Pakistan J. Zool., vol. 50(6), pp 2223-2228, 2018. DOI: http://dx.doi.org/10.17582/journal.pjz/2018.50.6.2223.2228

In vitro Antiviral Activity of *Nigella sativa* against Peste des Petits Ruminants (PPR) Virus

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Article Information
Received 22 April 2017
Revised 23 June 2017
Accepted 07 July 2017
Available online 05 October 2018

Authors' Contribution
KA, MuR, AA and AJ designed and planned the study. KA, RQ, FY and FY executed the experiments and provided technical help. SU and MLS performed statistical analysis of the data and wrote the article.

Key words
Peste des petits ruminants virus, *N. sativa*, Antiviral activity.

ABSTRACT

Peste des petits ruminants (PPR) is an important viral disease of sheep, goat and other small ruminants, imparting substantial economic losses. In current study, antiviral activity of six dilutions of *Nigella sativa* (*N. sativa*) alcoholic extracts against the PPRV was investigated in vitro. Vero cell lines infected with PPRV were treated with six dilutions alcoholic extracts of *N. sativa* (200, 100, 50, 25, 12.5 and 6.25 µg/mL). The antiviral effects were judged by 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide colorimetric (MTT) assay. Plaque reduction assay was used to test the mode of action. Out of six dilutions, three dilutions (50, 25, 12.5 µg/ml) showed significant antiviral activity and cell survival was more than 50%. Two dilutions of *N. sativa* (200 µg/ml and 100 µg/ml) extracts showed cytotoxicity to Vero cell lines. In the plaque reduction assay, alcoholic extracts of *N. sativa* (50 µg/ml) significantly reduced the plaques count as compared with the control negative (P<0.05) in all the tested modes of action. The current experiment revealed that the alcoholic extracts of *N. sativa* (50 µg/ml) could increase the viability of infected cells, and curtail the cytopathic effects of PPRV. *N. sativa* seed extracts should be explored in field conditions for the control of PPR.

INTRODUCTION

Peste des petits ruminants (PPR), also called as kata, goat plague, syndrome of stomatitis, pneumoenteritis and ovine rinderpest. It is a viral disease of both domesticated and wild small ruminants. It imparts great threats to food security and livelihood of livestock farmers in Middle East, Asia and Africa (Banyard *et al*., 2010). The infection is characterized by fever, pneumonia, discharge from oral cavity and nostrils, ulceration of the mucous membranes and inflammation reaction at the gastrointestinal tract, causing diarrhoea. This disease has been reported to be an emerging infection in various regions around the globe (Banyard *et al*., 2014). The etiological agent is Peste des Petits Ruminants Virus (PPRV) of genus *Morbillivirus* and family *Paramyxoviridae*. A genome size of 15948 bp makes it the largest member of this *Morbillivirus* (Bailey *et al*., 2005). Its genome codes for six proteins *i.e.* nucleoprotein (N), viral RNA-dependent polymerase (L), RNA-polymerase phosphoprotein co-factor (P), matrix protein (M), fusion protein (F) and hemagglutinin protein (H) (Diallo, 2003). It is transmitted through direct contact with already infected animals through secretions and fecal material (Truong *et al*., 2014). Although, only a single serotype of PPRV is reported to exist, phylogenetic analysis indicated that viral strains can be divided into four lineages. Out of which three occur in Africa only, while one exists in Africa and Asia (Parida *et al*., 2015). There is no standard medications protocol exists for the treatment of the disease, but there are vaccines (Abubakar *et al*., 2011).

Currently, feed industry is focusing on various substitutes for antiviral 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* are one of such alternatives that could be used as feed additives in order to reduce

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0030-9923/2018/0006-2223 $ 9.00/0

the pathogen load in animals. It has been reported that *N. sativa* or its constituents such as thymoquinone, nigellone, and thymohydroquinone demonstrated several pharmacological activities such as anti-inflammatory, anti-ischemic, antioxidant and antibacterial,antiviral antihistaminic, hepatoprotective and immunopotentiating effects (Hosseinzadeh *et al*., 2012, 2013). Thymoquinone has been found as the key bioactive component of *N. sativa* seeds. Recently, clinical and experimental studies have demonstrated many therapeutic effects of Thymoquinone including immunomodulatory, anti-inflammatory, anti-tumor, and antimicrobial effects (Ahmad *et al*., 2013; Azeem *et al*., 2014; Al-Mufarrej, 2014). Large number of biological targets and nearly no adverse side effects make *N. sativa* a key player for the cure of immunosuppressive diseases. The goal of this communication is to analyze the antiviral potential of various dilutions of alcoholic extract of the *N. sativa*

MATERIALS AND METHODS

Cells and viruses

Vero cell lines were propagated in Dulbecco's minimal essential medium (DMEM; Gibco-BRL, Grand Island, NY) enriched with 10% calf serum (CS; Gibco-BRL) and antibiotics. Vero cells were seeded at the rate of 10^4 cells/well on 96 wells cell culture plates and were incubated at 37°C in humid atmosphere having 5% CO_2. Cells were counted by haemocytometer (Ali *et al.*, 2017). After 48-72 h of incubation, the cell lines were examined through inverted microscope. The 80% to 90% confluent cell lines in each well was picked for further study.

Purified PPRV virulent strain was provided by Dr. Muhammad Anees (Veterinary Research Institute, Lahore, Pakistan). This virus was originally isolated from field samples and characterized using molecular techniques. Virus was propagated on Vero cell lines by subsequent passages. $TCID_{50}$ was measured using the statistic analysis by Reed and Muench method (Gialleonardo *et al*., 2011). PPRV with a titer of 10^3 $TCID_{50}$/50µl was used for antiviral and cytotoxicity activity.

Preparation N. sativa *extracts*

NS seeds were procured from herbal store in Lahore, Pakistan. The taxonomic identification of the plant seeds was done at the University of the Punjab, Lahore, Pakistan. The seeds were washed, dried and grounded into a coarse powder, and then weighed by analytical balance. Powdered seeds were extracted with 95% ethanol using soxhlet apparatus. After reduced pressure evaporation, dried powder was dissolved in 20mL sterilized water and filtered by a 0.22 µm millipore filter to remove bacteria (5.12 g/20mL of pure water). Extract was then dissolved in 2 mL media to obtain two 200 µg/mL dilutions for antiviral and cytotoxicity assay. Serial two fold dilution of 200 µg/mL was done to obtain 100, 50, 25, 12.5 and 6.25 µg/mL in duplicate manner. Sterility of extract was checked on Nutrient, Maconkey and Sabroud agar (Ulasli *et al*., 2014).

Antiviral activity assay

A suspension (50µL) having 10^3 median tissue culture infectious doses ($TCID_{50}$) virus were added to vero cells monolayer in 96 well plates and were incubated at 4°C for 60 min to permit attachment. After that unabsorbed virus was removed by aspirating the medium. After washing the cell monolayers with PBS, six dilutions of the test samples (200, 100, 50, 25, 12.5 and 6.25 µg/mL) were added in the plates. Infected cells without treatment and uninfected cells were kept as controls. The experiment was executed in triplicate with infected- untreated and uninfected-treated as controls. All the plates were incubated at 37°C in humidified 5% CO_2 and were observed daily by inverted microscope. Scoring of viral cytopathic effects (CPE) of the tests was performed under light microscopy in comparison to the virus control and cell control. Cell viability was observed by by 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide colorimetric (MTT) method assay (MTT colorimetric assay) for evaluation of antiviral activity as described previously (Bhanuprakash *et al*., 2007; Xu *et al*., 2012).

Cytotoxicity assay

The cytotoxic effects of extracts on Vero cell monolayers were evaluated by quantifying the viability of cell through MTT assay (Li *et al*., 2005). Briefly, Vero cell lines were grown into monolayer in 96-well plates and the medium was eliminated. One mL test sample with various concentration (suspension made in MEM supplemented with 2% CS) was poured in each well. The dilution medium was kept as control. This experiment was conducted in triplicate, and plates were incubated at 37°C in a humidified 5% CO_2. After 96 h, MTT solution (20 µL, 5 mg/mL in PBS) was added in each well and the plate was then again incubated for 4 h for MTT formazan formation. After removal of the medium, formazan crystals were dissolved using 100 µL dimethylsulfoxide. After 20 min, the contents were homogenized using microplate shaker. The optical densities (OD) were read at wavelengths of 490 nm through spectrophotometer. Cells were observed under inverted microscope for visible morphological changes and compared with controls. Maximum non-toxic concentration (MNTC) was calculated on the basis of cellular morphological changes in Vero cells (Bhanuprakash *et al*., 2007; Xu *et al*., 2012).

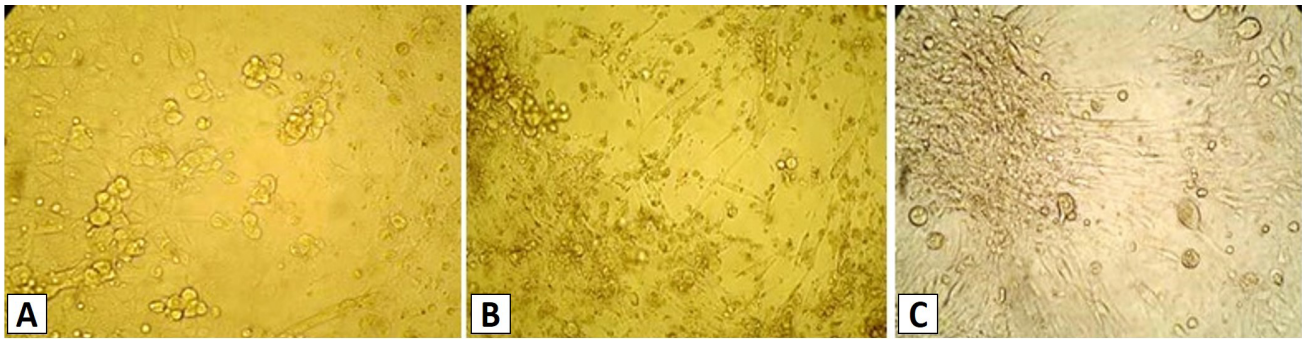

Fig. 1. Effect of extract from *N. sativa* on morphological changes of Vero cell lines caused by Peste des Petit ruminants virus (PPRV). Microscopic view of the inhibition of *N. sativa* extracts on cytopathic effect (CPE) formation at about 96 h postinfection by PPRV. A, CPE of Vero cells infected by PPRV; B, Vero cells infected by PPRV and *N. sativa* extract (100µg/ml); C, Vero cells infected by PPRV and *N. sativa* extract (50µg/ml).

Plaque reduction assay

The inhibitory effects of the extracts on the PPRV replication in vero cells were analyzed through plaque reduction assay, performed as described previously (Xu *et al*., 2012). The tests for the plaque reduction assay were performed in 24 well plates.

Virus inactivation assay

A concentrated virus suspension along with equal volumes of extract dilutions were incubated at 37°C for 60 min. The mixture alone, without any addition of test sample was kept as control. The remaining virus in the sample was diluted (100 fold) before placing on monolayers for 60 min. Cell monolayers were washed with PBS before overlaying with DMEM (2% CS and 1% methylcellulose). After 5 days of incubation, the cells were stained using 0.1% crystal violet in 20% methanol and plaques were enumerated after washing.

Inhibition of virus attachment

Extract dilution and virus suspension were placed on monolayers to allow virus to adsorb for 60 min at 37°C, with and without test samples. After that unabsorbed mixture was removed. Adsorption efficiency was determined by counting plaques as described for virus inactivation assay.

Intracellular inhibition assay

The cell monolayer were infected with virus and incubated at 37C for 1 h. The unabsorbed virus was removed by aspirating the medium. Before overlaying the cell monolayers extract dilutions, washing was done by PBS. The wells overlaid with DMEM without the addition of test sample were kept as control. The plate was incubated at 37°C for 120 min. Plaque reduction assay was done similar to the test above.

Pretreatment assay

Different dilutions of extract were added in cell monolayers and were set to be incubated at 37°C for 2 h. Washing of cell monolayers were done by PBS and overlaid virus. The plate was then incubated at 37°C for 60 min. The cell monolayers without test samples were kept as control. Plaque reduction assay were carried out as described for Inhibition of virus attachment.

Statistical analysis

Statistical analysis was performed using the Statistical Product and Service Solutions (version 17.0, SPSS Inc., Chicago, IL, USA). Data are shown as mean± SD and Duncan's multiple range tests were conducted to separate means.

RESULTS

Cell cytotoxicity and antiviral activity of the extracts

N. sativa extract dilutions (200 and 100 µg/mL) showed high cytotoxicity to Vero cells and low anti-PPRV activity. The MTT reduction assay indicated that the four extract dilutions of *N. sativa* (50, 25, 12.5 and 6.5 µg/mL) showed no cytotoxic effects on Vero cells. The CPE reduction assay indicated that the 50µg/mL yielded significant ($P<0.05$) antiviral activity. The MNTC value of the extract confirmed that cytotoxicity did not influenced the anti-PPRV activity of extracts. The changes in morphology of infected Vero cells infected with PPRV and treated with different dilutions of *N. sativa* were shown in Figure 1. The cells were dense and fine in normal Vero cells after humidified incubation (5% CO_2) at 37°C. The PPRV infected cells showed CPE on the whole monolayer of cells at about 72 h postinfection (Fig. 1A). The dense bundles of fibers, with an intact structure and well-defined

border with few CPE, indicated that 50 µg/mL and 25 µg/mL could inhibit CPE formation caused by PPRV (Fig. 1B, C). Moreover, extract dilution 50 µg/mL showed maximum percentage of survival cell followed by 25 µg/mL 12.5 µg/mL and 6.5 µg/mL (Fig. 2).

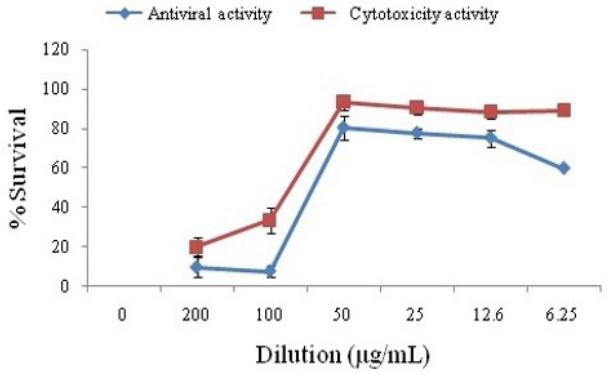

Fig. 2. Cell survival percentage for each dilution of of *N. sativa* extracts used for antiviral and cytotoxicity assay.

Table I.- Number of plaques in different plaque reduction assays.

Plaque reduction assay	Number of plaques (Mean±SD)
Virus inactivation	22.6^{a}±2.3
Inhibition of virus attachment	20.9^{a}±1.6
Intracellular inhibition	$13.b^{b}$±0.89
Pretreatment	15.4^{b}±1.5
Negative control	36.8^{c}±0.78

$^{a\text{-}c}$, different lowercase letters represent significant differences among groups ($P < 0.05$). The 50µg/ml N/sativa extract was used in plaque forming assay. The study was performed in triplicate.

Plaque reduction assay

N. sativa dilution (50µg/mL) was used in the mode of action study. The numbers of plaques under the various modes of action are shown in Table I. Numbers of plaques in all plaque reduction Assays differed significantly from the negative control ($P < 0.05$). Out of all the assays, intracellular inhibition assay yielded the best antiviral activity. Number of plaques had statistically significant difference ($P < 0.05$) from the virus inactivation assay and inhibition of virus attachment assay.

DISCUSSION

Peste des Petits Ruminants (PPR) is a serious disease of sheep and goat causing huge economic losses every year. As PPR is a viral disease, there exists no particular treatment for the disease and post-exposure therapeutic approaches for infection are not described much in the literature (Balamurugan *et al*., 2014). The antiviral activities of *N. sativa* seeds against several kinds of virus were reported in previous literatures (Dorucu *et al*., 2009; Shewita and Taha, 2011; Ahmad *et al*., 2013; Umar *et al*., 2015, 2016). Numerous studies reports the use of *N. sativa* as liver tonics, anti-diarrheal, analgesics, and anti-bacterial. Recently, clinical and experimental studies have demonstrated many therapeutic effects of thymoquinone including immunomodulatory, anti-inflammatory, anti-tumor, and antimicrobial (Al-Mufarrej, 2014). Due to a number of different biological targets and no side effects, NS has attained the potential therapeutic interest to cure viral diseases involving immunosuppression (Umar *et al*., 2016). The result of the antiviral activity of extracts from *N. sativa* against PPRV showed *N. sativa* extracts possessed the antiviral activity according the viability of infected cell assay. Antiviral activity assay results showed that alcoholic dilutions of *N. sativa* 200, 100, 50, 25, 12.5 and 6.25 µg/ml showed 9.4%,7.4%, 80.4%, 77.7%, 75.3% and 59.8% cell survival percentage, respectively. More than 50% cell survival percentage of Vero cells occurred in four dilutions after virus infection. Similarly, alcoholic dilutions of *N. sativa* (200, 100, 50, 25, 12.5 and 6.25 µg/ml) showed 20%, 33.4%, 93.6%, 90.7%, 88.9% and 89.6 % cell survival percentage respectively for cytotoxic activity assay. Alcholic extract of *N. sativa* showed toxicity at 200 and 100 µg/ml to Vero cells. Extracts of medicinal plants showed antiviral activity against goat pox virus (Bhanuperkash *et al*., 2008). In another study, *N. sativa* showed significant antiviral activity against Murine cytomegalo virus (MCMV) (Salem and Hossain, 2000) as compared to controls. It can be assumed that *N. sativa* seed may contain active compounds that would be effective in treatment of PPR virus infections in goats.

To study the mode of action of 50 µg/mL *N. sativa* extracts, a sequence of plaque reduction experiments were done in the current study, including inhibition of virus attachment assay, virus inactivation assay, intracellular inhibition assay and pretreatment assay (Li *et al*., 2005; Harden *et al*., 2009). The decrease in plaques count per well denoted the antiviral activity of the test dilution of *N. sativa* extract. The findings of all these plaque reduction procedures revealed that 50 µg/mL of *N. sativa* extracts significantly reduced the number of plaques caused by PPRV in all the four modes of actions as compared to the negative control. Results revealed that the plaques in intracellular inhibition assay and pretreatment assay were significantly lesser than the rest of the test groups. It can be attributed to the ability of test drug to pass through cell membranes to enter into other cells, and protects

them from the virus induced injury and inhibits the viral activity more effectively with the assistance of cells. The results of this study suggested that *N. sativa* seed extracts are a potential source of antiviral materials effective in controlling PPRV *in vitro*. *N. sativa* seed extracts should also be studied in field experiments as a control measure for PPR. It may be suggested that *N. sativa* seed extracts posses anti-PPRV activity and play a significant role in diminishing the pathogenic effects of PPRV in goats. This study strengthens our belief that *N. sativa* seed extracts (50 µg/mL) is safe and useful antiviral agent against PPRV.

ACKNOWLEDGEMENTS

We are grateful to Dr. Muhammad Anees (Veterinary Research Institute, Lahore, Pakistan) for providing virus and technical help for this study

Statement of conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

REFERENCES

Abubakar, M., Arshed, M.J., Zahur, A.B., Ullah, F., Ishfaq, F. and Ali, Q., 2011. Post outbreak profile of Peste des petits ruminants (PPR) virus antibodies in relation with vaccination in recovered goats. *Pak. J. Life Soc. Sci.*, **9**: 169-171.

Ahmad, A., Husain, A., Mujeeb, M., Khan, S.A., Najmi, A.K., Siddique, N.A., Damanhouri, Z.A. and Anwar, F., 2013. A review on therapeutic potential of Nigella sativa: A miracle herb. *Asian-Pac. J. Trop. Biomed.*, **3**: 337-352. https://doi.org/10.1016/S2221-1691(13)60075-1

Al-Mufarrej, S.I., 2014. Immune-responsiveness and performance of broiler chickens fed black cumin (*Nigella sativa* L.) powder. *J. Saudi Soc. agric. Sci.*, **13**: 75-80.

Ali, S., Neubauer, H., Melzer, F., Khan, I., Akhter, S., Jamil, T. and Umar, S., 2017. Molecular identification of bovine brucellosis at selected private farms located in Pothohar Plateau, Pakistan. *Paistan J. Zool.*, **49**: 1111-1114.

Azeem, T., Rehman, Z., Umar, S., Asif, M., Arif, M. and Rahman, A., 2014. Effect of *Nigella sativa* on poultry health and production: A review. *Sci. Lett.*, **2**: 76-82.

Bailey, D., Banyard, A., Dash, P., Ozkul, A. and Barrett, T., 2005. Full genome sequence of Peste des Petits ruminants virus, a member of the *Morbillivirus* genus. *Virus Res.*, **110**: 119-124. https://doi.org/10.1016/j.virusres.2005.01.013

Balamurugan, V., Krishnamoorthy, P., Raju, D.S.N., Rajak, K.K., Bhanuprakash, V., Pandey, A.B., Gajendragad, M.R., Prabhudas, K. and Rahman, H., 2014. Prevalence of Peste-des-petits-ruminant virus antibodies in cattle, buffaloes, sheep and goats in India. *Virus Dis.,* **25**: 85-90. https://doi.org/10.1007/s13337-013-0177-5

Banyard, A.C., Parida, S., Batten, C., Oura, C., Kwiatek, O. and Libeau, G., 2010. Global distribution of Peste des Petits ruminants virus and prospects for improved diagnosis and control. *J. Gen. Virol.*, **91**: 2885-2897. https://doi.org/10.1099/vir.0.025841-0

Banyard, A.C., Wang, Z. and Parida, S., 2014. Peste des Petits Ruminants virus, Eastern Asia. *Emerg. Infect. Dis.*, **20**: 2176-2177. https://doi.org/10.3201/eid2012.140907

Bhanuprakash, V., Hosamani, M., Balamurugan, V., Gandhale, P., Naresh, R., Swarup, D. and Singh, R.K., 2008. *In vitro* antiviral activity of plant extracts on goatpox virus replication. *Indian J. exp. Biol.*, **46**: 120-127.

Diallo, A., 2003. Control of Peste des Petits ruminants: Classical and new generation vaccines. *Dev. Biol. (Basel),* **114**: 113-119.

Dorucu, M., Ozesen Colak, S., Ispir, U., Altinterim, B. and Celayir, Y., 2009. The effect of black cumin seeds, Nigella sativa, on the immune response of rainbow trout, *Oncorhynchus mykiss*. *Medit. Aquacult. J.*, **2**: 27-33. https://doi.org/10.21608/maj.2009.2667

Gialleonardo, L., Migliaccio, P., Teodori, L. and Savini, G., 2011. The length of BTV-8 viraemia in cattle according to infection doses and diagnostic techniques. *Res. Vet. Sci.*, **91**: 316-320. https://doi.org/10.1016/j.rvsc.2010.12.017

Harden, E.H., Falshaw, R., Carnachan, S.M., Kern, E.R. and Prichard, M.N., 2009. Virucidal activity of polysaccharide extracts from four algal species against herpes simplex virus. *Antiviral Res.*, **83**: 282-289. https://doi.org/10.1016/j.antiviral.2009.06.007

Hosseinzadeh, H., Tafaghodi, M., Mosavi, M.J. and Taghiabadi, E., 2013. Effect of aqueous and ethanolic extracts of *Nigella sativa* seeds on milk production in rats. *J. Acupunct Meridian Stud.*, **1**: 18e23

Hosseinzadeh, H., Taiari, S. and Nassiri-Asl, M., 2012. Effect of thymoquinone, a constituent of *Nigella sativa* L. on ischemia-reperfusion in rat skeletal

muscle. *Naunyn-Schmiedeberg Arch. Pharmacol*., **385**: 503-508.

Li, Y., But, P.P. and Ooi, V.E., 2005. Antiviral activity and mode of action of caffeoylquinic acids from *Schefflera heptaphylla* (L.) Frodin. *Antiviral Res*., **68**: 1-9. https://doi.org/10.1016/j.antiviral.2005.06.004

Parida, S., Muniraju, M., Mahapatra, M., Muthuchelvan, D., Buczkowski, H. and Banyard, A.C., 2015. Peste des petits ruminants. *Vet Microbiol*., 1**8**1: 90-106. https://doi.org/10.1016/j.vetmic.2015.08.009

Salem, M.L. and Hossain, M.S., 2000. Protective effect of black seed oil from *Nigella sativa* against murine cytomegalovirus infection. *Int. J. Immunopharmacol*., **22**: 729-740. https://doi.org/10.1016/S0192-0561(00)00036-9

Shewita, R.S. and Taha, A.E., 2011. Effect of dietary supplementation of different levels of black seed (*Nigella sativa* L.) on growth performance, immunological, hematological and carcass parameters of broiler chicks. *World Acad. Sci. Eng. Technol.*, **77**: 788-794.

Truong, T., Boshra, H., Embury-Hyatt, C., Nfon, C. and Gerdts, V., 2014. Peste des Petits ruminants virus tissue tropism and pathogenesis in sheep and goats following experimental infection. *PLoS One*, **9**: e87145. https://doi.org/10.1371/journal.pone.0087145

Ulasli, M., Gurses, S.A., Bayraktar, R., Yumrutas, O., Oztuzcu, S., Igci, M., Igci, Y.Z., Cakmak, E.A. and Arslan, A., 2014. The effects of *Nigella sativa* (Ns), *Anthemis hyalina* (Ah) and *Citrus sinensis* (Cs) extracts on the replication of coronavirus and the expression of TRP genes family. *Mol. biol. Rep*., **41**: 1703-1711. https://doi.org/10.1007/s11033-014-3019-7

Umar, S., Munir, M.T., Subhan S., Azam, T., Nisa, Q., Khan, M.I., Umar, W., Rehman, Z., Saqib, A.S. and Shah, M.A., 2016. Protective and antiviral activities of *Nigella sativa* against avian influenza (H9N2) in turkeys. *J. Saudi Soc. agric. Sci*., https://doi.org/10.1016/j.jssas.2016.09.004

Umar, S., Rehman, A., Younus, M., Shahzad, M., Nisa, Q., Yaqoob, M., Ali, M. and Shah, A., 2015. Effects of *Nigella sativa* on immune responses and pathogenesis of avian influenza (H9N2) virus in turkeys. *J. appl. Poult. Res*., **25**: 95-103. https://doi.org/10.3382/japr/pfv070

Xu, J., Song, X., Yin, Z.Q., Cheng, A.C., Jia, R.Y., Deng, Y.X., Ye, K.C., Shi, C.F., Lv, C. and Zhang, W., 2012. Antiviral activity and mode of action of extracts from neem seed kernel against duck plague virus *in vitro*. *Poult. Sci*., **91**: 2802-2807. https://doi.org/10.3382/ps.2012-02468