Effects of *Nigella sativa* Oil Fractions on Reactive Oxygen Species and Chemokine Expression in Asthma Airway Smooth Muscle Cells

ABSTRACT

**Background/Aim:** Airway smooth muscle (ASM) cells are considered the major source of inflammatory mediators in asthma. Allergic asthma mediated through increased oxidative stress in asthma airways contribute towards the increased generation of reactive oxygen species. Globally, glucocorticoids (GC) have been the mainstay of asthma therapeutics. However, the two major limitations are increased toxicity with high doses and refractory response from severe patients. The limitation fuelled the research towards finding an alternative approach to asthma treatment. *Nigella sativa* L. (*N. sativa*) oil has been reported for therapeutic and pharmacological effects due to its anti-bacterial, anti-diabetic, anti-fungal, anti-oxidant, anti-inflammatory, anti-cancer, and immunomodulatory properties. However, its effects on ASM cell chemokines in resistant conditions remain unclear. Therefore, the current study investigates whether *N. sativa* total oil(TO) *and* oil fractions modulate cytokine-induced GC resistance in human ASM cells and what effect they have on reactive oxygen species in asthma patient samples. **Materials and Methods:** Cytotoxicity of *N. sativa* oil fractions was assessed using an MTT assay. Asthmatic ASM cells obtained from patient samples were treated with TNF-α/IFN-γ for 24 hours, and *N. sativa* oil fractions were added two hours before. ELISA assay indicated the effect of *N. sativa* oil fractions on chemokine production (RANTES, IP-10 IL-8). The scavenging effect of *N. sativa* oil fractions was evaluated using three reactive oxygen species ROS (O2•‾, OH, and H2O2). **Results and Discussion:** Results indicate that the unused concentrations (25 and 50 µg/ml) of different fractions did not affect cell viability. In TNFα/IFNγ-treated cells, all fractions of *N. sativa* oil inhibited RANTES, IL-8, and IP-10 in a concentration-dependent manner. Whereas TO is attributed to the most significant chemokines inhibition. Moreover, TO had the highest scavenging effect, i.e., the half maximal inhibitory concentration (IC50) of 0.105, at the concentration of 0.440 mg/ml. **Conclusion:** These results suggest that *N. sativa* oil modulates human ASM cells' expression of chemokines and demonstrates good free radical scavenging activity. Future, research studies should look into mechanistic that may play some beneficial roles in treating steroid-resistant patients with asthma.

Keywords:

Chemokines, Asthma, *Nigella sativa* oil, Airway smooth muscle cells, Inflammation, and Reactive Oxygen Species.

INTRODUCTION

Over the last 100 years, highly effective medications have been developed to treat asthmatic disease. Asthma is defined as a chronic inflammatory condition of the respiratory system (airways) characterized by airway inflammation, intermittent airflow obstruction, and bronchial hyperresponsiveness. Approximately 5 to 10% of patients with severe asthma are poorly responsive to high doses of glucocorticoids (GC) (oral or inhaled) (Gavrila et *al*., 2015). Airway inflammation and asthma severity are closely associated with contraction and structural changes of airway smooth cells mediated through airway responsiveness sensitivity towards stimuli causing airway remodeling. This is the fundamental mechanism implying inflammation’s role in asthma dysfunction (Padrid et *al*., 1995; Powell et *al*., 1994; Defnet et *al*., 2019). Treatment of Airway Smooth Muscle (ASM) cells with TNF-α and IFN-γ induces different pro-inflammatory proteins, which regulate airway inflammation using varied mechanisms (Tliba et *al*., 2008; Calzetta et *al*., 2021). Many inflammatory cells, such as T cells, helper T cells, CD + cells, mast cells, macrophages, basophils, and eosinophils, are involved in airway inflammation in asthma (Kelly et *al*., 1998) and produce more reactive oxygen species, which immediately induce the contraction of ASM cells. Oxidative stress is involved in the pathogenesis of asthma since the biochemical environment in asthmatic airways is favorable for free radical reactions (Park et *al*., 2009; Boldogh et *al*., 2005; Albano et *al*., 2022). Alternatively, increased oxidative stress may contribute to the progression or durability of existing airway inflammation through increased airway hyperresponsiveness and induction of various pro-inflammatory chemical mediators, chemokines, adhesion molecules, and eosinophil granule proteins (Tenscher et *al*., 1996; Chihara et *al*., 1994).Recent studies suggest ROS-mediated reactions can modify or induce specific inflammatory and cellular immunological responses by generating second messengers (Stone et *al*., 2006; Zhang et *al*., 2016; Sies et *al*., 2020; ). As mentioned previously, glucocorticoid is considered the first line of therapy in asthma therapeutics. ROS facilitates corticosteroid insensitivity by disrupting glucocorticoid receptor signaling. It has led to searching for alternative therapies having lesser side effects and greater efficacy, where *Nigella* *sativa* L. (*N. sativa*)*,* has been reported as a potent therapeutic agent having anti-Inflammatory, and immunomodulatory activities ([Kabir et](https://www.sciencedirect.com/science/article/pii/S2405844022005308%22%20%5Cl%20%22bib21)*[al](https://www.sciencedirect.com/science/article/pii/S2405844022005308%22%20%5Cl%20%22bib21)*[., 2020](https://www.sciencedirect.com/science/article/pii/S2405844022005308%22%20%5Cl%20%22bib21); [Islam et](https://www.sciencedirect.com/science/article/pii/S2405844022005308%22%20%5Cl%20%22bib20)*[al](https://www.sciencedirect.com/science/article/pii/S2405844022005308%22%20%5Cl%20%22bib20)*[., 2017](https://www.sciencedirect.com/science/article/pii/S2405844022005308%22%20%5Cl%20%22bib20))

*N. sativa* is a herbaceous plant. It is native to Southern Europe, North Africa, and Southeast Asia (Torequl Islam et *al*., 2016; Randhawa et *al*., 2002; Ghaznavi, 1991), and its taxonomical classification is shown in Table 1. This plant is traditionally used worldwide for culinary and medicinal purposes (Ahlatci et *al*., 2014; Sharma et *al*., 2005; Khare et *al*., 2004). It is commonly used as an immune-stimulatory, anti-inflammatory, hypoglycemic, antihypertensive, antiasthmatic, antimicrobial, antiparasitic, antioxidant, and anticancer agent (Ahmad et *al*., 2016; Mahdavi et *al*., 2016; Al-Attass et *al*., 2016; Ahmad et *al*., 2013; Toma et *al*., 2010).

*N. sativa* and its constituents have been traditionally used to relieve respiratory disorders such as asthma, bronchospasm, and chest congestion (Hajhashemi et *al*., 2004). The oil of this plant was shown to exert immunomodulatory and therapeutic effects on patients with allergic diseases (allergic rhinitis, bronchial asthma, and atopic eczema) (Salem, 2005; Lus et *al*., 2003) by inhibiting pro-inflammatory cytokines, down-regulating PGD2 and COX-2 expressions, and by reducing airway inflammatory cell infiltration.

In addition, many studies reported the beneficial role of *N. sativa* on guinea pig tracheal chains; it exerts a relaxant and functionally antagonistic effects on muscarinic receptors (Boskabady et *al*., 2002), an inhibitory effect on histamine (H1) receptors (Boskabady et *al*., 2004a), an inhibitory effect on calcium (Boskabady et *al*., 2004b), an opening effect on potassium channels (Boskabady et *al*., 2010), and a stimulatory effect on b-adrenoceptors (Brightling et *al*., 2005). A boiled extract of *N. sativa* indicated a bronchodilatory effect on asthmatic patients by increasing pulmonary function (Barnes et *al*., 2013).

To manage patients with severe asthma, the chronic administration of high doses of GC is required, often associated with serious adverse effects (Henderson et *al*., 2020). Although glucocorticoid resistance has been reported in a small fragment of the asthmatic population, it is still a serious medical issue warranting the need for novel therapies targeting glucocorticoid resistance asthma (Henderson et *al*., 2020). Clinical trials are oriented toward natural biomolecules extracted from the medicinal plant to treat various diseases (Palhares et *al*., 2015).

Based on previous results, we propose that *N. sativa* TO and its fractions could exert therapeutic effects in the inflammation of ASM cells in asthmatic subjects. The present study aimed to show the impact of *N. sativa* TO and its fractions on different chemokines produced in human ASM cells treated with pro-asthmatic cytokine (TNF-α/IFN-γ).

MATERIALS AND METHODS

MATERIALS

Collection of plant

Seeds of *N. sativa* used in this study were collected from Algerian Sahara, Becharin 2016. The plant material was identified by Dr. Houssine Laouar, Setif 1 University, Algeria. The voucher specimen was deposited at the Natural Biological Resources Development laboratory at Setif 1 University, Algeria. Seeds were cleaned and stored in darkness and at 4℃ until use.

Subjects

Asthmatic subjects (n= 6) and healthy controls (n =6) were recruited from Glenfield Hospital outpatients, staff, and local advertising. Selection criteria for asthmatic subjects were; type and severity of asthma, degree of airway inflammation, and type of treatment applied. Lung function was evaluated by spirometry, and normal subjects had no history of respiratory disease and normal spirometry. Dr. AMRANI Yacine, Leicester Biomedical Research Center Respiratory, Leicester, UK, provided ASM cells. Additional details regarding patients are shown in Table 2.

Cell culture

Tissue culture from the Human tracheal was obtained from lung transplant donors per procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. The culture of human ASM cells was performed as reported previously (Brightling et *al*.,2005).

Preparation of extracts

*N. sativa* seeds powder (30g) was extracted in methanol solvent (400 ml) using soxhlet for two hours at room temperature (Ramadan et *al*., 2002a). Methanol was evaporated at reduced pressure at 40°C using a rotary evaporator (BÜCHI 461). The resulting extract was mixed with 200 ml of hexane solvent in Bulb for settling, and two phases appeared; methanol and hexane. The latter phase was recovered, and hexane solvent was evaporated at 40°C to get total oil (TO) characterized by a greenish color.

The extracted oil fraction was then applied on a silica gel column to be separated into neutral lipids (NL), glycolipids (GL), phospholipids (PL), and unsaponifiable (IS). The eluting solvents for NL, GL, and PL were chloroform, acetone, and methanol. Solvents were evaporated with a rotary evaporator, and the percentage of each fraction was calculated as per the process reported earlier (Ramadan et *al*., 2002a).

Analysis of *Nigella sativa* L*.*total oilLipid components using GC-MS

Gas chromatography-mass spectrometry (GC/MS) analysis was used for the quantitative analysis of 18 lipid compounds of *N. sativa* TO. The test was performed according to the method of Morrisson and Smith (1964).

Superoxide anion radical (O2•‾) scavenging

Superoxide anion was produced with Xanthine/XOR system according to Robak and Gryglewski's method (1988).

Hydroxyl radical (OH•) scavenging

This experiment was carried out using Smirnoff and Cumbes method (1989). The final volume of the reactive medium was 3.0 ml. It contained 1.0 ml FeSO4 (1.5 mM), 0.7 ml hydrogen peroxide (H2O2) (6 mM), 0.3 ml salicylate sodium (20 mM), and 1 ml of different oil fractions. After incubation at 37°C for one hour, salicylate Hydroxyl complex absorption was measured at 562 nm. The percentage of OH• inhibition was calculated using the following formula:

Inhibition % = [1‐ (A1-A2) / A0] x 100

A0: Absorbance of control (without extrait).

A1: Absorbance of extracts.

A2**:** Absorbance without sodium salicylate.

Hydrogen peroxide scavenging

The ability of TO and NLF to scavenge H2O2 was determined according to Ruch et al. (1989).

Cell viability assay

Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Stoev et *al*., 2009). *N. sativa* TO and its fractions; NL, GL, PL, and unsaponifiable (IS) were dissolved in DMSO (0.01%). For each fraction, two standard concentrations were made, i.e. 25 and 50 µg/ml. The concentrations were made from the initial stock solution with a concentration of 100 mg of extract/1 ml of Dimethyl Sulfoxide (DMSO). The final concentration of 25 and 50 µg/ml was obtained by taking an appropriate amount of stock solution based on the volumetric equation C1V1 = C2V2.

This test was conducted using 24 well microplates. ASM cells were incubated with the medium for one week to ensure the adherence of cells to the microwells (mention the protocol used for cell adherence with details of the number of cells plated to the microwells or write the modulated protocol followed by XYZ et al. (year). The step is followed by careful aspiration of the superficial medium through a micro-pipette (mention the pipette volume in micro-liter). The incubated ASM cells were left for incubation for a period of 24 and 48 hours, having different concentrations of TO and their respective fractions NL, GL, PL, and IS ( 25 and 50 µg/ml). The superficial medium is slowly aspirated and 200 μL of a medium consisting of 5% FBS and 0.5 mg/ml of MTT solvent was added to each well. The plates were incubated for four hours. Post incubation, the previously added media was removed, and 200 μl of dimethyl sulfoxide (DMSO) was added to each well. The absorbance was read at 570 nm by an ELISA reader. To assess the effect of TNFα, IFNγ, and DMSO on cell viability, two negative controls were performed simultaneously with oil fractions:

Control 1: Cells+ TNFα and IFNγ,

Control 2:Cells+ TNFα and IFNγ+DMSO.

Measurement of RANTES, IP-10, and IL-8 secretion

According to the manufacturer's instructions, ELISA assays for IP-10, IL-8, and RANTES were performed using Duoset ELISAs (R&D Systems).

The cells were grown for seven days for confluence washed with PBS, and serum starved using DMEM supplemented with antibiotics, 4 mML-glutamine, 20 mM HEPES (pH 7.4), and 0.1% BSA. Cultured cells were treated in duplicate with NL, GL, PL, and IS of concentrations 25 and 50 µg/ml for two hours, followed by treatment with recombinant human TNF-α and INF- γ (R&D Systems, Minneapolis, MN). The cell culture plates were incubated for 24 hours in a (writhe type of incubator used). The supernatants were collected post-24-hour incubation for ELISA assay (Chachi et *al*., 2013; Tliba et *al*.,2004). The light absorbance was recorded at a wavelength of 450 nm for the three chemokines.

Statistical Analysis

The statistical analysis was performed using Prism six software. The statistical tests employed include one-way ANOVA was used to assess the significant differences between the different groups. *Post hoc* tests (Bonferonni) or paired or unpaired *t-*tests with values of p 0.05 were used to determine the groups that differ from each other.

RESULTS

Extraction yield

The TO yield was 11.79 g/ml, which corresponds to a representation of 13.61% of the seed's weight. Results of TO fractionation using the chromatography column are summarized in Table 3.

Analysis of *Nigella sativa* L*.*oil lipid components using GC/MS

The TO of *N. sativa* was analyzed by Gas Chromatography-Mass Spectrophotometer. The obtained chromatogram exhibited multiple peaks at various retention times and intensities (Figure 1).

Compounds corresponding to these peaks were identified based on molecular weight. The chemical compositions of *N. sativa* TO identified by GC-MS are listed in Table 4. In total, 18 constituents were identified in TO obtained from Algerian *N. sativa* seeds using the GC-MS technique.

To determine the efficiency of *N. sativa* oil fractions, anti-radical, anti-inflammatory, and cell viability assays were performed. The half-maximal inhibitory concentration (IC50) values of *N. sativa* oil fractions were determined.

Anti-radical assays

Anti-radical properties of *N. sativa* oil fractions were compared using different reactive oxygen species (ROS) (O2•‾, OH, and H2O2). All assays showed a similar trend in the scavenging of free radicals. Figure 2 indicates that TO has the highest scavenging effect, followed by PL, GL, and NL.

For superoxide anion radical, TO present the best scavenging activity with an IC50 of 0.0172 mg/ml, followed by PL, GL, and NL fractions with an IC50 of 0.0197, 0.0261, and 0.0680 mg/ml, respectively. The Same pattern was also reported for Hydroxyl radical and H2O2 scavenging capacity, when TO shows the highest anti-radical activity with an IC50 of 0.105, at the concentration of 0.440 mg/ml, followed by PL 0.132, at the concentration of 0.465 mg/ml, GL 0.330, at the concentration of 0.626 mg/ml and, NL 0.460, at 0.930 mg/ml respectively.

MTT assay

To investigate the effect of *N. sativa* oil fractions on cell viability, ASM cells were treated with 25 and 50 µg/ml ( *N. Sativa* TO and fractions)for 24 hours and 48 hours and subjected to the MTT colorimetric assay.

The percent cell viability of ASM cells against *N. sativa* oil fractions, as observed by MTT assay, is presented in Figure 3. The result shows that all fractions of *N. sativa* oil demonstrate no toxicity or any adverse effect on the proliferation of ASM cells at the dosages used and the treatment periods.

Anti-inflammatory activity of *Nigella sativa* oil fractions

In the present study, we aimed to investigate the effect of *N.sativa* oil fractions on the expression of chemokines in ASM cells treated with TNF-α 10 ng/ml and IFN γ 25 ng/ml. However, no reports have analyzed the anti-inﬂammatory potential of different fractions of *N. sativa* oil on human ASM cells chemokine.

As shown in Figure 4, chemokine production was significantly increased in ASM cells from subjects with asthma compared to unstimulated ASM cells (the basal). After treatment with the different fractions, significant inhibition was observed with dose-dependent concentration, and the best inhibition was achieved at 50 µg/ml for all fractions. In ASM-treated cells, TO Inhibited RANTES secretion at 43.33%, IP-10 secretion at 40.03%, and IL-8 secretion at 47.62%. NF inhibited RANTES secretion with 41.66%, IP-10 secretion with 36.25%, and IL-8secretion with 39.53%. PL Inhibited RANTES secretion with 40.05%, IP-10 secretion with 31.25%, and IL-8secretion with 29.48%. GL Inhibited RANTES secretion with 36.33%, IP-10 secretion with 37.50%, and IL-8secretion with 42.26%. IS inhibited RANTES secretion with 38.02%, IP-10 secretion with 35.62%, and IL-8secretion with 28.11% (Figure 3).

DISCUSSION

The present study aimed to explore the anti-inflammatory effect of different fractions of *N. sativa* oil in terms of chemokines secretion in human ASM cells. The study found that *N. sativa* TO and fractions have a potential anti-inflammatory effect on chemokine expression and ROS scavenging activity. Similarly, the *N. sativa* TO and fractions have no adverse effects on the cells, irrespective of the concentration used and incubation period.

The chemical composition of *N. sativa* TO identified by GC-MS indicated the presence of Linoleic acid (18:2n-6), Oleic acid (18:1n-9), Palmitic acid (16:0), and Stearic acid based on comparative value for the standards present in the database. Comparing our results with those reported by Ramadan et *al*. (2003), which used Egyptian *N. sativa*, indicated that linoleic acid, oleic acid, and Palmitic acid measured for Egyptian TO were higher than that of Algerian TO.

Another study showed that the concentrations of fatty acids in Algerian *N. sativa* oil were higher than those of Indian and Ethiopian plants (Thilakarathnaet *al*., 2018) (Table 5).

Oxidative stress is involved in the pathophysiology of many human diseases, including asthma. In asthma, oxidative damage of biomolecules has a definite and major role in asthmatic inflammation. In the airways and systemic circulation, asthma is associated with oxidative stress since the oxidative damage of biomolecules is strongly implicated in asthmatic inflammation (Sugiura et *al*., 2008; Riedl et *al*., 2008).

ASM has been considered an important player in the pathogenesis of asthma through the airway remodeling and inflammation seen in asthma (Oliver et *al*., 2006; Chung, 2005). ASM cells can be a source of chemokines in airway inflammation that ultimately recruit and retain inflammatory cells (Tliba et *al*., 2008a). In cultured ASM cells, TNF-α cooperates with IFN-γ synergistically to induce the expression of different pro-inflammatory mediators, including cytokines (IL-6 and IL-1) and chemokines (RANTES, IL-8) (Halayko et al., 2003; John et al., 1997). RANTES is a chemokine expressed in smooth muscle bundles of bronchial biopsies in asthmatic subjects. It attracts monocytes, eosinophils, and T cells during inflammation and immune response (Schall et *al*., 1990). In asthmatic subjects, IP-10 was expressed and activated T cells, NK cells, and mast cells (Brightling et *al*., 2005; Luster et *al*.,1985). Interleukin-8 (IL-8) is a part of the CXC chemokine, and it may evoke the migration of neutrophils (Konstan et *al*., 1997), monocytes (van Schaik et *al*., 2000), and eosinophils (Proud et *al*., 2006), to the inflammation sites.

The current study determines, *in vitro,* the capacity of *N. sativa* oil fractions to scavenge O2•‾, OH•, and H2O2 in a dose-dependent manner. TO showed the highest scavenging capacity in all assays, followed by PL, GL, and NL. The observation of anti-radical assays agrees with previous studies showing that TO and PL presented a potent source of antioxidant compounds.

The treatment of ASM cells of asthmatic subjects with TNFα/IFNγ induced an increase in RANTES, IP-10, and IL8 compared with non-treated asthmatic subjects. The application of different fractions of *N. sativa* oil showed an anti-inflammatory effect mainly due to the inhibition of chemokines secretion. However, TO showed the most significant inhibition of all chemokines. The maximum preventive effect of the plant extract was obtained at the highest concentration applied. The results also suggest that *N. sativa*, with the concentration used in this work, didn’t present any cytotoxicity.

Various fractions of *N. sativa* indicated a difference in antioxidant and anti-inflammatory activities, and the wide variance can be explained based on the difference in functional groups, structures, and fatty acid composition (Khan et *al*., 2000). The anti-radical and anti-inflammatory capacity of N. sativa TO can be interpreted as the combined action of endogenous antioxidants.

The therapeutic effect of *N. sativa* on smooth muscles might be explained by several mechanisms; (1) Spasmolytic effect mediated through calcium channel blocking activity (Ghayur et *al*., 2012; Parvardeh et *al*., 2007), (2) Opening effect on potassium channels (Buckle et *al*., 1993), (3) Anticholinergic and muscarinic receptor inhibitory effects (Boskabady et *al*., 2011a; Boskabady et *al*., 2003), (4) Histaminic antagonistic activity (Boskabady et *al*., 2002), and (5) Stimulatory effect on β2-adrenoceptors (Boskabady et *al*., 2011b).

Brightling et al.(2005) have reported the relaxant effect of *N. sativa’s* fixed and volatile oils on tracheal smooth muscle. Essential oil of *N. sativa* exhibited an inhibitory effect on the cyclooxygenase and 5-lipoxygenase pathways of arachidonic acid metabolism and membrane lipid peroxidation. Similarly, a study by Proud et *al*., 2006 on guinea pigs using different *N. sativa* fractions (n-hexane, dichloromethane, methanol, and aqueous) showed a significant relaxant effect on tracheal smooth muscle, and the most potent relaxant effect was detected for methanol and dichloromethane fractions. The therapeutic effect of *N. sativa* oil on patients with allergic diseases (allergic rhinitis, bronchial asthma, and atopic eczema) has also been demonstrated (Khan et *al*., 2000). It’s important to point out that results are valid on experimental conditions used in the current study; the amount of *N. sativa* oil fractions and application duration.

CONCLUSION

The present study intended to determine the potential effects of *N. sativa* oils on pro-inflammatory responses in ASM cells. Results demonstrate that *N. sativa* TO, NL, GL, PL, and IS differentially inhibit the secretion of chemokines used in this work; RANTES, IP-10, and IL-8. Understanding how different fractions decrease the level of chemokines could offer a novel therapeutic approach to asthma. Future studies are therefore needed to demonstrate the effect of fractions on the expression of the chemokines in ASM cells *via* transcriptional and post-transcriptional mechanisms.