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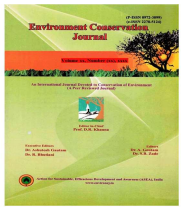

Immunostimulatory potential of *Nigella sativa* seeds extract on chicken macrophage function

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ABSTRACT

In present study, the effect of *Nigella sativa* seeds extract on the chicken peripheral blood mononuclear cells (PBMCs) was investigated. The chicken PBMCs were stimulated with two different doses (125µg/ml; 250µg/ml) of *Nigella sativa* seeds extract and the cells were harvested at different time points till 48h post-stimulation for analysis of iNOS gene expression by quantitative PCR and nitric oxide (NO) estimation at 24 and 48h post-stimulation. High dose (250µg/ml) of *Nigella sativa* seed extract showed remarkable induction of iNOS transcripts expression and NO production at 48h post-stimulation, which were 12.8040±1.03347 folds and 5.7089±.64535µM respectively. The results indicated the immunostimulatory potential of the *Nigella sativa* seed extract on the chicken PBMCs.

Introduction

Natural product obtained from the plants widely used in the treatment of different diseases. *Nigella sativa* belongs to Ranunculaceae family and known as black cumin. *Nigella sativa* has been widely used as food additive, food preservative or as spice in many cultures. In the field of medicine due to its various therapeutic potentials *Nigella sativa* broadly used in the treatment of many infectious diseases Yimer *et al.* (2019). The seeds of *Nigella sativa* showed remarkable benefits due to the presence of a variety of biochemical and pharmacological components present into it. The various active components of black cumin have been reported to show characteristics like antimicrobial, antioxidant, anti-malarial, anti-inflammatory, analgesic, antihypertensive, antidiabetic, antineoplastic Darmawan *et al.* (2019) and Hwang *et al.* (2021) and immune-potentiating effects Manoharan *et al.* (2021). Macrophages act as scavenger cell; provide the first line of defense against microbial invasion and activate different types of signaling pathways Chen *et al.* (2017) and Ren *et al.* (2017). Macrophages constitute important participation in the bi-directional interaction between innate and specific immunity Carnagarin *et al.* (2019). The macrophage act as scavenger cell and performs many functions such as engulfment of deformed cells, attacking the invaders and providing response to infections, tumors and inflammation Wang *et al.* (2019). Kiboneka (2021) reported that macrophages play important role in innate and adaptive immunity and kill pathogenic microorganisms via phagocytosis process and initiate an immune response by antigen processing and presentation to T- and B-lymphocytes. They also modulate adaptive immune system by producing various inflammatory and anti-inflammatory cytokines. As per Tejero *et al.* (2019), macrophages also release immunologically important effector biomolecules, such as reactive oxygen species (ROS) and nitric oxide (NO). Macrophages produce NO by oxidizing the

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guanidine nitrogen of L-arginine by an enzyme, nitric oxide synthase (NOS). This enzyme is inducible in macrophages by external stimulators like bacterial endotoxins (lipopolysaccharide) or by lymphokines and is termed as inducible NOS (iNOS) Gordon (2016). Macrophages also secrete cytokines IFN-γ and IL-2 (Th1-type cytokines) and IL-13, IL-4 (a Th2-type cytokine) and aid the development of the adaptive immune responses Khan *et al.* (2017). Recently, Mehkri *et al.* (2021) demonstrated the potentiation effects of Thymoquinone (*Nigella sativa*) oil on mouse macrophages.

Elmowalid *et al.* (2013) revealed out the direct effects of *Nigella sativa* seed purified aqueous extract on sheep monocytes-derived- macrophages (MDM) culture. They reported that the *Nigella sativa* effect is not species specific and confirmed and suggested that *Nigella sativa* may serve as potent immunomodulator of sheep macrophage functions that could contribute to the overall host defense by preventing host cell microbial infections. AlMahmud *et al.* (2017) demonstrated that *Nigella sativa* active component Thymoquinone (TQ) modulates nitric oxide production and also inhibited the replication of intracellular *Mycobacterium tuberculosis* in macrophages. *Nigella sativa* oil alone or in combination with albendazole showed anti-echinoncoccosis effect by increasing IL-5 and NO production and reducing TNF- α in mice model Kishik *et al.* (2020). Bahrami *et al.* (2021) reveled out that ethanolic extract of *Nigella sativa* significantly decreased the level of NO in peritoneal murine macrophages and RAW264.7 cells. They also demonstrated that *Nigella sativa* ethanolic extract significantly increased IL-10 expression, while IL-6 and TNF-α expression was significantly decreased in RAW264.7 cells. *Nigella sativa* extract was used by many workers as an immunomodulating agent and its immunostimulatory activity has been reported in sheep Elmowalid *et al.* (2013), in human Koshak *et al.* (2018) and in mice Kishik *et al.* (2020), but not yet explored in chicken. There is very little known about the immunostimulatory effect of *Nigella sativa* seed extract on chicken immune cells. Here, we demonstrated the effect of *Nigella sativa* extract with two doses (Low and high) on the PBMCs that helped in understanding the mode of action and molecular mechanisms involved in the macrophage function. We analyzed the transcriptional levels of inducible nitric oxide synthase (iNOS) as well as NO production in the cell culture supernatant on stimulation with two doses; lower and higher dose of *Nigella sativa* in chicken PBMCs to understand the effect on macrophage functions.

Material and Methods

Reagents

RPMI 1640 with L-glutamine and 25 mM HEPES buffer were purchased from HiMedia Pvt. Ltd. India. Fetal bovine serum (FBS) was purchased from Hyclone (Logan, USA) and inactivated by heating at 56 °C for 30 min.

Seeds Extract Preparation

Seeds of *Nigella sativa* were purchased from authenticated Local herb store available at Bareilly, UP (India). The Seeds were dried at 45 °C for removal of moisture and powdered. In 70% methanol (100ml), Ten grams of powdered seed was stirred overnight and centrifuged at 10,000 rpm at 4 °C for 10 min, the supernatant was collected. Methanol was removed by evaporation, and obtained yield was 12% w/w. The seed extract was suspended in DMSO for further *in vitro* studies. The extract was sterilized by filtration using 0.22 µm syringe filters and stored at -20 °C till use.

Primers

Published primers specific to chicken were synthesized from M/S Integrated DNA Technologies, Iowa, USA and used in the study (Table 1).

Isolation of Chicken PBMCs

Blood was collected with heparin (20 IU/ml of blood) from the experimental birds: Chicken (n=6) maintained in the Avian Immunology laboratory, Immunology Section, ICAR-IVRI, Izzatnagar Bareilly U.P. (India). The blood was layered on to the equal amount of Ficoll Hypaque (Sigma, MO, USA) with specific gravity 1.077 g/ml and PBMCs were isoloated by density gradient centrifugation at 500 × g for 45 min. The PBMCs were washed twice with sterile PBS (pH 7.2) and the cells were re-suspended in RPMI-1640 medium containing 2 % fetal bovine serum. The viability of cells was determined by trypan blue dye exclusion method and cells were adjusted to a concentration of 1×10^7 cells/ml and stored on ice.

Table: 1 Primer used for quantitative real time PCR

Target Gene	Primer sequence (5'-3')	Product size (bp)	Reference
GAPDH	F: AGCACCCGCATCAAAGG R: CATCATCCCAGCGTCCA	283	Kuo *et al.* (2017)
iNOS	F: TGGGTGGAAGCCGAAATA R:GTACCAGCCGTTGAAAGGAC	241	Zhang *et al.* (2019)

Stimulation of macrophages for Nitric oxide (NO) estimation

The PBMCs (2×10^6 cells/ml) were resuspended in RPMI 1640 medium containing L-arginine (5 mM) and stimulated with two different doses (125 and 250µg/ml) of *Nigella sativa* extract in triplicate. The supernatants were collected at 24 and 48h post-stimulation and stored at −20 °C until further processing. NO production was determined by measuring the concentration of nitrite in the supernatant by using Griess assay. Briefly, 50 µL of standard and samples was added with 50 µL of Griess reagent (Sigma, MO, USA) followed by incubation at 37 °C for 30 min. Optical density was determined at 550 nm in a spectrophotometer. Different concentrations of sodium nitrite were used as standard and the concentration of NO was calculated from the standard curve.

Stimulation of chicken PBMCs with *Nigella sativa* extract

The PBMCs (1 x 10^6 cells) were stimulated with two different doses of (low dose, 125µg/ml; high dose, 250µg/ml) of *Nigella sativa* seed extract by incubation at 40 °C, 5% CO_2 environment Swamy and Tan (2000) and Gholamnezhad *et al*. (2015). Then, the cells were harvested at different time points till 48h post-stimulation for analysis of iNOS gene expression by qPCR at 3, 6, 12, 24 and 48h post-stimulation.

Total RNA isolation from PBMCs

Treated and untreated PBMCs were centrifuged for 5 min and supernatant was discarded. Into the pellet 750 µl of QIAzol lysis reagent (Qiagen, CA, USA) and 250 µl of chloroform were added and vortexed for 30 s. For separation of phases, the tubes were centrifuged at 12000 × g for 20 min at 4 °C. The supernatant aqueous phase rich in RNA were precipitated with 400 µl of isopropanol at 12000 × g for 15 min and washed with 1 ml of 70% ethanol. Then, the tubes were air dried by inverting on to the clean filter paper for about 10 min for the removal of excess ethanol. The RNA pellet was dissolved in nuclease free water (20 µl). The purity of RNA was checked by measuring the absorbance at 260 and 280 nm in a Nanodrop UV spectrophotometer (Thermo Scientific, USA) Bashir *et al*. (2019).

Complementary DNA (cDNA) preparation

Total RNA (2µg) was used for preparation of cDNA employing Revertaid™ First Strand cDNA Synthesis Kit (Thermo Scientific, USA), following manufacturer's instructions. For the synthesis of cDNA, 2 µg of RNA and 1 µL of random hexamer (Thermo Scientific, USA) were added to the 9.5 µL of nuclease-free water (total volume 12.5 µL) and incubated at 65 °C for 10 min. Then, the following reagents were added to the tubes: 5 X reaction buffer (4 µL), RNase inhibitor (0.5 µL), 10mM dNTP mix (2 µL), and reverse transcriptase (1 µL) and mixed and then incubated at 25 °C for 10 min followed by 50 °C for 50 min for cDNA synthesis and by heating at 85 °C for 5 min, reaction was terminated. The cDNA was stored at -20 °C till further use.

Quantitative Real-Time PCR (qPCR)

Expression levels of iNOS was analyzed by real-time PCR (Agilent Technologies AriaMx Real-Time PCR System) using the Quanti fast SYBR Green qPCR kit (Agilent) (Fig: 1).The chicken specific primers (Table 1) were used. The qPCR mixture consisted of 2 µl cDNA, 10 µl of QuantiFast® SYBR Green Master Mix (Qiagen, CA, USA), primers (0.5 µl each, 10 pmol concentration) and nuclease free water to a volume of 20 µl. Real time PCR was carried out with the following programme: 1 cycle at 95 °C for 5 min, followed by 40 cycles each of 94 °C for 30 s, 60 °C for 45 s, 70 °C for 45 s and 1 cycle of 94 °C for 30 s. All the samples were carried out in duplicates on the same plate. iNOS transcripts expression level was calculated relative to the expression of the GAPDH gene Boeglin *et al.* (2011) and Berzi *et al.* (2014) and expressed as n-fold changes relative to the control samples Pfaffl (2001) (Fig: 2). The cycle in which reporter dye concentration crossed a

preset threshold was recorded as cycle threshold (Ct) value. The data of qPCR were analyzed by $2^{-\Delta\Delta Ct}$ method of Pfaffl (2001) to derive the relative fold change in mRNA of the target gene.

Statistical analysis

GraphPad Prism 8.0 was used for the statistical analyses. The relative fold change in the expression of immune response genes were analyzed by using one way analysis of variance (ANOVA); when the F ratio was significant, least significant difference (LSD) was used as *post hoc* test. * ($P \leq 0.05$) and ** ($P \leq 0.01$) indicate statistically significant.

Results and Discussion

Effect of *Nigella sativa* seed extract on iNOS gene expression and NO production in the chicken PBMCs

In present study, we have analyzed the transcriptional levels of iNOS as well as NO production in the supernatant of cell culture in the chicken PBMCs on stimulation with two doses of methanolic *Nigella sativa* seed extract to understand its effect on macrophage functions. The enzyme iNOS is involved in the production of NO, which has antimicrobial and antiviral properties. Out of three essential enzymes which produced NO from L- arginine, iNOS play very crucial role in infection, inflammation and blood pressure regulation Matsubara *et al*. (2015).

The higher dose of *Nigella sativa* extract significantly ($P \leq 0.01$) up-regulated iNOS expression in chicken PBMCs at 12, 24 and 48h post-stimulation and a peak expression of 12.80±1.033 folds was observed at 48h interval in the table 2 and figure 3. Tripathi *et al.* (2012) also studied that methanolic fraction of *Nigella sativa* on rat-peritoneal-macrophage culture

Table 2: Quantitative real time PCR analysis of *Nigella Sativa* extract induced iNOS expression in Chicken

Treatments	Relative fold change in iNOS expression				
	3h	6h	12h	24h	48h
Low dose	2.5226±1.04606	2.3591±.95981	.8843±.25474	2.3347±.68389	7.9943±2.87686**
High dose	1.4970±.55634	1.1401±.41510	6.3746±1.29504**	6.8396±.90284**	12.8040±1.03347**

**** indicate statistically significant difference at P≤0.01**

Table: 3 Nitric oxide production by chicken PBMCs after *Nigella sativa* extract (Low dose and high dose) stimulation

Treatments	24h	48h
Low dose	1.8433±.06642*	1.7489±.42986*
High dose	3.3633±.55827**	5.7089 ±.64535**

*** indicate statistically significant difference at P≤0.05 and ** indicate statistically significant difference at P≤0.01**

indicated the significantly induction of iNOS expressions and NO production, which is in favor of present study conducted on macrophage of chicken PBMCs. The lower dose of the extract showed significant ($P \leq 0.01$) up-regulation of the iNOS transcripts only at 48h post-stimulation period in the chicken PBMCs. Further, the higher dose of the extract also induced the maximum level of NO production, which was 3.36±.56 and 5.71±.65 µM at 24 and 48 h post-stimulation, respectively as shown in table 3 and figure 4. Miliani *et al*. (2018) also observed enhanced production of iNOS in necrotic Jurkat T cell line lysates-pulsed macrophage co-culture system with thymoquinone treatment. In contrast to our finding, Thymoquinone treatment to *Mycobacterium tuberculosis* infected RAW264.7 cells inhibited expression expression of iNOS gene and reduced production of NO and eventually diminished pathogen-derived stress in host cells Al Mahmud *et al*. (2017). Moreover, recently, Bahrami *et al*. (2021) reported that ethanolic extract of *Nigella sativa* seeds reduced NO production and reduced IL-10 expression in RAW264.7 cells and peritoneal murine macrophages while in present study up-regulation of iNOS transcripts in the chicken PBMCs along with the significant production NO by extract of *Nigella sativa* seeds indicated its potential for enhancing the macrophage functions in chicken.

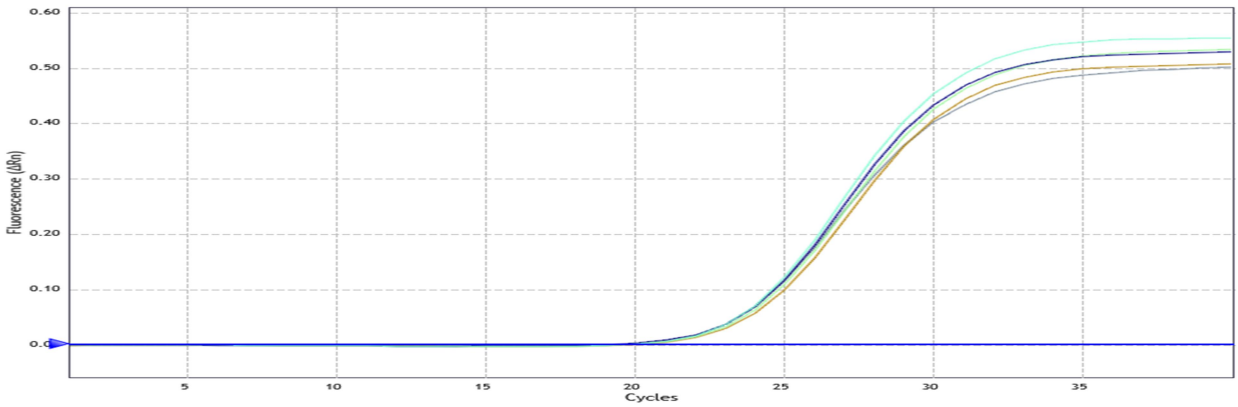

Figure 1: Amplification plot of qPCR assay for iNOS gene from Non-stimulated Control and stimulated samples

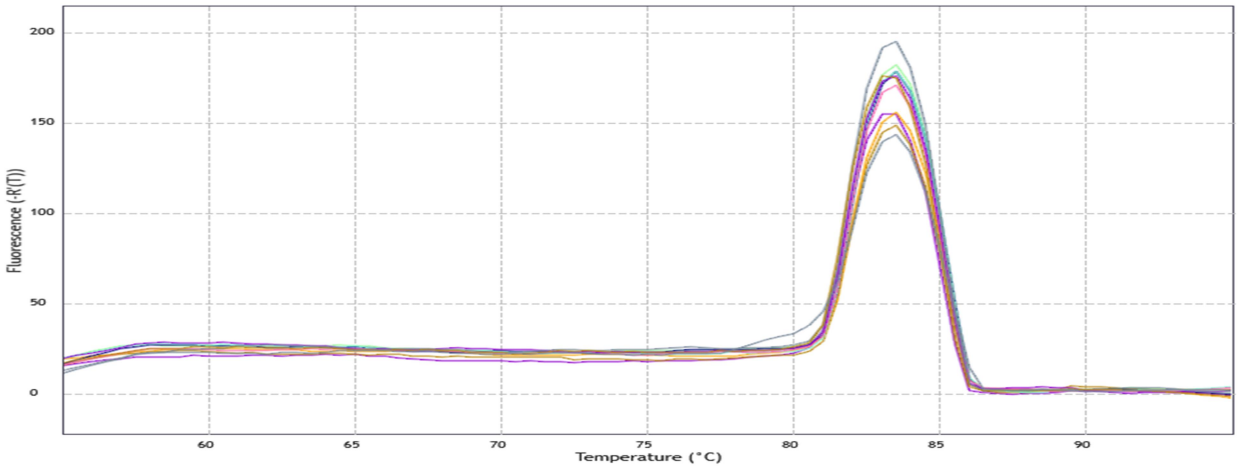

Figure 2: Melt curve of qPCR assay for iNOS gene from Non-stimulated Control and stimulated samples

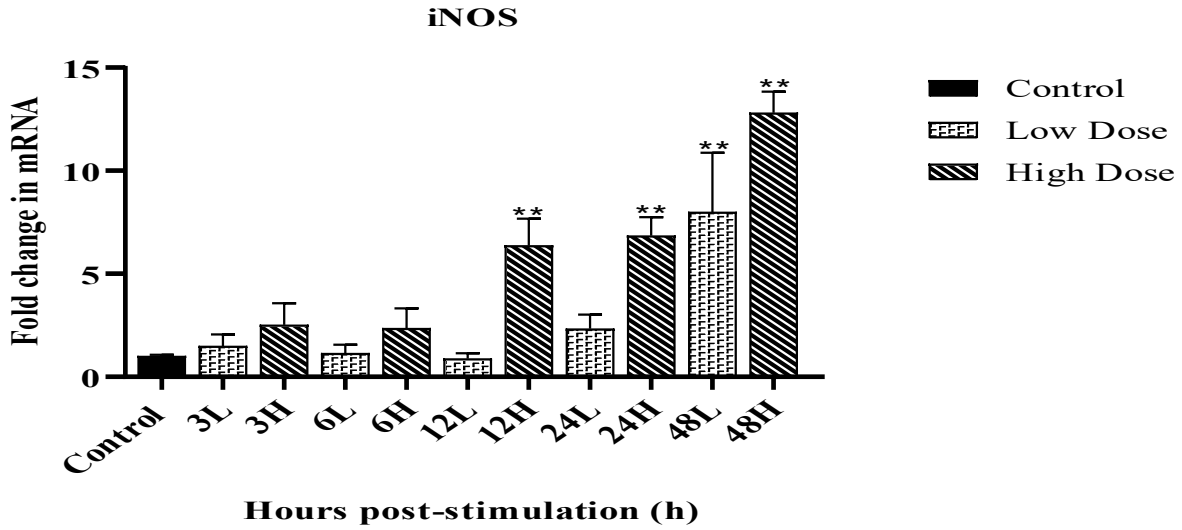

Figure 3: Relative expression of iNOS transcript in chicken PBMCs stimulated with Low dose (125µg/ml) and High dose (250 µg/ml) of *Nigella sativa* seed extract over a period of 48h

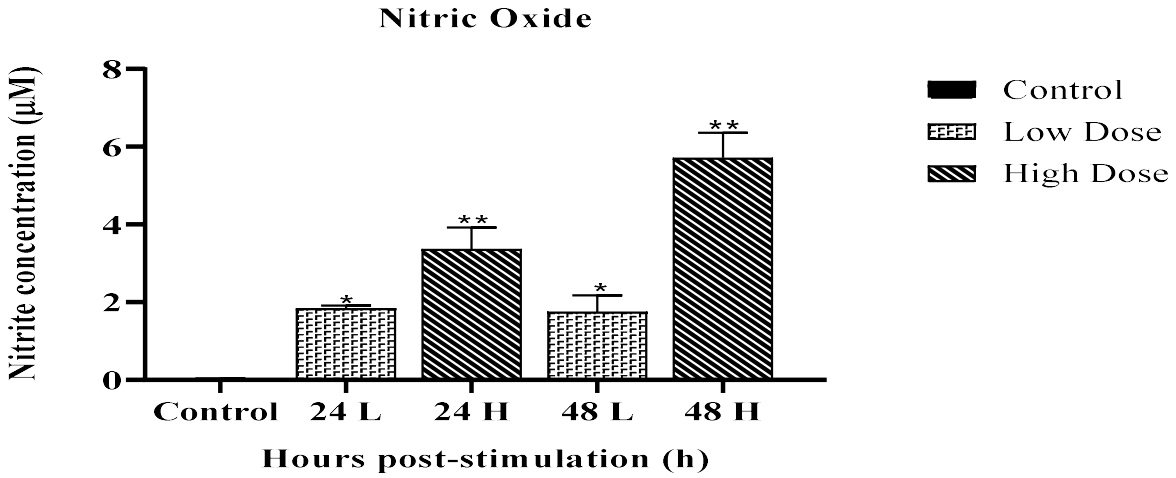

Figure 4: Production of nitric oxide (µM) in chicken PBMCs stimulated with Low dose (125µg/ml) and High dose (250µg/ml) of *Nigella sativa* seed extract stimulated at 24 and 48hr, un-stimulated PBMCs as control

Conclusion

The *Nigella sativa* methanolic extract induced the expression of iNOS transcripts and Nitric oxide production in the chicken PBMCs. The results of the present study also indicated the ability of the *Nigella sativa* methanolic extract to enhance macrophage functions that could regulate adaptive immunity and control infectious diseases. Hence, the *Nigella sativa* methanolic extract can be tried as an adjuvant with any vaccine or antigen in the chicken.

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References

Al Mahmud, H., Seo, H., Kim, S., Islam, M. I., Nam, K. W., Cho, H. D., & Song, H. Y. (2017). Thymoquinone (TQ) inhibits the replication of intracellular Mycobacterium tuberculosis in macrophages and modulates nitric oxide production. *BMC complementary and alternative medicine*, *17*(1), 1-8.

Bahrami, M., Ghazavi, A., Ganji, A., & Mosayebi, G. (2021). Anti-Inflammatory Activity of *S. Marianum* and *Nigella sativa* Extracts on Macrophages. *Reports of Biochemistry & Molecular Biology*, *10*(2), 288.

Bashir, K., Kappala, D., Singh, Y., Dar, J. A., Mariappan, A. K., Kumar, A., & Ramakrishnan, S. (2019). Combination of TLR2 and TLR3 agonists derepress infectious bursal disease virus vaccine-induced immunosuppression in the chicken. *Scientific reports*, *9*(1), 1-16.

Berzi, A., Varga, N., Sattin, S., Antonazzo, P., Biasin, M., Cetin, I., & Clerici, M. (2014). Pseudo-mannosylated DC-SIGN ligands as potential adjuvants for HIV vaccines. *Viruses*, *6*(2), 391-403.

Boeglin, E., Smulski, C. R., Brun, S., Milosevic, S., Schneider, P. & Fournel, S. (2011). Toll-like receptor agonists synergize with CD40L to induce either proliferation or plasma cell differentiation of mouse B cells. *PLoS One*, *6*(10), e25542.

Carnagarin, R., Matthews, V., Zaldivia, M. T., Peter, K., & Schlaich, M. P. (2019). The bidirectional interaction between the sympathetic nervous system and immune mechanisms in the pathogenesis of hypertension. *British journal of pharmacology*, *176*(12), 1839-1852.

Chen, S. M., Shen, H., Zhang, T., Huang, X., Liu, X. Q., Guo, S. Y., & An, M. M. (2017). Dectin-1 plays an important role in host defense against systemic *Candida glabrata* infection. *Virulence*, *8*(8), 1643-1656.

Darmawan, W., Farida, R., & Redjeki, S. (2019). The effect of nigella sativa (Black cumin) seed extract on *Candida albicans* viability. *International Journal of Applied Pharmaceutics*, *11*, 88-91.

Elmowalid, G., Amar, A. M., & Ahmad, A. A. M. (2013). *Nigella sativa* seed extract: 1. Enhancement of sheep macrophage immune functions in vitro. *Research in veterinary science*, *95*(2), 437-443.

Gholamnezhad, Z., Rafatpanah, H., Sadeghnia, H. R., & Boskabady, M. H. (2015). Immunomodulatory and cytotoxic effects of *Nigella sativa* and thymoquinone on rat splenocytes. *Food and Chemical Toxicology*, *86*, 72-80.

Gordon, S. (2016). Phagocytosis: an immunobiologic process. *Immunity*, *44*(3), 463-475.

Hwang, J. R., Cartron, A. M., & Khachemoune, A. (2021). A review of *Nigella sativa* plant-based therapy in dermatology. *International Journal of Dermatology*.

Khan, J., Noboru, N., Young, A., & Thomas, D. (2017). Pro and anti-inflammatory cytokine levels (TNF-α, IL-1β, IL-6 and IL-10) in rat model of neuroma. *Pathophysiology*, *24*(3), 155-159.

Kiboneka, A. (2021). Principals of innate and adaptive immunity. Immunity to microbes & fundamental concepts in immunology. *World Journal of Advanced Research and Reviews*, *10*(3), 188-197.

Kishik, S., Nagati, I., El Hayawan, I., Ali, I., Fawzy, M., & Ali, H. (2020). Efficacy of *Nigella sativa* oil and its chitosan loaded nanoparticles on experimental cystic echinoncoccosis with immunological assessment. *Parasitologists United Journal*, *13*(3), 172-178.

Koshak, A. E., Yousif, N. M., Fiebich, B. L., Koshak, E. A., & Heinrich, M. (2018). Comparative immunomodulatory activity of *Nigella sativa* L. preparations on proinflammatory mediators: A focus on asthma. *Frontiers in pharmacology*, *9*, 1075.

Kuo, S. M., Chen, C. J., Chang, S. C., Liu, T. J., Chen, Y. H., Huang, S. Y., & Shih, S. R. (2017). Inhibition of avian influenza A virus replication in human cells by host restriction factor TUFM is correlated with autophagy. *MBio*, *8*(3), e00481-17.

Manoharan, N., Jayamurali, D., Parasuraman, R., & Govindarajulu, S. N. (2021). Phytochemical composition, therapeutical and pharmacological potential of *Nigella sativa*: a review. *Tradit Med Res*, *6*(4), 32.

Matsubara, K., Higaki, T., Matsubara, Y., & Nawa, A. (2015). Nitric oxide and reactive oxygen species in the pathogenesis of preeclampsia. *International journal of molecular sciences*, *16*(3), 4600-4614.

Mehkri, S., Chandrasagar, K., Ashok, G. and Bopanna, K.2021. Evaluation of In Vitro phagocytic property of macrophages in presence of Thymopure™ (*Nigella Sativa*) oil. *International Journal of Biology Research,* 6(2):18-21.

Miliani, M., Nouar, M., Paris, O., Lefranc, G., Mennechet, F., & Aribi, M. (2018). Thymoquinone potently enhances the activities of classically activated macrophages pulsed with necrotic jurkat cell lysates and the production of antitumor Th1-/M1-related cytokines. *Journal of Interferon & Cytokine Research*, *38*(12), 539-551.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic acids research*, *29*(9), e45-e45.

Ren, D., Lin, D., Alim, A., Zheng, Q., & Yang, X. (2017). Chemical characterization of a novel polysaccharide ASKP-1 from Artemisia sphaerocephala Krasch seed and its macrophage activation via MAPK, PI3k/Akt and NF-κB signaling pathways in RAW264. 7 cells. *Food & function*, *8*(3), 1299-1312.

Swamy, S. M. K., & Tan, B. K. H. (2000). Cytotoxic and immunopotentiating effects of ethanolic extract of *Nigella sativa* L. seeds. *Journal of ethnopharmacology*, *70*(1), 1-7.

Tejero, J., Shiva, S., & Gladwin, M. T. (2019). Sources of vascular nitric oxide and reactive oxygen species and their regulation. *Physiological reviews*, *99*(1), 311-379.

Tripathi, Y. B., Chaturvedi, A. P., & Pandey, N. (2012). Effect of *Nigella sativa* seeds extracts on iNOS through antioxidant potential only: crude/total extract as molecular therapy drug.

Wang, L. X., Zhang, S. X., Wu, H. J., Rong, X. L., & Guo, J. (2019). M2b macrophage polarization and its roles in diseases. *Journal of leukocyte biology*, *106*(2), 345-358.

Yimer, E. M., Tuem, K. B., Karim, A., Ur-Rehman, N., & Anwar, F. (2019). *Nigella sativa* L.(black cumin): a promising natural remedy for wide range of illnesses. *Evidence-Based Complementary and Alternative Medicine*, *2019*.

Zhang, B., Gan, L., Shahid, M. S., Lv, Z., Fan, H., Liu, D., & Guo, Y. (2019). In vivo and in vitro protective effect of arginine against intestinal inflammatory response induced by Clostridium perfringens in broiler chickens. *Journal of animal science and biotechnology*, *10*(1), 1-14.