Curr. Protoc. Cytom. 44 (9.25) (2008), https://doi.org/10.1002/0471142956.cy0925s44.

[30] S.K. Tiruppur Venkatachallam, H. Pattekhan, S. Divakar, U.S. Kadimi, Chemical composition of Nigella sativa L. seed extracts obtained by supercritical carbon dioxide, J. Food Sci. Technol. 47 (2010) 598–605, https://doi.org/10.1007/s13197-010-0109-y.

[31] E. Sieniawska, R. Sawicki, J. Golus, M. Swatko-Ossor, G. Ginalska, K. Skalicka-Wozniak, Nigella damascena L. essential oil—a valuable source of β-elemene for antimicrobial testing, Molecules 23 (2018) 256, https://doi.org/10.3390/molecules23020256.

[32] L. Kokoska, J. Havlik, I. Valterova, H. Sovova, M. Sajfrtova, I. Jankovska, Comparison of chemical composition and antibacterial activity of nigella sativa seed essential oils obtained by different extraction methods, J. Food Prot. 71 (2008) 2475–2480, https://doi.org/10.4315/0362-028x-71.12.2475.

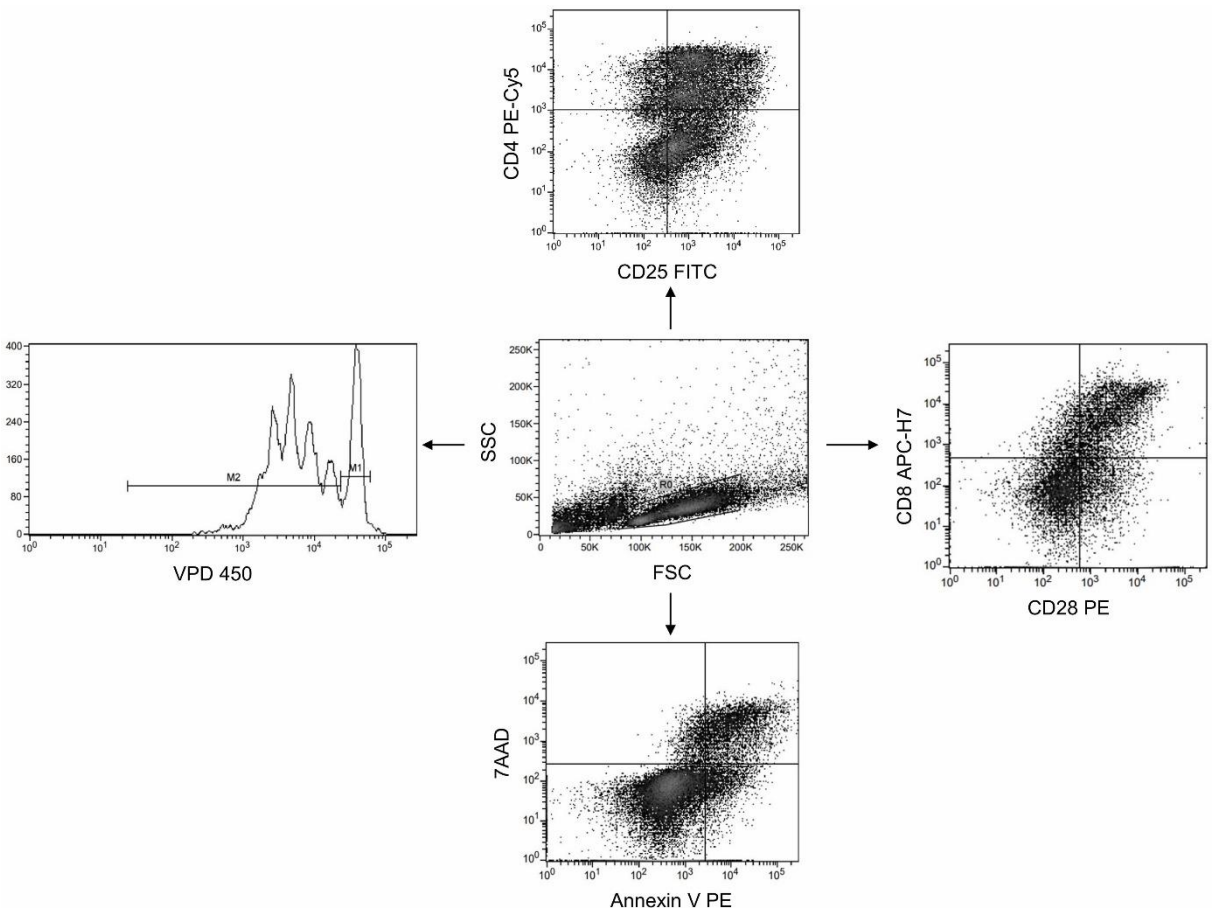
[33] A. Wajs, R. Bonikowski, D. Kalemba, Composition of essential oil from seeds ofNigella sativaL. cultivated in Poland, Flavour Fragr. J. 23 (2008) 126–132, https://doi.org/10.1002/ffj.1866.

[34] O. Gaudin, F. Toukal, C. Hua, N. Ortonne, H. Assier, A. Jannic, E. Giménez-Arnau, P. Wolkenstein, O. Chosidow, S. Ingen-Housz-Oro, Association between severe acute contact dermatitis due to nigella sativa oil and epidermal apoptosis, JAMA Dermatol. 154 (2018) 1062, https://doi.org/10.1001/jamadermatol.2018.2120.

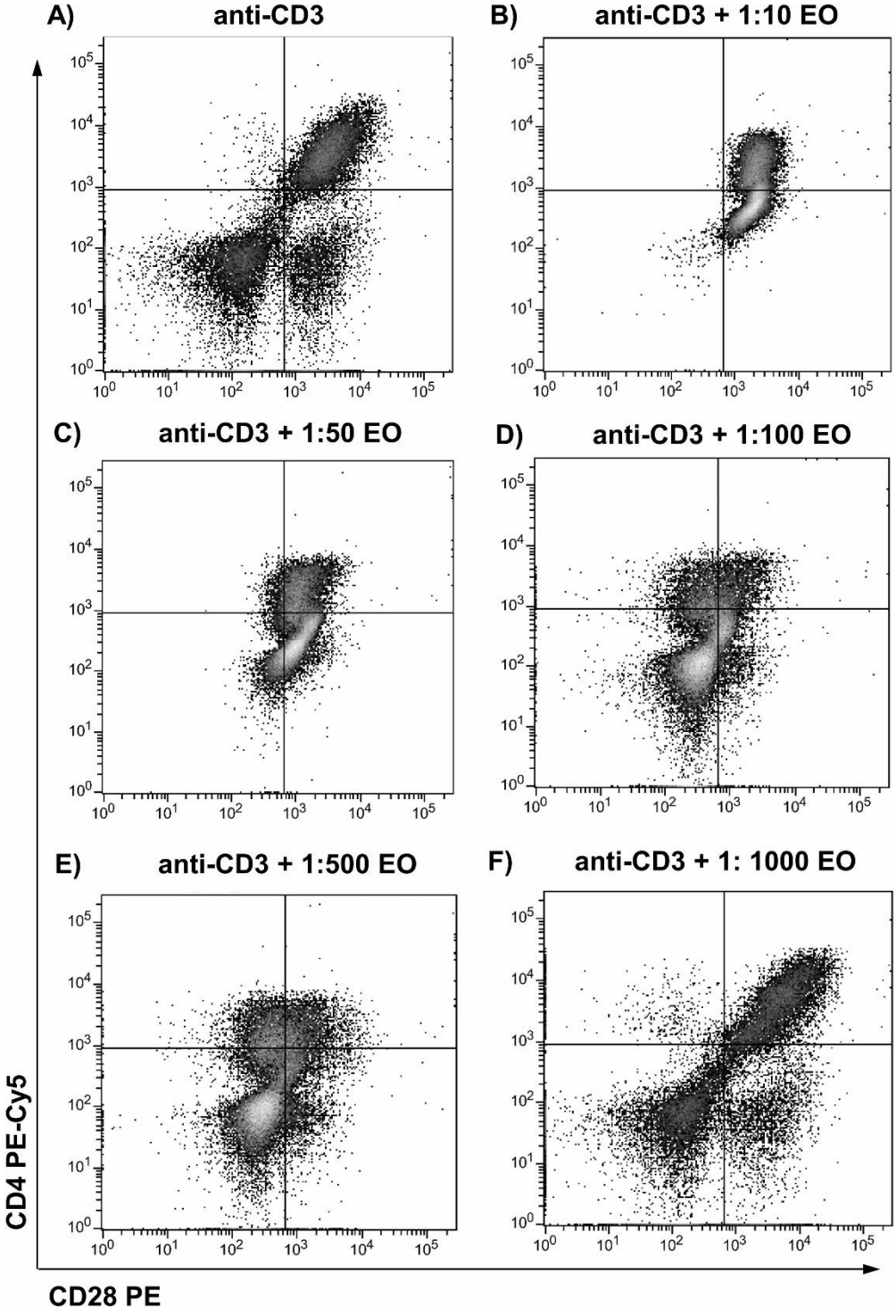
[35] U. Glamoclija, L. Mahmutovic, E. Bilajac, V. Soljic, K. Vukojevic, M. Suljagic, Metformin and thymoquinone synergistically inhibit proliferation of imatinib-resistant human leukemic cells, Front. Pharmacol. 13 (2022), 867133, https://doi.org/10.3389/fphar.2022.867133.

[36] R.O. Formiga, E.B. Alves Júnior, R.C. Vasconcelos, G.C.B. Guerra, A. Antunes de Araújo, T.G. Carvalho, V.B. Garcia, R.F. de Araújo Junior, F.A.A.F. Gadelha, G. C. Vieira, M.V. Sobral, J.M. Barbosa Filho, F. Spiller, L.M. Batista, p-cymene and rosmarinic acid ameliorate TNBS-induced intestinal inflammation upkeeping ZO-1 and MUC-2: role of antioxidant system and immunomodulation, Int. J. Mol. Sci. 21 (2020) 5870, https://doi.org/10.3390/ijms21165870.

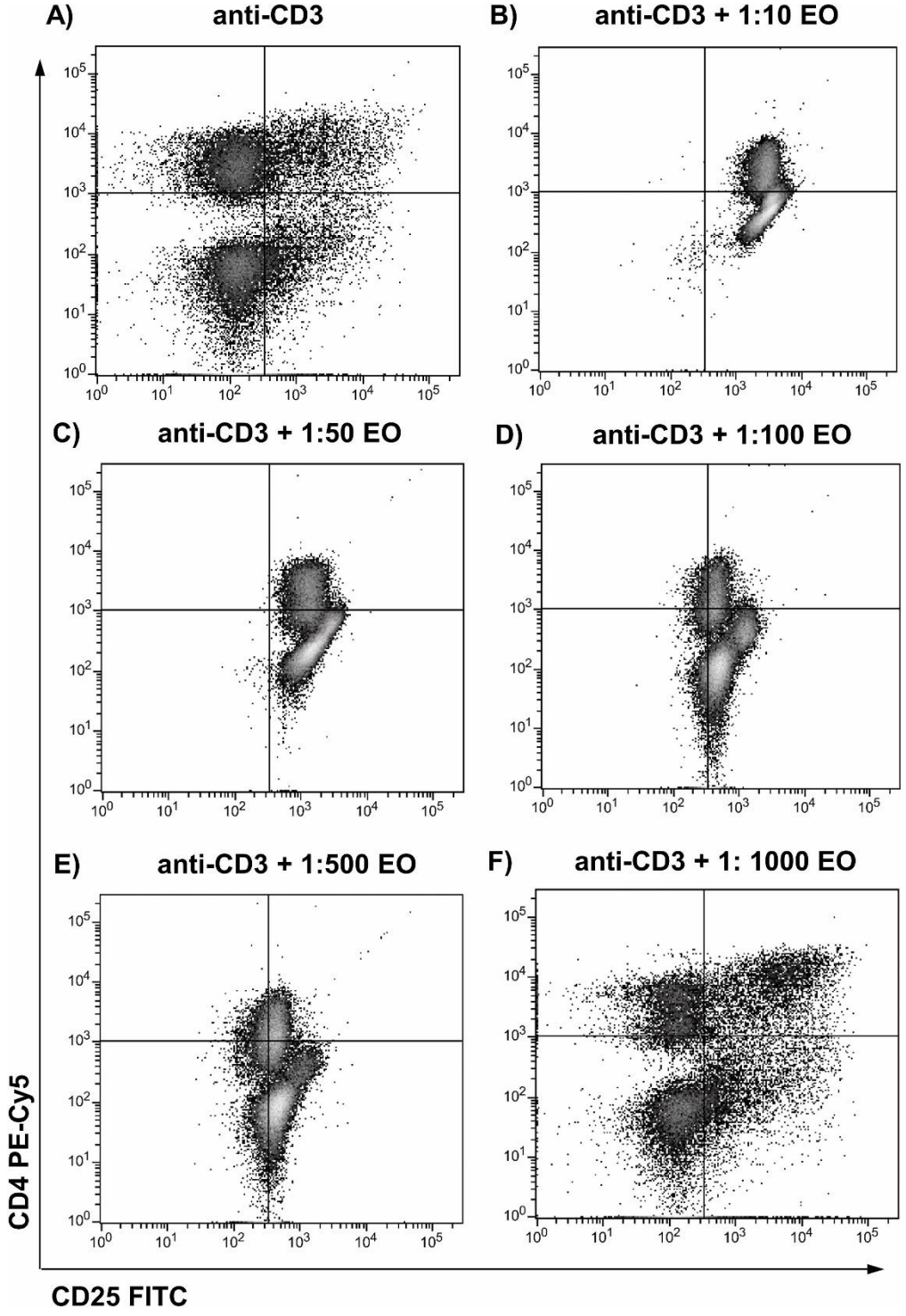
Supplementary Figures



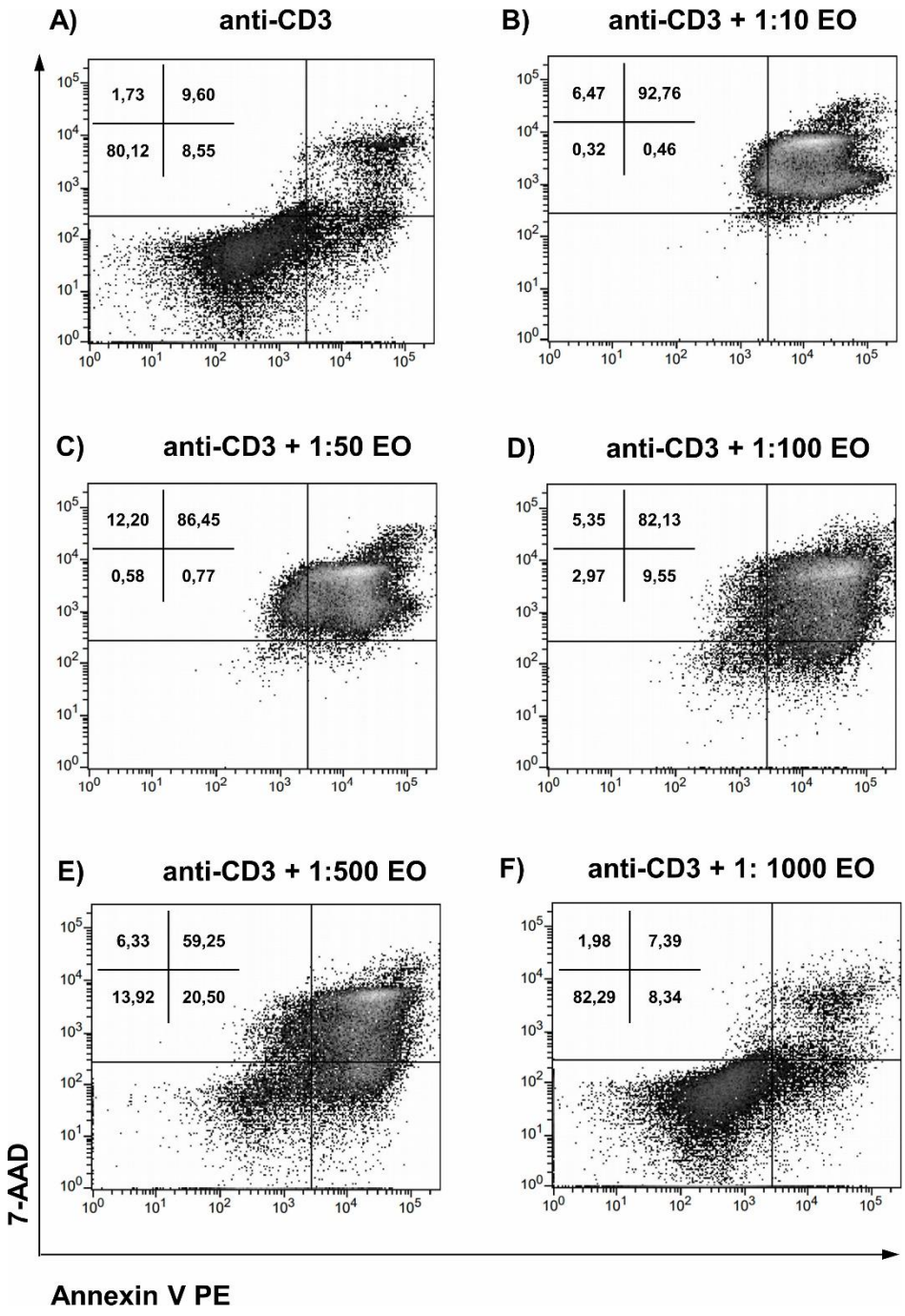
1. Representative dot-plots showing cytometric analysis of lymphocyte proliferation, apoptosis and necrosis, and expression of CD28 and CD25 antigens on $CD4^+$ and $CD8^+$ cells (explanation in Material and methods section).



2. Representative dot-plots showing CD4 and CD28 antigens' expression on lymphocytes stimulated for 72 hours with an anti-CD3 antibody with different NSEO dilutions. Graph A shows cells stimulated with anti-CD3 alone, B – cells stimulated with anti-CD3 and 1:10 NSEO dilution, C – cells stimulated with anti-CD3 and 1:50 NSEO, D – cells stimulated with anti-CD3 and 1:100 NSEO, E – cells stimulated with anti-CD3 and 1:500 NSEO, and F – cells stimulated with anti-CD3 and 1:1000 NSEO.



3. Representative dot-plots showing CD4 and CD25 antigens' expression on lymphocytes stimulated for 72 hours with an anti-CD3 antibody with different NSEO dilutions. Graph A shows cells stimulated with anti-CD3 alone, B – cells stimulated with anti-CD3 and 1:10 NSEO dilution, C – cells stimulated with anti-CD3 and 1:50 NSEO, D – cells stimulated with anti-CD3 and 1:100 NSEO, E – cells stimulated with anti-CD3 and 1:500 NSEO, and F – cells stimulated with anti-CD3 and 1:1000 NSEO.



4. Representative dot-plots showing percentages of living, apoptotic and necrotic lymphocytes stimulated for 72 hours with an immobilized anti-CD3 antibody with different NSEO dilutions. Graph A shows cells stimulated with anti-CD3 alone, B – cells stimulated with anti-CD3 and 1:10 NSEO dilution, C – cells stimulated with anti-CD3 and 1:50 NSEO, D – cells stimulated with anti-CD3 and 1:100 NSEO, E – cells stimulated with anti-CD3 and 1:500 NSEO, and F – cells stimulated with anti-CD3 and 1:1000 NSEO. Cells negative for annexin V and 7-AAD were considered alive, cells in early apoptosis were annexin V-positive and 7-AAD-negative, in late apoptosis – were positive for annexin V and 7-AAD, and necrotic cells were only 7-AAD-positive.

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The Impact of *Nigella sativa* Essential Oil on T Cells in Women with Hashimoto's Thyroiditis

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Abstract: Background: Hashimoto's thyroiditis (HT) is an autoimmune disease mediated by T cells. It is characterized by the presence of thyroid autoantibodies in the serum, such as anti-thyroid peroxidase antibodies (TPO-Ab) and anti-thyroglobulin antibodies (TG-Ab). The essential oil extracted from *Nigella sativa* seeds is rich in bioactive substances, such as thymoquinone and cymene. Methods: Therefore, we examined the effect of essential oil from *Nigella sativa* (NSEO) on T cells from HT patients, especially their proliferation capacity, ability to produce cytokines, and susceptibility to apoptosis. Results: The lowest ethanol (EtOH) dilution (1:10) of NSEO significantly inhibited the proliferation of $CD4^+$ and $CD8^+$ T cells from HT patients and healthy women by affecting the percentage of dividing cells and the number of cell divisions. In addition, 1:10 and 1:50 NSEO dilutions induced cell death. Different dilutions of NSEO also reduced the concentration of IL-17A and IL-10. In healthy women, the level of IL-4 and IL-2 significantly increased in the presence of 1:10 and 1:50 NSEO dilutions. NSEO did not influence the concentration of IL-6 and IFN-γ. Conclusions: Our study demonstrates that NSEO has a strong immunomodulatory effect on the lymphocytes of HT patients.

Keywords: *Nigella sativa*; essential oil; Hashimoto's thyroiditis; apoptosis; proliferation; immunomodulation

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1. Introduction

Hashimoto's thyroiditis (HT), also known as chronic autoimmune thyroiditis, is an autoimmune disease characterized by lymphocytic infiltration into and gradual damage to the thyroid parenchyma and the production of organ-specific anti-thyroid antibodies: anti-thyroid peroxidase (TPO-Ab) and anti-thyroglobulin (TG-Ab) [1–3]. Studies using reverse polymerase chain reaction to assess cytokine gene expression within the thyroid gland showed increased mRNA levels of IL-2, IL-4, IL-10, and INF-γ [4]. As with other diseases of autoimmune etiology, Hashimoto's thyroiditis is caused by a combination of genetic and environmental factors, leading to a loss of immune tolerance and resulting in the destruction of thyroid tissue [5]. Based on epidemiological studies, HT most commonly affects women between 30 and 50 years [1].

The etiology of Hashimoto's thyroiditis is complex. A critical moment in the pathogenesis of HT is the formation of autoreactive cells directed against the thyroid gland, which may result from a loss of immunological tolerance to its tissues. Immune tolerance is developed during the perinatal period. At that time, immature lymphocytes are exposed to autoantigens, and self-tolerance to autoantigens is conditioned by the clonal deletion and induced anergy of autoreactive T cells [6]. However, these mechanisms may fail, and some cells recognizing autoantigens enter the bloodstream, which leads to a breakdown of auto-tolerance, in this case, against thyroid antigens. This process may be caused by

overexposure to thyroid antigens and exposure to environmental antigens similar to autoantigens [7]. The central role in this process is attributed to T helper 1 (Th1) cells, a $CD4^+$ effector T cell lineage. Th1 cells are responsible for the lymphocytic inflammatory infiltration into the thyroid gland, which at a later stage causes thyroiditis and damage to the thyroid gland. They produce IFN-γ and activate the infiltration of macrophages into the thyroid gland, thereby inducing the secretion of various cytokines and the progressive damage of thyrocytes by apoptosis. Activated cytotoxic lymphocytes and macrophages are directly responsible for destroying thyroid follicular cells [8]. Furthermore, through stimulation with cytokines, mainly interleukin-1 (IL-1) secreted by antigen-presenting cells (APCs) and Th1 cells, follicular thyroid cells are induced to express molecules involved in the regulation of programmed cell death, including Fas and FasL. The result of this stimulation can be cell apoptosis [9].

In addition, $CD8^+$ (cytotoxic) T cells (CTLs) may cause thyrocyte destruction through the Fas–FasL pathway [9]. CTLs produce cytotoxic granules, such as perforin and granzymes (including granzyme B) [10]. The perforin molecule forms a pore in the cellular membrane of target cells, and granzyme B activates proapoptotic molecules, such as caspases and cytochrome c [11]. CTLs detected in HT patients could be one of the causative factors for thyrocyte destruction and hypothyroidism [12]. TPO- and Tg-specific $CD8^+$ T cells are present in the peripheral blood and thyroid gland of HT patients, where they induce the lysis of target cells [13].

In addition to Th1 cells, Th2 cells are also involved in the pathogenesis of HT. They stimulate B cells to produce antibodies against thyroid antigens, such as TPO and TG, thereby leading to thyroid tissue inflammation [14]. Furthermore, Th2 cells produce cytokines, such as IL-4, IL-5, IL-6, and IL-10, further stimulating the production of antithyroid antibodies. Hence, thyroid cells have been shown to produce cytokines, such as IL-1, IL-6, IL-8, TNF-α, and TGF-β, leading to their destruction [15].

There is increasing interest worldwide in alternative therapies based on herbal medicine or herbal extracts and their supplementation in chronic diseases, including Hashimoto's thyroiditis. Among the medicinal herbs gaining more and more attention is *Nigella sativa* (NS), also known as black cumin or black seed, which belongs to the Ranunculaceae family, cultivated in many regions, such as the central and eastern Mediterranean, southern Europe, India, Pakistan, Syria, and Turkey [16]. Black seeds are a source of active ingredients characterized by antioxidant, anti-inflammatory, and immunomodulatory properties [17]. NS seed supplementation has been shown to improve the efficacy of specific immunotherapy in patients with allergic rhinitis [18], rheumatoid arthritis (RA) [19], or asthma [20]. In women with RA, treatment with NS seed oil led to a significant reduction in serum C-reactive protein (CRP) levels and an improvement in the number of swollen joints compared to the control group [19]. In addition, a reduction in the percentage of $CD8^+$ (cytotoxic) T cells and an increase in $CD4^+CD25^+$ cells were also observed compared to the placebo. In young patients with asthma, supplementation with NS seed oil caused a significant reduction in Th17 cells and increased regulatory T cells (Tregs) [20].

The presented studies demonstrate the strong therapeutic potential and use of NS in treating autoimmune diseases. In hypothyroidism, the therapeutic effect of NS is attributed to its antioxidant action [21,22]. In a study by Farhangi et al. [23], an 8-week powdered NS treatment caused a decrease in TSH and TPO-Ab and increased serum triiodothyronine (T3) levels in HT patients. Similar results were obtained by Tajmiria et al. [24], who also investigated the effect of NS powder supplementation on thyroid function. Additionally, the authors demonstrated a reduction in serum IL-23, an inflammatory cytokine involved in amplifying and stabilizing Th17 cells. NS seed oil was also shown to have antioxidant activity that may reduce propylthiouracil (PTU)-induced oxidative stress and damage to thyroid follicles, which may have important therapeutic implications in hypothyroidism [25].

The reduction in serum TPO-Ab levels after treatment with NS seed oil or powder in different studies can be explained by its immunomodulatory effects, which have been previously confirmed by its protective role against several autoimmune diseases, including

type 1 diabetes and experimental autoimmune encephalomyelitis (EAE) [26,27]. Furthermore, Avci et al. [28] demonstrated that it is the essential oil (EO) obtained from NS (NSEO) that is responsible for increasing the concentration of total triiodothyronine (tT3) in hypothyroid and hyperthyroid models in rats. Still, there are not enough studies verifying the therapeutic effect of Nigella sativa in patients suffering from autoimmune diseases. Therefore, in order to explain the potential immunomodulatory properties of *Nigella sativa*, this study investigates the influence of NSEO on activated T cells, including their proliferation capacity, ability to produce cytokines, and susceptibility to apoptosis, in women with Hashimoto's thyroiditis.

2. Materials and Methods

2.1. Study Group

The study group consisted of 9 women diagnosed with Hashimoto's thyroiditis, with a mean age of 39 ± 8 years. The control group consisted of 9 healthy women with a mean age of 36 ± 8 years. The control group did not take any medication that could influence the immune system. All participants were informed about the purpose of the study and provided their written informed consent to participate in the study. The Independent Bioethics Committee for Scientific Research approved the study (consent no. NKBBN/417-233/2019, received on 10 April 2019). All experiments were conducted following relevant guidelines and regulations.

2.2. Plant Material and Essential Oil Isolation

The seeds of black cumin were obtained from Makar Bakalie, Poland. The material was stored in the dark in a hermetically sealed container and processed with convection drying (105 °C for 4 h, Binder FD oven, Tuttlingen, Germany). For NSEO isolation, a 100 g sample of NS seeds was frozen in liquid nitrogen. Next, the still-frozen material was moved into a round-bottom flask, water with an antifoam agent (Sigma Aldrich Inc., Saint Louis, MO, USA) was added, and the flask was connected to an etheric oil distillation apparatus (Carl-Roth, Karlsruhe, Germany). 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. The details of the processes and parameters are described in a previous paper [29].

2.3. PBMC Isolation and Stimulation

About 25 mL of peripheral venous blood was collected from each subject into EDTA blood collection tubes. Peripheral blood mononuclear cells (PBMCs) were next isolated by centrifuging the blood in a gradient on Histopaque®-1077 (Sigma Aldrich Inc., Saint Louis, MO, USA). Next, PBMCs were stained with Violet Proliferation Dye 450 (VPD450; Becton Dickinson, Franklin Lakes, NJ, USA) for 12 min in the dark at 37 °C according to the manufacturer's protocol. Afterward, the cells were washed in phosphate-buffered saline (PBS) (EURx, Gdańsk, Poland), after which they were resuspended in complete culture medium (RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin) at a concentration of 1.5 million cells per 1.5 medium.

The cells were then incubated with an immobilized (tissue culture plate-bound) anti-CD3 monoclonal antibody (BD Pharmingen, San Diego, CA, USA) for 24 h to activate the lymphocytes. Four (1:10, 1:50, 1:100, 1:500, and 1:1000) ethanol (EtOH) dilutions of NSEO were prepared. After 24 h, 3.75 µL of each EtOH NSEO dilution was added to preactivated cells. The final NSEO concentrations in the cell culture were 1:10–0.025%, 1:50–0.005%, 1:100–0.0025%, 1:500–0.0005%, and 1:1000–0.00025%. The controls consisted of cells stimulated with an anti-CD3 antibody (in experiments assessing proliferation, apoptosis, and necrosis) and unstimulated (US) cells (in experiments assessing cytokine production).

Stimulated cells were harvested after 72 h and 120 h. They were then stained with the following antibodies conjugated with fluorescent dyes: PerCP-conjugated anti-CD4

and APC-H7-conjugated anti-CD8 (BD Pharmingen, San Diego, CA, USA). In addition, cells were also stained with PE-conjugated annexin V and 7-aminoactinomycin D (7-AAD) according to the manufacturer's protocol (BD Pharmingen, San Diego, CA, USA) and analyzed with flow cytometry using a FACSVerse instrument (Becton Dickinson, Franklin Lakes, NJ, USA).

2.4. Measurement of Cytokines in Cell Culture Supernatants

We performed quantitative cytometric fluorescence analysis with the FACSAria III cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokines Kit (BD Biosciences, San Jose, CA, USA) was used according to the manufacturer's protocol to determine the level of seven different cytokines in the cell culture supernatant samples from HT patients and healthy controls: IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, IL-17A. The kit performance was optimized to analyze physiologically relevant concentrations (pg/mL levels) of specific cytokine proteins in tissue culture supernatants and serum samples. The limit of detection for IL-2 was 2.6 pg/mL; IL-4, 4.9 pg/mL; IL-6, 2.4 pg/mL; IL-10, 4.5 pg/mL; TNF, 3.8 pg/mL; IFN-γ, 3.7 pg/mL; and IL-17A, 18.9 pg/mL.

2.5. Analysis and Statistics

Lymphocytes were sorted based on forward scatter (FSC) and side scatter (SSC) characteristics and their positivity for surface antigens (CD4, CD8), as previously described [29]. Cytometric analysis was performed using FCSalyzer software (copyright © 2012–2019 Sven Mostböck).

The dividing cell tracking (DCT) method was applied to examine the cell proliferation kinetics [30]. It uses VPD450, which becomes fluorescent and covalently binds to proteins within the cells after cleavage by esterase within viable cells. As viable cells divide, the VPD450 dye is distributed uniformly between daughter cells, so each daughter cell retains approximately half of the VPD450 fluorescence intensity of its parent cell. In Figure 1, non-dividing cells are indicated with marker 1 (M1), while proliferating cells are indicated with M2. The number of divisions, on the contrary, is shown as the number of division peaks on the histogram within cells marked with M2. Annexin V and 7-AAD staining was used to identify cells as alive (cells negative for both 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).

Data, including the standard curve range for a given cytokine (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, or IL-17A), were statistically analyzed using GraphPad Prism software, version 9 (GraphPad Software, San Diego, CA, USA). Significance tests were selected according to the data distribution, with a significance level of $p < 0.05$.

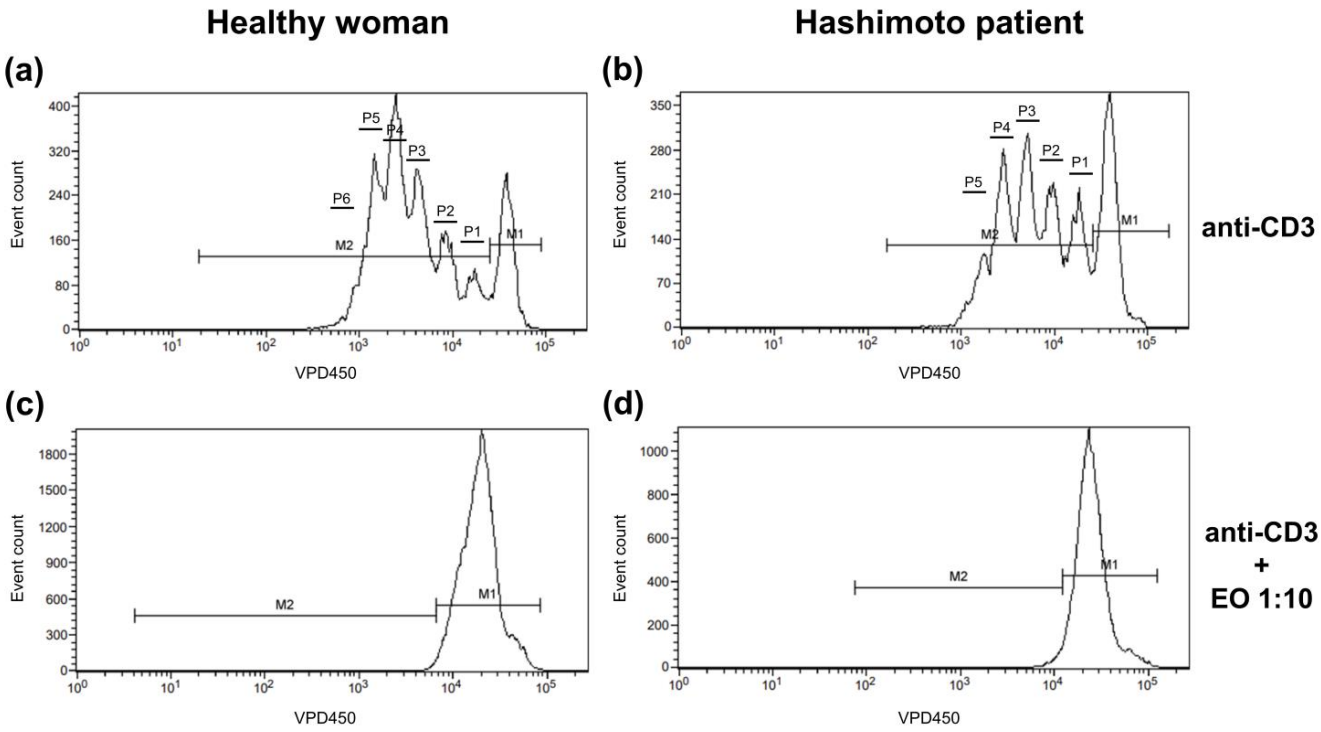

Figure 1. Representative histograms showing the proliferation pattern of $CD4^+$ cells stimulated with anti-CD3 antibody alone (**a**,**b**) or with 1:10 NSEO (**c**,**d**). Graphs show cell proliferation measured as VPD450 fluorescence. Marker 1 (M1) indicates non-diving cells; M2, the proportion of cells proliferating (daughter cells) in response to stimulation; and P1–P6, subsequent cell divisions.

3. Results

3.1. Influence of NSEO on T Cell Proliferation in HT Patients and Healthy Women

Figure 2 presents the number of cell divisions of lymphocytes stimulated with immobilized monoclonal anti-CD3 antibody in the presence of different EtOH dilutions of NSEO. In healthy women, a decrease in the number of cell divisions of $CD4^+$ (Figure 2a) and $CD8^+$ (Figure 2c) cells after 72 h of stimulation was observed in the presence of the lowest (1:10) dilution of NSEO compared to cells stimulated with anti-CD3 antibody alone. In HT patients, there was also a decrease in the number of cell divisions of $CD4^+$ and $CD8^+$ cells in the presence of 1:10 NSEO dilution after 72 h of stimulation. Additionally, there was a decrease in the number of cell divisions of $CD8^+$ cells in the presence of 1:50 and 1:100 NSEO in HT patients after 72 h (Figure 2c). After 120 h, there was also a decrease in the number of cell divisions of $CD4^+$ (Figure 2b) and $CD8^+$ (Figure 2d) cells from HT patients and healthy women when cells were incubated with 1:10 NSEO dilution. Significant differences were also seen between higher NSEO dilutions (1:500 and 1:1000) and 1:10 NSEO dilution, especially in healthy women.

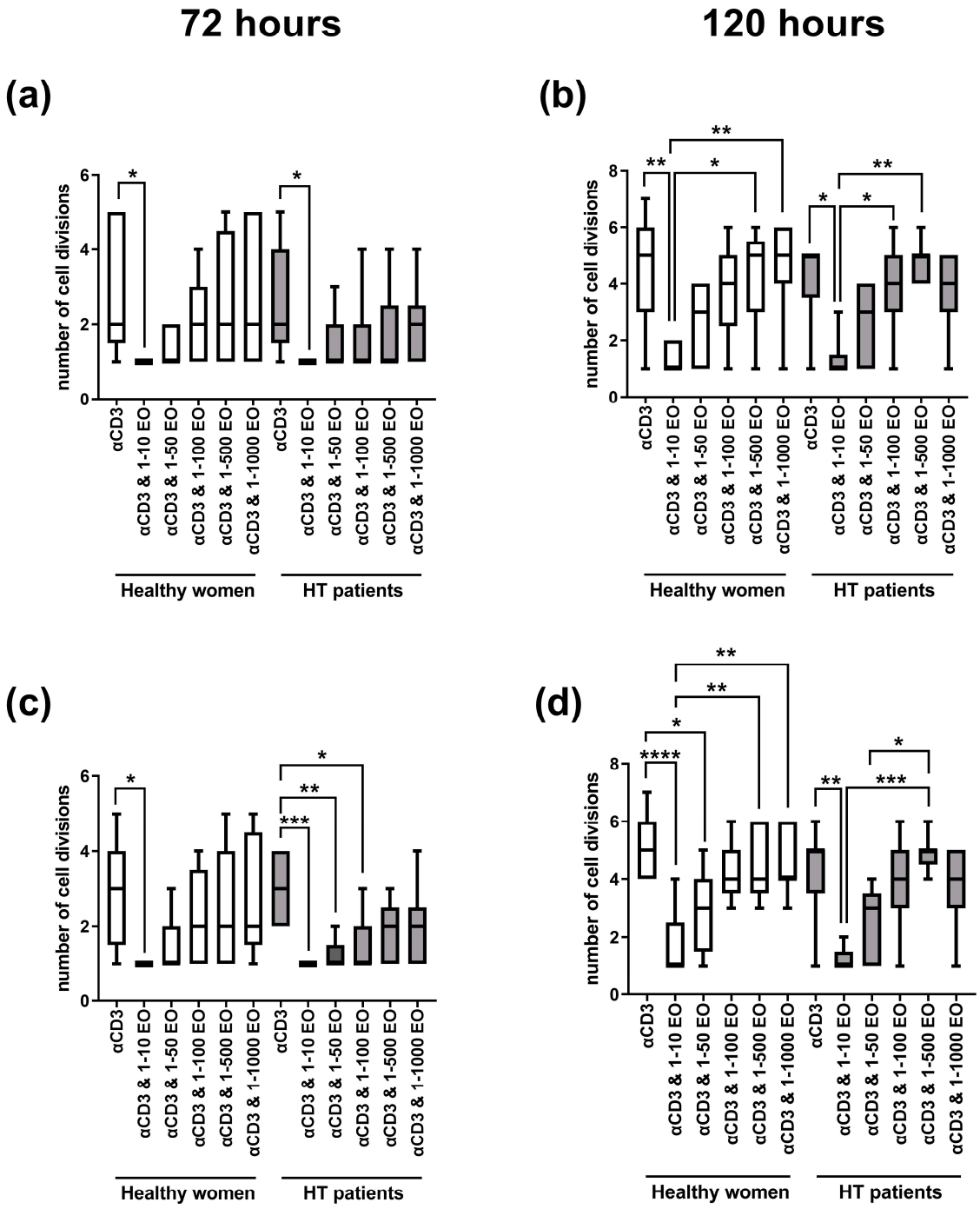

Figure 2. Comparison of the number of cell divisions of $CD4^+$ (**a**,**b**) and $CD8^+$ (**c**,**d**) cells stimulated with anti-CD3 antibody alone (control) or with different dilutions of NSEO for 72 (**a**,**c**) and 120 (**b**,**d**) hours in healthy people and HT patients. Graphs show the median, percentiles with the maximum and minimum values, and ANOVA Friedmann with Dunn's post hoc test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3 shows the changes in the percentage of dividing $CD4^+$ and $CD8^+$ cells. After 72 h, we observed a decrease in the percentage of proliferating cells in the presence of 1:10 NSEO in healthy women and HT patients for each T cell subpopulation ($CD4^+$ and $CD8^+$); see Figure 3a,c. In HT patients, the decrease in the percentage of proliferating CD4+ cells was significant compared to cells incubated with anti-CD3 antibody alone (Figure 3a). Meanwhile, in healthy women, the decrease in the percentage of proliferating

$CD8^+$ cells was significant compared to cells incubated with anti-CD3 antibody alone and cells incubated in the presence of 1:500 and 1:1000 NSEO dilutions (Figure 3c). After 120 h, there was also a decrease in the percentage of proliferating $CD4^+$ and $CD8^+$ cells in healthy women and HT patients when cells were incubated with 1:10 NSEO dilution compared to cells incubated with anti-CD3 antibody alone (Figure 3b,d). In healthy women, the decrease was also significant when compared to cells incubated with 1:100 or 1:500 NSEO dilution. In HT patients, the decrease was significant compared to cells incubated with 1:500 NSEO dilution but not when cells were incubated with 1:100 NSEO dilution.

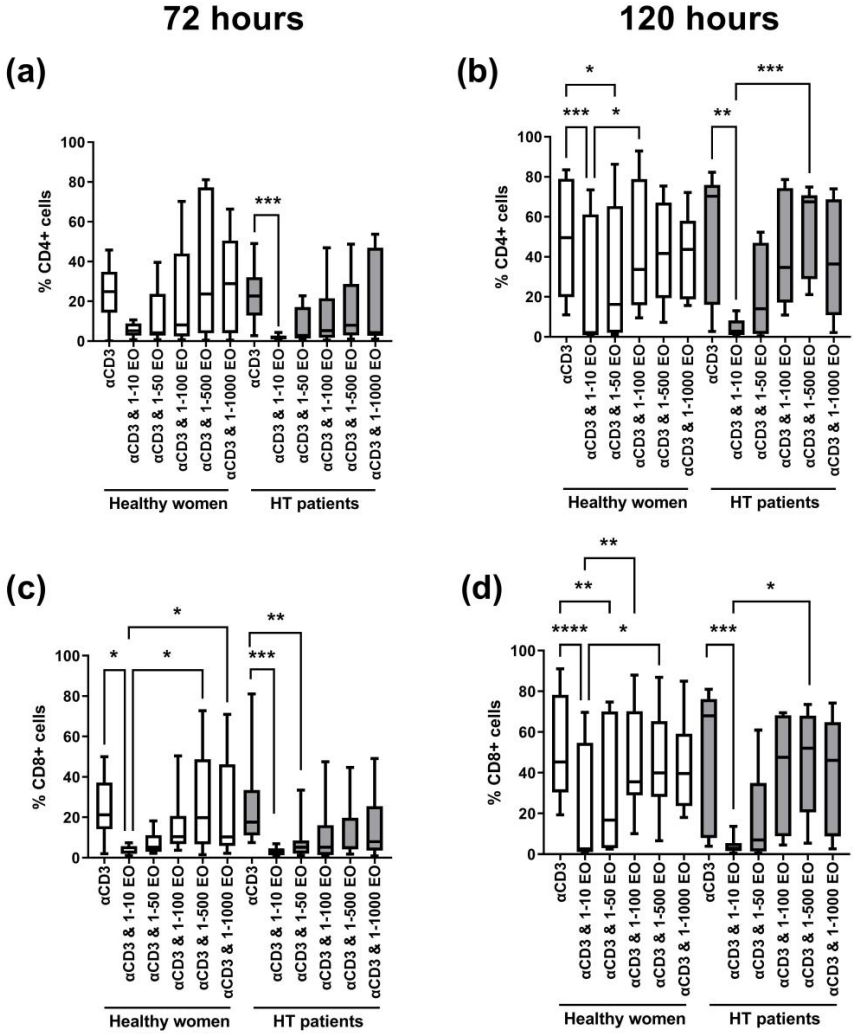

Figure 3. Comparison of the percentage of proliferating $CD4^+$ (**a**,**b**) and $CD8^+$ (**c**,**d**) cells stimulated with anti-CD3 antibody alone (control) or with different dilutions of NSEO for 72 (**a**,**c**) and 120 (**b**,**d**) hours in healthy people and HT patients. Graphs show the median, percentiles with the maximum and minimum values, and ANOVA Friedmann with Dunn's post hoc test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2. Influence of NSEO on Lymphocyte Apoptosis in HT Patients and Healthy Women

The most significant differences in the percentages of live, apoptotic, or necrotic cells were seen between lymphocytes stimulated only with anti-CD3 antibody and cells incubated in the presence of 1:10 and 1:50 dilutions of NSEO. After 72 h, in healthy women and HT patients, there was a significant decrease in the percentage of living cells after incubation with 1:10 and 1:50 NSEO (Figure 4a), with a simultaneous increase in cells in late apoptosis (Figure 4c) when compared with cells incubated with anti-CD3 antibody alone or in combination with higher dilutions (1:100, 1:500, and 1:1000). The percentage

of cells in early apoptosis decreased after 72 h incubation with 1:10 dilution of NSEO in healthy women and HT patients (Figure 4b). In healthy people, there was no change in the percentage of necrotic cells after NSEO treatment (Figure 4d). In HT patients, there was an increase in necrotic cells after incubation with 1:10 and 1:50 NSEO dilutions compared to cells incubated with anti-CD3 antibody alone or in combination with higher dilutions of NSEO.

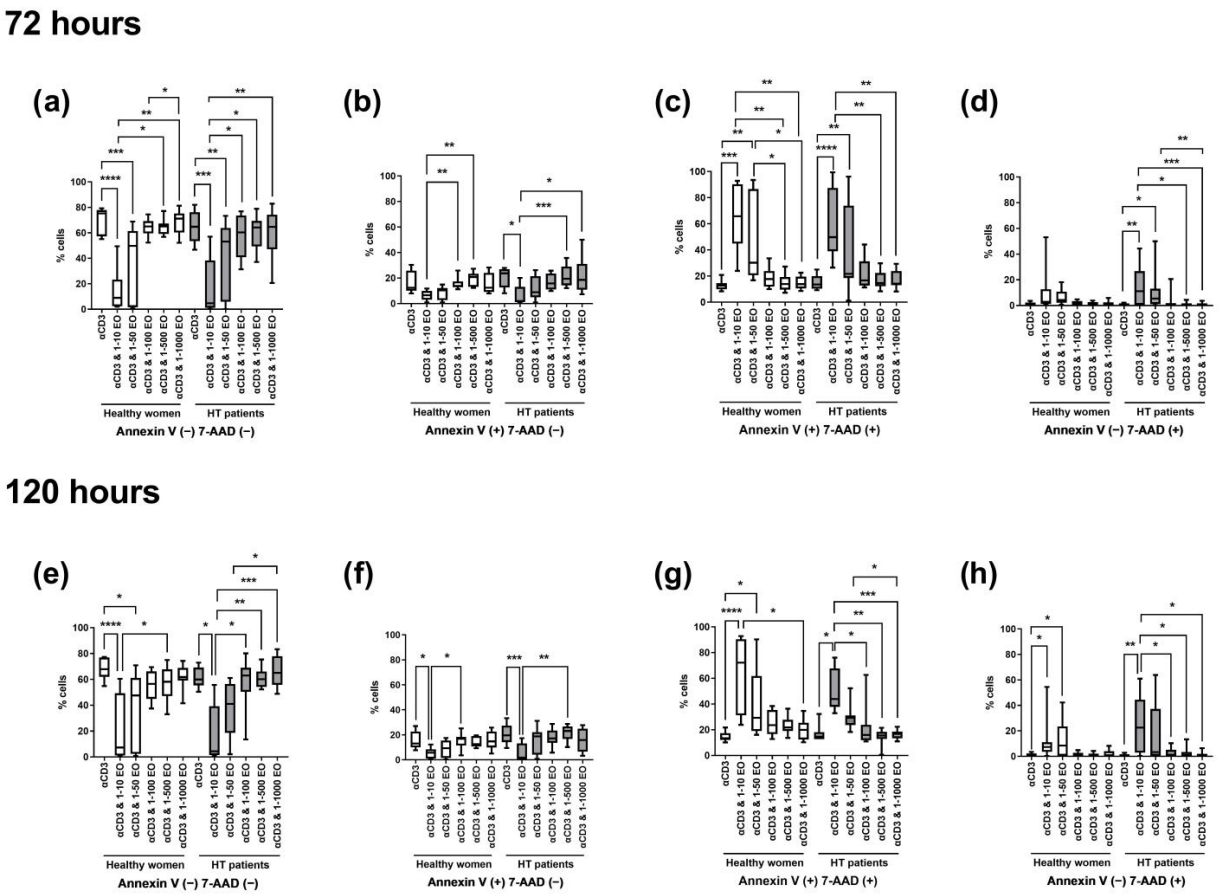

Figure 4. Comparison of the percentage of living, apoptotic (early and late), and necrotic lymphocytes stimulated for 72 (**a**–**d**) and 120 (**e**–**h**) hours with anti-CD3 antibody (control) with different dilutions of NSEO in healthy people and HT patients. Graphs show the median, percentiles with the maximum and minimum values, and ANOVA Friedman with Dunn's post hoc test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

After 120 h, there was a decrease in the percentage of living cells after incubation with the lowest NSEO dilution (1:10) compared to other stimulation variants in healthy women and HT patients. There were significant differences between the lowest dilutions (1:10 and 1:50) in healthy controls and between 1:10 and higher dilutions, such as 1:100, 1:500, and 1:1000, in HT patients (Figure 4e). The percentages of cells in late apoptosis (Figure 4g) and necrosis (Figure 4h) were higher in the presence of 1:10 and 1:50 NSEO dilutions in healthy women and in the presence of 1:10 NSEO in HT patients. There was a considerable statistical difference in those variants, especially between the control variant and a low dilution of NSEO and between the 1:10 variant and higher dilutions, such as 1:100, 1:500, and 1:1000. The changes in the percentage of cells in early apoptosis were similar to those observed after 72 h. There was a significant difference between the control variant and 1:10 NSEO in healthy controls and HT patients. There was also a significant difference between the 1:10 and 1:100 variants in healthy controls and 1:500 dilution in HT patients (Figure 4f).

3.3. Influence of NSEO on the Production of Cytokines in HT Patients and Healthy Women

We also compared the concentrations of cytokines (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A) in cell culture supernatants after 72 h of stimulation with immobilized monoclonal anti-CD3 antibody in the presence of different EtOH dilutions of NSEO. In addition, supernatants from wells where cells were incubated without stimulation served as an unstimulated (US) control.

In both healthy women and HT patients, IL-2 decreased after stimulation with anti-CD3 antibody alone (Figure 5a). In healthy women, the level of IL-2 significantly increased in the presence of 1:10 and 1:50 NSEO dilutions and decreased in the presence of 1:100, 1:500, and 1:1000 NSEO dilutions. Meanwhile, in HT patients, the level of IL-2 significantly decreased in the presence of 1:50, 1:100, and 1:1000 NSEO dilutions.

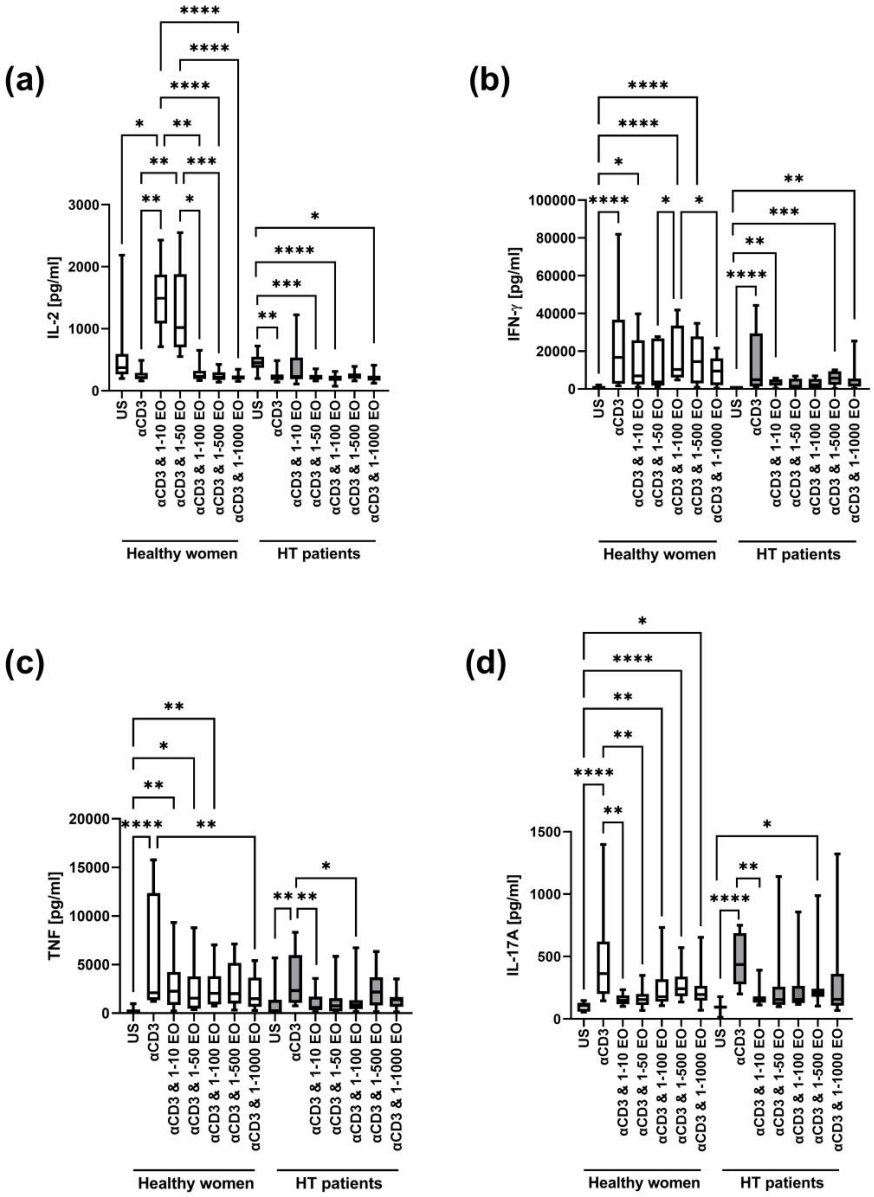

Figure 5. Comparison of Th1 and Th17 cytokine levels produced by cells stimulated for 72 h with anti-CD3 antibody with different dilutions of NSEO in healthy controls and HT patients. Graphs show the levels of IL-2 (**a**), INF-γ (**b**), TNF (**c**), and IL-17A (**d**). Graphs show the median, percentiles with the maximum and minimum values, and ANOVA Friedmann with Dunn's post hoc test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. US—unstimulated.

In healthy women, the IFN-γ level increased after stimulation with anti-CD3 antibody alone or in the presence of 1:10, 1:100, and 1:500 NSEO dilutions compared to the unstimulated control (Figure 5b). In HT patients, the IFN-γ level also increased after stimulation with anti-CD3 antibody alone compared to the unstimulated control. In addition, in the presence of 1:10, 1:500, and 1:1000 NSEO dilutions, the IFN-γ level also increased compared to the unstimulated control, but it was visibly lower compared to anti-CD3 antibody alone.

In addition, in both study groups, the TNF level increased after stimulation with anti-CD3 antibody compared to the unstimulated control (Figure 5c). In healthy women, the level of TNF significantly increased in the presence of 1:10, 1:50, and 1:100 NSEO dilutions compared to the unstimulated control. Meanwhile, in HT patients, the level of TNF significantly decreased in the presence of 1:10 and 1:100 NSEO dilutions compared to anti-CD3 antibody stimulation.

In healthy women and HT patients, there was an increase in IL-17A levels after stimulation with anti-CD3 antibody compared to the unstimulated control (Figure 5d). In healthy women, IL-17A levels decreased in the presence of 1:10 and 1:50 NSEO dilutions compared to anti-CD3 antibody. In addition, IL-17A levels increased in the presence of 1:100, 1:500, and 1:1000 NSEO compared to the unstimulated control, but the levels were visibly lower compared to anti-CD3 antibody alone. In HT patients, IL-17A levels decreased in the presence of 1:10 NSEO, but in the presence of 1:500 NSEO, IL-17A levels increased compared to the unstimulated control.

The level of IL-4 in supernatants after stimulation with anti-CD3 antibody alone did not change significantly compared to the unstimulated control in HT patients and healthy women (Figure 6a). However, the IL-4 level increased in the presence of 1:10 and 1:50 NSEO in healthy women and in the presence of 1:10 NSEO in HT patients. However, the level of IL-4 in the presence of 1:10 NSEO increased to a lesser extent in HT patients compared to healthy women ($p = 0.0002$, Mann–Whitney U test). In healthy women, there were differences between the unstimulated control and cells stimulated with 1:10 and 1:50 NSEO dilutions; between cells stimulated only with anti-CD3 antibody and with 1:10 and 1:50 NSEO dilutions; between cells stimulated with 1:10, 1:100, 1:500, and 1:1000 NSEO dilutions; and between cells stimulated with 1:50, 1:500, and 1:1000 NSEO dilutions. In HT patients, the difference was only between the unstimulated control and cells stimulated with 1:10 NSEO dilution.

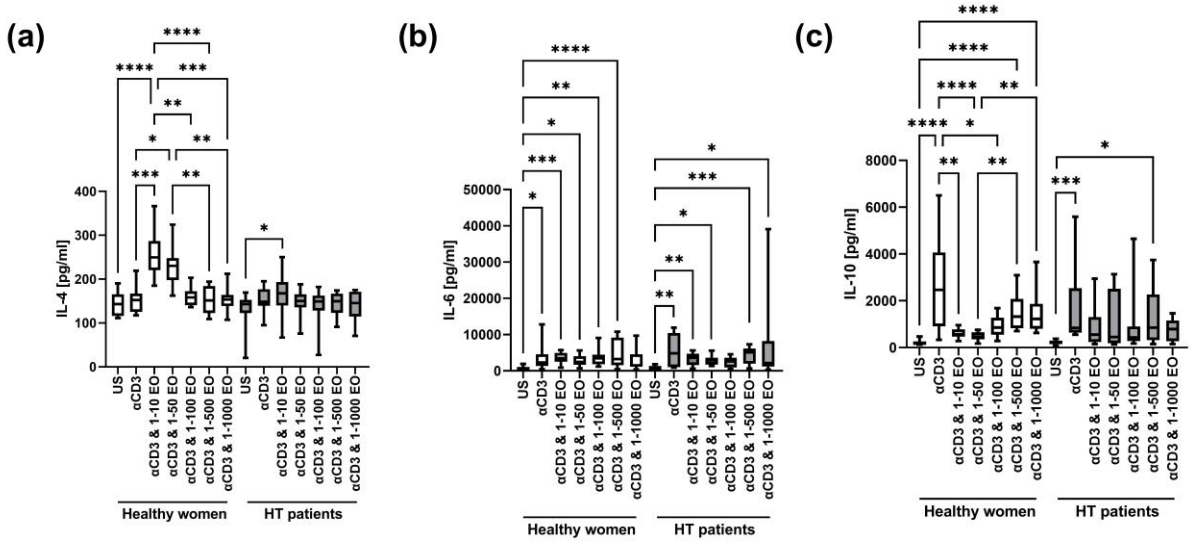

Figure 6. Comparison of Th2 cytokine levels produced by cells stimulated for 72 h with anti-CD3 antibody with different dilutions of NSEO in healthy controls and HT patients. Graphs show the levels of IL-4 (**a**), IL-6 (**b**), and IL-10 (**c**). Graphs show the median, percentiles with the maximum and minimum values, and ANOVA Friedmann with Dunn's post hoc test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. US—unstimulated.

There was an increase in IL-6 levels after stimulation with anti-CD3 antibody compared to the unstimulated control in both study groups (Figure 6b). In healthy women, the level of IL-6 also increased in the presence of 1:10, 1:50, 1:100, and 1:500 NSEO dilutions compared to the unstimulated control. In HT patients, IL-6 levels increased in the presence of 1:10, 1:50, 1:500, and 1:1000 NSEO dilutions compared to the unstimulated control.

There was an increase in the IL-10 level after stimulation with anti-CD3 antibody compared to the unstimulated control in both study groups (Figure 6c). In healthy women, the IL-10 level decreased in the presence of 1:10 and 1:50 NSEO dilutions compared to anti-CD3 antibody alone. Compared to the unstimulated control, the IL-10 level increased in the presence of 1:500 and 1:1000 NSEO dilutions. In HT patients, the level of IL-10 was significantly lower in unstimulated cells than in those exposed to 1:500 NSEO dilution.

4. Discussion

The pathogenesis of HT is determined by a disturbed immune system, manifested by the accumulation of autoreactive lymphocytes and macrophages with the simultaneous loss of immune tolerance to one's own tissues, which in turn leads to the destruction of the thyroid gland and the development of hypothyroidism [2]. $CD4^+$ T cells play a significant role in HT immunopathogenesis. When they get activated, they differentiate into different subtypes, depending on the cytokines released. By secreting interferon-gamma (IFN-γ), Th1 cells control macrophage-dependent cell-mediated immunity. Meanwhile, Th2 cells secrete IL-4, IL-5, or IL-10 and regulate B cell responses [31]. IL-12 is crucial for activating Th1 responses, while IL-6 promotes the IL-4-dependent induction of Th2 differentiation. In addition, $CD8^+$ T cells may cause thyrocyte destruction through the Fas–FasL pathway [9] or perforin and granzymes [10]. Suppression of T cell responses could play a clinical role in the development and course of Hashimoto's thyroiditis, even at the stage of full progression of the disease, because the thyroid tissue of HT patients is infiltrated by $CD69^+$ and $CD25^+$, with moderate numbers of Foxp3+ cells. In addition, the number and function of peripheral Tregs decrease, indicating that suppression of the T cell response is still clinically crucial at the stage of full progression of the disease [32].

The effect of NS on T cells in patients with Hashimoto's thyroiditis has not been studied so far. However, Farhangi et al. [24] showed that supplementation with powdered NS significantly reduces serum TSH and TPO-Ab concentrations in HT patients. Therefore, we decided to analyze the influence of NSEO on some essential properties of T cells from HT patients. We focused on examining T cells' proliferation capacity, susceptibility to apoptosis and necrosis, and ability to produce cytokines. To do that, we prepared an experimental model in which human PBMCs from healthy women and HT patients were preactivated by 24 h stimulation with an immobilized monoclonal anti-CD3 antibody. Next, different NSEO dilution variants were added to the cell culture. The purpose of such an experimental protocol was to mimic in vivo conditions in HT patients, where lymphocytes are continuously activated upon contact with an autoantigen. The incredible advantage of this model was the use of PBMCs, which are a mixture of different cells; in addition to T cells, there are also B cells, dendritic cells (DCs), and monocytes. In HT, the role of not only T cells but also of other cells that participate in the presentation of autogens and stimulate antibody production is essential.

Our findings showed that the proliferation of $CD4^+$ and $CD8^+$ T cells from healthy women and HT patients is significantly inhibited mainly in the presence of the lowest (1:10) NSEO dilution; the number of cell divisions reduced, and the percentage of dividing cells decreased. In our previous work, we used a different stimulation protocol in which NSEO was added simultaneously with anti-CD3 antibody, which resulted in a much more significant reduction in the percentage of proliferating T cells from healthy people, observed even at higher NSEO dilutions (1:50 and 1:100) [29]. Thus, adding NSEO before stimulation has a much stronger effect, probably due to the inhibition of T cell activation through the TCR/CD3 complex. Meanwhile, the effect of NSEO on previously activated T cells is weaker due to earlier activation of specific signaling pathways essential for their function.

Our results would explain the observation made by Kheirouri et al. [19], who demonstrated that oral supplementation with NS oil decreases the percentage of blood $CD8^+$ T cells in RA patients. It could also explain a reduction in RA symptoms after oral NS supplementation. Similar observations were made in other immune-related diseases; symptoms reduced after consuming NS seeds or oil in patients with asthma [33] and allergic rhinitis [18,34]. The common feature of these diseases is the malfunction of $CD4^+$ T cells, which react to antigens that usually do not cause any reaction in healthy people (allergens) or autoantigens present in the body and then induce a response of $CD8^+$ T cells and B cells.

A reduction in the percentage of proliferating T cells in the presence of 1:10 NSEO dilution in cell culture was related to intensified cell apoptosis and necrosis. Cell death was also induced in the presence of 1:50 NSEO. Similar effects were seen in HT patients and healthy women. Similar cytotoxic activity was also observed for methanolic NS seed extract in human lymphoma U937 cells [35], SiHa human cervical cancer cells [36], and MCF- 7 breast cancer cells [37]. NS inhibited cell proliferation and stimulated apoptosis by activating p53 and caspases. However, there are some papers describing the protective effect of NS. For instance, Tripathi and Pandey [38] showed that the methanolic extract from NS seeds protects human lymphocytes stimulated with phorbol myristate acetate (PMA) from apoptosis. Salem et al. [39] showed that NS oil increases the numbers of $CD4^+$ T cells but not $CD8^+$ T cells in mice.

It has been suggested that thymoquinone could be the primary bioactive constituent of NSEO as it induces mitochondria-mediated apoptosis of leukemic cell lines through the Bax pathway [40,41]. Another proposed active compound is α-hederin, which causes the formation of membrane pores and changes in the cell membrane, including vacuolization of the cytoplasm, leading to cell death in cultures of cancerous (melanoma) and non-cancerous mouse 3T fibroblast cells [42,43]. However, essential oils of plants are a mixture of volatile substances of different natures and complex chemical compositions. Their rich chemical composition determines their multidirectional biological activity. As substances with high lipophilicity, they easily penetrate the cell wall and membranes of various microorganisms, disrupting the integrity of these structures. One of the proposed mechanisms of toxicity against cells of EOs or their terpenoid components is the coagulation of the cytoplasm and, above all, the permeabilization of the cell membrane, which causes excessive loss of ions, which consequently lowers the membrane potential, disrupting the functioning of proton pumps and the associated reduction in the pool of intracellular ATP [44].

In Hashimoto's thyroiditis, the immune response of Th1 lymphocytes predominates, which, together with macrophages, produce mainly pro-inflammatory cytokines, including INF-γ, TNF-α, IL-1, and IL-6. This mechanism activates the apoptosis of thyroid cells through FasL co-expressed on the cell surface [45]. In our study, we observed that cells from HT patients produced high levels of IL-10, TNF, IFN-γ, and IL-17A after stimulation with anti-CD3 antibody alone. Most of the listed cytokines are involved in developing Th1 and Th17 responses. While the immunopathogenesis of HT is mainly associated with excessive activity of Th1 cells and CTLs, it has been shown that HT patients have increased levels of circulating Th17 cells and that Th17 cells are found in the thyroid gland [46,47]. IL-17, a pro-inflammatory cytokine, induces the production of other pro-inflammatory cytokines, such as IL-1β and IL-6 [48]. An increase in the same cytokines was also observed in healthy women in this study, indicating a strong activation of Th1 and Th17 responses under culture conditions.

Our results also demonstrated that Th1, Th2, and Th17 cells do not produce cytokine synchronously, regardless of health status. The dynamics of cytokine secretion vary [49], which explains why IL-10, TNF, IL-6, IFN-γ, and IL-17A increased in cell culture supernatants after 72 h of stimulation with anti-CD3 antibody alone, while IL-2 and IL-4 were low. The secretion of IL-2 is a critical and early event in the activation program of T cells. The production of this critical cytokine is subject to multifactorial regulation at the level of chromatin remodeling, transcription, message stability, and possibly even translation. In vivo studies using animal models have demonstrated that the transcript can be detected

in naive T cells as early as 1 h after stimulation and peaks at 4–6 h [50]. However, maximal intracellular accumulation of the protein in vivo has been reported to take as long as 12–14 h [51]. Next, IL-2 production terminates quickly, and secretion is undetectable by 20–24 h in either cell type [52]. This explains why IL-2 in the supernatants was low after anti-CD3 antibody stimulation in HT patients and healthy women in this study. Studies show that the secretion of IL-4 is generally low compared to IFN or IL-17 [49], which we also saw in our model.

Noteworthy was the significant decrease in IL-17A production in the presence of the broad spectrum of NSEO dilutions in HT patients and healthy women. It was recently shown in a mouse model that B cells induce the activation of Th17 cells [53], and these cells are inhibited by brodalumab in the treatment of various autoimmune diseases, such as systemic sclerosis [54]. In our study model, among PBMCs, B cells were found. From our observations, B cell proliferation is also inhibited in the presence of NSEO. Therefore, they probably contribute to IL-17A secretion in both healthy women and HT patients.

In healthy women, the presence of 1:10 and 1:50 NSEO dilutions in cell culture stimulated the release of IL-2 and IL-4 into cell culture supernatants. In HT patients, the concentration of IL-4 was higher in the presence of 1:50 NSEO, without affecting the IL-2 levels. These results could suggest that NSEO shifts the T cell response toward Th2, as evidenced by the increased IL-4 levels. Barlianto et al. [55] obtained the opposite effect while examining the effect of NS oil supplementation in children with asthma. The authors demonstrated that 8 weeks of NS oil supplementation increases serum IFN-γ levels and reduces IL-4 levels, thus redirecting the T cell response toward Th1. The discrepancy in the results may be because HT is dominated by the Th1 response, which under the influence of NSEO is redirected toward Th2. Meanwhile, in asthma, the Th2 response is dominant, which under the influence of NS may have been redirected toward Th1, proving the immunomodulatory properties of NSEO. However, it should be emphasized that the authors analyzed serum cytokine levels after NS oil supplementation, while we assessed the effect of NSEO in vitro. Therefore, it seems more likely that the release of IL-2 and IL-4 into cell culture supernatants resulted from the cytotoxic effect of NSEO on lymphocytes in the tested dilutions.

Still, the phenomenon cannot be only explained by intensified cell death because some cytokines (e.g., IFN-γ, IL-6) were unaffected by NSEO and some (e.g., IL-17A) decreased in the broad spectrum of NSEO dilutions, even those that did not cause apoptosis. In addition, the sensitivity of cells to NSEO was also dependent on health status. For example, TNF was not significantly affected by NSEO in healthy women, while it decreased in the presence of 1:10 and 1:100 NSEO dilutions in HT patients. In contrast, IL-10 decreased in the presence of 1:10–1:100 NSEO dilutions in healthy women, but in HT patients, it remained unaffected.

5. Conclusions

Even though the size of the study groups was relatively small, we demonstrated that essential oil sourced from *Nigella sativa* seeds has a proapoptotic and antiproliferative effect on activated T cells in patients with Hashimoto's thyroiditis under cell culture conditions. In addition, NSEO also affects the Th1/Th2/Th17 cytokine production by reducing the concentration of some cytokines important for regulating inflammatory processes, such as TNF and IL-10, and the Th1 response, i.e., IFN-γ, and increasing the production of IL-4 associated with the Th2 response in healthy women and HT patients.

It is unquestionably true that the differences in the T cell reaction between HT patients and healthy participants are relatively small. The exceptions are changes in the concentrations of IL-2 and IL-4 in cell culture supernatants in response to NSEO. However, we believe that even those modest differences may reflect a changing trend and potentially affect Hashimoto's thyroiditis. Furthermore, HT patients are predisposed to other autoimmune conditions, which means potential autoreactive cells are present among blood lymphocytes. Further research with a broader range of measures and a larger study group

is needed to fully understand the significance of these differences between the study and control groups.

To the best of our knowledge, this is the first scientific study that explains the mechanisms of action of *Nigella sativa* on immune cells. Furthermore, it shows that NSEO has a strong immunomodulatory effect that could favorably affect the abnormal response of T cells in immune-related diseases, such as allergies or autoimmune syndromes. Still, additional studies are necessary to explain how exactly NSEO influences lymphocytes and what signaling pathways it affects.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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References

1. Ahmed, R.; Al-Shaikh, S.; Akhtar, M. Hashimoto Thyroiditis. *Adv. Anat. Pathol.* **2017**, *19*, 181–186. [CrossRef] [PubMed]
2. Dayan, C.M.; Daniels, G.H. Chronic Autoimmune Thyroiditis. *N. Engl. J. Med.* **1996**, *335*, 99–107. [CrossRef] [PubMed]
3. Ragusa, F.; Fallahi, P.; Elia, G.; Gonnella, D.; Paparo, S.R.; Giusti, C.; Churilov, L.P.; Ferrari, S.M.; Antonelli, A. Hashimotos' thyroiditis: Epidemiology, pathogenesis, clinic and therapy. *Best Pract. Res. Clin. Endocrinol. Metab.* **2019**, *33*, 101367. [CrossRef]
4. Lee, H.J.; Li, C.W.; Hammerstad, S.S.; Stefan, M.; Tomer, Y. Immunogenetics of autoimmune thyroid diseases: A comprehensive review. *J. Autoimmun.* **2015**, *64*, 82–90. [CrossRef] [PubMed]
5. Brix, T.H.; Kyvik, K.O.; Hegedüs, L. A population-based study of chronic autoimmune hypothyroidism in Danish twins. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 536–539. [CrossRef]
6. Janeway, C.A., Jr.; Travers, P.; Walport, M.; Shlomchik, M.J. *Immunobiology: The Immune System in Health and Disease*, 5th ed.; Garland Science: New York, NY, USA, 2001.
7. Martin, A.; Davies, T.F. T cells and human autoimmune thyroid disease: Emerging data show lack of need to invoke suppressor T cell problems. *Thyroid* **1992**, *2*, 247–261. [CrossRef]
8. Ben-Skowronek, I.; Szewczyk, L.; Kulik-Rechberger, B.; Korobowicz, E. The differences in T and B cell subsets in thyroid of children with Graves' disease and Hashimoto's thyroiditis. *World J. Pediatr.* **2013**, *9*, 245–250. [CrossRef]
9. Giordano, C.; Stassi, G.; De Maria, R.; Todaro, M.; Richiusa, P.; Papoff, G.; Ruberti, G.; Bagnasco, M.; Testi, R.; Galluzzo, A. Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. *Science* **1997**, *275*, 960–963. [CrossRef]
10. Peters, P.J.; Borst, J.; Oorschot, V.; Fukuda, M.; Krähenbühl, O.; Tschopp, J.; Slot, J.W.; Geuze, H.J. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J. Exp. Med.* **1991**, *173*, 1099–1109. [CrossRef]
11. Stassi, G.; De Maria, R. Autoimmune thyroid disease: New models of cell death in autoimmunity. *Nat. Rev. Immunol.* **2002**, *2*, 195–204. [CrossRef]
12. Wu, Z.; Podack, E.R.; McKenzie, J.M.; Olsen, K.J.; Zakarija, M. Perforin expression by thyroid-infiltrating T cells in autoimmune thyroid disease. *Clin. Exp. Immunol.* **1994**, *98*, 470–477. [CrossRef] [PubMed]
13. Ehlers, M.; Thiel, A.; Bernecker, C.; Porwol, D.; Papewalis, C.; Willenberg, H.S.; Schinner, S.; Hautzel, H.; Scherbaum, W.A.; Schott, M. Evidence of a combined cytotoxic thyroglobulin and thyroperoxidase epitope-specific cellular immunity in Hashimoto's thyroiditis. *J. Clin. Endocrinol. Metab.* **2012**, *97*, 1347–1354. [CrossRef]

14. Berghi, N.O. Immunological Mechanisms Implicated in the Pathogenesis of Chronic Urticaria and Hashimoto Thyroiditis. *Iran J. Allergy Asthma Immunol.* **2017**, *16*, 358–366. [PubMed]
15. Luty, J.; Ruckemann-Dziurdzińska, K.; Witkowski, J.M.; Bryl, E. Immunological aspects of autoimmune thyroid disease—Complex interplay between cells and cytokines. *Cytokine* **2019**, *116*, 128–133. [CrossRef] [PubMed]
16. Ahmad, A.; Husain, A.; Mujeeb, M.; Khan, S.A.; Najmi, A.K.; Siddique, N.A.; Damanhouri, Z.A.; Anwar, F. A review on therapeutic potential of *Nigella sativa*: A miracle herb. *Asian Pac. J. Trop. Biomed.* **2013**, *3*, 337–352. [CrossRef]
17. Ghosheh, O.A.; Houdi, A.A.; Crooks, P.A. High performance liquid chromatographic analysis of the pharmacologically active quinones and related compounds in the oil of the black seed (*Nigella sativa* L.). *J. Pharm. Biomed. Anal.* **1999**, *19*, 757–762. [CrossRef]
18. Işik, H.; Cevikbaş, A.; Gürer, U.S.; Kiran, B.; Uresin, Y.; Rayaman, P.; Rayaman, E.; Gürbüz, B.; Büyüköztürk, S. Potential adjuvant effects of *Nigella sativa* seeds to improve specific immunotherapy in allergic rhinitis patients. *Med. Princ. Pract.* **2010**, *19*, 206–211. [CrossRef]
19. Kheirouri, S.; Hadi, V.; Alizadeh, M. Immunomodulatory Effect of *Nigella sativa* Oil on T Lymphocytes in Patients with Rheumatoid Arthritis. *Immunol. Investig.* **2016**, *45*, 271–283. [CrossRef]
20. Barlianto, W.; Wulandari, D.; Chusniyah, M.; Chandra Kusuma, H.; Prawiro, S.R. Improvement of Th17/Treg balance and asthma control test score by *Nigella sativa* supplementation in asthmatic children: A new approach to managing asthma. *Tur. J. Immunol.* **2018**, *6*, 1–7. [CrossRef]
21. Houcher, Z.; Boudiaf, K.; Benboubetra, M.; Houcher, B. Effects of methanolic extract and commercial oil of *Nigella sativa* L. on blood glucose and antioxidant capacity in alloxan-induced diabetic rats. *Pteridines* **2007**, *18*, 8–18. [CrossRef]
22. Panahi, M.; Namjoyan, F.; Shakerin, Z. Evaluation of antioxidant effects of *Nigella sativa* on the ultrastructure of neural tube defects in diabetic rats offspring. *Jundishapur J. Nat. Pharm. Prod.* **2011**, *6*, 16–23.
23. Farhangi, M.A.; Dehghan, P.; Tajmiri, S.; Abbasi, M.M. The effects of *Nigella sativa* on thyroid function, serum Vascular Endothelial Growth Factor (VEGF)—1, Nesfatin-1 and anthropometric features in patients with Hashimoto's thyroiditis: A randomized controlled trial. *BMC Complement. Altern. Med.* **2016**, *16*, 471. [CrossRef] [PubMed]
24. Tajmiri, S.; Farhangi, M.A.; Dehghan, P. *Nigella Sativa* treatment and serum concentrations of thyroid hormones, transforming growth factor β (TGF-β) and interleukin 23 (IL-23) in patients with Hashimoto's Thyroiditis. *Eur. J. Integr. Med.* **2016**, *8*, 576–580. [CrossRef]
25. Ahmed Khalawi, A.; Ahmed Al-Robai, A.; Mohamed Khoja, S.; Shaker Ali, S. Can *Nigella sativa* Oil (NSO) Reverse Hypothyroid Status Induced by PTU in Rat? Biochemical and Histological Studies. *Life Sci. J.* **2013**, *10*, 802–811.
26. Hmza, A.J.A.; Osman, M.T.; Adnan, A.; Omar, E. Immunomodulatory effect of *Nigella sativa* oil in the disease process of type 1 diabetic rats. *Res. J. Pharm. Biol. Chem. Sci.* **2013**, *4*, 980–988.
27. Noor, N.A.; Fahmy, H.M.; Mohammed, F.F.; Elsayed, A.A.; Radwan, N.M. *Nigella sativa* amliorates inflammation and demyelination in the experimental autoimmune encephalomyelitis-induced Wistar rats. *Int. J. Clin. Exp. Pathol.* **2015**, *8*, 6269.
28. Avci, G.; Ulutas, E.; Ozdemir, V.; Kivrak, I.; Bulbul, A. The positive effect of black seed (*Nigella sativa* L.) essential oil on thyroid hormones in rats with hypothyroidism and hyperthyroidism. *J. Food Biochem.* **2021**, *46*, e13801. [CrossRef]
29. Ciesielska-Figlon, K.; Daca, A.; Kokotkiewicz, A.; Łuczkiewicz, M.; Zabiegała, B.; Witkowski, J.M.; Lisowska, K.A. The influence of *Nigella sativa* essential oil on proliferation, activation, and apoptosis of human T lymphocytes in vitro. *Biomed. Pharmacother.* **2022**, *153*, 113349. [CrossRef]
30. Witkowski, J.M. Advanced application of CFSE for cellular tracking. *Curr. Protoc. Cytom.* **2008**, *44*, 9.25.1–9.25.8. [CrossRef]
31. Na, H.; Cho, M.; Chung, Y. Regulation of Th2 Cell Immunity by Dendritic Cells. *Immune Netw.* **2016**, *16*, 1. [CrossRef]
32. Marazuela, M.; García-López, M.A.; Figueroa-Vega, N.; de la Fuente, H.; Alvarado-Sánchez, B.; Monsiváis-Urenda, A.; Sánchez-Madrid, F.; González-Amaro, R. Regulatory T cells in human autoimmune thyroid disease. *J. Clin. Endocrinol. Metab.* **2006**, *91*, 3639–3646. [CrossRef] [PubMed]
33. Boskabady, M.H.; Javan, H.; Sajady, M.; Rakhshandeh, H. The possible prophylactic effect of *Nigella sativa* seed extract in asthmatic patients. *Fundam. Clin. Pharmacol.* **2007**, *21*, 559–566. [CrossRef] [PubMed]
34. Nikakhlagh, S.; Rahim, F.; Aryani, F.H.N.; Syahpoush, A.; Brougerdnya, M.G.; Saki, N. Herbal treatment of allergic rhinitis: The use of *Nigella sativa*. *Am. J. Otolaryngol.* **2011**, *32*, 402–407. [CrossRef]
35. Arslan, B.A.; Isik, F.B.; Gur, H.; Ozen, F.; Catal, T. Apoptotic Effect of *Nigella sativa* on Human Lymphoma U937 Cells. *Pharmacogn. Mag.* **2017**, *13*, S628–S632. [CrossRef]
36. Hasan, T.N.; Shafi, G.; Syed, N.A.; Alfawaz, M.A.; Alsaif, M.A.; Munshi, A.; Lei, K.Y.; Alshatwi, A. A Methanolic Extract of *Nigella sativa* Seed Inhibits SiHa Human Cervical Cancer Cell Proliferation Through Apoptosis. *Nat. Prod. Commun.* **2013**, *8*, 213–216. [CrossRef] [PubMed]
37. Alhazmi, M.I.; Hasan, T.N.; Shafi, G.; Al-Assaf, A.H.; Alfawaz, M.A.; Alshatwi, A.A. Roles of p53 and caspases in induction of apoptosis in MCF-7 breast cancer cells treated with a methanolic extract of *Nigella sativa* seeds. *Asian Pac. J. Cancer Prev.* **2014**, *15*, 9655–9660. [CrossRef]
38. Tripathi, Y.B.; Pandey, N. Preventive Effects of Methanolic Extract of Seeds of *Nigella sativa* Linn. on PMA Induced Apoptosis in Human Blood Lymphocytes. *Cell Biol. Res. Ther.* **2015**, *4*, 1. [CrossRef]
39. Salem, M.L.; Hossain, M.S. Protective effect of black seed oil from *Nigella sativa* against murine cytomegalovirus infection. *Int. J. Immunopharmacol.* **2000**, *22*, 729–740. [CrossRef]

40. Salim, L.Z.; Othman, R.; Abdulla, M.A.; Al-Jashamy, K.; Ali, H.M.; Hassandarvish, P.; Dehghan, F.; Ibrahim, M.Y.; Omer, F.A.; Mohan, S. Thymoquinone inhibits murine leukemia WEHI-3 cells in vivo and in vitro. *PLoS ONE* **2014**, *9*, e115340. [CrossRef]
41. Salim, L.Z.; Mohan, S.; Othman, R.; Abdelwahab, S.I.; Kamalidehghan, B.; Sheikh, B.Y.; Ibrahim, M.Y. Thymoquinone induces mitochondria-mediated apoptosis in acute lymphoblastic leukaemia in vitro. *Molecules* **2013**, *18*, 11219–11240. [CrossRef]
42. Lorent, J.; Lins, L.; Domenech, Ò.; Quetin-Leclercq, J.; Brasseur, R.; Mingeot-Leclercq, M.P. Domain formation and permeabilization induced by the saponin α-hederin and its aglycone hederagenin in a cholesterol-containing bilayer. *Langmuir* **2014**, *30*, 4556–4569. [CrossRef] [PubMed]
43. Lorent, J.; Le Duff, C.S.; Quetin-Leclercq, J.; Mingeot-Leclercq, M.P. Induction of highly curved structures in relation to membrane permeabilization and budding by the triterpenoid saponins, α- and δ-Hederin. *J. Biol. Chem.* **2013**, *288*, 14000–14017. [CrossRef]
44. Gardner, J.F.; Peel, M.M. *Principles of Chemical Disinfection: Introduction to Sterilization, Disinfection and Infection Control*, 3rd ed.; Churchill Livingstone: London, UK, 1991.
45. Rydzewska, M.; Jaromin, M.; Pasierowska, I.E.; Stozek, K.; Bossowski, A. Role of the T and B lymphocytes in pathogenesis of autoimmune thyroid diseases. *Thyroid Res.* **2018**, *11*, 2. [CrossRef]
46. Pyzik, A.; Grywalska, E.; Matyjaszek-Matuszek, B.; Roliński, J. Immune disorders in Hashimoto's thyroiditis: What do we know so far? *J. Immunol. Res.* **2015**, *2015*, 979167. [CrossRef] [PubMed]
47. Figueroa-Vega, N.; Alfonso-Pérez, M.; Benedicto, I.; Sánchez-Madrid, F.; González-Amaro, R.; Marazuela, M. Increased Circulating Pro-Inflammatory Cytokines and Th17 Lymphocytes in Hashimoto's Thyroiditis. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 953–962. [CrossRef] [PubMed]
48. Annunziato, F.; Cosmi, L.; Liotta, F.; Maggi, E.; Romagnani, S. Defining the human T helper 17 cell phenotype. *Trends Immunol.* **2012**, *33*, 505–512. [CrossRef] [PubMed]
49. Duechting, A.; Przybyla, A.; Kuerten, S.; Lehmann, P.V. Delayed Activation Kinetics of Th2- and Th17 Cells Compared to Th1 Cells. *Cells* **2017**, *6*, 29. [CrossRef]
50. Bruniquel, D.; Schwartz, R.H. Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nat. Immunol.* **2003**, *4*, 235–240. [CrossRef]
51. Khoruts, A.; Mondino, A.; Pape, K.A.; Reiner, S.L.; Jenkins, M.K. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J. Exp. Med.* **1998**, *187*, 225–236. [CrossRef]
52. Sojka, D.K.; Bruniquel, D.; Schwartz, R.H.; Singh, N.J. IL-2 secretion by CD4+ T cells in vivo is rapid, transient, and influenced by TCR-specific competition. *J. Immunol.* **2004**, *172*, 6136–6143. [CrossRef]
53. Fukasawa, T.; Yoshizaki, A.; Ebata, S.; Yoshizaki-Ogawa, A.; Asano, Y.; Enomoto, A.; Miyagawa, K.; Kazoe, Y.; Mawatari, K.; Kitamori, T.; et al. Single-cell-level protein analysis revealing the roles of autoantigen-reactive B lymphocytes in autoimmune disease and the murine model. *Elife* **2021**, *10*, e67209. [CrossRef] [PubMed]
54. Fukasawa, T.; Yoshizaki, A.; Ebata, S.; Fukayama, M.; Kuzumi, A.; Norimatsu, Y.; Matsuda, K.M.; Kotani, H.; Sumida, H.; Yoshizaki-Ogawa, A.; et al. Interleukin-17 pathway inhibition with brodalumab in early systemic sclerosis: Analysis of a single-arm, open-label, phase 1 trial. *J. Am. Acad. Dermatol.* **2023**, *29*, S0190-9622(23)00516-9. [CrossRef] [PubMed]
55. Barlianto, W.; Rachmawati, M.; Irawan, M.; Wulandari, D. Effects of *Nigella sativa* oil on Th1/Th2, cytokine balance, and improvement of asthma control in children. *Paediatr. Indones.* **2017**, *57*, 223. [CrossRef]

antioxidants

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Review

The Immunomodulatory Effect of *Nigella sativa*

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Abstract: Background: For thousands of years till nowadays, *Nigella sativa* (NS) has served as a common spice and food preservative. Its seed extracts, seed oil, and essential oil in traditional medicine have been used to remedy many ailments such as headaches, fever, gastric complaints, and even rheumatism. In addition, the antibacterial, virucidal, fungicidal, and antiparasitic properties of NS are well known. However, studies on the possible immunomodulatory effects of black cumin are relatively scarce. This article discusses in vitro and in vivo research supporting the immunomodulatory role of NS. Methods: The review is based on articles, books, and conference papers printed until September 2022, found in the Web of Science, PubMed, Wiley Online Library, and Google Scholar databases. Results: Experimental findings were reported concerning the ability of NS to modulate inflammation and immune responses or cytotoxic activity. Conclusions: All results suggest that NS can potentially be employed in developing effective therapeutic agents for regulating immune reactions.

Keywords: *Nigella sativa*; essential oil; immunomodulation; antioxidant

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1. Introduction

The long-standing interest in natural products has led to the emergence of plant substances in medicine. This branch of medicine is growing rapidly due to the increased effectiveness of new medicines of plant origin and the development of alternative therapies using plant products as support for traditional therapies for the treatment of various diseases. The use of plants as medicinal substances is culturally well-established and dates back to the earliest years of human activity [1]. It is known that people of different cultures have used the same plants for similar health problems, which have been repeatedly written down and passed down from generation to generation. Today, taking advantage of the possibilities of science, many of these plants have been studied, and substances isolated from them have shown beneficial therapeutic effects, including anticancer, antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory effects. In the present article, we focused on the immunomodulatory potential of NS, describing studies using extracts, fixed and essential oil obtained from black seeds in vitro and in vivo models. We traced the articles published between 1995 and 2022. The literature included studies using animals, humans, and cell lines.

Nigella sativa (NS), a flowering plant that belongs to the Ranunculaceae family originates from south and southwest Asia. Presently, it is cultivated in several other regions including middle Europe, Mediterranean countries, and western Asia [2]. The plant grows up to a height of 20–30 cm and has linear leaves that are finely divided. Its flowers typically have 5–10 petals and come in pale blue, pink, or white colors, and bloom from May to September [3]. The fruit of the plant is a large and inflated capsule with three to seven united follicles, each containing seeds. The seeds are obtained when the plant reaches full maturity, which usually occurs in mid-August. They are small, black, and velvety, measuring 1–5 mm in size, and have a pungent taste resembling pepper, with a spicy aroma.

The seeds are often used to season tender meats such as lamb and poultry and are also a great addition to salads, marinades, and cottage cheese [4]. In addition, cumin seeds are also used in bread and dairy products [5].

Seeds and oil from *Nigella sativa* have long been used in medicine and cooking [6]. This plant called the "gold of the Pharaohs", was considered a panacea for many diseases in antiquity. Additionally, many ancient cultures, especially in Asia, used NS oil for the treatment of various allergies [7]. In Islamic countries, NS is extensively used in traditional medicine for healing numerous gastrointestinal and respiratory diseases [8]. In traditional and alternative medicine, NS is considered a panacea for various conditions, such as diarrhea and asthma [9]. Other uses include headache, amenorrhea, anorexia, cough, rheumatism, eczema, bronchitis, fever, influenza, as a diuretic, lactagougue, vermifuge, and various health care issues [10–14].

1.1. Composition of Seeds from NS

NS seeds contain proteins (26%), fat (28%), carbohydrates (25%), crude fiber (8.4%), and ash (4.8%) [15]. High levels of carotene and minerals (copper, zinc, phosphorous, and iron) are also found [15]. The high content of macromolecules, such as proteins, is crucial for the plant's properties. Worth mentioning is a group of defense proteins, such as thionins and defensins, which are intracellular low-molecular proteins. Their beneficial properties are described by some studies, focusing on antibacterial or antifungal effects [16,17] and are even antiproliferative and pro-apoptotic against cancerous cell lines [18]. Furthermore, seeds hold 36–38% fixed oil, alkaloids, and saponins [10,19,20]. The oil obtained from NS seeds contains fatty and essential oil fractions [21]. The percentage of component content depends on time, location, and method of harvesting. NS seeds and their oils generally have very low toxicity [22].

The oil contains a fatty oil rich in unsaturated fatty acids, mostly linoleic acid (50–60%), oleic acid (20%), dihomolinoleic acid (10%), and eicodadienoic acid (3%). Saturated fatty acids (palmitic and stearic acid) amount to about 30% [23–25]. The oil contains alkaloids (nigellicines and nigelledine), saponins, tocopherols, phytosterols, flavonoids, and essential oil (EO) (0.4–2.5%) [26]. Most health-promoting properties are mainly attributed to quinone constituents, with thymoquinone (TQ) as a more abundant compound, which is also likely to be involved in pharmaceutical properties [27].

EO is utilized in food (as flavoring), perfumes, and pharmaceuticals [28]. Numerous active NS essential oil (NSEO) compounds have been isolated, identified, and reported in many experiments. Gas chromatography and gas chromatography-mass spectrometry analysis of volatile oil resulted in the identification of bioactive compounds representing even 98% of NSEO total amount [5,29]. In terms of quantity, the main identified compounds were p-cymene (50–60%) [5,29,30] and α-thujene (15%). Thymoquinone, thymohydroquinone, dithymoquinone, and thymol were the key phenolic compounds detected [31]. Traces of the esters of saturated and unsaturated fatty acids were also identified in NSEO [19]. Two monoterpenoids, cis- and trans-4-methoxythujane, were found in the essential oil [5]. Four terpenoids: cis-sabinene hydrate methyl ether, trans-sabinene hydrate methyl ether, 1,2-epoxy-menth-4(8)-ene, and 1,2-epoxy-menth-4-ene were identified in NSEO by nuclear magnetic resonance [32].

Figure 1 shows the chemical structures of the most important compounds of *Nigella sativa*.

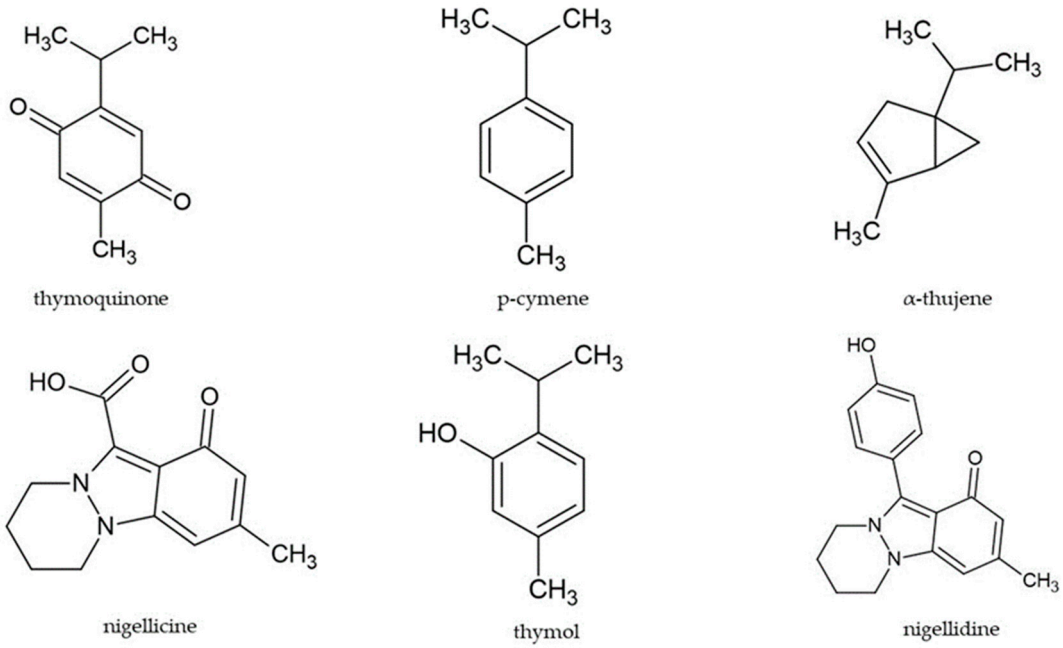

Figure 1. The chemical structures of the main compounds of *Nigella sativa*.

1.2. Antioxidant Activity of the Seeds NS Extracts

Many studies using different experimental models highlight the antioxidant activity of NS extracts. Bordoni et al. [33] verified fixed oil's antioxidant properties in an in vitro model of inflamed adipose tissue using Simpson–Golabi–Behmel syndrome (SGBS) human preadipocytes and monocytic leukemia cell line. The authors used different spectrophotometric and chemiluminescent assays to measure the antioxidant of NS oil. The total antioxidant activity (TAA) measured in the supernatant of preadipocytes showed that NS oil has very high residual activity. Tiji et al. [34] compared the antioxidant properties of NS seed hexane and acetone extract and fractions. The authors confirmed that the extracts are characterized by the presence of fractions with different levels of antioxidant activity, which probably depends on specific secondary metabolites present in the fractions, such as polyphenols. Another study showed that methanolic extract from NS seeds has higher antioxidant activity than aqueous extract [35]. Ouattar et al. [36] showed that NS extracts have a dose-dependent antiradical activity, with crude extract having an activity lower than n-butanol or ethyl acetate extracts. As can be seen, NS exhibits antioxidant activity, which was confirmed by various methods. However, the level of this activity depends on the type of extract and the fraction tested.

1.3. The Most Efficient Methods for Obtaining and Detecting the Composition of Oils and Essences from Nigella sativa

Many extraction techniques have been developed to extract oils from *Nigella sativa* seeds. A relevant aspect worth mentioning is that the different forms of extraction of NS seed oils are one of the main factors influencing their final properties. Therefore, it is crucial to adapt the extraction technique to the specific application [37].

Some methods allow the isolation of macromolecules, such as proteins. In 2016, innovative research was conducted on the proteome of *Nigella sativa* seeds [38]. The researchers analyzed the protein profile of NS seeds with a proteome mapping technique using one-dimensional gel electrophoresis followed by liquid chromatography and tandem mass spectrometry strategies. Next, the vanillin method, for example, can be used to extract such compounds.

Cold extraction, also known as cold press, is among the conventional oil extraction methods. It does not require auxiliary chemicals, so it is preferred by those concerned

about natural and safe foods [39]. However, this method is characterized by poor overall efficiency [40]. Additionally, the residual meal contains only about 11% of oil, which may severely limit usage in the food industry [40].

The hydrodistillation (HD) method is among the traditional techniques for extracting essential oils and volatile components together with steam distillation (SD). In this method, the hydrolysis temperature is essential for improving the borage oil extraction yield by cold pressing. Presently, HD is mainly used as a comparative method for newly developed methods for extracting volatile components of NS [5,40,41].

According to the literature, NS oil has often been produced by hot solvent extraction (SE) [42]. Among the most common solvents used to extract essential and solid oils from NS are hexane, chloroform, acetone, and methanol [5,20,40,43–45]. At the same time, its disadvantage is the requirement for high temperatures, which can degrade the desired components of the oils [46].

Considering the lack of selectivity of the previously mentioned methods due to insufficient control of the individual parameters of both techniques, a new supercritical extraction (SFE) method was developed [47]. Compared to other conventional techniques, higher yields of antioxidants, greater purity of the extracts, and more considerable retention of bioactivity are the main advantages of SFE. To date, many extractions of NS seed oils have been performed using the SFE method. Thus, in a study, Kokoska et al. [48] compared the chemical composition and antimicrobial activity of the essential oil extracted by the SFE-SD method and the traditional HD and SD methods. The results showed that the extract obtained by the SFE-SD method differed in composition and higher bioactivity compared to traditional methods. Additionally, a study performed by Ismail et al. [49] showed that NSO extracted using the SFE method contained significantly higher amounts of TQ than the oil extracted using the solvent extraction method.

Another technique is microwave-assisted extraction (MAE), belonging to alternative methods for extracting essential oils, aromatics, pesticides, phenols, and other organic compounds. Microwave heating offers the advantage of breaking weak hydrogen bonds promoted by the dipole rotation of the molecules [50]. A study by Benkaci-Ali et al. [51] showed that extracting essential oil from NS seeds using MAE had a shorter time to obtain the oil and significant energy savings compared to the HD technique.

Meanwhile, gas chromatography (GC) is among the most commonly used methods to identify and determine the chemical composition of essential oils and lipids obtained from the extraction of NS seeds. Using a combination of the GC-MS technique with iterative and non-iterative resolution methods, the known volatile components in NS seeds increased from 39 to 98 [52]. Interestingly, the GC technique has also been used to study differences in chemical composition between ancient and modern *Nigella sativa* seeds. As a result, it was shown that the composition of both seeds was very similar in the content of essential oils in the plants [53].

2. Immunomodulatory Properties of *Nigella sativa*

Immunomodulation is defined as modifying the immune response by regulating the crosstalk among different components, such as interactions between neutrophils and macrophages and T and B cells. Immunomodulators can help support immune function by stimulating or suppressing the immune system [54]. Below we discuss the available literature data regarding the immunomodulatory effect of NS seed extracts, fixed oil, and essential oil.

2.1. Immunomodulatory Properties of NS Seed Extracts

2.1.1. Studies In Vitro

Haq and colleagues [55] examined the effect of phosphate buffer saline (PBS) extract from NS seeds in 50, 5, and 0.5 µg/mL concentrations on human lymphocytes and polymorphonuclear (PMNs) leukocytes. Peripheral blood mononuclear cells (PBMCs) were stimulated with concanavalin A (ConA), phytohemagglutinin (PHA), or pokeweed mitogen

(PWM) in the presence of several concentrations of NS extract. The high concentrations of NS extract suppressed the lymphocyte response to all mitogens due to increased cell death. Furthermore, at the highest concentration, NS extract also suppressed the phagocytic activity of PMNs. At the same time, the extract stimulated lymphocytes to secrete interleukin 1beta (IL-1β) and IL-3 but not IL-2. Majdalawieh and colleagues [56] examined how NS influences splenocyte proliferation, macrophage function, and natural killer (NK) cell activity in C57/BL6 and BLAB/c cells. The authors demonstrated that the aqueous extract from NS seeds in four doses (1, 10, 50, and 100 g/mL) increased splenocyte proliferation and the secretion of Th2 cytokines responsible for humoral immune responses in a dose-dependent manner by splenocytes. At the same time, it suppressed the secretion of key proinflammatory mediators, that is, tumor necrosis factor-alpha (TNF-α), IL-6, and nitric oxide (NO) by macrophages. Finally, the authors showed that the extract significantly augmented NK cell cytotoxicity against YAC-1 tumor cells. This study showed that the effect of the NS extract depends on the type of immune cells, as some immune subpopulations were stimulated in the NS presence to proliferate and produce cytokines, while others were inhibited.

Hexane and methanol extracts from NS seeds in a concentration of 10 mg/mL were found to prevent the formation of protein carbonyl and depletion of glutathione (GSH) in L929 fibroblasts exposed to toluene, which confirms that NS has antioxidant potential [57]. In 2013, Elmowalid and colleagues [58] examined the immunomodulatory effect of an aqueous extract concentration of 10 mg/mL from NS seeds on sheep macrophage functions in vitro. The authors showed that the addition of NS extract caused an increase in phagocytic activity and the capacity to produce NO. In the study by Gholamnezhad et al. [59], ethanolic extract from NS seeds in concentrations of 100, 500, and 1000 µg/mL was examined to see whether it affected rat splenocytes, especially their viability, proliferation, and cytokine secretion. NS extract inhibited the proliferation and decreased the viability of splenocytes stimulated with PHA or ConA in a dose-dependent manner. Higher concentrations of the extract also reduced the secretion of IL-4 and interferon-gamma (IFN-γ) and increased the IFN-γ/IL-4 ratio. In a recent study, Singh et al. [60] investigated the immunomodulatory effect of methanolic extract from NS seed in two doses, 125 and 250 µg/mL, on chicken PBMCs. The authors demonstrated that the extract stimulated the expression of inflammatory genes coding IL-1β, IL-4, IL-10, IL-12, IL-13, IFN-β, and IFN-γ.

However, scarce studies on human material have been reported. For example, Koshak and colleagues [61] compared the effects of ethanol, aqueous, and supercritical fluid (SCF) extracts from NS seeds in concentrations of 10 and 100 µg/mL on asthma-related mediators of inflammation in human T cells and monocytes. SCF had the most robust suppressive properties; the extracts reduced the release of IL-2, IL-6, and prostaglandin E2 (PGE2) from T cells and monocytes. Alshatwi [62] showed that methanolic extract in concentrations of 2.5 and 5.0 µg/mL from NS seeds inhibited the proliferation of T cells stimulated with PHA for 48 h in a dose-dependent manner. However, without PHA stimulation, NS extract stimulated T cells. The NS extract also decreased the expression of genes coding TNF-α, IL-6, and IL-8 in human PBMCs stimulated with PHA. Conversely, in the absence of PHA stimulation, gene expression was increased in the presence of NS extract.

2.1.2. Studies In Vivo

The in vivo immunomodulatory properties of extracts from NS seed were also examined in different animal models. For example, intraperitoneal administration of NS methanolic extract increased the total amount of white blood cells (WBC) in BALB/c mice [63]. In addition, hexane and methanol extracts prevented the loss of hepatic GSH in male Wistar rats exposed to toluene [57].

Boskabady and colleagues [64] studied the immunomodulatory properties of ethanolic extract from NS seeds in guinea pigs sensitized to ovalbumin (OVA) as an animal model of asthma. The authors showed that NS extract significantly decreased pathological changes in the lungs of animals. The infiltration of eosinophils and lymphocytes, as well as local epithelial necrosis, was reduced. Simultaneously, serum IL-4 and IFN-γ were increased

in animals treated with NS extract compared to control animals. These results confirmed the preventive effect of NS extract on lung inflammation. In the latest study by Hikmah and colleagues [65], the influence of ethanol NS extract on renal tissue damage in the pristane-lupus (PIL) mice model has been investigated. The results showed that the percentage of Th17 cells responsible for inflammatory responses, regulatory T cells (Tregs), and macrophages producing IL-6 and IL-23 in lupus mice treated with NS extract was lower compared to PIL mice treated with placebo or steroids. Additionally, the serum anti-dsDNA antibodies were lower in these mice. Additionally, the renal injury was smaller in lupus mice treated with NS extract. These results showed that the ethanolic extract from NS seeds has immunomodulatory properties and prevents kidney injury in lupus mice.

In another study, the immunomodulatory properties of ethanol extract from NS seeds were assessed in dexamethasone-induced immune-suppressed male rabbits [66]. Animals were treated for six weeks orally with water, NS extract, and dexamethasone (Dex) in different combinations. The authors reported a significant decrease in the phagocytic activity of the cells in rabbits treated with Dex alone. However, the administration of NS extract simultaneously with Dex or after three weeks of treatment with Dex improved the phagocytic activity. The authors also showed that NS extract improved bone marrow mitotic activity after treatment with Dex.

The properties of NS were also studied in fish [67]. In 2012 Elkamel and Mosaad published work that investigated the modulation of the immune system of *Nile tilapia* by NS seeds added as a food additive by comparing its properties with fish basic or a CloSTAT diet (diet with the addition of Bacillus subtilis PB6). The authors investigated immune parameters, including serum globulins, WBC counts, and phagocytic activities. Results showed that adding NS seeds significantly increased the serum globulins, WBC count, and phagocytic activity and reduced fish mortality compared with the standard or CloSTAT diet. These effects were even significantly higher when fish were treated with NS seeds combined with the CloSTAT diet.

In another study, the prophylactic properties of water extract from NS seeds were studied in a group of asthmatic patients [68]. The study group received boiled NS seed extract for 3 months. Asthma severity and frequency of symptoms per week, and wheezing were analyzed three times: in the beginning, 45 days after treatment, and at the end of the study. All asthma symptoms, frequency of attacks, chest wheezing, and pulmonary function test (PFT) values significantly improved in the second and third visits compared with the beginning of the experiment.

2.2. Immunomodulatory Properties of NS Oil

2.2.1. Studies In Vitro

Only a few studies describe the immunomodulatory properties of NS cold-pressed oil in vitro. In 2005, Buyukozturk et al. [69] examined how NS oil influences cytokine production by splenic mononuclear cells (MNCs) in mice. First, BALB/c mice have been give daily 0.3 mL of NS oil through an oro-esophageal cannula for a month. Then, in the third week of the study, all mice were sensitized using intraperitoneal injections of 20 µg of OVA. Finally, splenic MNCs from mice were cultured with OVA or ConA. The authors showed that the cytokine production did not significantly differ between mice treated with NS and control mice treated only with saline solution. In the second study, the authors examined the influence of NS oil on human PBMCs stimulated with an immobilized monoclonal anti-CD3 antibody in the presence of serial (1:1, 1:10, 1:100, and 1:1000) ethanol (EtOH) dilutions of NS oil [70]. The lowest dilutions (1:10 and 1:50) of NS oil inhibited the proliferation of lymphocytes and reduced the percentage of living cells by inducing apoptosis.

2.2.2. Studies In Vivo

More studies demonstrate the influence of NS oil in vivo in animals and humans [71]. In one of the studies, the authors studied the influence of NS oil in a rat model of allergic

airway inflammation. Rats were first treated with intraperitoneally administered oil and then exposed to OVA. The authors measured serum levels of total immunoglobulin E (IgE), IgG1, and OVA-specific IgG1 and analyzed the proliferation of T cells in the spleen. They also analyzed the expression of genes coding different cytokines, including IL-4, IL-5, IL-6, and transforming growth factor-beta 1 (TGF-β1). The results showed that NS oil suppressed the Th2-type response in rats by preventing inflammatory cell infiltration and pathological lung lesion formation. NS oil also significantly decreased NO production in bronchoalveolar lavage fluid (BALF), total serum IgE, IgG1, OVA-specific IgG1, and IL-4, IL-5, IL-6, and TGF-β1 gene expression. Treatment with NS oil also inhibited the proliferation of T cells in the spleen. Balaha and colleagues [72] investigated the anti-inflammatory and immunomodulatory effects of oral administration of NS oil in a mouse model of allergic asthma. The authors examined the airway function, the presence of inflammatory infiltrates in the airways, local cytokine production in BALF, serum immunoglobulin concentrations, and histopathological changes in lung tissues. They showed that oral treatment with NS oil at a dose of 4 mL/kg/day in OVA-sensitized mice significantly improved airway reactivity, decreased the number of WBC, macrophages, and eosinophils, reduced the production of Th2 cytokine (IL-4, IL-5, and IL-13), and significantly increased IFN-γ with the abrogation of histological changes in lung tissues in a dose-dependent manner. Sheir and colleagues in 2015 [73] examined the immune mechanisms possibly involved in ameliorating histopathological changes in the livers of mice infected with *Schistosoma mansoni* and treated with NS oil. The authors measured total serum IgG and cytokines IL-2, IL-12, and TNF-α. Mice treated with NS oil were characterized by increased serum IgG, IL-2, IL-12, and TNF-α compared with infected mice. Additionally, histological observation of liver tissue of infected mice treated with NS oil combined with antiparasitic drugs showed some improvement compared with infected mice.

The authors of another study examined the influence of NS oil on CD4+ T cells in Sprague-Dawley rats exposed to dimethylbenzanthracene (DMBA), a carcinogenic immunosuppressive agent [74]. Animals were fed different doses of NS oil for 14 days before and during DMBA induction. At week 27, peripheral blood was taken to measure the number of CD4+ and CD4+ CD25+ T cells. NS oil administration increased the absolute number of CD4+ T cells and CD4+ CD25+ cells (Tregs) count in a dose-independent manner.

It has been shown that NS cold-pressed oil also influences the human immune system. In 2016 Kheirouri et al. [75] published a study that investigated the immunomodulatory effect of NS oil on selected subsets of T cells in women with rheumatoid arthritis (RA). Forty-three female RA patients were recruited and received 1 g of NS oil or starch capsule (placebo) in two dosages for two months. The Disease Activity Scores in 28 joints (DAS28) were calculated, and percentages of blood CD4+, and CD8+ T cells were analyzed. Treatment with NS oil significantly decreased the serum concentration of high-sensitivity C-reactive protein (hs-CRP) and reduced the DAS-28 score. Patients also reported a reduction in the number of swollen joints. No changes were observed in the percentage of CD4+ T cells before and after treatment. NS oil administration reduced the percentage of CD8+ T cells and increased CD4+CD25+ T cells and the CD4+/CD8+ ratio compared to RA patients treated with placebo and baseline results. Another study showed that NS oil affects Th1/Th2 cytokine balance and improves asthma control in children [76]. Children in this study were given 15–30 mg/kg/day of NS oil for eight weeks. The asthma control test (ACT) score was used to assess the improvement of asthma control. Numbers of blood Th1 and Th2 cells and serum IFN-γ and IL-4 were analyzed. The authors demonstrated that children treated with NS oil had significantly elevated serum IFN-γ and reduced IL-4 compared with the placebo group. However, the ACT score was not significantly different between groups at the end of the study. In another trial, the benefits of NS oil supplementation in asthma patients were studied [77]. Each participant received capsules of 500 mg twice a day for 4 weeks. Compared with the placebo, patients supplemented with NS showed a significant improvement in mean ACT score and a significant reduction in blood eosinophils. Hidayati et al. [78] analyzed the immunomodulatory properties

of NS oil in active smoker volunteers. The authors showed that people receiving 3 × 1, 3 × 2, and 3 × 3 capsules/day for 30 days were characterized by increased IL-2 expression in CD4+ cells. Laily et al. [79] examined the effect of NS oil in a smoking group on serum IL-1β levels and neutrophil percentage. The study group received three-dose NS oil for 30 days. The authors showed that the average IL-1β levels did not differ between placebo and study groups. The neutrophil percentage, however, decreased after 30 days compared with the placebo group.

2.3. Immunomodulatory Properties of NS Essential Oil

2.3.1. Studies In Vitro

Only one study examines the immunomodulatory properties of NS essential oil (NSEO) in vitro [29]. The authors stimulated human PMBCs with an immobilized monoclonal anti-CD3 antibody in the presence of serial (1:10, 1:50, 1:100, 1:500, or 1:1000) EtOH dilutions of NSEO. Their results showed that 1:10, 1:50, and 1:100 NSEO strongly inhibited the proliferation of CD4+ and CD8+ T cells and induced their apoptosis and necrosis in a dose-dependent manner. Additionally, the authors observed reduced surface expression of CD28 and CD25 antigens, which are essential for lymphocyte activation. The results obtained explained the immunomodulating effect of NS cold-pressed oil observed earlier by the authors.

2.3.2. Studies In Vivo

In 2004, Nazrul Islam et al. [80] published a study that examined the immunosuppressive and cytotoxic properties of NSEO in a rat model. The authors challenged Long-Evans rats with typhoid antigen and injected them with NSEO for 30 days; then analyzed peripheral immune cells (lymphocytes, monocytes, neutrophils, and eosinophils) and serum immunoglobulins. NSEO significantly decreased neutrophil counts and increased lymphocytes and monocytes in the experimental animals. Meanwhile, serum immunoglobulins were decreased in animals treated with NSEO.

Figure 2 shows the potential immunomodulatory effect of *Nigella sativa*, and Table 1 summarizes its known immunomodulatory properties.

Table 1. The summary of the immunomodulatory properties of *Nigella sativa*.

Type of Material	In Vitro	In Vivo
Seed extracts	- PBS extract of 50 μg/mL NS induces cell death of human PBMCs, stimulates IL-1β and IL-3 secretion, and suppresses the phagocytic activity of PMNs [55] - aqueous extract in concentrations 1, 10, 50, and 100 g/mL of NS seeds increases splenocyte proliferation and secretion of Th2 cytokines, inhibits the secretion of IL-6, TNF-α, and NO, and increases the cytotoxic activity of NK cells against YAC-1 tumor cells [56] - the aqueous extract in a concentration of 10 mg/mL increases phagocytic activity and the NO production capacity of macrophages [58] - the ethanolic extracts in concentrations of 500 and 1000 μg/mL reduce the viability and inhibit the proliferation of rat splenocytes [59] - methanolic extracts in two doses of 125 and 250 μg/mL stimulate the expression of genes coding IL-1β, IL-4, IL-10, IL-12, IL-13, IFN-β, and IFN-γ in chicken PBMCs [60] - SCF extract in concentrations 10 and 100 μg/mL reduces the release of IL-2, IL-6, and PGE2 from human T cells and monocytes [61] - methanolic extracts in concentrations of 2.5 and 5.0 μg/mL inhibit human T cell proliferation and reduce the expression of genes encoding IL-6, IL-8, and TNF-α [62]	- the methanolic extract increases total WBC count in BALB/c mice [63] - the ethanolic extract reduces lung infiltration of eosinophils and lymphocytes and increases serum IL-4 and IFN-γ in OVA-sensitized guinea pigs [64] - the ethanolic extract reduces kidney damage, percentages of Th17 and macrophages in PIL mice [65] - the ethanolic extract improves bone marrow mitotic activity in rabbits with Dex-induced immunodeficiency [66] - seeds increase WBC count and phagocytic activity in *Nile tilapia* [67] - the aqueous extract reduces clinical symptoms of asthma patients [68]

Table 1. *Cont.*

Type of Material	In Vitro	In Vivo
Fixed oil	- oil in ethanolic dilutions 1:1, 1:10 inhibits human T cell proliferation and induces their apoptosis [70]	- oil inhibits Th2-type response and prevents allergic airway inflammation in OVA-sensitized rats [71] - the oil improves airway reactivity, decreases blood WBC, macrophages, and eosinophils, inhibits Th2 cytokine production, and increases IFN-γ in OVA-sensitized mice [72] - oil increases serum IL-2, IL-12, and TNF-α in mice infected with Schistosoma mansoni [73] - oil increases CD4+ T cell and Tregs counts in Sprague-Dawley rats exposed to DMBA [74] - oil reduces serum hs-CRP levels, DAS-28 scores, and the number of swollen joints in RA patients; it also reduces the percentage of CD8+ T cells and increases CD4+CD25+ T cells and the CD4+/CD8+ ratio [75] - oil reduces serum IL-4 and increases IFN-γ in asthma children [76] - the oil improves ACT scores and reduces eosinophil counts in adult patients with asthma [77] - oil increases IL-2 gene expression in CD4+ T cells and decreases neutrophil percentage in smokers [78]
Essential oil	- EO in ethanolic dilutions 1:10, 1:50, and 1:100 inhibits the proliferation of CD4+ and CD8+ cells, induces their apoptosis, and decreases the expression of CD28 and CD25 antigens [29]	- EO induced a significant decrease in neutrophil counts and increased lymphocytes and monocytes in rats exposed to typhoid antigen [80]

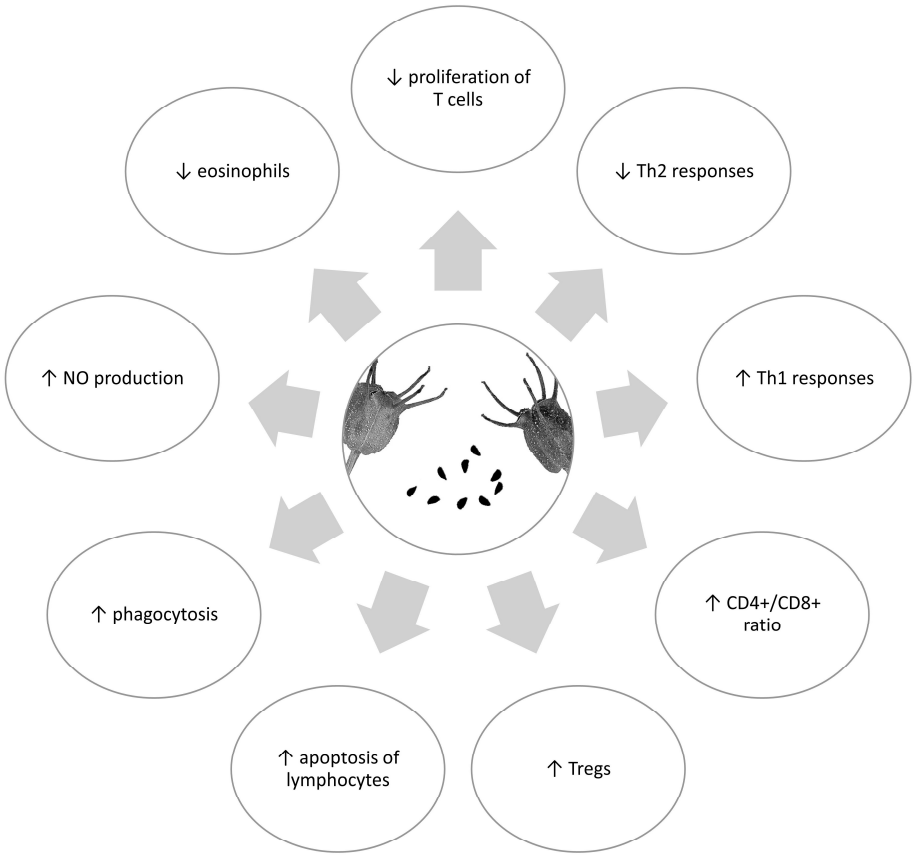

Figure 2. Potential immunomodulatory effects of *Nigella sativa*.

3. Conclusions

Since the 1950s, the phytochemicals and therapeutic properties of *Nigella sativa* have been studied exhaustively. Its antibacterial, antiviral, and antifungal properties are well-

known and widely described, but we know little about its effects on the immune system. The research results described in this review demonstrate that the NSEO influences immune cells by modulating their parameters such as the ability to produce cytokines or nitric oxide, phagocytic and cytotoxic activity, splenocyte and T-cell proliferation, and susceptibility to apoptosis and necrosis both in vivo and in vitro. Therefore, NS is a promising source of active ingredients that could be implemented in different clinical settings. However, further studies are necessary to explore the mechanisms of cold-pressed oil and essential oil from *Nigella sativa*, particularly in humans at the cellular and molecular levels. This review could be considered a compass for upcoming studies and the future development of therapeutic agents to help reduce symptoms of immune-related diseases, such as asthma or rheumatoid arthritis.

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References

1. Fong, H.H.S. Integration of Herbal Medicine into Modern Medical Practices: Issues and Prospects. *Integr. Cancer Ther.* **2002**, *1*, 287–293. [CrossRef]
2. Zohary, D.; Hopf, M.; Weiss, E. *Domestication of Plants in the Old World: The Origin and Spread of Domesticated Plants in Southwest Asia, Europe, and the Mediterranean Basin*; Oxford University Press: Oxford, UK, 2012.
3. Warrier, P.K. *Indian Medicinal Plants: A Compendium of 500 Species*; Orient Blackswan: Hyderabad, India, 1993; Volume 5.
4. Yimer, E.M.; Tuem, K.B.; Karim, A.; Ur-Rehman, N.; Anwar, F. *Nigella sativa* L. (Black Cumin): A Promising Natural Remedy for Wide Range of Illnesses. *Evid. Based Complement Alternat. Med.* **2019**, *2019*, 1528635. [CrossRef]
5. Wajs, A.; Bonikowski, R.; Kalemba, D. Composition of essential oil from seeds of *Nigella sativa* L. cultivated in Poland. *Flavour Fragr. J.* **2008**, *23*, 126–132. [CrossRef]
6. Saad, B.; Azaizeh, H.; Said, O. Tradition and Perspectives of Arab Herbal Medicine: A Review. *Evid. Based Complement Alternat. Med.* **2005**, *2*, 475–479. [CrossRef] [PubMed]
7. Kalus, U.; Pruss, A.; Bystron, J.; Jurecka, M.; Smekalova, A.; Lichius, J.J.; Kiesewetter, H. Effect of *Nigella sativa* (black seed) on subjective feeling in patients with allergic diseases. *Phytother. Res.* **2003**, *17*, 1209–1214. [CrossRef] [PubMed]
8. Riaz, M.; Syed, M.; Chaudhary, F.M. Chemistry of the medicinal plants of the genus Nigella. *Hamdard Med.* **1996**, *39*, 40–45.
9. Benhaddou-Andaloussi, A.; Martineau, L.; Vuong, T.; Meddah, B.; Madiraju, P.; Settaf, A.; Haddad, P.S. The In Vivo Antidiabetic Activity of *Nigella sativa* Is Mediated through Activation of the AMPK Pathway and Increased Muscle Glut4 Content. *Evid. Based Complement Alternat. Med.* **2011**, *2011*, 538671. [CrossRef] [PubMed]
10. Lautenbacher, L.M. Schwarzkummelol: Eine Neue Quelle Ungesattigter Fettsauren. *Deutsche Apotheker-Zeitung* **1997**, *137*, 68–69.
11. Eschborn, M.I. Pruefung von Schwarzkuemmeloel. *Pharmazeutische Zeitung* **1997**, *142*, 46–48.
12. Gholamnezhad, Z.; Keyhanmanesh, R.; Boskabady, M.H. Anti-inflammatory, antioxidant, and immunomodulatory aspects of *Nigella sativa* for its preventive and bronchodilatory effects on obstructive respiratory diseases: A review of basic and clinical evidence. *J. Funct. Foods* **2015**, *17*, 910–927. [CrossRef]
13. Muthu, C.; Ayyanar, M.; Raja, N.; Ignacimuthu, S. Medicinal plants used by traditional healers in Kancheepuram District of Tamil Nadu, India. *J. Ethnobiol. Ethnomed.* **2006**, *2*, 43. [CrossRef] [PubMed]
14. Ramadan, M. Nutritional value, functional properties and nutraceutical applications of black cumin (*Nigella sativa* L.): An overview. *Int. J. Food Sci. Technol.* **2007**, *42*, 1208–1218. [CrossRef]
15. Al-Jassir, M.S. Chemical composition and microflora of black cumin (*Nigella sativa* L.) seeds growing in Saudi Arabia. *Food Chem.* **1992**, *45*, 239–242. [CrossRef]
16. Vasilchenko, A.S.; Smirnov, A.N.; Zavriev, S.K.; Grishin, E.V.; Vasilchenko, A.; Rogozhin, E. Novel Thionins from Black Seed (*Nigella sativa* L.) Demonstrate Antimicrobial Activity. *Int. J. Pept. Res. Ther.* **2017**, *23*, 171–180. [CrossRef]
17. Rogozhin, E.A.; Oshchepkova, Y.I.; Odintsova, T.I.; Khadeeva, N.V.; Veshkurova, O.N.; Egorov, T.A.; Grishin, E.V.; Salikhov, S.I. Novel antifungal defensins from *Nigella sativa* L. seeds. *Plant Physiol. Biochem.* **2011**, *49*, 131–137. [CrossRef]
18. Khurshid, Y.; Syed, B.; Simjee, S.U.; Beg, O.; Ahmed, A. Antiproliferative and apoptotic effects of proteins from black seeds (*Nigella sativa*) on Human breast MCF-7 cancer line. *BMC Complement Med. Ther.* **2020**, *20*, 5. [CrossRef]
19. Burits, M.; Bucar, F. Antioxidant activity of *Nigella sativa* essential oil. *Phytother. Res.* **2000**, *14*, 323–328. [CrossRef]
20. Singh, G.; Marimuthu, P.; de Heluani, C.S.; Catalan, C. Chemical constituents and antimicrobial and antioxidant potentials of essential oil and acetone extract of *Nigella sativa* seeds. *J. Sci. Food Agric.* **2005**, *85*, 2297–2306. [CrossRef]

21. Javed, S.; Shahid, A.A.; Haider, M.S.; Umeera, A.; Ahmad, R.; Mushtaq, S. Nutritional, phytochemical potential and pharmacological evaluation of Nigella Sativa (Kalonji) and Trachyspermum Ammi (Ajwain). *J. Med. Plants Res.* **2012**, *6*, 768–775.
22. Ali, B.H.; Blunden, G. Pharmacological and toxicological properties of *Nigella sativa*. *Phytother. Res.* **2003**, *17*, 299–305. [CrossRef]
23. Cheikh-Rouhou, S.; Besbes, S.; Lognay, G.; Blecker, C.; Deroanne, C.; Attia, H. Sterol composition of black cumin (*Nigella sativa* L.) and Aleppo pine (Pinus halpensis Mill.) seed oils. *J. Food Comp. Anal.* **2008**, *21*, 162–168.
24. Mehta, B.K.; Verma, M.; Gupta, M. Novel lipid constituents identified in seeds of *Nigella sativa* (Linn). *J. Braz. Chem. Soc.* **2008**, *19*, 458–462. [CrossRef]
25. Bourgou, S.; Ksouri, R.; Bellila, A.; Skandrani, I.; Falleh, H.; Marzouk, B. Phenolic composition and biological activities of Tunisian *Nigella sativa* L. shoots and roots. *Comptes Rendus Biol.* **2008**, *331*, 48–55. [CrossRef] [PubMed]
26. Ghahramanloo, K.; Kamalidehghan, B.; Akbari Javar, H.; Teguh Widodo, R.; Majidzadeh, K.; Noordin, M.I. Comparative analysis of essential oil composition of Iranian and Indian *Nigella sativa* L. extracted using supercritical fluid extraction and solvent extraction. *Drug Des. Devel. Ther.* **2017**, *11*, 2221–2226. [CrossRef]
27. D'Antuono, L.F.; Moretti, A.; Lovato, A.F.S. Seed yield, yield components, oil content and essential oil content and composition of *Nigella sativa* L. and *Nigella damascena* L. *Ind. Crops Prod.* **2002**, *15*, 59–69. [CrossRef]
28. Burt, S. Essential oils: Their antibacterial properties and potential applications in foods—A review. *Int. J. Food Microbiol.* **2004**, *94*, 223–253. [CrossRef] [PubMed]
29. Ciesielska-Figlon, K.; Daca, A.; Kokotkiewicz, A.; Łuczkiewicz, M.; Zabiegała, B.; Witkowski, J.M.; Lisowska, K.A. The influence of *Nigella sativa* essential oil on proliferation, activation, and apoptosis of human T lymphocytes in vitro. *Biomed. Pharmacother.* **2022**, *153*, 113349. [CrossRef]
30. Harzallah, H.; Kouidhi, B.; Flamini, G.; Bakhrouf, A.; Mahjoub, T. Chemical composition, antimicrobial potential against cariogenic bacteria and cytotoxic activity of Tunisian *Nigella sativa* essential oil and thymoquinone. *Food Chem.* **2011**, *129*, 1469–1474. [CrossRef]
31. Tiruppur Venkatachallam, S.K.; Pattekhan, H.; Divakar, S.; Kadimi, U.S. Chemical composition of *Nigella sativa* L. seed extracts obtained by supercritical carbon dioxide. *J. Food Sci. Technol.* **2010**, *47*, 598–605. [CrossRef] [PubMed]
32. Bourgou, S.; Pichette, A.; Lavoie, S.; Marzouk, B.; Legault, J. Terpenoids isolated from Tunisian *Nigella sativa* L. essential oil with antioxidant activity and the ability to inhibit nitric oxide production: Active terpenoids from Tunisian *Nigella sativa*. *Flavour Fragr. J.* **2012**, *27*, 69–74. [CrossRef]
33. Bordoni, L.; Fedeli, D.; Nasuti, C.; Maggi, F.; Papa, F.; Wabitsch, M.; De Caterina, R.; Gabbianelli, R. Antioxidant and Anti-Inflammatory Properties of *Nigella sativa* Oil in Human Pre-Adipocytes. *Antioxid. Act.* **2019**, *25*, 51. [CrossRef]
34. Tiji, S.; Benayad, O.; Berrabah, M.; El Mounsi, I.; Mimouni, M. Phytochemical Profile and Antioxidant Activity of *Nigella sativa* L Growing in Morocco. *Sci. World J.* **2021**, *20*, 6623609. [CrossRef]
35. Pop, R.M.; Bocsan, I.C.; Buzoianu, A.D.; Chedea, V.S.; Socaci, S.A.; Pecoraro, M.; Popolo, A. Evaluation of the Antioxidant Activity of *Nigella sativa* L. and *Allium ursinum* Extracts in a Cellular Model of Doxorubicin-Induced Cardiotoxicity. *Molecules* **2020**, *25*, 5259. [CrossRef]
36. Ouattar, H.; Zouirech, O.; Kara, M.; Assouguem, A.; Almutairi, S.M.; Al-Hemaid, F.M.; Rasheed, R.A.; Ullah, R.; Abbasi, A.M.; Aouane, M.; et al. In Vitro Study of the Phytochemical Composition and Antioxidant, Immunostimulant, and Hemolytic Activities of *Nigella sativa* (Ranunculaceae) and Lepidium sativum Seeds. *Molecules* **2022**, *27*, 5946. [CrossRef] [PubMed]
37. Mohammed, N.K.; Abd Manap, M.Y.; Tan, C.P.; Muhialdin, B.J.; Alhelli, A.M.; Meor Hussin, A.S. The Effects of Different Extraction Methods on Antioxidant Properties, Chemical Composition, and Thermal Behavior of Black Seed (*Nigella sativa* L.) Oil. *Evid Based Complement Alternat. Med.* **2016**, *2016*, 6273817. [CrossRef] [PubMed]
38. Alanazi, I.O.; Benabdelkamel, H.; Alfadda, A.A.; AlYahya, S.A.; Alghamdi, W.M.; Aljohi, H.A.; Almalik, A.; Masood, A. Proteomic Analysis of the Protein Expression Profile in the Mature *Nigella sativa* (Black Seed). *Appl. Biochem. Biotechnol.* **2016**, *179*, 1184–1201. [CrossRef]
39. Kiralan, M.; Özkan, G.; Bayrak, A.; Ramadan, M.F. Physicochemical properties and stability of black cumin (*Nigella sativa*) seed oil as affected by different extraction methods. *Ind. Crops Prod.* **2014**, *57*, 52–58. [CrossRef]
40. Soto, C.; Chamy, R.; Zuniga, M. Enzymatic hydrolysis and pressing conditions effect on borage oil extraction by cold pressing. *Food Chem.* **2007**, *102*, 834–840. [CrossRef]
41. Toma, C.C.; Simu, G.; Hanganu, D.; Olah, N.K.; Vata, F.M.G.; Hammami, C.; Hammami, M. Chemical composition of the Tunisian *Nigella sativa*. note II. Profile on fatty oil. *Farmacia* **2010**, *58*, 458–464.
42. Mildner-Szkudlarz, S.; Różańska, M.; Siger, A.; Kowalczewski, P.Ł.; Rudzińska, M. Changes in chemical composition and oxidative stability of cold-pressed oils obtained from by-product roasted berry seeds. *LWT* **2019**, *111*, 541–547. [CrossRef]
43. Bourgou, S.; Bettaieb, I.; Saidani, M.; Marzouk, B. Fatty Acids, Essential Oil, And Phenolics Modifications of Black Cumin Fruit under NaCl Stress Conditions. *J. Agric. Food Chem.* **2010**, *58*, 12399–12406. [CrossRef]
44. Rao, M.V.; Al-Marzouqi, A.H.; Kaneez, F.S.; Ashraf, S.S.; Adem, A. Comparative Evaluation of SFE and Solvent Extraction Methods on the Yield and Composition of Black Seeds (*Nigella sativa*). *J. Liq. Chromatogr. Relat. Technol.* **2007**, *30*, 2545–2555. [CrossRef]
45. Zúñiga, M.E.; Soto, C.; Mora, A.; Chamy, R.; Lema, J.M. Enzymic pre-treatment of Guevina avellana mol oil extraction by pressing. *Process Biochem.* **2003**, *39*, 51–57. [CrossRef]
46. Pokorny, J.; Korczak, J. *Preparation of Natural Antioxidants—Antioxidants in Food*, 1st ed.; Woodhead: Cambridge, UK, 2001.

47. Lang, Q. Supercritical fluid extraction in herbal and natural product studies—A practical review. *Talanta* **2001**, *53*, 771–782. [CrossRef] [PubMed]
48. Kokoska, L.; Havlik, J.; Valterova, I.; Sovova, H.; Sajfrtova, M.; Jankovska, I. Comparison of Chemical Composition and Antibacterial Activity of *Nigella sativa* Seed Essential Oils Obtained by Different Extraction Methods. *J. Food Prot.* **2008**, *71*, 2475–2480. [CrossRef] [PubMed]
49. Ismail, M.; Al-Naqeep, G.; Chan, K.W. *Nigella sativa* thymoquinone-rich fraction greatly improves plasma antioxidant capacity and expression of antioxidant genes in hypercholesterolemic rats. *Free Radic. Biol. Med.* **2010**, *48*, 664–672. [CrossRef]
50. Kaufmann, B.; Christen, P. Recent extraction techniques for natural products: Microwave-assisted extraction and pressurised solvent extraction. *Phytochem. Anal.* **2002**, *13*, 105–113. [CrossRef] [PubMed]
51. Benkaci-Ali, F.; Baaliouamer, A.; Meklati, B.Y. Kinetic Study of Microwave Extraction of Essential Oil of *Nigella sativa* L. Seeds. *Chromatographia* **2006**, *64*, 227–231. [CrossRef]
52. Jalali-Heravi, M.; Zekavat, B.; Sereshti, H. Use of gas chromatography–mass spectrometry combined with resolution methods to characterize the essential oil components of Iranian cumin and caraway. *J. Chromatogr. A* **2007**, *1143*, 215–226. [CrossRef]
53. Salih, B.; Sipahi, T.; Dönmez, E.O. Ancient nigella seeds from Boyalı Höyük in north-central Turkey. *J. Ethnopharmacol.* **2009**, *124*, 416–420. [CrossRef]
54. Bascones-Martinez, A.; Mattila, R.; Gomez-Font, R.; Meurman, J.H. Immunomodulatory drugs: Oral and systemic adverse effects. *Med. Oral Patol. Oral Cirugia Bucal* **2014**, *19*, e24. [CrossRef]
55. Haq, Z.; Abdullatif, M.; Lobo, P.I.; Khabar, K.S.A.; Sheth, K.V.; Al-Sedairy, S.T. *Nigella sativa*: Effect on human lymphocytes and polymorphonuclear leukocyte phagocytic activity. *Immunopharmacology* **1995**, *30*, 147–155. [CrossRef]
56. Majdalawieh, A.F.; Hmaidan, R.; Carr, R.I. *Nigella sativa* modulates splenocyte proliferation, Th1/Th2 cytokine profile, macrophage function and NK anti-tumor activity. *J. Ethnopharmacol.* **2010**, *131*, 268–275. [CrossRef]
57. Ashraf, S.S.; Rao, M.V.; Kaneez, F.S.; Qadri, S.; Al-Marzouqi, A.H.; Chandranath, I.S.; Adem, A. *Nigella Sativa* Extract as a Potent Antioxidant for Petrochemical-Induced Oxidative Stress. *J. Chromatogr. Sci.* **2011**, *49*, 321–326. [CrossRef] [PubMed]
58. Elmowalid, G.; Amar, A.M.; Ahmad, A.A.M. *Nigella sativa* seed extract: 1. Enhancement of sheep macrophage immune functions in vitro. *Res. Vet. Sci.* **2013**, *95*, 437–443. [CrossRef] [PubMed]
59. Gholamnezhad, Z.; Rafatpanah, H.; Sadeghnia, H.R.; Boskabady, M.H. Immunomodulatory and cytotoxic effects of *Nigella sativa* and thymoquinone on rat splenocytes. *Food Chem. Toxicol.* **2015**, *86*, 72–80. [CrossRef] [PubMed]
60. Singh, Y.; Prakashbhai, P.N.; Saxena, B.; Ramakrishnan, S. Immunomodulatory Effects of *Nigella sativa* Extract on Chicken Peripheral Blood Mononuclear Cells: In-vitro. *J. Anim. Res.* **2021**, *11*, 579–586. [CrossRef]
61. Koshak, A.E.; Yousif, N.M.; Fiebich, B.L.; Koshak, E.A.; Heinrich, M. Comparative Immunomodulatory Activity of *Nigella sativa* L. Preparations on Proinflammatory Mediators: A Focus on Asthma. *Front. Pharmacol.* **2018**, *9*, 1075. [CrossRef]
62. Alshatwi, A.A. Bioactivity-guided identification to delineate the immunomodulatory effects of methanolic extract of *Nigella sativa* seed on human peripheral blood mononuclear cells. *Chin. J. Integr. Med.* **2014**. [CrossRef]
63. Ghonime, M.; Eldomany, R.; Abdelaziz, A.; Soliman, H. Evaluation of immunomodulatory effect of three herbal plants growing in Egypt. *Immunopharmacol. Immunotoxicol.* **2011**, *33*, 141–145. [CrossRef]
64. Boskabady, M.H.; Keyhanmanesh, R.; Khameneh, S.; Doostdar, Y.; Khakzad, M.R. Potential immunomodulation effect of the extract of *Nigella sativa* on ovalbumin sensitized guinea pigs. *J. Zhejiang Univ. Sci. B* **2011**, *12*, 201–209. [CrossRef]
65. Hikmah, Z.; Endaryanto, A.; Ugrasena, I.D.G.; Rahaju, A.S.; Arifin, S. *Nigella sativa* L. as immunomodulator and preventive effect on renal tissue damage of lupus mice induced by pristane. *Heliyon* **2022**, *8*, e09242. [CrossRef] [PubMed]
66. Saaidi, J.A.A.; Dawood, K.A.; Latif, A.D. Immunomodulatory effect of *Nigella sativa* seed extract in male rabbits treated with dexamethasone. *Iraqi J. Vet. Sci.* **2012**, *26*, 141–149. [CrossRef]
67. Elkamel, A. Immunomodulation of Nile Tilapia, Oreochromis niloticus, by *Nigella sativa* and Bacillus subtilis. *J. Aquac. Res. Dev.* **2012**, *3*, 1000147.
68. Boskabady, M.H.; Javan, H.; Sajady, M.; Rakhshandeh, H. The possible prophylactic effect of *Nigella sativa* seed extract in asthmatic patients. *Fundam. Clin. Pharmacol.* **2007**, *21*, 559–566. [CrossRef] [PubMed]
69. Büyüköztürk, S.; Gelincik, A.; Özşeker, F.; Genç, S.; Savran, F.O.; Kiran, B.; Yillar, G.; Erden, S.; Aydin, F.; Colakoğlu, B.; et al. *Nigella sativa* (black seed) oil does not affect the T-helper 1 and T-helper 2 type cytokine production from splenic mononuclear cells in allergen sensitized mice. *J. Ethnopharmacol.* **2005**, *100*, 295–298. [CrossRef]
70. Ciesielska-Figlon, K.; Lisowska, K.A.; Mikosik-Roczynska, A.; Witkowski, J.M. *Nigella sativa* oil inhibits proliferation and stimulates apoptosis of human lymphocytes in vitro. *Hum. Immunol.* **2021**, *82*, 608–614. [CrossRef] [PubMed]
71. Shahzad, M.; Yang, X.; Raza Asim, M.B.; Sun, Q.; Han, Y.; Zhang, F.; Cao, Y.; Lu, S. Black seed oil ameliorates allergic airway inflammation by inhibiting T-cell proliferation in rats. *Pulm. Pharmacol. Ther.* **2009**, *22*, 37–43. [CrossRef]
72. Balaha, M.F.; Tanaka, H.; Yamashita, H.; Abdel Rahman, M.N.; Inagaki, N. Oral *Nigella sativa* oil ameliorates ovalbumin-induced bronchial asthma in mice. *Int. Immunopharmacol.* **2012**, *14*, 224–231. [CrossRef]
73. Sheir, S.K.; Maghraby, A.M.; Mohamed, A.H.; Osman, G.; Al-Qormuti, S.A. Immunomodulatory and ameliorative role of *Nigella sativa* oil on Schistosoma mansoni infected mice. *Can. J. Pure Appl. Sci.* **2015**, *9*, 3345–3355.
74. Hidayati, T.; Akrom, A.; Indrayanti, I.; Sagiran, S. Black cumin seed oil increase leucocyte and CD4Thelper number in sprague-dawley rats induced with dimethylbenzanthracene. *Int. J. Public Health Sci.* **2019**, *8*, 238. [CrossRef]

75. Kheirouri, S.; Hadi, V.; Alizadeh, M. Immunomodulatory Effect of *Nigella sativa* Oil on T Lymphocytes in Patients with Rheumatoid Arthritis. *Immunol. Invest.* **2016**, *45*, 271–283. [CrossRef] [PubMed]
76. Barlianto, W.; Rachmawati, M.; Irawan, M.; Wulandari, D. Effects of *Nigella sativa* oil on Th1/Th2, cytokine balance, and improvement of asthma control in children. *Paediatr. Indones.* **2018**, *57*, 223. [CrossRef]
77. Koshak, A.; Wei, L.; Koshak, E.; Wali, S.; Alamoudi, O.; Demerdash, A.; Qutub, M.; Pushparaj, P.N.; Heinrich, M. *Nigella sativa* Supplementation Improves Asthma Control and Biomarkers: A Randomized, Double-Blind, Placebo-Controlled Trial: *Nigella sativa* in asthma. *Phytother. Res.* **2017**, *31*, 403–409. [CrossRef] [PubMed]
78. Hidayati, T.; Akrom, A.; Apriani, L.; Sun, S. Study of Black Cumin Seed Oil (BCSO) (*Nigella sativa* L.) as an Immunomodulator in The Healthy Active Smoker Volunteer. *IOP Conf. Ser. Earth Environ. Sci.* **2021**, *810*, 012036.
79. Laily, S.A.; Hidayati, T.; Akrom, A.; Sofia, V. The Effect of Black Cumin Seed Oil on Active Smokers Based on Interleukin-1β Activity and Neutrophil. *Indones. J. Pharm.* **2021**, *18*, 476–484. [CrossRef]
80. Nazrul Islam, S.K.; Begum, P.; Ahsan, T.; Huque, S.; Ahsan, M. Immunosuppressive and cytotoxic properties of *Nigella sativa*. *Phytother. Res.* **2004**, *18*, 395–398. [CrossRef]