International Journal of Biology Research
www.biologyjournal.in
ISSN: 2455-6548
Received: 02-10-2021, Accepted: 15-10-2021, Published 28-10-2021
Volume 6, Issue 4, 2021, Page No. 5-10

Evaluation of thymopure™ *(Nigella sativa)* oil on the *In vitro* expression levels of selected cytokines in mouse macrophages cells

S Mehkri[1], K Chandrasagar[2], G Ashok[3], Krathish Bopanna[4*]
[1] Director, Bio-Gen Extracts Pvt Ltd, Bangalore, Karnataka, India
[2] Study Director, Radiant Research Services, Bangalore, Karnataka, India
[3] Research Director, Radiant Research Services, Bangalore, Karnataka, India
[4] Consultant Pharmacologist, Ctejhana Consulting LLP, Bangalore, Karnataka, India

Abstract
Nigella sativa has been shown to exhibit anti-inflammatory and anti-oxidant effects on cells in literature. Increased concentrations of TNF-α, COX-2, iNOS are found in acute and chronic inflammatory conditions. These mediators have been shown to play a key role in inflammatory process and thus can be a potential target for therapeutic intervention.
The current study was designed to evaluate the effects of ThymoPure™ (*Nigella sativa*) oil on the in-vitro expression level of cytokines TNF-α, COX-2 and iNOS in mouse macrophage cells.
ThymoPure™ (*Nigella sativa*) oil was initially studied for cytotoxicity on mouse macrophage cells by exposing them to different concentrations of the test substance. Further two non-toxic doses of 125 mg/ml and 62.5 mg/ml were selected for the study. Mouse macrophage cells were treated with 125 mg/ml and 62.5 mg/ml of the test substance along with LPS (1 µg/ml) and were incubated for 24 hours. The cells were then harvested for studying the gene expression level of TNF-α, COX-2 and iNOS by Quantitative RT-PCR analysis. The study results showed a decrease in mRNA gene expression of TNF-α (0.97 & 1.17 folds), *i*NOS (0.91 & 0.99 folds) and COX-2 (1.05 & 1.17 folds) on exposure to lower and higher dose of the test substance respectively when compared to the cell control. The level of expression was observed to decrease when the cells were treated with higher dose (125 µg/ml) in comparison to lower dose (62.5 µg/ml) of ThymoPure™ (*Nigella sativa*) oil for 24 hours.
In the current in-vitro study ThymoPure™ (*Nigella sativa*) oil has shown potential anti-inflammatory effects as it reduced the expression of TNF-α, COX-2 and *i*NOS in an accelerated dose response.

Keywords: thymopure™, gene expression, *Nigella sativa*, mouse macrophage cells

Introduction
Nigella sativa (NS) also known as black cumin, is a potent anti-inflammatory and immunomodulatory substance. Notably, the seed's biological activity has been associated with its thymoquinone (TQ) content [1, 2]. NS has shown to inhibit inflammatory mediators (IL-6, IL-2, PGE_2) in human primary T-lymphocyte cells, human primary monocytes, and A549 lung epithelial cells substantiating its effect on the respiratory system[2,3]. The review of literature revealed that NS inhibits the release of PGE_2, which is thought to have a broncho-dilatory effect on the bronchial epithelium [2, 3]. This suppressive effect on the PGE_2 release from T-lymphocytes may be considered to be a beneficial effect due to the general pro-inflammatory properties of PGE_2 and its effect of PGE_2 on Th2 and IgE responses. Interestingly, there is a positive relationship between the TQ level in the body and the inhibition of inflammatory mediators that includes IL-2, IL-6, and PGE_2 [2-4]. TQ has shown the ability to modulate or inhibit inflammatory responses e.g. IL-1, IL-6, IL-10, IL-18, TNF-α, and NF-κB [5].
Inflammatory cytokines secreted from immune cells like tumor necrosis factor (TNF)-alpha and interleukin (IL)-6 are commonly seen in an active immune response and as a part of cytokine storm. TNF promotes inflammatory response, which may result in the clinical symptoms associated with diseases like inflammatory lung disease, rheumatoid arthritis, crohn's disease, psoriasis etc [6, 7, 8, 9]. In a study by Partsch *et al* high levels of TNF-α and other proinflammatory cytokines were seen the synovial fluid in psoriatic arthritis. The level of TNF-alpha, IL-1beta, and IL-8 were significantly higher in the synovial fluid of psoriatic arthritis than in osteoarthritis, although lower than in rheumatoid arthritis [10]. TQ has shown the ability to inhibit the production of pro-inflammatory cytokines through a variety of methods. In a study by Arjumand *et al* TQ downregulated TLR2, TLR4, TNF-α, IL-1 and NFkb mRNA expression in rats with rheumatoid arthritis, thus exhibiting antiarthritic effect [11].
Pro-inflammatory mediators like nitric oxide (NO) and prostaglandins when released in high concentration, increases inflammation. Immunostimulatory cytokines or bacterial pathogens activate the enzyme inducible nitric oxide synthase (iNOS) and generate high concentration of NO through the activation of inducible nuclear factors including NFkb [12]. Overproduction of NO is catalysed by iNOS. The expression of iNOS expression has been described in rheumatoid arthritis, multiple sclerosis, Alzheimer's disease and tumour of brain, breast etc [13, 14, 15, 16, 17]. Black seed oil has shown the ability to inhibit nitric oxide (NO) production and synthesis, thus helps mediate the inflammatory process [5]. In an invitro study by Mahmood *et al*, the inhibitory effect of aqueous extract of *Nigella*

sativa seeds on nitric oxide (NO) production by murine macrophages was demonstrated [18]. TQ has also shown to suppress the expression of inducible nitric oxide synthase (iNOS) and transforming growth factor-β1 (TGF-β1) mRNAs, preventing inflammatory modifications associated with asthma [19].

Prostaglandins are generated through arachidonic acid metabolism by cyclooxygenase-1 and -2 enzymes (COX-1 and COX-2) [20]. COX-2 is rapidly expressed in several cell types in response to growth factors, cytokines, and pro-inflammatory molecules [21]. In allergic airway inflammation, TQ has been shown to suppress COX-2 expression and the generation of prostaglandins. In the study on mice with allergic airway inflammation sensitized by ovalbumin (OVA), there was a significant increase in PGD2 and PGE2 production in the airways, an increase in the inflammatory cell numbers and Th2 cytokine levels in the bronchoalveolar lavage (BAL) fluid, lung airway eosinophilia and goblet cell hyperplasia, as well as the induction of COX-2 protein expression in the lung. Intraperitoneal injection of TQ for 5 days before the first OVA challenge attenuated airway inflammation as demonstrated by the significant decrease in Th2 cytokines, lung eosinophilia, and goblet cell hyperplasia. This attenuation of airway inflammation was concomitant to the inhibition of COX-2 protein expression and PGD2 production [20]. Al Wafai *et al* showed that supplementation with TQ and N Sativa in streptozotocin (STZ) stimulated diabetic rats prevented COX-2 enzyme expression in pancreatic tissue [22].

In our previous studies with ThymoPure™ (*Nigella sativa*) oil on mouse macrophages, the dendritic cell maturation, survival, and cytokine release (TNF-alpha, IL-6, IL-1-b and IL-17) was seen to be counteracted following LPS exposure [23]. In another study with ThymoPure™ (N. sativa) oil potentiation effects on phagocytosis were seen on mouse macrophages [24].

The current in-vitro study was designed to evaluate the effects of ThymoPure™ (*Nigella sativa*) oil on the gene expression levels of TNF-α, iNOS and COX-2 in mouse macrophage cells. These mediators play a key role in inflammatory processes and thus an understanding of the mRNA gene expression level under inflammatory conditions may be useful as potential targets for therapeutic intervention in number of pathological conditions.

Materials and Methods

Test substance information

ThymoPure™ (*Nigella sativa)* oil was manufactured by Bio-gen Extracts Pvt Ltd, Bangalore, INDIA. This was stored at room temperature with a shelf life of about three years. ThymoPure™ (*Nigella sativa)* oil, was manufactured by super-critical-fluid-extraction (SCFE) technology.

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 effect on the mRNA gene expression of TNF-α, iNOS and COX-2 on mouse macrophage cells.

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 foetal 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 reduction assay in semi confluent monolayer cultures. The solution of the test substance were added to cells and incubated at 37^0C in 5% CO_2 atmosphere. After 72 hr of incubation, plates were centrifuged at 500g and the solutions of test substance in the wells were carefully removed and 100 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37 °C in 5% CO_2 atmosphere. The supernatant was removed as mentioned above and 100 µl of DMSO 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. Cells were exposed to different concentrations of the ThymoPureTM (N. sativa) oil. Dilutions of 1000mg/ml, 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5mg/ml were studied (Table 1). ThymoPureTM (N. sativa) oil demonstrated a CTC50 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 studies.

Treatment for gene expression

Mouse macrophage cells (1.5 x 10^6) were seeded into 60 mm petri dishes and were grown in DMEM media until confluency. When the cells reached about 70-80% confluency, they were treated with the non-toxic concentrations of the test substance along with LPS (1 µg/ml). The petri dishes were incubated for 24 h at 37 ºC and thereafter the cells were harvested for gene expression studies.

RNA isolation and cDNA synthesis

The mouse macrophage cells treated with test substance were subjected to cell lysis by treating with Tri-extract reagent. Chloroform was added, to isolate the total RNA from the sample which was then subjected to centrifugation. Out of the three distinct layers observed, upper layer was collected in a fresh tube and an equal volume of isopropanol was added and incubated at -20^0C for 10mins. After the incubation followed by centrifugation, appropriate volume of ethanol was added to resuspend the pellet. After incubation and centrifugation, the pellet was air dried and

appropriate volume of TAE buffer was added. The isolated total RNA was further used for cDNA synthesis. cDNA was synthesized by priming with oligo dT primers followed by reverse transcriptase enzyme treatment according to manufacturer's protocol (Thermo scientific). The cDNA thus synthesized was taken up for PCR for the amplification of TNF-α, *i*NOS and COX-2 and GAPDH (internal control).

RT-PCR Procedure

The mRNA expression levels of TNF-α, *i*NOS and COX-2 were determined using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). 50µl of the reaction mixture was subjected to PCR for amplification of TNF-α, *i*NOS and COX-2. cDNAs using specifically designed primers procured from Eurofins, India and as an internal control GAPDH (housekeeping gene) was co-amplified with each reaction.

Amplification conditions for TNF-*α*, *i*NOS and COX-2 genes

95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing Tm for 30 seconds and extension at 72°C for 45 seconds. This was followed by final extension at 72°C for 10 min.

Table 1

Gene	Primers	Expected product size
TNF-alpha	Forward: 5' GCA TGA TCC GCG ACG TGG AA 3'	135bp
	Reverse: 5' TTC CAC GTC GCG GAT CAT GC 3'	
iNOS	Forward: 5' CAC CTC ACT GTG GCC GTG GT 3'	360 bp
	Reverse: 5' CAC CTG GAA CAG CAC TCT CT 3'	
COX-2	Forward: 5' ATT CAA CAC ACT CTA TCA CT 3'	610bp
	Reverse: 5' TTG CAC ATT GAA AGA AGT GA 3'	

Results

ThymoPure™ (*Nigella sativa)* oil was initially studied for cytotoxicity on Raw 264.7 macrophage cells by MTT assay *in vitro*. Macrophage cells were exposed to different concentrations of the ThymoPure™ (*Nigella sativa)* oil. Dilutions of 1000 mg/ml, 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml were studied (Table 1). The compound demonstrated a CTC_{50} value of 210.5±2.45; consequently, the non-toxic concentrations (125 µg/ml and 62.5 µg/ml) were identified and selected for further gene expression studies. Mouse macrophage cells were treated with the non-toxic concentrations (125 µg/ml and 62.5 µg/ml) of the test substance along with LPS (1 µg/ml). The petri dishes were incubated for 24 h at 37 ºC and thereafter the cells were harvested for gene expression studies. The mRNA expression levels of TNF-α, *i*NOS and COX-2 were determined using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). *TNF*-α, iNOS and COX-2 genes were amplified from cell control, LPS control and the test substance ThymoPure™ (*Nigella sativa*) oil with mouse macrophage cell line (Figure 1). First lane in the distribution was assessed as cell control (untreated), test substance (62.5 µg/ml) in Lane 3 and (125µg/ml) in Lane 4 was compared with LPS control (Lane 2). The data obtained was normalised with reference to GAPDH values. There was a reduction of TNF-*a* gene expression in macrophage cells treated with the test substance ThymoPure™ (*Nigella sativa*) oil at concentrations of 62.5 µg/ml and 125µg/ml as compared to LPS control and cell control (Table-2). Reduction of TNF alpha gene expression was about 22% (125µg/ml) and 6.8% (62.5 µg/ml) compared to LPS control. This reduction of TNF alpha gene expression was higher in highest concentration compared to lower concentration as exhibited in the RT-PCR data. The gene expression level of *i*NOS was reduced in macrophage cells treated with the test substance ThymoPure™ (*Nigella sativa*) oil at concentrations of 62.5 µg/ml and 125µg/ml as compared to LPS control and cell control (Table-2). Reduction of *i*NOS gene expression was about 19.7% (125µg/ml) and 16.8% (62.5 µg/ml) compared to LPS control. This reduction of *i*NOS gene expression was higher in highest concentration compared to lower concentration as exhibited in the RT-PCR data. A reduction of COX-2 gene expression was seen reduced in macrophage cells treated with the test substance ThymoPure™ (*Nigella sativa*) oil at concentrations of 62.5 µg/ml and 125µg/ml as compared to LPS control and cell control (Table-2). Reduction of COX-2 gene expression was about 28.57% (125µg/ml) and 20.4% (62.5 µg/ml) compared to LPS control. There was rapid reduction of COX-*2* gene expression amongst test substance which was much higher with higher doses compared to low doses of ThymoPure™ (*Nigella sativa*) oil.

Table 2: Cytotoxic properties of ThymoPure™ (*Nigella sativa)* oil against Raw 264.7 cells

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

Table 3: Modulatory effect of ThymoPure™ *(Nigella sativa)* oil on TNF-a, *i*NOS and COX-2 gene expression in mouse macrophages

Test Sample	Regulation in Terms of Folds		
	TNF-a	*i*NOS	COX-2
Cell control	1.00	1.00	1.00
LPS control	1.25	1.19	1.47
RR200568- (62.5 µg/ml)	1.17	0.99	1.17
RR200568- (125 µg/ml)	0.97	0.91	1.05

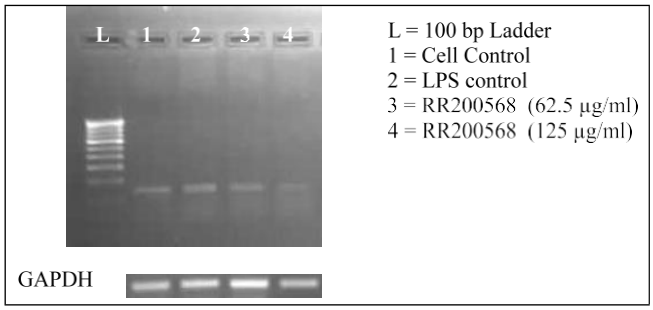

Fig 1: RT-PCR profile of TNF-α gene amplified from test substance ThymoPure™ *(Nigella sativa)* oil treated Mouse Macrophage cell line: Effect of the ThymoPure™ on TNF-*α* transcripts in mouse macrophage L: 100bp marker, Lane 1: Cell Control (Untreated), Lane 2: LPS control (1 µg/ml), Lane 3: (62.5 µg/ml), Lane 4: (125µg/ml).

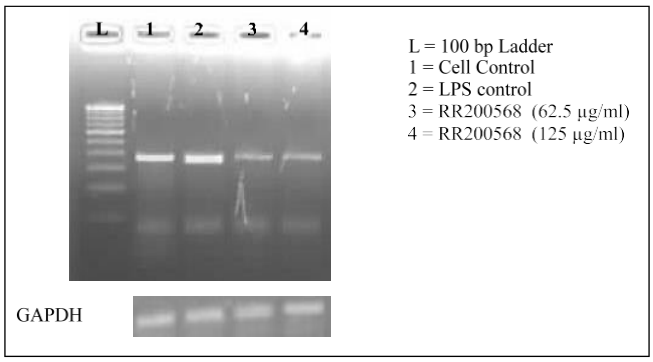

Fig 2: RT-PCR profile of *i*NOS gene amplified from test substance ThymoPure™ *(Nigella sativa)* oil treated Mouse Macrophage cell line: Effect of the ThymoPure™ (RR200568) on *i*NOS transcripts in mouse macrophage L: 100bp marker, Lane 1: Cell Control (Untreated), Lane 2: LPS control (1 µg/ml), Lane 3: RR200568 (62.5 µg/ml), Lane 4: RR200568 (125µg/ml)

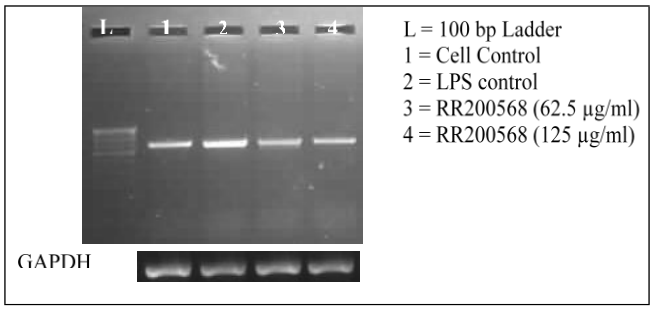

Fig 3: RT-PCR profile of COX-2 gene amplified from Thymo Pure™ *(Nigella sativa)* Oil treated Mouse Macrophage cell line: Effect of the ThymoPure™ (RR200568) on COX-2 transcripts in mouse macrophage L: 100bp marker, Lane 1: Cell Control (Untreated), Lane 2: LPS control (1 µg/ml), Lane 3: RR200568 (62.5 µg/ml), Lane 4: RR200568 (125µg/ml)

Discussion and Conclusion

The immune system is complex and it reacts in ways designed to protect the body from foreign aggressors. This complex activity is an orchestra of cells and proteins that provide a combination of powerful offensive capabilities alongside solid-defence mechanisms. Cytokines are relatively low-molecular-weight proteins produced by many cell types and are pharmacologically active, exhibiting both beneficial and pathologic effects on the target cells. Imbalanced expression of cytokines has been implicated in the progression of many diseases [25]. Overexpression of TNF-α, *i*NOS have been seen in inflammatory lung disease [6], rheumatoid arthritis [7, 13] etc. In a study by Fathy M *et al* [24]. overproduction of the inflammatory mediators (NO, TNF-a, and IL-6) and stimulation of *i*NOS enzyme activity and expression was reported in DENA-induced hepatocarcinogenesis[26].Overall cytokines such as TNF-*α*, COX-2 and *i*NOS play a huge role in triggering chronic inflammatory responses as a part of the inflammatory cascade. Inhibition of these cytokines can be a potential target for compounds synthesized for boosting the immune system.

The present study was conducted to evaluate the effect of ThymoPure™ (*Nigella sativa*) oil on the mRNA gene expression levels of cytokines TNF-α, COX-2 and *i*NOS on mouse macrophage cells. Quantitative RT-PCR analysis revealed that the mRNA gene expression of TNF-α was decreased on exposure to ThymoPure™ (*Nigella sativa*) oil at lower (62.5 µg/ml) and higher concentration (125 µg/ml) compared to the cell control over a period of 24 hrs. ThymoPure™ (*Nigella sativa*) oil at the concentration of 62.5 µg/ml and 125 µg/ml down-regulated the TNF-*α* gene expression levels compared to the control by 1.17 and 0.97 folds respectively. There was a two-fold difference between the expression levels of TNF- *α* in the cells treated with ThymoPure™ (*Nigella sativa*) oil compared to the cell control. Salem [25] reported that *N. sativa* inhibited the inflammatory cytokines IL-1 and TNF-*α*. It further enhanced IL-8 expression in human Peripheral Blood Mono-nuclear Cells (PBMC) through an alteration of trafficking of inflammatory cells. This was done by modulating the expression of chemokines and or other adhesion molecules. Howard *et al* [26] reported that *N. sativa* helps to balance the inflammatory response during endotoxemia. Velagapudi *et al* [27] demonstrated that TQ administration in rats abrogated LPS-stimulated increase in PGE_2, TNF-α, IL-6, and IL-1β. In another study TQ downregulated TLR2, TLR4, TNF-α, IL-1 and NF-kb mRNA expression in rats with rheumatoid arthritis, thus exhibiting antiarthritic effect [11].

Estimation of *i*NOS mRNA gene expression by quantitative RT-PCR analysis revealed a decrease in gene expression level on exposure to ThymoPure™ (*Nigella sativa*) oil compared to the cell control. ThymoPure™ (*Nigella sativa*) oil in higher concentration (125 µg/ml) decreased the expression by 0.91 folds, while in lower concentration (62.5 µg/ml) decreased the *i*NOS expression by 0.99 folds as compared to the cell control over a period of 24 hrs. Ibrahim *et al* [28]. showed that NS inhibited hepatotoxicity induced by carbon tetra chloride by down regulation of CYP2E and CYP3A protein expressions due to reduction of nitrous oxide through downregulation of inducible nitrous oxide synthase (*i*NOS) expression. In another study TQ inhibited the increase in *i*NOS mRNA expression induced by LPS in rat peritoneal macrophages, and the inhibitory effects were confirmed by decreased immunoreactivity for *i*NOS by immunofluorescence staining as compared to control LPS-stimulated cells. They concluded that TQ suppresses the production of NO by macrophages; an effect which may be useful in ameliorating the inflammatory and autoimmune conditions [31]. Fathy M *et al* [24] in their study on the *In vivo* modulation of *i*NOS pathway in hepatocellular carcinoma by *Nigella sativa* observed a significant increase in serum AFP, NO, TNF-a, and IL-6 levels and iNOS enzyme activity in rats treated with DENA. Significant up-regulation of liver *i*NOS mRNA and protein

expression was also observed. Subsequent treatment with NSEE (*Nigella sativa* Ethanolic Extract) significantly reversed these effects and improved the histopathological changes in malignant liver tissue. They concluded that attenuation of the iNOS pathway and suppression of the inflammatory response mediated by TNF-a, and IL-6 could be implicated in the antitumor effect of NSEE [26].

Quantitative RT-PCR analysis of COX-2 m-RNA gene expression revealed that the expression of COX-2 was decreased on exposure to ThymoPure™ (*Nigella sativa*) oil at lower (62.5 µg/ml) and higher concentration (125 µg/ml) as compared to the cell control over a period of 24 hrs. ThymoPure™ (*Nigella sativa*) oil at higher concentration (125 µg/ml) decreased the expression by 1.05 fold and at lower concentration (62.5 µg/ml) the decreased COX-2 expression by 1.17 fold in comparison to the cell control. In mice with allergic airway inflammation, TQ has been shown to suppress COX-2 expression and the ensuing generation of prostaglandins[31]. Wafai *et al* [20]. Showed that supplementation with TQ and N. Sativa in streptozotocin (STZ)-stimulated diabetic rats prevented COX-2 enzyme expression in pancreatic tissue [22]. Another study determined the expression of *cox-2* gene in human Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) and the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on both anchorage-dependent and independent growth of GEP-NET cells. They found 87.8% of GEP-NET tissues stained positive for COX-2 and the PGE2 (prostaglandin E2) amounts quantified in the supernatants of NET cells matched to *cox-2* expression level.

NS is known to have anti-inflammatory and immunomodulatory effects in literature. Several studies published in literature have confirmed the pharmacological action of NS in regulating inflammatory cytokines during obstructive respiratory disorders e.g. Thymoquinone suppresses mRNA expression which downregulates interferon gene and other inflammatory responses [3]. Similarly, α-hederin suppresses the miRNA-126 expression which consequently interfere with IL-13 secretion pathway [30]. Molecular docking results have showed the superiority of these compounds on FDA approved drugs [31]. In the current *in vitro* study ThymoPure™ (*Nigella sativa*) oil has produced anti-inflammatory effects as it has reduced the level of expression of TNF-*α*, COX-2 and *i*NOS gene in an accelerated dose response. The study results have given some understanding of expression of cytokines TNF-*α*, COX-2 and *i*NOS *in vitro*. However, these studies have to be repeated in a clinical program to elucidate the effects of ThymoPure™ (*Nigella sativa*) oil on the expression of these cytokines.

References

1. Abidi A, Robbe A, Kourda N, Khamsa S Ben, Legrand A. *Nigella sativa*, a traditional Tunisian herbal medicine, attenuates bleomycin-induced pulmonary fibrosis in a rat model. *Biomed. Pharmacother*,2017:90:626-637.
2. 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.
3. Abbas AT, AbdelAziz MM, Zalata KR, Abd AlGalel TE. Effect of dexamethasone and *Nigella sativa* on peripheral blood eosinophil count, IgG1 and IgG2, cytokine profiles and lung inflammation in murine model of allergic asthma. Egypt. J. Immunol,2005:12:95-102.
4. Kulyar MF, Li R, Mehmood K, Waqas M, Li K, Li J. Potential influence of Nagella sativa (Black cumin) in reinforcing immune system: A hope to decelerate the COVID-19 pandemic. *Phytomedicine*,2021:85:153277.
5. Sharma JN, Al-Omran A, Parvathy SS. "Role of nitric oxide in inflammatory diseases." Inflammopharmacology,2007:15(6):252-259.
6. Lundblad LK, Thompson-Figueroa J, Leclair T, Sullivan MJ, Poynter ME, Irvin CG, Bates JH. Tumor necrosis factor-alpha overexpression in lung disease: a single cause behind a complex phenotype. Am J Respir Crit Care Med,2005:171(12):1363-70.
7. Brennan FM, Maini RN, Feldmann M. TNF alpha–a pivotal role in rheumatoid arthritis? Br J Rheumatol,1992:31:293.
8. Plevy SE, Landers CJ, Prehn J, Carramanzana NM, Deem RL, Shealy D, *et al*. A role for TNF-alpha and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. J Immunol,1997:159:6276.
9. Mease P. TNFα therapy in psoriatic arthritis and psoriasis. Annals of the Rheumatic Diseases,2004:63:755-758.
10. Partsch G, Steiner G, Leeb BF, Dunky A, Bröll H, Smolen JS. Highly increased levels of tumor necrosis factor-alpha and other proinflammatory cytokines in psoriatic arthritis synovial fluid. J Rheumatol,1997:24(3):518-523.
11. Arjumand Sabeen, Shahzad Muhammad, Shabbir, Arham, Yousaf, Muhammad Zubair. Thymoquinone attenuates rheumatoid arthritis by downregulating TLR2, TLR4, TNF-α, IL-1, and NFκB expression levels. Biomedicine & Pharmacotherapy,2019:111:958-963.
12. Sakurai H, Kohsaka H, Liu MF, Higashiyama H, Hirata Y, Kanno K, Saito I, *et al*. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest,1995:96:2357-63.
13. Bagasra O, Michaels FH, Zheng YM, Bobroski LE, Spitsin SV, Fu ZF, *et al*. Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis. Proc Natl Acad Sci USA,1995:92:12041-5.
14. Vodovotz Y, Lucia MS, Flanders KC, *et al*. Inducible nitric oxide synthase in tangle-bearing neurons of patients with Alzheimer's disease. J Exp Med,1996:184:1425-33.
15. Cobbs CS, Brenman JE, Aldape KD, Bredt DS, Israel MA. Expression of nitric oxide synthase in human central nervous system tumors. Cancer Res,1995:55:727-30.
16. Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S. Nitric oxide synthase activity in human breast cancer. Br J Cancer,1995:72:41-44.
17. Mahmood MS, Gilani AH, Khwaja A, Rashid A, Ashfaq MK. The *in vitro* effect of aqueous extract of *Nigella sativa* seeds on nitric oxide production. Phytother. Res,2003:17:921-924.
18. Ammar ESM, Gameil NM, Shawky NM, Nader MA. Comparative evaluation of anti-inflammatory properties of thymoquinone and curcumin using an asthmatic murine model, Int. Immunopharmacol, 2011:11:2232-2236.

19. RababEl Mezayen *et al*. Effect of thymoquinone on cyclooxygenase expression and prostaglandin production in a mouse model of allergic airway inflammation Cyclooxygenase-2 (COX-2) in Inflammatory and Degenerative Brain Diseases. *Journal of Neuropathology & Experimental Neurology*,2004:63:901-910.
20. Al Wafai, Rana J. *Nigella sativa* and Thymoquinone Suppress Cyclooxygenase-2 and Oxidative Stress in Pancreatic Tissue of Streptozotocin-Induced Diabetic Rats. Pancreas,2013:42(5):841-849
21. Mehkri S, Chandrasagar K, Ashok G, Krathish Bopanna. Evaluation of *In vitro* immuno modulatory activity of Thymopure™ *Nigella sativa* oil in dendritic cells. International Journal of Biology Research,2021:6(2):53-57
22. Mehkri S, Chandrasagar K, Ashok G, Krathish Bopanna. Evaluation of *In vitro* phagocytic property of macrophages in presence of Thymopure™ (*Nigella sativa*) oil. International Journal of Biology Research,2021:6(2):18-21
23. Soliman MM, El-Fattah El-Senosi YA, El-Hamid OMA, El-Desouki Abd El-Mageed A, Ismaeil RS, El-Maqsoud Ali HA. *Nigella sativa* Modulates Cytokines Expression in Mature Bovine Adipocytes. Asian Journal of Biochemistry,2009:4:60-67.
24. Moustafa Fathy, Toshio Nikaido. *In vivo* modulation of iNOS pathway in hepatocellular carcinoma by *Nigella sativa*. Environ Health Prev Med,2013:18:377-385
25. Salem ML. Immunomodulatory and therapeutic properties of the *Nigella sativa* L. seed. Int. Immunopharmacol,2005:5:1749-1770.
26. Howard M, Muchamuel T, Andrade S, Menon S. Interleukin 10 protects mice from lethal endotoxemia. J. Exp. Med,1993:177:1205-1208.
27. Velagapudi R, Kumar A, Bhatia HS, El-Bakoush A, Lepiarz I, Fiebich BL, Olajide OA. Inhibition of neuroinflammation by thymoquinone requires activation of Nrf2/ARE signalling, Int. Immunopharmacol,2017:48:17-29.
28. Ibrahim S, Ishizuka M, Soliman M, El-Bohi K, Sobhy K *et al*. Protection by *Nigella sativa* against carbon tetrachloride induced downregulation of hepatic cytochrome P450 isozymes in rats. Japanese J. Vet. Res,2008:56:119-128.
29. Fallahi M, Keyhanmanesh R, Khamaneh AM, Ebrahimi Saadatlou MA, Saadat S, Ebrahimi H. Effect of Alpha-Hederin, the active constituent of *Nigella sativa*, on miRNA-126, IL-13 mRNA levels and inflammation of lungs in ovalbumin-sensitized male rats. Avicenna J Phytomed,2016:6(1):77-79
30. Gao F, Zafar MI, Jüttner S, Höcker M, Wiedenmann B. Expression and Molecular Regulation of the Cox2 Gene in Gastroenteropancreatic Neuroendocrine Tumors and Antiproliferation of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs). Med Sci Monit,2018:24:8125-8140
31. Shaterzadeh-Yazdi H, Noorbakhsh MF, Samarghandian S, Farkhondeh T. An Overview on Renoprotective Effects of Thymoquinone. Kidney Dis (Basel),2018:4(2):74-82.
32. Alyavi AL, Sabirjanova ZT, Tulyaganova DK, Radjabova DI, Nuritdinova SK, Khan T. Relationship between cytokines and the remodeling of the structural and functional state of the left ventricle in patients with coronary artery disease.