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Evaluation of *In vitro* immuno modulatory activity of thymopure™ *Nigella sativa* oil in dendritic cells

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Abstract
Nigella sativa or black seed has been reviewed in greater detail in the past decade, for its role on immunomodulatory and immunotherapeutic potential. Studies have shown it to have potential anti-inflammatory effects on several inflammation-based models. The current study evaluated the effects of Thymo Pure ™ (Nigella sativa) oil on LPS-induced dendritic cells maturation, survival and cytokine release.
Thymo Pure ™ (Nigella sativa) oil was initially examined for cytotoxicity against dendritic cells, in vitro by MTT assay. The dendritic cells were exposed to different concentrations of the test substance. Non-toxic concentrations were identified and were used for further efficacy studies.
Thymo Pure ™ (Nigella sativa) oil inhibited LPS induced TNF-α in dendritic cells by 47.75% ± 1.6% and 38.77% ± 0.9%, IL-1- β levels by 40.60% ± 2.70% and 36.36% ± 3.08%, IL-6 by 53.48% ± 2.1% and 26.24% ± 1.6% and IL-17 by 43.98% ± 2.1% and 21.45% ± 2.6%, over the control at test doses of 125 µg/ml and 62.5 µg/ml, respectively.
Thymo Pure ™ (Nigella sativa) oil counteracts DC maturation. Upregulation of several maturation markers like TNF-a, IL-6, IL-1beta and IL-17 following LPS exposure, was inhibited in the presence of ThymoPure™ (Nigella sativa) oil in the current study, which further substantiates its immuno modulatory activity.

Keywords: immuno modulatory activity, *Nigella sativa* oil, dendritic cells

Introduction

Seeds of *Nigella sativa* (NS), have been used in traditional medicine from centuries due to their medicinal properties. Thymoquinone (TQ) has been reviewed in great detail for its roleon immunomodulatory and immunotherapeutic potential [2, 3]. TQ has shown to have potent anti-inflammatory effects on several inflammation-based models including experimental encephalomyelitis, colitis, peritonitis, oedema, and arthritis through suppression of the inflammatory Mediators prostaglandins and leukotrienes. The beneficial immunomodulatory properties of NS was shown by augmenting the T cell and natural killer cell (NKC)-mediated immune responses. The anti-inflammatory effect of TQ is believed to be mainly on the function of dendritic cells (DCs). Dendritic cells are sentinel cells responsible for the recognition of pathogens and signal tissue damage[2]. DC maturation and cytokine release is triggered by bacterial components such as lipopolysaccharides (LPS). This includes LPS-induced cell swelling, reactive oxygen species (ROS) production, TNF-α release and migration which is regulated by the Na (+)/H (+) exchanger (NHE).

Despite its powerful influence on inflammation and its immunomodulatory effects, little is known about its effect on how NS or TQ work on dendritic cells (DCs). Xuan *et al* (2010) [2] studied mouse bone marrow derived DCs which were treated with LPS and different concentrations of thymoquinone and the surface expression of CD11c, CD86, MHCII, CD54 and CD40 and the formation of the interleukins 10 (IL-10) and 12 (IL-12p70),TNF-α, caspase activation, Akt and ERK1/2. LPS increased the percentage of CD11c, CD86, CD11c, MHCII, CD11c, CD40, CD11c, CD54 cells and stimulated the release of IL-10, IL-12p70 and TNF-α. These effects were blunted by thymoquinone in a concentration dependent manner (1-20 µM). Moreover, LPS decreased and thymoquinone increased caspase 3 and caspase 8 activation and annexin V binding. Overall, thymoquinone compromises the maturation, cytokine release and survival of DCs.

Yang *et al* (2012) [3] explored, whether thymoquinone influences NHE (Na^+/H^+ Exchange) activity in DCs derived from mouse bone marrow. DCs were treated with LPS in the absence and presence of thymoquinone (10 µM). Exposure of DCs to LPS (1 µg/ml) resulted in transient increase of NHE activity within four hours. LPS increased ROS formation, cell volume and triggered TNF-α release and migration These effects were blunted in the presence of thymoquinone. Thymoquinone blunted LPS induced NHE activity, cell swelling, oxidative burst, cytokine release and migration of bone marrow derived murine dendritic cells. NHE inhibition may thus contribute to the anti-inflammatory action of thymoquinone

Given their potent immunomodulatory effects[4], studies are required to explore effects of TQ on the professional antigen presenting cells (APC), including macrophages and dendritic cells, as well as its modulatory effects upon Th1- and Th2-mediated inflammatory immune diseases. It is envisaged that results emerging from these studies may substantially improve the immunotherapeutic application of TQ in clinical settings.

The current study evaluated the effects of ThymoPure™ (*Nigella sativa*) oil on LPS-induced DC maturation, survival and cytokine release.

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.

The non-toxic concentrations of the test substance were selected to evaluate its Immuno-boosting activity against LPS induced toxicity on dendritic cells. After dendritic cells were stimulated by LPS and treated with the test substance, the cell supernatants were used for estimation of the immune markers by ELISA kits

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).

Isolation of DC from mouse spleen

The C57 mouse was anaesthetized with an overdose of isoflurane (adjust flow rate of isoflurane to >5% and continue exposure at least 2 min after breathing stops) and dissected to separate the spleen under aseptic conditions. The spleen was separated and transferred to a fresh petri dish, washed with RPMI medium and was cut into small (~0.2 cm^2) pieces using a surgical scalpel. The pieces were ncubated for 30 min at 37 °C with 10 ml of collagenase solution. The partially digested suspension of the splenic tissue was poured onto a 70 µm cell strainer. Using a 5 ml syringe the contents were compressed. The mesh was washed with 10 ml of fresh medium and the filter was discarded. The suspension was centrifuged at 300 x g for 10 min at 4 °C to pellet the cells. The supernatant was aspirated, and the cells were resuspended in 2 ml of RBC lysis buffer and incubated at RT for 10 min. The suspension was centrifuged at 300 x g for 5 min at 4 °C.

To examine the capacity of the spleen cells[5] to proliferate in response to GM-CSF, 2 x 10^6 cells were placed in each well of a 24-well plate in 1 ml of complete medium, supplemented with 10% FBS and with 4 ng/ml mouse recombinant GM-CSF (Sigma, #G0282). Until day 4, the cells were gently washed after every 48 h and the media was replenished with GM-CSF. After 4 days, the non-adherent cells were harvested and were collected by day 10. The dendritic cells were isolated by negative selection method using FACS [6]. Dendritic cells were cultured in DMEM-HG/RPMI media 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 stock cultures were grown in 25 cm^2 culture flasks and experiments were carried out in 96 microtiter plates for cytotoxicity.

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 [7-,9]. The drug solutions were added to cells and incubated at 37ºC 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.

Immune modulatory Studies

The isolated primary cells, 2 x 106 cells were seeded onto a 6 well plate and incubated for 24 h. After 24h, cell supernatants were replaced with non-toxic doses of test substances and LPS (1 µg/ml). Cell control and standard control (Dexamethasone) were maintained. After 4 h of incubation the supernatant was collected and centrifuged at 500g for 10 min and stored at -80 °C until further use. Before estimation of the immune markers, the supernatants were brought to room temperatures and processed for immune markers TNF-alpha, IL-1 beta, IL-6 and IL-17 by ELISA kit method according to the manufacturer's instructions (Elabscience Biotechnology Inc, USA).

Results

ThymoPure™ (*N. sativa)* oil was initially studied for cytotoxicity on dendritic cells by MTT assay *in vitro*. Dendritic cells were exposed to different concentrations of 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 immunemodulating studies (Table 1).

Table 1: Cytotoxic properties of ThymoPure™ (*Nigella sativa)* oil against mouse spleen dendritic cells

Sl. No	Name of Test substance	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1.	*Nigella sativa* Oil (Dendritic cells)	1000	91.87 ± 0.1	206.99 ± 6.3
		500	82.23 ± 0.8	
		250	58.95 ± 1.7	
		125	32.82 ± 3.9	
		62.5	23.80 ± 2.5	

LPS induction is carried in mouse spleen dendritic cells by using dexamethasone which served as a control in the immune modulating studies. There is a substantial reduction of inflammatory mediators that includes TNF-a, IL-1-b, IL -6 and IL-17 in the mouse spleen dendritic cells.

Thymo Pure™ (*N. sativa)* oil was shown to reduce TNF-a, IL-1-b, IL -6 and IL-17 when compared to control (Table 2).

- TNF-a was reduced by 48% (125 mg/ml) and 39% (62.5 mg/ml) in a dose response manner compared to that of control.
- IL-1-b was reduced by 41% (125 mg/ml) and 37% (62.5 mg/ml) in a dose response manner compared to that of control.
- IL-6 was reduced by 53% (125 mg/ml) and 26% (62.5 mg/ml) in a dose response manner compared to that of control.
- IL-17 was reduced by 44% (125 mg/ml) and 21% (62.5 mg/ml) in a dose response manner compared to that of control.

Table 2: Immuno- modulatory effect of Thymo Pure™ (*Nigella sativa)* oil on TNF-α levels in mouse spleen dendritic cells

Sl. No	Samples	Concentration tested	% TNF- α inhibition over Control	% IL-1- β inhibition over Control	% IL-6 inhibition over control	% IL-17 inhibition over control
1.	*Nigella sativa* oil (RR200568)	125 µg/ml 62.5 µg/ml	47.75 ± 1.60 38.77 ± 0.90	40.60 ± 2.70 36.36 ± 3.08	53.48 ± 2.1 26.24 ± 1.6	43.98 ± 2.1 21.45 ± 2.6
2.	Dexamethasone (Std. control)	100 µM	74.82 ± 0.80	75.74 ± 1.10	80.45 ± 8.9	84.17 ± 6.3

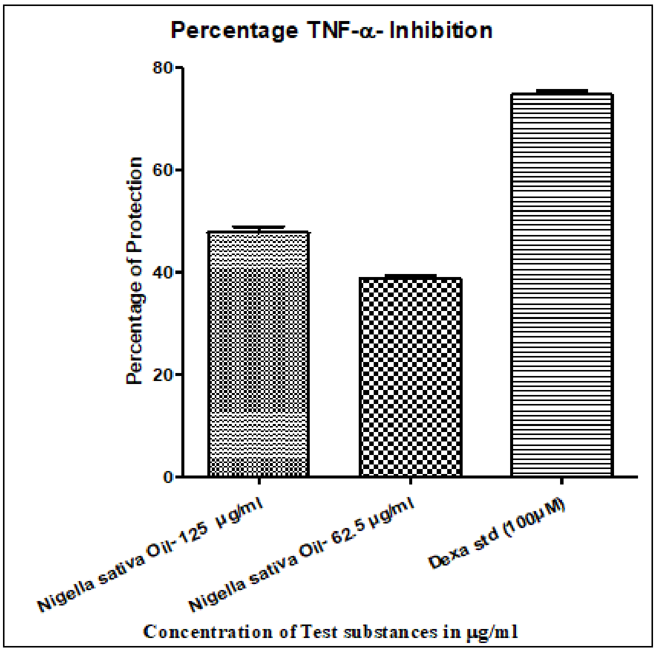

Fig 1: Immunomodulatory effect of the ThymoPure™ (*Nigella sativa)* Oil on LPS induced TNF-α generation in mouse spleen dendritic cells. (P < 0.0001).

The percentage inhibition of the cytokines is based on the comparison to the cell control. The values are normalised to cell control. Therefore, the cell control data values are not shown in the graphs.

In the aforesaid figure ThymoPure™ (*N. sativa)* oil has shown to protect to the tune of 48% (125 mg/ml) and 39% (62.5 mg/ml) in LPS induced inflammation (TNF-a) by dexamethasone on mouse spleen dendritic cells. The difference observed when compared to the control is highly significant (P<0.001) and the doses used in the study are able to produce necessary anti-inflammatory response in immunomodulatory studies conducted on ThymoPure™ (*N. sativa)* oil.

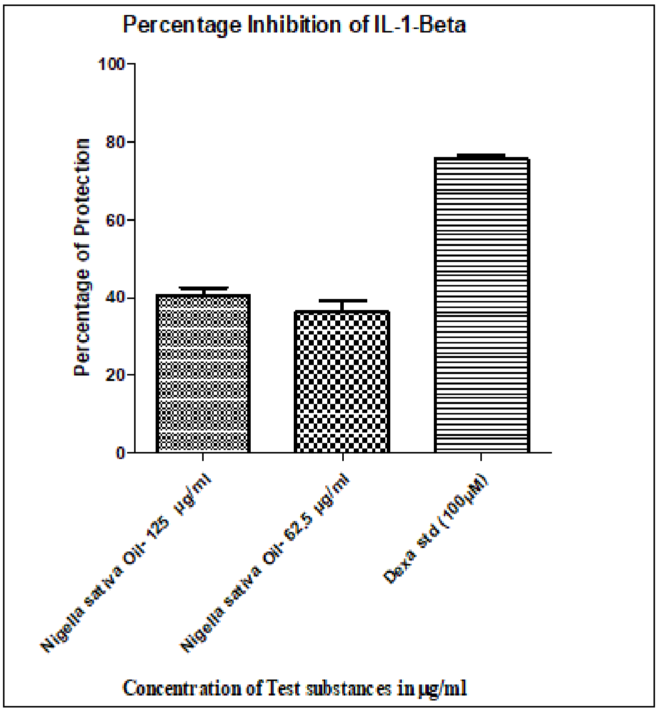

Fig 2: Immunomodulatory effect of the ThymoPure™ (*Nigella sativa)* on LPS induced IL-1- β generation in mouse spleen dendritic cells. (P <0.0001).

The percentage inhibition of the cytokines are based on the comparison to the cell control. The values are normalised to

cell control. Therefore, the cell control data values are not shown in the graphs.
In the aforesaid figure (Fig.2), ThymoPure™ (*N. sativa)* oil has shown to protect to the tune of 41% (125 mg/ml) and 37% (62.5 mg/ml) in LPS induced inflammation (IL-1-b) by dexamethasone on mouse spleen dendritic cells. The difference observed when compared to the control is highly significant (P<0.001) and the doses used in the study are able to produce necessary anti-inflammatory response in immunomodulatory studies conducted on ThymoPure™ (*N. sativa)* oil.

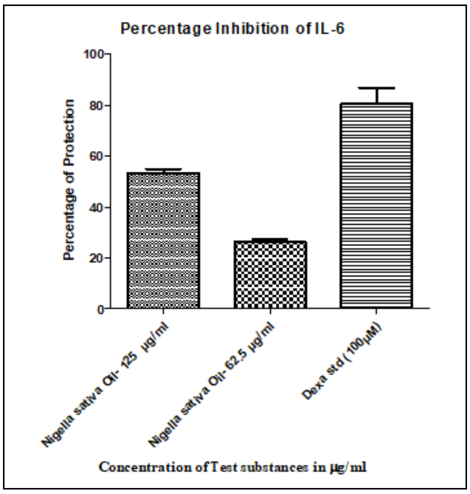

Fig 3: Immunomodulatory effect of the ThymoPure™ (*Nigella sativa)* Oil on LPS induced IL-6 generation in mouse spleen dendritic cells. (P < 0.0001).

The percentage inhibition of the cytokines are based on the comparison to the cell control. The values are normalised to cell control. Therefore, the cell control data values are not shown in the graphs. In the below mentioned figure (Fig.3), ThymoPure™ (*N. sativa)* oil has shown to protect to the tune of 53% (125 mg/ml) and 26% (62.5 mg/ml) in LPS induced inflammation (IL-6) by dexamethasone on mouse spleen dendritic cells. The difference observed when compared to the control is highly significant (P<0.001) and the doses used in the study are able to produce necessary anti-inflammatory response in immunomodulatory studies conducted on ThymoPure™ (*N. sativa)* oil.

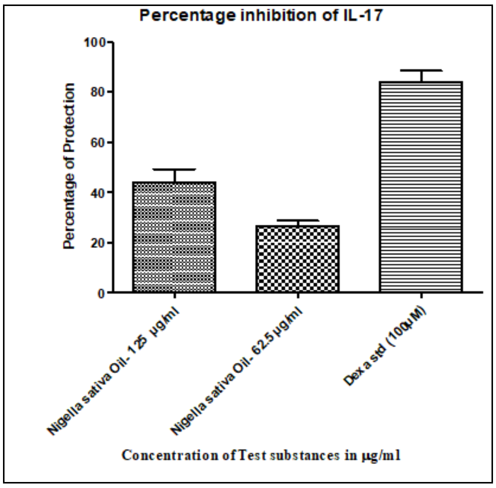

Fig 4: Immuno-modulatory effect of the ThymoPure™ (*Nigella sativa)* Oil on LPS induced IL-17 generation in mouse spleen dendritic cells (P <0.0001).

The percentage inhibition of the cytokines are based on the comparison to the cell control. The values are normalised to cell control. Therefore, the cell control data values are not shown in the graphs.
In the aforesaid figure (Fig.4), ThymoPure™ (*N. sativa)* oil has shown to protect to the tune of 44% (125 mg/ml) and 21% (62.5 mg/ml) in LPS induced inflammation (IL-17) by dexamethasone on mouse spleen dendritic cells. The difference observed when compared to the control is highly significant (P<0.001) and the doses used in the study are able to produce necessary anti-inflammatory response in immunomodulatory studies conducted on ThymoPure™ (*N. sativa)* oil.

Discussion
Dendritic cells (DCs) are responsible for the initiation of adaptive immune responses and hence function as the 'sentinels' of the immune system. DCs are specialised to capture and process antigens, converting proteins to peptides that are presented on major histocompatibility complex (MHC) molecules recognised by T cells. Cytokines such as TNF-α, IL-6, IL-1-β and IL-17 are messengers of pathogenesis in the immune system and trigger chronic inflammatory responses. Inhibiting these cytokines can be a potential target for compounds synthesized for boosting the immune system.
The results from the current study suggest that ThymoPure™ *(Nigella sativa)* oil counteracts DC maturation, survival and cytokine release. LPS exposure is followed by the upregulation of several maturation markers, such TNF-alpha, IL-6, IL-1-b and IL-17, an effect blunted in the presence of ThymoPure™ (*Nigella sativa*). This is in line with the earlier report[10] that thymoquinone (Nigella sativa) inhibited the production of IL-6 in LPS-activated murine macrophage-like RAW264.7 cells. NS fixed oil significantly suppressed mRNA IL-6 in whole lung tissue of rats, expression of IL-6 from *M. tuberculosis* infected A549 cells, inhibitory IL-6 expression in human proximal tubular epithelial cells. It is assessed that suppression of IL-6 may possess beneficial anti-inflammatory effects in bronchial epithelial cells of asthmatic patients.[11-13] Increased PGE_2 release by TQ-rich NS preparations may have a favourable effect in the context of asthma as several studies showed that PGE_2 may possess beneficial local protective and bronchodilator effects in the airways of the lung.[14-15]Similar to what has been shown in other cells, LPS-induced activation through dexamethasone on the dendritic cells may activate Na+ /H+ exchanger leading to swelling of the cell. ThymoPure™ may induce spleen dendritic cell apoptosis by inhibiting LPS induced cell inflammation or swelling through inhibition of TNF-α, IL-6, IL-1-β and IL-17.

Conclusion
In conclusion, thymoquinone derived from ThymoPure™ (*Nigella sativa*) oil inhibits LPS-induced stimulation through Na+/H+ activity, an effect resulting in blunted LPS induced cell swelling, ROS formation, TNF-α release and migration. The effect of thymoquinone on Na+/H+ activity may contribute to the known anti-inflammatory effect of this nutrient.

References

1. Ahmad A, Husain A, Mujeeb M, Khan SA, Najmi AK,

Siddique NA. *et al*. A review on therapeutic potential of Nigella sativa: a miracle herb. Asian Pac. J. Trop. Biomed,2013:3:337-352. 10.1016/S2221
2. Xuan NT, Shumilina E, Qadri SM, Götz F, Lang F. Effect of Thymoquinone on Mouse Dendritic Cells. Cell Physiol Biochem,2010:25:307-314.
3. Yang W, Bhandaru M, Pasham V, Bobbala D, Zelenak C, Jilani K *et al*. Effect of thymoquinone on cytosolic pH and Na+/H+ exchanger activity in mouse dendritic cells. Cell Physiol Biochem,2012:29(1-2):21-30.
4. Burits M, Bucar F. Antioxidant Activity of Nigella sativa Essential Oil. Phytother. Res,2000:14(5):323-328.
5. Stagg AJ, Burke F, Hill S, Knight SC. Isolation of mouse spleen dendritic cells. Methods Mol Med,2001:4:9-22.
6. Arora P, Porcelli SA. An Efficient and High Yield Method for Isolation of Mouse Dendritic Cell Subsets. *J Vis Exp*, 2016, (110).
7. Francis D, Rita L. Rapid Coloro metric Assay for Cell Growth and survival modifications to the Tetrazolium Dye Procedure Giving Improved Sensitivit y and Reliability. J. Immunol. Methods,1986:89:271.
8. Stockert JC, Horobin RW, Colombo LL, Blázquez-Castro A. Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging, and labeling perspectives" (PDF). *Acta Histochemica*,2018:120(3):159-167.
9. Berridge MV, Herst PM, Tan AS. "Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction". *Biotechnology Annual Review*,2005:11:127-52.
10. Koshak AE, Yousif NM, Fiebich BL, Koshak EA, Heinrich M. Comparative Immunomodulatory Activity of *Nigella sativa* L. Preparations on Proinflammatory Mediators: A Focus on Asthma. *Front Pharmacol*,2018:9:1075. Published 2018 Oct 2.
11. Shahzad M, Yang X, Raza Asim MB, Sun Q, Han Y, Zhang F *et al*. Black seed oil ameliorates allergic airway inflammation by inhibiting T-cell proliferation in rats. Pulm. Pharmacol. Ther,2009:22:37-43.
12. Mahmud HA, Seo H, Kim S, Islam MI, Nam K-W, Cho H-D. *et al*. Thymoquinone (TQ) inhibits the replication of intracellular Mycobacterium tuberculosis in macrophages and modulates nitric oxide production. *BMC Complement. Altern. Med*,2017:17:279. 10.1186/s12906-017-1786-0
13. Sayed AAR, Morcos M. Thymoquinone decreases AGE-induced NF-kappaB activation in proximal tubular epithelial cells. Phyther. Res,2007:21:898-899. 10.1002/ptr.2177
14. Torres R, Picado C, de Mora F. The PGE2-EP2-mast cell axis: an antiasthma mechanism. Mol. Immunol,2015:63:61-68.
15. Chung KF. Evaluation of selective prostaglandin E2 (PGE2) receptor agonists as therapeutic agents for the treatment of asthma. Sci. STKE, 2005, e47. 10.1126/stke.3032005pe47