Journal of Animal Research: v.11 n.4, p. 579-586. August 2021

DOI: 10.30954/2277-940X.04.2021.3

Immunomodulatory Effects of *Nigella sativa* Extract on Chicken Peripheral Blood Mononuclear Cells: *In-vitro*

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Received: 07 July, 2021 **Revised:** 13 July, 2021 **Accepted:** 18 July, 2021

ABSTRACT

Nigella sativa has been used since ancient time for curing various infectious diseases due to its miraculous biochemical and pharmacological properties. It belongs to the Ranunculaceae family and generally used as food additives and also known as black cumin. The active components in black cumin have shown remarkable characteristics like antimicrobial, anti-diabetics, anti-cancerous, anti-tumoral, analgesic, anti-fungal, anti-inflammatory, and immunological and immune-potentiating effects. In the present study, we investigated the immunomodulatory effect of *Nigella sativa* seeds extract on the chicken peripheral blood mononuclear cells (PBMCs). The chicken PBMCs were stimulated with two different doses (125 µg/ml; 250 µg/ml) of *Nigella sativa* seeds extract and the cells were harvested at different time points till 48 h post-stimulation for analysis of various immune gene expression by quantitative real-time PCR. High dose (250 µg/ml) of *Nigella sativa* seed extract showed remarkable induction of immune response genes (IL-1β, IFN-β, IFN-γ, IL-4, IL-10, IL-13, IL-12 transcripts) expression at 48h post-stimulation. The results indicated the immunomodulatory activity of the *Nigella sativa* seed extract on the chicken PBMCs and suggested the potential use as an adjuvant with various vaccines in chicken.

HIGHLIGHTS

- Immunomodulatory effect of *Nigella sativa* extract was observed in chicken PBMCs.
- *Nigella sativa* seed extract enhanced expression of immune response genes in chicken PBMCs.

Keywords: *Nigella sativa*, PBMCs, immunomodulator, immune response genes, chicken

Natural products that are used traditionally will help in the rediscovery of useful drugs. Herbal products are widely used in the treatment of various human diseases for centuries. *Nigella sativa* is a very popular plant from Ranunculaceae family with medicinal values. *Nigella sativa* have been broadly used as liver tonic, anti-diarrheal, antifungal, antibacterial and also used in the cure of skin disorders. Many researchers confirmed its wide pharmacological actions that includes: anti-diabetic, anti-bacterial, antiviral, antioxidant, anti-malarial (Okeola *et al.,* 2011), anti-inflammatory, anticancer **(**Dilshad *et al.,* 2012**)** and gastro-protective (Bukar *et al*., 2017). The therapeutic properties of *Nigella sativa* seed oil are due to the presence of a bioactive component thymoquinone (Ahmad *et al*., 2013). Treatment of *Nigella sativa* to the sensitized guinea pigs significantly reduced pathological changes in lungs and increased level of IFN-γ (Boskabady *et al.,* 2011). It has been also observed that seed oil and extract of *Nigella sativa* reduce the viability of human lung cancer cells (Al-Sheddi *et al.,* 2014). In Islamic literature *Nigella sativa* is mentioned as a miracle plant because it is a very effective herbal remedy for many diseases (Hussain and Hussain, 2016). Recently, antibacterial activity of

How to cite this article: Singh, Y., Prakashbhai, P.N., Saxena, B. and Ramakrishnan, S. (2021). Immunomodulatory Effects of *Nigella sativa* Extract on Chicken Peripheral Blood Mononuclear Cells: *In-vitro. J. Anim. Res.,* **11**(04): 579-586.

Source of Support: None; **Conflict of Interest:** None

Nigella sativa has been observed against multidrug resistance strains of *Staphylococcus aureus* (Salman and Khan, 2016). The extract of *Nigella sativa* was used by many workers as an immunomodulating agent. Recent research reveals that increment in dose of ethanolic extract of *Nigella sativa* significantly reduced the levels of TNFα in mice pre-eclampsia model (Zainiyah *et al*., 2018). Further, ethanol extract of *Nigella sativa* showed excessive antioxidant activity and also observed rich in antioxidants together with gallic acid, eucalyptol and levomenthol that play very imperative function in the enhancement of hepato-renal health in rats (Akinwumi *et al*., 2020). *Nigella sativa* play an important role in balancing the Th1/Th2 cytokines production and asthma control in asthmatic children (Barlianto *et al*., 2017). There is a requirement of studies to shed more light on the immunomodulatory effects of *Nigella sativa* on Th1/Th2 cytokine by using different model in both *in vitro* and *in vivo* conditions. Th1 cytokines mediates cell mediated immune response, whereas Th2 cytokines play important role in humoral immunity and regulate allergic inflammatory response. The effect of *Nigella sativa* extract on chicken immune system is not yet explored. This knowledge would help in understanding the mode of action and molecular mechanisms involved in the immunomodulatory activities of *Nigella sativa* extract in the chicken model. Hence, we aimed to explore the immunomodulatory activity of purified extract from *Nigella sativa* seeds on chicken PBMCs.

MATERIALS AND METHODS

Reagents

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

Extract preparation

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

Primers

Published oligonucleotide primers specific to chicken genes viz., GAPDH, IL-1β, IFN-β, IFN-γ, IL-4, IL-10, IL-12, IL-13 were synthesized from M/S Integrated DNA Technologies, Iowa, USA and used in the study (Table 1).

Chicken peripheral blood mononuclear cells (PBMCs) isolation

Blood was collected with heparin (20 IU/ml of blood) from the experimental birds (n = 6) maintained in the Avian Immunology laboratory, Immunology Section, ICAR-IVRI, Izatnagar. 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.

Determination of the *in vitro* Cytotoxic activity of *Nigella sativa* to chicken PBMCs

The PBMCs (1×10^6 cells) were incubated with various concentrations of *Nigella sativa* extract for 48 h at 40 ˚C, 5% CO_2 environment with cells containing medium alone as the negative control. Twenty microlitre of MTT (5 mg/ml) was added (Cole, 1986; Campling *et al.,* 1991) and further incubated in the same condition for 4 h. The absorbance value was recorded at 570 nm and the level of cytotoxicity was assessed by the following formula:

Stimulation index = OD of the stimulated cells with the extract/OD of the unstimulated cells

Table 1: Primers used for quantitative real time PCR

Target Gene	Primer sequence (5'-3')	Product size (bp)	Reference
GAPDH	F: AGCACCCGCATCAAAGG	283	Kuo *et al.,* 2017
	R: CATCATCCCAGCGTCCA		
IL-1β	F: GGATTCTGAGCACACCACAGT	272	Ramakrishnan *et al.,* 2015
	R: TCTGGTTGATGTCGAAGATGTC		
IFN-β	F: GCTCACCTCAGCATCAACAA	187	Ramakrishnan *et al.,* 2015
	R: GGGTGTTGAGACGTTTGGAT		
IFN-γ	F: TGAGCCAGATTGTTTCGATG	152	Ramakrishnan *et al.,* 2015
	R: CTTGGCCAGGTCCATGATA		
IL-4	F: GTGCCCACGCTGTGCTTAC	82	Huang *et al.,* 2019
	R: AGGAAACCTCTCCCTGGATGTC		
IL-10	F: CGCTGTCACCGCTTCTTCA	88	Zhang *et al.,* 2019
	R: TCCCGTTCTCATCCATCTTCTC		
1L-12	F:CGAAGTGAAGGAGTTCCCAGAT	123	Liu *et al.,* 2010
	R: GACCGTATCATTTGCCCATTG		
IL-13	F:GCTGAGGGTGAAGTTTGAGGAA	123	Liu *et al.,* 2010
	R: GAAGCGCAGCATCTCTGACA		

Stimulation of chicken PBMCs with *Nigella sativa* extract

The PBMCs (1 × 10^6 cells) were stimulated with two different doses of (low dose, 125 µg/ml; high dose, 250 µg/ml) of *Nigella sativa* seed extract (Swamy and Tan, 2000 and Gholamnezhad, *et al*., 2015) by incubation at 40ºC, 5% CO_2 environment. Then, the cells were harvested at 0, 3, 6, 12, 24 and 48h post-stimulation for the evalution of immune response genes by quantitative real-time PCR.

Isolation of total RNA 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 4ºC. The supernatant aqueous phase rich in RNA was 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).

Preparation of complementary DNA (cDNA)

For preparation of cDNA, total RNA (2µg) was taken by using Revertaid^{TM} First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's instructions. For the synthesis of cDNA, two µg of RNA and 1 µL of random hexamer (Thermo Scientific, USA) were added to the 9.5 µL of nuclease-free water (total 12.5 µL volume) and incubated at 65 ºC for 10 min. Further, following reagents were added to the tubes: 5 X reaction buffer (4 µL), Ribolock RNase inhibitor (0.5 µL), 10mM dNTP mix (2 µL), and Revert Aid reverse transcriptase (1 µL) and mixed and then incubated at 25 ºC for 10 min accompanied by 50 ºC for 50 min for cDNA synthesis and the reaction was terminated by heating at 85 ºC for 5 min. cDNA was stored at -20 ºC.

Quantitative Real-Time PCR (qPCR)

Differential expression of immune response genes such as IL-1β, IFN-β, IFN-γ, IL-4, IL-13, IL-10 IL-12 were

analyzed by real-time PCR (Agilent Technologies AriaMx Real- Time PCR System). 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 RNase free water to a volume of 20 µl. Real time PCR was carried out by using the following programme: 1 cycle at 95 ºC for 5 min, followed by 40 cycles each of 94 ºC for 30 sec, 60 ºC for 45 sec, 70ºC for 45 sec and 1 cycle of 94 ºC for 30 sec. To determine the specificity of amplicon melting curve was carried out as the final step. All the samples including treated and controls were carried out in duplicates. Expression levels of IL-1β, IFN-β, IFN-γ, IL-4, IL-13, IL-10 and IL-12 transcripts were calculated relative to the expression of the GAPDH gene (Boeglin *et al.,* 2011; Berzi *et al.,* 2014) and mentioned as n-fold increase or decrease relative to the control samples (Pfaffl, 2001). The Ct (cyclic threshold) value was recorded as the cycle in which reporter dye concentration crossed a preset threshold. The data of real time PCR were analyzed by $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) to derive the relative fold change in mRNA of the target gene.

Statistical analysis

The relative fold change in the expression of immune response genes were analyzed by using analysis of variance (ANOVA) one way; 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. GraphPad Prism 8.0 was used for the statistical analyses.

RESULTS AND DISCUSSION

Effect of *Nigella sativa* seed extract on pro-inflammatory cytokine expression in the chicken PBMCs

IL-1β belongs to the β-trefoil family of cytokines. The role of IL-1β in the chicken's immune response to disease will result in a pro-inflammatory response. IL-1β is expressed as a propeptide, subsequently cleaved by the action of caspase-1 in a position near a conserved aspartic acid residue in viral infection, IL-1 activity was increased in macrophage supernatants from birds suffering from poultry enteritis and mortality syndrome (Heggen *et al*., 2000). IL-1β mRNA levels, of course, do not necessarily correspond to an effective release of biologically active protein. There were significantly ($P \leq 1.01$) higher IL-1β transcripts in the PBMCs treated with high dose of *Nigella sativa* extract at 12, 24 and 48 h post-stimulation, which were 7.3932 ± 2.01284, 9.9547 ± 2.14836 and 17.4484 ± 3.16608 folds, respectively (Fig. 1). However, there was no significant expression of IL-1β transcripts in the PBMCs treated with the low dose of the extract. The results showed that the *Nigella sativa* extract has the potential to stimulate inflammatory responses in the chicken PBMCs.

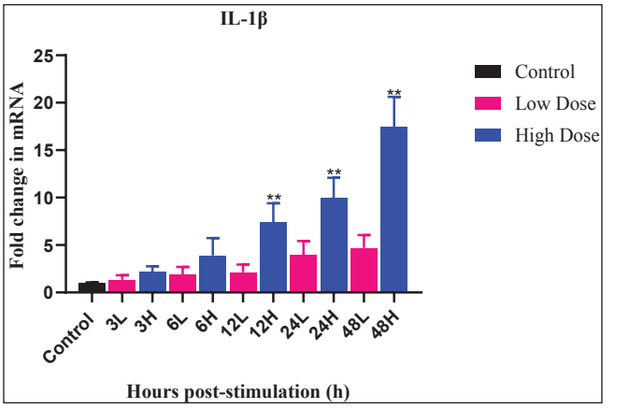

Fig. 1: Relative expression of IL-1β transcript in PBMCs stimulated with Low dose (125 µg/ml) and High dose (250 µg/ml) of *Nigella sativa* seed extract over a period of 48 h

Effect of *Nigella sativa* seed extract on antiviral gene expression in the chicken PBMCs

Interferons (IFN) are characterized by antiviral properties. Macrophages, monocytes, T cells, B cells and NK cells are predominant producer of type I IFNs which in turn activates IFN stimulatory genes (Seo and Hahm, 2010). IFN-α and IFN-β have antiviral activity and type I IFNs generally induce DC maturation that effectively enhance the adaptive immunity (Rahman and Eo, 2012). The present findings showed significantly ($P \leq 0.01$) higher IFN-β transcripts in the chicken PBMCs when stimulated with high dose of *Nigella sativa* extract at 24 and 48 h post-stimulation, which were 10.0688 ± 2.59787 and 18.3659 ± 2.83 folds, respectively (Fig. 2). Further, the results indicated the ability of *Nigella sativa* extract to stimulate the type I IFN in the chicken PBMCs.

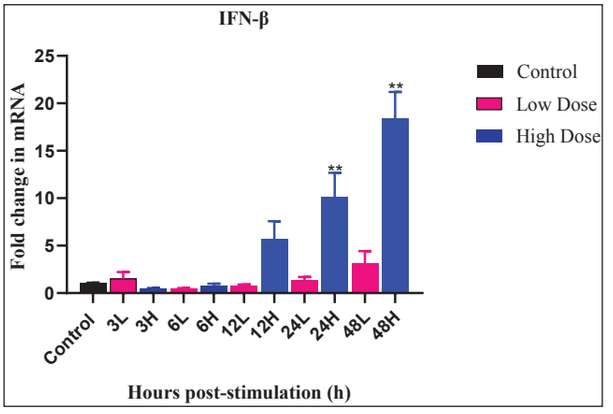

Fig. 2: Relative expression of IFN-β transcript in chicken PBMCs stimulated with Low dose (125 µg/ml) and High dose (250 µg/ml) of *Nigella sativa* seed extract over a period of 48 h

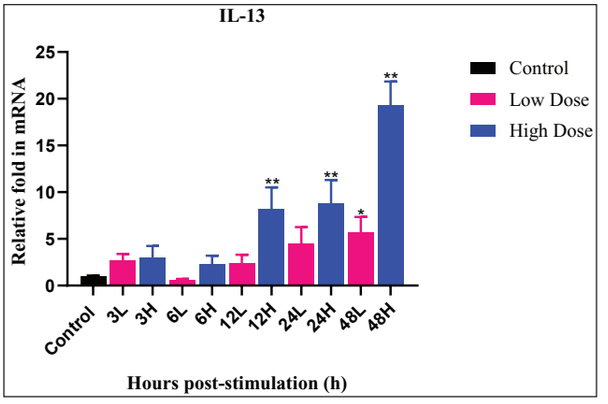

Fig. 3: Relative expression of IL-13 transcript in chicken PBMCs stimulated with Low dose (125 µg/ml) and High dose (250 µg/ml) of *Nigella sativa* seed extract over a period of 48 h

Effect of *Nigella sativa* seed extract on Th1 cytokine gene expression in the chicken PBMCs

CD4+ Th1 cells, CD8+ T cells, NK cells, macrophages and DCs produce IFN-γ and regulate it in an autocrine fashion (Schroder *et al.,* 2004; Frucht *et al.,* 2001; Carvalho-Pinto *et al.,* 2002). IFN-γ possess the antiviral activity by inducing CD8+ T cells, NK cells and interferon inducible genes (Romagnani, 1997; Samuel, 2001) and plays an essential role in innate as well as adaptive immunity (Samuel, 2001). In our current study, the high dose of *Nigella sativa* up-regulated IFN-γ transcripts significantly at 12 (P≤0.05), 24 and 48 h (P≤0.01) post-stimulation in the chicken PBMCs, which were to the tune of 11.5359 ± 2.11920, 11.7301 ± 3.64259 and 18.2217 ± 2.77 folds, respectively (Fig. 4).

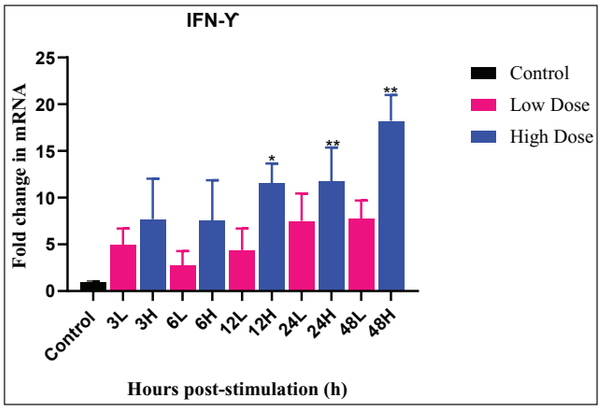

Fig. 4: Relative expression of IFN-Ɣ transcript in chicken PBMCs stimulated with Low dose (125 µg/ml) and High dose (250 µg/ml) of *Nigella sativa* seed extract over a period of 48 h

The high dose of *Nigella sativa* seed extract significantly (P≤0.01) up-regulated the IL-12 transcripts in the chicken PBMCs at 6, 12, 24 and 48h post-stimulation with a peak level of 37.47±5.22 folds at 48 h time interval (Fig. 5).

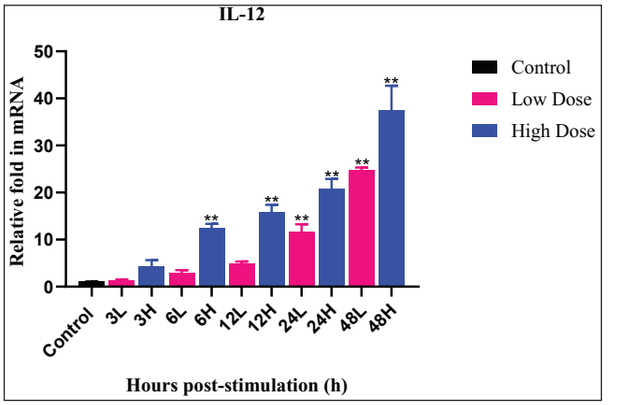

Fig. 5: Relative expression of IL-12 transcript in chicken PBMCs stimulated with Low dose (125 µg/ml) and High dose (250 µg/ml) of *Nigella sativa* seed extract over a period of 48 h

Further, the low dose of *Nigella sativa* seed extract significantly (P≤0.01) up-regulated the IL-12 transcripts in the chicken PBMCs at 24 and 48h post-stimulation only. The present findings suggested the potential of *Nigella sativa* seed extract in inducing the Th1 cytokine responses

in the chicken PBMCs, which can be further tested as an adjuvant with any antigen or vaccine in chickens for the possible enhancement of cell mediated immune responses.

Effect of *Nigella sativa* seed extract on Anti-inflammatory and Th2 cytokine gene expression in the chicken PBMCs

The high dose of *Nigella sativa* seed extract induced both IL-4 and IL-13 transcripts in the chicken PBMCs at 12, 24 and 48h post-stimulation and the low dose was able to induce them only at 48 h post-stimulation. The level of IL-4 expression was 9.01 ± 2.88, 11.00 ± 3.28 and 17.88 ± 2.93 folds at 12, 24 and 48 h post-stimulation, respectively (Fig. 6).

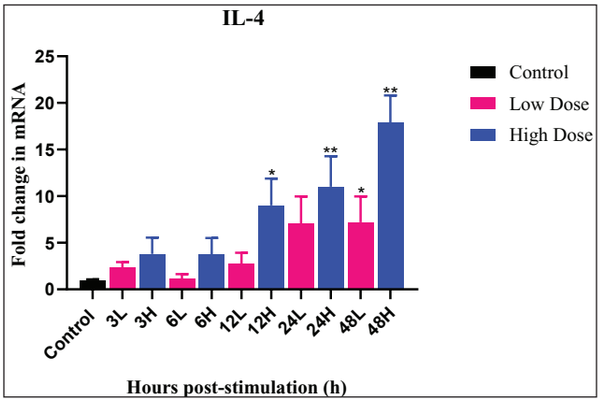

Fig. 6: Relative expression of IL-4 transcript in chicken PBMCs stimulated with Low dose (125 µg/ml) and High dose (250 µg/ml) of *Nigella sativa* seed extract over a period of 48 h

While the level of IL-13 transcripts was 8.1994 ± 2.31790, 8.7327 ± 2.57686 and 19.24 ± 2.6 folds at 12, 24 and 48 h post-stimulation, respectively (Fig. 3). The high dose of *Nigella sativa* seed extract also induced significantly (P≤0.01) higher level of IL-10 transcripts in the chicken PBMCs, which were 8.06 ± 2.13; 7.45 ± 2.52 and 11.57 ± 2.23 folds, respectively at 12, 24 and 48 h post-stimulation (Fig. 7). Previous studies have revealed out the anti-inflammatory potential of *Nigella sativa.* IL-13 and IL-4 are predominantly produced by Th2 cells and also by CD8+ T cells, mast cells, eosinophils and basophils. Majdalawieh *et al.,* 2010 tested *Nigella sativa* aqueous extract on splenocytes and finds that it was potentially effective and significantly enhanced the secretion of IL-4 and IL-10 which corroborated the present findings. Further, the results of the present study indicated the potential of *Nigella sativa* seed extract to induce Th2 cytokine responses in the chicken PBMCs, which could possibly help for further enhancement of antigen specific antibody responses in the chicken when used with any antigen or vaccine.

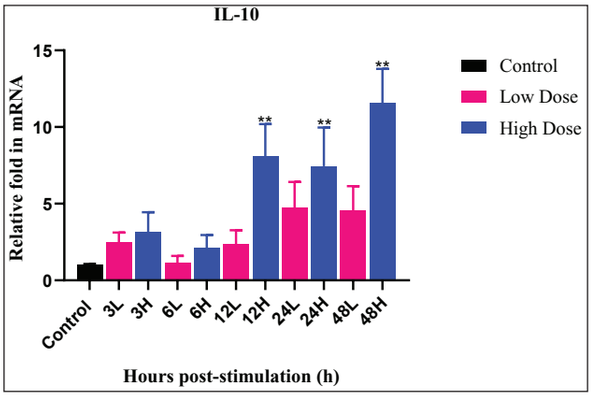

Fig. 7: Relative expression of IL-10 transcript in chicken PBMCs stimulated with Low dose (125 µg/ml) and High dose (250 µg/ml) of *Nigella sativa* seed extract over a period of 48 h

CONCLUSION

In conclusion, *Nigella sativa* methanolic extract induced the expression of IL-1β, IFN-β, IFN-γ, IL-4, IL-10, IL-13, and IL-12 transcripts in the chicken PBMCs. The results of the present study indicated the ability of the *Nigella sativa* methanolic extract to induce pro-inflammatory, antiviral, Th1 and Th2 cytokine responses. Hence, the *Nigella sativa* methanolic extract can be tried as an adjuvant with any vaccine or antigen in the chicken.

ACKNOWLEDGEMENTS

The authors acknowledge the help and necessary facilities provided by The Director, ICAR - Indian Veterinary Research Institute, Izatnagar-243 122, Bareilly-Uttar Pradesh (India) for this investigation.

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