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FULL LENGTH ARTICLE

# Protective and antiviral activities of *Nigella sativa* against avian influenza (H9N2) in turkeys

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**KEYWORDS**

*Nigella sativa*;
Avian influenza;
H9N2;
Antiviral activity;
Turkey

**Abstract** The main objective of this study was to determine the possible effects of *Nigella sativa* on immune-response and pathogenesis of H9N2 avian influenza virus in turkeys. The experiment was performed on 130 non-vaccinated mixed-sex turkey poults, divided into five experimental groups of 30 birds each. Group A was kept as non-infected and a non-treated negative control while group B was kept as infected and non-treated positive control. Turkeys in groups A and B received normal commercial feed while turkeys of groups C, D and E were fed on diets containing 2%, 4% and 4% NS seeds, respectively, from day one through the entire experiment period. All groups were challenged with H9N2 AIV at 4th week of age except group A. Infected turkeys showed clinical signs of different severity, showing the most prominent disease signs in turkeys of the group B. All infected turkeys showed positive results for virus shedding; however, the pattern of virus shedding was different, and with turkeys of the group B showing pronounced virus secretion than the turkeys in other groups receiving different levels of NS. Moreover, significantly higher antibody titer against H9N2 AIV in turkeys fed 6% NS seeds shows the immunomodulatory nature of NS. Similarly, increased cytokine gene expression suggests antiviral behavior of NS especially in dose dependent manner, leading to suppressed pathogenesis of H9N2 viruses. However, reduced virus shedding and enhanced immune responses were more pronounced in those turkeys received NS at the rate

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of 4% and 6%. This study showed that supplement of NS would significantly enhance immune responsiveness and suppress pathogenicity of influenza viruses in turkeys.

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## 1. Introduction

Avian influenza virus (**AIV**) subtype H9N2 is becoming a serious threat to poultry birds. H9N2 AIV is an emerging respiratory problem, isolated from different birds from a number of countries and has been reported to have zoonotic potential (Swayne, 2012; Ahad et al., 2013; Umar et al., 2016a, 2016b). H9N2 AIV has been isolated from live bird markets and poultry farms as well as from poultry workers from different parts of India, Pakistan and Egypt (Alexander et al., 2009; Afifi et al., 2013; Umar et al., 2015c, 2016c, 2016d). Highly pathogenic avian influenza (**HPAI**) viruses are known to cause systemic infection and death in chickens, while low pathogenic avian influenza (**LPAI**) viruses usually cause mild infection in poultry. However, H9N2 LPAI viruses have shown diverse pathogenic behavior of varying clinical signs in chicken largely depending upon the viral strains and other co-infecting pathogens in field conditions (Ullah et al., 2013; Umar et al., 2015a). Recently, H9N2 AIV isolated from Korean poultry farms has been reported that has a novel genotype and potential to infect humans due to antigenic shift and drift (Lee et al., 2007 and Abid et al., 2016). It has been reported that H9N2 circulating in Pakistan poultry has attained a novel genotype by exchanging some genes with HPAI H7N3 and H5N1 viruses (Iqbal et al., 2009; Chaudhry et al., 2015). Moreover, the Egyptian H9N2 strains have the ability to become highly pathogenic by a genetic change to acquire basic amino acids on the HA cleavage site (Alexander et al., 2009; Afifi et al., 2013).

Currently, the feed industry is focusing on various substitutes for antimicrobial drugs (Al-Mufarrej, 2014). Antimicrobial agents of plant origin such as essential oils, plants extracts, and complete plant substances are considered as alternatives to the traditional antimicrobial feed additives. *Nigella Sativa* (**NS**) are one of such alternatives that could be used as feed additives in order to reduce the pathogen load in poultry. Thymoquinone (TQ) has been found as the main bioactive constituent of the volatile oil of NS seeds. Recently, clinical and experimental studies have demonstrated many therapeutic effects of TQ including immunomodulatory, anti-inflammatory, anti-tumor, and antimicrobial (Ahmad et al., 2013; Azeem et al., 2014; Al-Mufarrej, 2014). Due to a vast number of biological targets and virtually no side effects, NS has achieved the potential therapeutic interest to cure immunosuppressive viral diseases.

Host cell mediated immune response is considered important in the pathogenesis of avian influenza viruses and plays a big role in recovery from viral infection (Wells et al., 1981). However, there have been a limited number of studies associated with the immunomodulatory effects of NS in poultry. To the best of our knowledge, there is no study comprising the role of NS on immune responses, pathogenesis, and antiviral activity against LPAI H9N2 in turkeys. In the present study, we have evaluated the effect of NS on immune responses and pathogenesis of H9N2 AIV in turkeys. We hypothesized that the use of NS has significant anti-viral activity against H9N2 AIV and can be an economical and efficient way to enhance cellular and humoral immunity against infectious diseases in poultry in general and in turkeys in particular.

## 2. Materials and methods

### *2.1. Experimental turkeys and virus*

One-day-old mixed-sex turkey poults were purchased from a local hatchery and grown up to 4th wk of age. The study was conducted on 4 wk old 130 mixed-sex turkeys. The birds were kept in separate high efficiency particulate arresting (HEPA) isolators with wire flooring under standard management conditions. Experimental study protocol was approved by the Animal care and research committee of Veterinary Medical council, Pakistan, and experimentation was carried out according to the guidelines of the committee. Avian influenza A virus, A/chicken/Pakistan/10RS3039-284-48/2010 (H9N2) is a reassortant field isolate obtained from National Agriculture Research Council (NARC) Islamabad, Pakistan. Viral stocks were prepared and titrated in 9-day-old to 10-day-old chicken embryonated eggs and the median embryo infectious dose ($EID_{50}$) was calculated using previously reported methods (Reed and Muench, 1938). The viral stocks were diluted in medium containing antimicrobials to yield a final titer of $10^6$ $EID_{50}$/ml. All turkeys were declared serologically naïve and free from influenza virus by hemagglutination inhibition (HI) and virus isolation (VI) in eggs using standard methods (Iqbal et al., 2009).

### *2.2. Preparation of N. sativa seeds for addition to feed*

Commercial turkey feed having 22% total protein and 3000 kcal $kg^{-1}$ metabolizable energy was prepared without addition of any toxin binder, vitamins, mineral supplements, or antibiotics. Prior to use, each batch of the basal feed was analyzed for aflatoxin, ochratoxin, and zearalenone (Howel and Taylor, 1981). NS seeds were obtained from a local herbal store in Lahore, Pakistan. The taxonomic identity of the plant seeds was verified at the University of Agriculture, Faisalabad, Pakistan. The seeds were then carefully washed with distilled water to remove any extraneous materials, dried under shade at room temperature, grounded into a coarse powder using an electronic grinder, weighed by analytical balance, and added to the diets at the rate of 2%, 4% and 6% of total feed for groups C, D and E respectively, while groups A and B were fed with the commercial feed without NS (Al-Mufarrej, 2014).

### *2.3. Experimental design and treatments*

The birds were divided into five groups (A, B, C, D and E) with 30 birds each (with 2 replicates of 15 birds each). Group A served as negative control that was non-treated and

**Table 1** Experimental design.

| Groups | No. of birds | Treatment/inoculation |
|---|---|---|
| A (ctrl) | 30 | Non-treated, non-infected negative control |
| B (H9N2) | 30 | $10^6$ $EID_{50}$/ml (IT) only |
| C (H9N2 + 2% NS) | 30 | $10^6$ $EID_{50}$/ml (IT) + NS (2%) |
| D (H9N2 + 4% NS) | 30 | $10^6$ $EID_{50}$/ml (IT) + NS (4%) |
| E (H9N2 + 6% NS) | 30 | $10^6$ $EID_{50}$/ml (IT) + NS (6%) |

IN = intranasal; NS = *Nigella sativa.*

non-infected. Group B was infected with H9N2 AIV and served as positive control. Groups C, D and E were infected with H9N2 AIV and treated with NS as descried earlier (Table 1). All the experimental groups were offered *ad libitum* feed and water throughout the experiment. H9N2 AIV ($10^6$$EID_{50}$/ml/bird) was inoculated intra-tracheally (**IT**) to the groups B, C, D, and E at 4th wk of age. The turkeys were observed twice daily for clinical signs, such as sickness, sneezing, nasal discharge, diarrhea, head swelling, and mortality, up to 12 days post-infection (**DPI**).

### 2.4. Determination of virus shedding

Buccal (**B**) and cloacal (**C**) swabs were collected daily from all turkeys from 1 to 8 DPI to assess virus shedding in 1 ml of PBS containing 1% gentamycin and kept at −80 °C until further used. RNA was extracted using the QIAamp viral RNA isolation kit (Qiagen, Valencia, CA, USA) according to the instructions of the manufacturer. The virus titer of each sample was determined by using quantitative real-time reverse transcriptase polymerase chain reaction (**qRT-PCR**) as described in previous studies (Umar et al., 2015a), qRT-PCR reactions targeting the influenza virus M gene (Lee et al., 2007; OIE, 2012) were conducted using QuantiTect® SYBR Green RT-PCR Kit (Qiagen, Germany). The quantitative RT-PCR reactions were performed on a light cycler®480, Real-Time PCR system (Roche diagnostics, Switzerland) with following program: the reverse transcriptase step conditions for primer sets were one cycle at 50 °C for 30 min, 95 °C for 15 min followed by 45 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 15 s. A standard curve for virus quantification was established with RNA extracted from the challenge virus, and the $C_t$ values of samples were converted into $EID_{50}$/ml as described in previous studies (OIE, 2012; Umar et al., 2015a). The virus titer was presented by the mean ± SD of the virus titer per ml of sample.

### 2.5. Quantification of cytokine gene expression

Blood samples were taken 1–8 DPI in EDTA coated tubes and quantification of $IFN_\gamma$ gene expression was determined by using QuantiTect TM Probe RT-PCR (Qiagen, Valencia, CA, USA). Different primer sequences for $IFN_\gamma$ gene were used in qRT-PCR as reported previously (Kaiser et al., 2003; Loa et al., 2001). For endogenous control, 28S rRNA probe was used as described previously (Giulietti et al., 2001). The light cycler® 480, Real-Time PCR system (Roche diagnostics, Switzerland) was used for qRT-PCR with the following conditions: one cycle at 50 °C for 30 min, 95 °C for 15 min followed by 45 cycles of 94 °C for 20 s, and 59 °C for 60 s. Data were analyzed using the Light cycler® 480 software Version 1.5 SW.

### 2.6. Serology

Blood samples were taken before virus inoculation considered as d 0 and 4, 8 and 12 DPI for estimation of antibody titer against AIV using HI assays (OIE, 2012). Highest serum dilutions showing complete inhibition of chicken red blood cells agglutination were used for titer calculation. Serum titers of 1:8 ($2^3$) or lower were considered negative for antibodies against AIV (OIE, 2012; Umar et al., 2015b).

### 2.7. Statistical analysis

One way analysis of variance (ANOVA) with Tukey post-test was used to analyze HI titer, body weight, and IFN titer. For statistical purposes, all qRT-PCR negative oropharyngeal and cloacal swabs were given a numeric value of $10^{0.5}$ $EID_{50}$/ml for AIV. All HI-negative serum was given a value of $3\log_2$. These values represent the lowest detectable level of antibodies in these samples based on the methods used. The number of birds shedding virus was tested for statistical significance using Fisher's exact test and two-way ANOVA was used to evaluate virus titers in swabs. P-values less than 0.05 were considered as significant. A statistical software package GraphPad Prism 6 (GraphPad Software Inc. La Jolla, CA, USA) was used to analyze the data. Results were expressed as mean ± SD.

## 3. Results

### 3.1. Clinical signs

No clinical signs were observed in turkeys of group A. As expected, clinical sign was severe in turkeys of group B than in those of groups C, D, and E. Signs of facial swelling, diarrhea, sneezing and depression were more prominent in the group B followed the turkeys of groups C and D. On the other hand clinical signs were restricted to depression and decreased food consumption of group E (Table 2). Furthermore, the mean body weight of the turkeys in group E was found significantly higher ($P < 0.05$) than that of group B. However, mean body weight of turkeys in group B was found significantly lower ($P < 0.05$) than that of group A. Moreover, turkeys in the groups C and D also showed significant ($P < 0.05$) increase in body weight than those of group B. No mortality was observed in turkeys of all groups. Similarly, necropsy observations revealed reduced gross pathological lesions in turkeys fed NS in dose dependent manner than those of group B (data not shown).

### 3.2. Infection and viral shedding

All turkeys were negative for H9N2 AIV by serology and virus isolation in eggs prior to inoculation. All turkeys in inoculated

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**Table 2** Number of infected turkeys showing clinical disease signs.

| Clinical signs | Groups A (30) | Group B (30) | Group C (30) | Group D (30) | Group E (30) |
|---|---|---|---|---|---|
| General sickness | – | + + (30) | + (24) | + (21) | + (11) |
| Depression | _ | + (30) | + (21) | + (9) | + (7) |
| Decrease in feed intake | _ | + (30) | + (21) | + (7) | + (11) |
| Sneezing | _ | + (16) | + (5) | – | – |
| Respiratory sounds | _ | + (18) | + (9) | + (6) | + (1) |
| Ocular /nasal discharge | _ | + (13) | + (4) | + (2) | – |
| Head swelling | _ | + (11) | + (6) | + (4) | – |
| Loose droppings | _ | + (9) | + (4) | + (2) | – |
| Mortality | _ | _ | _ | – | – |
| Mean body weight* (g ± S.D.) | 1989 ± 19.5[a] | 1675 ± 55.4[d] | 1738 ± 13.7[c] | 1766 ± 12.5[c] | 1846 ± 16.0[b] |

Infected turkeys were inoculated with A/chicken/Pakistan/10RS3039-284-48/2010 (H9N2) at a dose rate of $10^6$ $EID_{50}$ through intra tracheal route. Clinical signs were observed between 1 and 12 dpi. The number in parentheses is the total number of turkeys in a group showing clinical signs. (–) indicates no apparent clinical disease signs. + mild, + + moderate, + + + severe.
[a–d]Mean values in the same row that do not share a common letter differ significantly ($P < 0.05$).
* Body weight was measured 12 days post-infection.

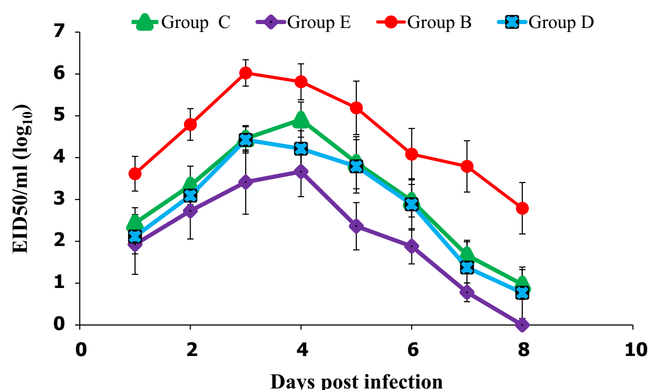


**Figure 1** Mean Virus titer values ($log_{10}$ EID50/ml) of AIV detected in buccal swabs per day post H9N2 AIV inoculation in different groups. The Error bar shows the standard deviation for 15 samples from fifteen turkeys.

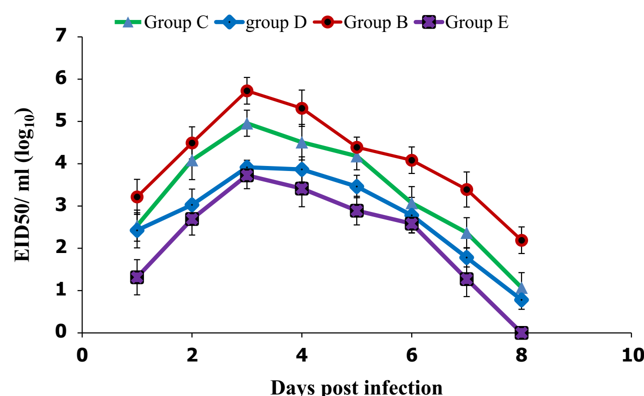


**Figure 2** Mean Virus titer values ($log_{10}$ EID50/ml) of AIV detected in cloacal swabs per day post H9N2 AIV inoculation in different groups. The Error bar shows the standard deviation for 15 samples from fifteen turkeys.

groups became infected with H9N2 AIV as determined by detection of the H9N2 AIV matrix gene in B and C swabs. As shown in Figs. 1 and 2, turkeys in the group B had higher level of virus shedding ($P < 0.05$), than those in the groups C, D, and E post-infection. Turkeys fed NS (6%) showed significantly lower ($P < 0.05$) virus titer than those in group B. Furthermore, turkeys in group D showed lower virus titer in swabs than turkeys of group C but the difference was no significant ($P > 0.05$). However, C swabs revealed different patterns of virus shedding, showing less virus shedding in all groups than B swabs post-infection. These results suggest that virulence of H9N2 LPAI virus was suppressed in turkeys fed NS than that of group B. The time to peak H9N2 AIV shedding in swabs varied between groups as determined by RT PCR results (Figs. 1 and 2). Turkeys in the group B had peaks for AIV shedding in B and C swabs at 3 DPI. Moreover, virus shedding period was shorter in turkeys fed NS than that in the group B.

### *3.3. Quantification of cytokines mRNA extracted*

To evaluate the effect of NS treated feed and H9N2 virus infection on the cytokine gene expression, IFNγ mRNA was quantified by real-time RT-PCR. There was an early increase ($P < 0.05$) in the expression levels of IFNγ mRNA in turkeys of group D and E than turkeys of the group B at 1–7 DPI (Fig. 3). However, a non-significant increase ($P > 0.05$) in the expression levels of IFNγ mRNA was observed in turkeys of the group C than the turkeys of the group B after post-infection ($P > 0.05$).

### *3.4. Serological studies*

All birds in the infected groups were serologically positive for the inoculated virus by HI test at 4, 8 and 12 DPI (Table 3). The HI titer for turkeys of groups C, D and E was higher than turkeys of the group B. This increase was more pronounced in turkeys fed NS (6%) followed by those fed NS (2% or 6%) compared to turkeys in group B ($P < 0.05$). Collectively, the geometric mean titer was lower ($P < 0.05$) in turkeys of the group B than other infected groups at 8 and 12 DPI. In our study, the geometric mean titers of C, D, and E groups ranged from $Log_2$ 5.8 at 4th DPI to $Log_2$ 7.4 at 12th DPI, $Log_2$ 6.6 at 4th DPI to $Log_2$ 7.3 at 12th DPI and $Log_2$ 6.9 at 4th DPI to $Log_2$ 8.8 at 12th DPI, respectively, whereas the group B ranged from $Log_2$ 4.6 at 4th DPI to $Log_2$ 6.4 at 12th DPI.

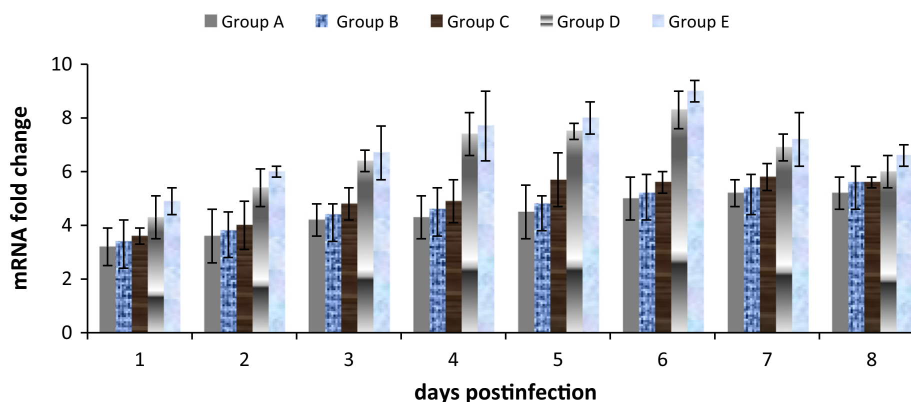


**Figure 3** Quantification of $IFN_{\gamma}$ mRNA extracted from peripheral blood mononuclear cells of turkeys in different groups after H9N2 LPAI virus experimental infection. The Error bar shows the standard deviation for 15 samples from fifteen turkeys.

**Table 3** HI titer of sera collected from virus inoculated turkeys in different groups.

| Groups | Pre infection HI titer $\log_2$ HI titer (GMT) | Number of turkeys positive for HI/total turkeys $\log_2$ HI titer range (GMT; Mean ± SD) | | |
|---|---|---|---|---|
| | | 4 DPI | 8DPI | 12DPI |
| Group B | <2 | 15/15 (4.6 ± 0.5)[c] | 15/15 (5.7 ± 0.2)[c] | 15/15 (6.4 ± 0.3)[c] |
| Group C | <2 | 15/15 (5.8 ± 0.2)[b] | 15/15 (6.7 ± 0.9)[b] | 15/15 (7.4 ± 0.5)[b] |
| Group D | <2 | 15/15 (6.6 ± 0.5)[a] | 15/15 (7.1 ± 0.7)[a] | 15/15 (7.3 ± 0.4)[bc] |
| Group E | <2 | 15/15 (6.9 ± 0.8)[a] | 15/15 (8.3 ± 0.4)[a] | 15/15 (8.8 ± 0.7)[a] |

GMT, Geometric mean titer, DPI, days post infection. HI titers 1:8 ($2^3$) or lower were considered negative for sero-conversion. Infected turkeys were inoculated with A/chicken/Pakistan/10RS3039-284-48/2010 (H9N2) at a dose rate of $10^6$ $EID_{50}$ through intranasal route.
[a–c]Mean values in the same column that do not share a common letter differ significantly ($P < 0.05$).

## 4. Discussion

The effects of NS on immune responses and pathogenicity of reassortant H9N2 virus A/chicken/Pakistan/10RS3039-284-48/2010 in turkey poults were studied. Infection of H9N2 virus produced mild to moderate clinical signs in infected birds but clinical signs were less pronounced in turkeys fed different levels of NS and inoculated with H9N2 AIV (Groups C, D and E). These findings are in line with previous studies that revealed mild infections when H9N2 LPAI virus inoculated experimentally in poultry (Lee et al., 2007; Umar et al., 2015a). Decreased severity in clinical signs of NS fed turkeys might be due to enhanced immunity and antiviral properties of NS as described previously (Dorucu et al., 2009; Shewita and Taha, 2011; Ahmad et al., 2013; Umar et al., 2015b). Therefore, it can be assumed that NS can suppress the severity of clinical signs of LPAI H9N2 especially when fed in combination. Moreover, there was a significant increase in body weight of those turkeys fed NS (6%) (E group) compared to those infected with H9N2 only (group B) at the end of experiment. The body weight increased ($P > 0.05$) in groups fed NS (2% or 4%) compared with the H9N2 group. In this study, NS supplementation caused no adverse effects with any of the levels used in the experiment. The effects of dietary NS on body weight of the turkeys are similar to other studies. El-Bagir et al. (2006) showed that dietary NS supplementation at 2% or 4% significantly increased final body weight of laying hens. However, other studies showed that the addition of NS seeds into the diet significantly decreased body weight of the chickens (Majeed et al., 2010). On the other hand, the results of the present study showed that supplementation of the diet with NS had a positive impact on body weight and did not negatively influence the final body weight of turkeys. Shewita and Taha (2011) showed that inclusion of *N. Sativa* in the diets of broiler chickens improved body weight and feed conversion ratio (**FCR**) at a lower dose while a higher inclusion rate showed no significant differences in comparison with the control group. The findings in the present study with regard to the performance are in agreement with those of Majeed et al. (2010). In contrast to the present study, however, El-Nattat and El-Kady (2007) reported that supplementation of black cumin meal at the level of 17% into the diet decreased broiler chicken performance. Furthermore, enhanced activities of trypsin and amylase in pancreas and small intestine of broiler chickens fed diets supplemented with NS have been reported (Durrani et al., 2007; Umar et al., 2015b). Therefore, improved body growth rates in our study can be partly attributed to the effects of NS on bile and digestive enzymes production and secretion and consequently better digestion and absorption of the dietary nutrients.

In addition, H9N2 LPAI infection in turkeys fed NS characterized by lower viral load in swabs and increased in $IFN_{\gamma}$ levels, may suggest that there was increase in cytotoxic T-cells of cell mediated immunity for the clearance of virus in

order to protect turkeys from infection of H9N2 LPAI virus. Interestingly, higher expression of $IFN_\gamma$ mRNA was observed in turkeys of group D than that of group E ($P > 0.05$). It has been described that NS enhances bone marrow cellularity, T-helper and cytotoxic T cells, leading to inhibition of influenza virus propagation (Ahmad et al., 2013; Al-Mufarrej, 2014; Umar et al., 2015b). Furthermore, NS has been reported to enhance splenocyte proliferation and interferon expression, decreased pro-inflammatory cytokines, viral proteins (integrase, protease) and RNA polymerase II, which play central roles in viral replication and thus minimizing harmful effects of viral pathogens in dose dependent manner (Ahmad et al., 2013).

From the results, we can speculate that NS induced T cell stimulation and proliferation to allow the high viral clearance and this low viral load in B and C swabs correlates with NS antiviral and immunomodulatory activities. These findings are in agreement with studies reported previously indicating that cytotoxic T cells can decrease viral shedding by clearing LPAI H9N2 virus, thus playing a major role by protecting poultry during early viral infections (Dorucu et al., 2009; Shewita and Taha, 2011; Al-Mufarrej, 2014; Umar et al., 2015b). All turkeys in the infected groups were serologically positive for the inoculated virus by HI test. The HI test revealed higher HI titer in NS treated groups and lower in the group B. Collectively, the geometric mean titer was significantly higher ($P < 0.05$) and developed faster in those groups fed different levels of NS. Immunostimulatory and antioxidant properties of NS may be the possible reason for this change (Umar et al., 2015b). These results also agree with those of Shewita and Taha (2011) who reported significant dose-dependent improvement in antibody titer against Newcastle disease virus (**NDV**) in response to NS. Similarly, significantly higher antibody titers were observed against NDV, infectious bursal disease virus (**IBDV**), and AIV in poultry fed different levels of NS (Ahmad et al., 2013; Umar et al., 2015b). Feeding NS at 40 g/kg enhanced antibody production against NDV and IBDV in broiler chickens (Durrani et al., 2007). In addition to these, NS significantly enhanced the immune system through increased lymphocyte production, and inhibited development of advanced dysplastic changes after topical application of DMBA (7,12-Dimethylbenz(a) anthracene) in hamsters to induce immuno-suppression (Al-Jawfi et al., 2008). However, the total immunoglobulin levels were significantly ($P < 0.05$) higher in NS fed turkeys (group E) than the control group indicating immune stimulating effects of NS. Dorucu et al. (2009) and Abdelwahab and El-Bahr (2012) recommended NS use in fish feed to enhance immunity against some pathogens. Furthermore, NS can be used as immunostimulant adjuvants in vaccines and can be a promising novel approach for new vaccine development (Mady et al., 2013).

## 5. Conclusion

In the present study, NS exhibited immune-stimulant effects as depicted by enhanced cell-mediated, humoral immune responses and cytokine gene expression leading to early viral clearance, reduced pathogenicity of H9N2 in turkeys possibly by inhibiting replication of viruses. It can be suggested that NS has anti-influenza virus activity, can induce efficient immune responses and play a significant role in diminishing the pathogenic effects of H9N2 in turkeys. This study strengthens our belief that NS (6%) is safe and useful immunomodulator for the immune system.

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