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Evaluation of *In Vitro* phagocytic property of macrophages in presence of Thymopure™ (*Nigella Sativa)* oil

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Abstract
Black cumin or *Nigella sativa* (*N. sativa)* has been used for its medicinal properties for centuries in many parts of the world. Studies have shown that *Nigella sativa* helps reinforce the body's defence responses through antioxidant and anti-inflammatory properties. Thymoquinone, a benzoquinone compound which is the main active component, exhibits anti-inflammatory activity through Toll-Like Receptor 4 (TLR-4) and is able to increase the activity of macrophages against invading foreign bodies. Mouse macrophages which mimic natural macrophages were used in the current study to investigate the benefits of ThymoPure™ (*N. sativa*) oil on potentiation of phagocytosis. ThymoPure™ (*N. sativa)* oil was initially studied for its *in vitro* cytotoxicity, by exposing the cells to different concentrations of the test substance. Non-toxic concentrations of 125 µg/ml and 62.5 µg/ml were identified and selected for the phagocytosis study. Macrophage (Raw 264.7) cells were treated with the test substance for 1 h then exposed to green *E.coli* slurry. The amount of phagocytosis observed in macrophage cells was estimated by fluorescent microscopy and flow cytometry. ThymoPure™ (*Nigella Sativa*) oil increased the phagocytic capacity of cells compared to the cell control. Further, flow cytometry analysis showed the increase in phagocytic capacity of the cells in both lower (18.7%) and higher concentrations (39.1%) of the test substance. ThymoPure™ (*N. sativa)* oil has shown to have some potentiation effects on phagocytosis on mouse macrophages in our study.

Keywords: Nigella sativa, ThymoPure, phagocytic activity, anti-oxidant, anti-inflammatory and mouse macrophages

Introduction
Black cumin or *Nigella sativa* (*N. sativa*) is widely used herbal medicine as a tonic, with anti-cancer, analgesic and anti-asthma effects [1-3]. Studies have shown that *N. sativa* helps reinforce the body's defence responses through antioxidant and anti-inflammatory properties. It is known to inhibit inflammation of bronchi and decrease asthma by inhibiting mRNA expression of IL-4, IL-5, IL-6 and TGF-β in the allergens-induced experimental animal models. *N. sativa* is known to contain fixed oil, essential oil, proteins, alkaloids and saponins. The most active component Thymoquinone is a benzoquinone compound. It has a chemical structure identical to other anti-inflammatory compounds and exhibits its anti-inflammatory activity through Toll-Like Receptor 4 (TLR-4) and is able to increase the activity of macrophages. Thymoquinone decreases inflammatory reactions in mice bronchi, IgE and IgG specific-OVA, IL-5, IL-4, IL-13 and increases IFN-γ in ovalbumin-induced mice [4-6].
Oxidative stress, inflammation, increased bronchial epithelial cell apoptosis, and deficient phagocytic clearance by the alveolar macrophages are characteristics of airway inflammatory disease. Thymoquinone, has been found to attenuate allergic inflammation by inhibiting T helper 2 (Th2) cells and eosinophil infiltration in the airways [6, 7]. Kalus *et al*. [7] have shown that the oil of *N. sativa* was effective in relieving symptoms of allergic diseases. *N. sativa* seed supplementation reduced symptom levels through peripheral blood polymorphonuclear leukocyte (PMN) functions (phagocytic and intracellular killing activity) and lymphocyte subsets in patients with allergic rhinitis. Akrom and Mustofa [8] in their study have shown that black cumin seed oil increased phagocytic activity and secretion of IL-2 by macrophages. Antioxidant and anti-inflammatory agent, thymoquinone, could improve macrophage phagocytosis via modulation of the S1P system and then protect bronchial epithelial cells from cigarette smoke or lipopolysaccharide (LPS)-induced apoptosis. Cigarette smoke and LPS have found to decrease phagocytosis and increase S1P receptor (S1PR)-5 mRNA expression in THP-1 macrophages. Thymoquinone was found to enhance efferocytosis and phagocytic ability, antagonize the effects of cigarette smoke extract and LPS on phagocytosis and S1PR5, and protect bronchial epithelial cells from cigarette smoke-induced apoptosis. Black seed oil[9] has shown to increase phagocytic activity by increasing secretion of Interleukin-12 (IL-12). IL-12 has found to be regulating innate and adaptive immune responses produced by macrophages.
In the current *in vitro* study, we have investigated the benefits of ThymoPure™ (*N. sativa*) oil on the potentiation of phagocytosis on mouse macrophages.

Materials and Methods
Test substance information
ThymoPure™ (*Nigella sativa)* oil (Batch no. NS00820002)

was manufactured by Bio-gen Extracts Pvt Ltd, Bangalore, INDIA. This is stored at room temperature with a shelf life of about three years (Date of manufacture, Aug 2020). ThymoPure™ (*Nigella sativa)* oil, is manufactured by super-critical-fluid-extraction (SCFE) technology where the seeds of *Nigella sativa* are extracted using carbon dioxide

Preparation of test solution

ThymoPure™ (Nigella sativa) oil, 10 mg was weighed and separately dissolved in DMEM-HG (Dulbecco′s modified eagle′s medium-high glucose) supplemented with 2% inactivated FBS (fetal bovine serum). The volume was made up with media to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two-fold dilutions were prepared from the stock solution to perform cytotoxic studies and further efficacy studies.

Cell line and Culture medium

Raw 264.7 (Mouse macrophage) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose (TPVG) solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96-well microtiter plates (Tarsons India Pvt. Ltd., Kolkata, India).

Cytotoxicity Studies

The cell viability was assessed by MTT (tetrazolium dye MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reduction assay in semi confluent monolayer cultures [10, 11]. The drug solutions were added to cells and incubated at 37 ºC in 5% CO_2 atmosphere. After 72 h of incubation, plates were centrifuged at 500g. Drug solutions in the wells were carefully removed and 100 µl of MTT in PBS (phosphate buffer saline) was added to each well. The plates were gently shaken and incubated for 3 h at 37 °C in 5% CO_2 atmosphere. Supernatant was removed as mentioned above and 100 µl of DMSO (dimethyl sulfoxide) was added and the plate was gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The plates were protected from light throughout the procedure. The percentage growth inhibition was calculated using the standard formula and concentration of test substances, needed to inhibit the growth of the cell by 50% i.e., CTC_{50} values were generated from the dose-response curves. The inhibition was expressed as the percentage relative to the cell control.

Phagocytosis Assay Studies

Phagocytosis in mammals serves as an important first line defence mechanism against invading pathogens. It is also essential for continuous clearance of dying cells, tissue remodelling, and acquisition of nutrients for some cells. Phagocytosis is a specific form of endocytosis initiated by recognition and binding of foreign particles by cell surface receptors, followed by their engulfment, and formation of phagosomes. Maturing phagosomes transform to phagolysosomes which destroy the pathogen through enzymes and toxic peroxides. *E. coli* and other bacterial strains are often used as a pathogen in phagocytosis assays. BioVision's EZCell™ Phagocytosis Assay Kit (Red *E. coli*), #K963 utilizes heat-killed, fluorescently pre-labeled *E. coli* particles as a tool for rapid and accurate detection and quantification of *in vitro* phagocytosis by fluorescent microscope, spectrophotometer or flow cytometry. The kit provides a robust screening system for activators and/or inhibitors of phagocytosis and Toll-like receptors (TLR) ligands.

The phagocytosis assay was processed according to the manufacturers' instructions for analysis with fluorescent microscopy and flow cytometry. Briefly, 100 µl the macrophage cells (1 x 10^6 cells/ml) were seeded onto a 96-well plate and incubated for 24 h. After 24h, cell supernatants were replaced with non-toxic doses of test substances and incubated for 1 h at 37 °C. After 1 h of incubation, 5 µl of *E. coli* slurry was added to each well and the plate was immediately incubated for 3 h at 37 °C. After incubation, 50 µl of quenching solution was added to each well and incubated for 2 min at room temperature. The wells were washed properly with phagocytosis assay buffer and observed under fluorescence microscope.

For analysis with flow cytometry, 500 µl the macrophage cells (1 x 10^6 cells/ml) were seeded onto a 24 well plate and incubated for 24h. After 24h, cell supernatants were replaced with 400 µl of non-toxic doses of test substances and incubated for 1 h at 37 °C. After 1 h of incubation, 20 µl of *E. coli* slurry was added to each well and immediately the plate was incubated for 3 h at 37 °C. After incubation, 200 µl of quenching solution was added to each well and incubated for 2 min at room temperature. The wells were washed with phagocytosis assay buffer and scraped with cell scraper. The cells were transferred into a fresh tube and centrifuged at 400g for 5 min. The phagocytosis assay buffer was replaced with 200 µl of ice-cold fresh assay buffer.100 µl of cell suspension was transferred into 900 µl of the phagocytosis assay Buffer and immediately analyzed with flow cytometry under excitation laser at 488 nm under the FL1 channel.

To calculate the net phagocytosis subtract the average RFU (relative fluorescence) of the no-cell negative-control wells from all positive control and experimental wells. The phagocytosis response to the experimental effector (% Effect) can be expressed as follows: % Effect = Net experimental phagocytosis × 100% Net positive control phagocytosis.

Results

ThymoPure™ (*N. sativa)* oil was initially studied for cytotoxicity on Macrophage cells by MTT assay *in vitro*. Macrophage cells were exposed to different concentrations of the ThymoPure™ (*N. sativa)* oil. Dilutions of 1000mg/ml, 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5mg/ml were studied (Table 1). ThymoPure™ (*N. sativa)* oil demonstrated a CTC_{50} value of 210.5±2.45. Furthermore, the non-toxic concentrations of 125 µg/ml and 62.5 µg/ml were identified and selected for further phagocytosis studies (Table 1).

Analysis of phagocytic property of the test substance was done by flow cytometry method. A minimum of 9,000 events within the macrophage gate was acquired. The positive phagocytic cells that had internalized labeled *E.coli* particles was obtained by cell quest software 6.0. The flow

cytometry analysis (Fig.2) showed the increase in phagocytic capacity of the cells with the lower (18.7%) and higher concentrations (39.1%) of the test substance.

The observation of cells under fluorescent microscope (Fig. 1) shows that the ThymoPure™ (*N. Sativa*) oil increases the capacity of cells to undergo phagocytosis by about 39.16% at higher concentration (125 µg/ml) when compared to the cell control.

Table 1: Cytotoxic properties of ThymoPure™ (*N. sativa)* oil against Raw 264.7 cells

Sl. No	Name of Test substance	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1.	*Nigella sativa* Oil (Raw 264.7)	1000 500 250 125 62.5	64.99 ± 0.6 62.97 ± 0.5 60.39 ± 1.1 27.56 ± 0.6 16.62 ± 1.4	210.5 ± 2.45

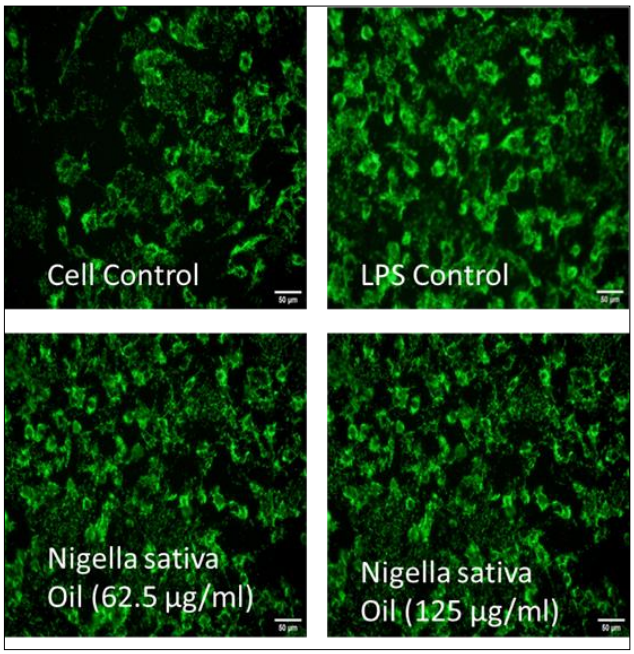

Fig 1: Estimation of phagocytosis property of the ThymoPure ™ (*N. sativa)* Oil on Macrophages by fluorescent microscopy: Phagocytosis was conducted for three hours and the amount of engulfed *E. coli* was determined by fluorescent microscopy.

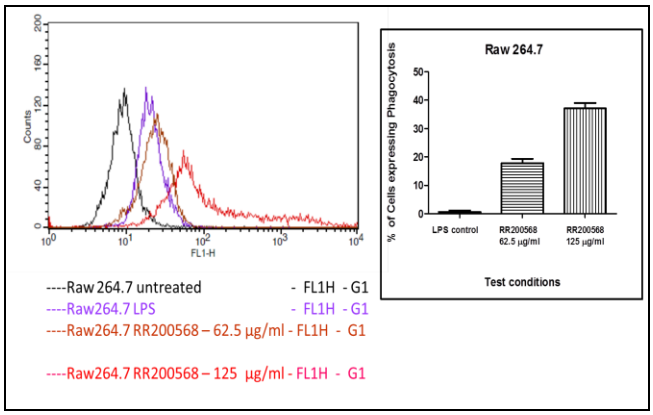

Fig 2: Estimation of phagocytosis property of the ThymoPure ™ (*N. sativa)* Oil on Macrophages by flow cytometry. Black line: untreated control cells; Purple line: LPS treated macrophages with engulfed E. coli particles; Brown line: phagocytosis by test substance at 62.5 (µg/ml) and Red line: phagocytosis by test substance at 125 (µg/ml). P value is < 0.0001

Discussion

Macrophages are professional phagocytes that act as antigen presenting cells (APCs) and are the main effectors in cellular innate and adaptive immune response. When a foreign pathogen infects higher organisms, phagocytosis serves as a first line defence mechanism. Compounds which promote phagocytosis are thought to help in clearing the infecting pathogen. Haq *et al* (12) demonstrated that *N. sativa seeds* activated T lymphocytes to secrete IL-3 and to cause enhanced IL-1 production. In a later study, the same authors[13] had fractionated *N. sativa* proteins by ion exchange chromatography and showed that some proteins have suppressive and other stimulatory properties in lymphocyte cultures. Currently, only one study has explored the effects of *N. sativa* on the leukocyte phagocytic activity of PMNs *in vitro*. In that study, no effect of *N. sativa* or its fractions was noticed on the phagocytic or killing activities of PMNs in the presence of *S. aureus* bacteria. On the other hand, *N. sativa* seed supplementation with specific immunotherapy increased CD8 cell count, which was decreased after the 1st month of specific immunotherapy. The mechanism of *N. sativa* stimulation of the increase in PMN functions and CD8 cells is unclear. It may be speculated that an important number of cytokines induced by *N. sativa* produce immunomodulatory changes [14, 15].

N. sativa also increased the PMN functions in healthy controls, indicating that this effect is not specific to allergic patients. The augmented PMN function by *N. sativa* seed supplementation can explain the beneficial effect of this supplementation on allergic rhinitis patients[14]. Although there is no confirmed evidence that allergic rhinitis patients are more susceptible to upper respiratory tract infections than normal, it has been observed that such infections lead to symptom aggravation in rhinitis patients, last longer and tend to become chronic. From this point of view, it can be speculated that augmented PMN functions stimulated by *N. sativa* supplementation may improve the response to microorganisms in rhinitis patients. Using Black seed[13] with food causes increased phagocytic activity in mononuclear cells and increasing of phagocytosis activity for uptake of foreign particles and pathogens. Thus, this substance can be used to define pathogens through activating monocyte-macrophage pathway, that is, the connector between the innate and adaptive immune systems. In another study Akrom and Mustofa [8] showed black cumin seed oil (BCSO) increased phagocytic activity, secretion of IL-12 by macrophage, and the expression of TLR4. The highest phagocytosis percent (56.83 ± 6.37%) and phagocytosis index (5.18 ± 0.39) was performed by 0.25 BCSO group and did not significantly differ with thymoquinone group. The highest level of IL-12 was in 2.5 BCSO group (66.33 ± 2.11 µM). In the current study, the flow cytometry analysis showed increase in phagocytic capacity of the cells by 18.7% and 39.1% with the lower (62.5 µg/ml) and higher concentrations (125 µg/ml) of the test substance, as well as increase in the capacity of cells to undergo phagocytosis by about 39.16% at higher concentration (125 µg/ml) by observation of cells under fluorescent microscope when compared to the cell control.

Conclusion

ThymoPure™ (*N. sativa)* oil has shown to have some potentiation effects on phagocytosis on mouse macrophages in the current study. As suggested earlier in studies, *N.*

sativa oil may have some beneficial immunomodulatory and antiallergic effects on allergic patients and healthy subjects as well. Our results show that ThymoPure™ (*N. sativa)* oil may potentiate first line defence on immune system through its phagocytic activity.

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