Research Article

Amelioration of Immune Response Induced Cytokine Imbalance by MERS-CoV Antigen in *Gallus gallus domesticus* Model by Ethanolic Extract of *Nymphaea caerulea*

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ABSTRACT

Background: Middle East Respiratory Syndrome Coronavirus (MERS-CoV) surfaced in late 2012 and was responsible for widespread infection and death throughout the globe. According to research reports, there is no potential medicine or vaccination available for the treatment or prevention of MERS-CoV. **Aim:** Therefore, in this particular study, we intended to explore the anti-viral activity of *Nymphaea caerulea* flower extract against MERS-CoV spike protein antigen-induced pathogenicity in fertilized egg model of *Gallus gallus domesticus* in terms of homeostasis of cytokine imbalance and morphological changes. **Materials and Methods:** Different experimental sets were prepared with 14th day fertilized chick eggs and they were aseptically harvested on the 16th day. Allantoic fluids were collected to study relative fold change in gene expression assay of targeted cytokines-Interferons (IFN) α, β, γ and Interleukins-IL-6, IL-8, IL-10, IL-1β concerning β actin. The ethanolic extract of *N. caerulea* was also scanned in the UV-vis range to identify the phytochemicals. **Results:** The well-known delayed stimulation of the IFN-α showing anti-viral activity following the MERS virus was found marginally improved by blue lotus extract. Moreover, the pro-inflammatory effect of cytokine IL-8 was found markedly suppressed by the blue lotus extract in the preventive set. Up-regulated IL-1β gene expression by MERS antigen was also significantly suppressed both in the curative and preventive sets. **Conclusion:** Thus, this novel study revealed many cytokine gene expression homeostasis by ethanolic extract of *N. caerulea* flower against MERS virus antigen-induced immune-pathogenic changes.

Keywords: Middle East respiratory syndrome corona virus, *Nymphaea caerulea,* Cytokines, *Gallus gallus domesticus.*

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INTRODUCTION

SARS-CoV-2 surfaced during December 2019 and produced catastrophic consequences throughout the globe. The infection is characterized by Cytokine Storm (CS) in many cases leading to Acute Respiratory Distress Syndrome (ARDS) and collateral damage to the tissues in different organs.[1] However, before that in 2012, another severe acute respiratory syndrome-producing Coronavirus Middle East respiratory syndrome coronavirus (MERS-CoV) caused the deaths of 858 infected persons in 27 countries (WHO, MERS-CoV bulletin).[2] However, there are reports showing differences in the CS profile between SARS-CoV and MERS-CoV, though the reason behind this phenomenon is still not clear. CS causes extensive damage to different tissues by hyperactivation of immune cells and elevated levels of freely circulating cytokines.[3] A similar pathophysiological condition also occurs in the terminal stages of many other diseases

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including cancer, fulminating infections, advanced auto-immune conditions, and monogenic disorders.[3]

MERS-CoV enters the host system, through a special receptor known as Dipeptidyl Peptidase-4 (DPP4) which cleaves some immunoregulatory peptides, releases chemotactic factors, promotes tethering and movement of innate immune cells and thus plays the key role in the activation of the acquired innate immune system signaling pathway among the infected patients.[4] It was conceptualized that with the addition of potent adjuvants along with the viral antigen, one may trigger the anti-viral response specifically against the MERS virus and initiate the production of neutralizing antibodies.[5,6] As mentioned by Mubarak *et al.*, there is no potential medicine or vaccination available for the treatment or prevention of any future catastrophe by MERS-CoV. So, research in this field is considered to be of utmost importance.[7]

In the case of MERS-CoV, the virus causes infection and replicates within the dendritic cells, monocyte–macrophage, and activated T cells resulting in apoptosis and up-regulation of cytokines genes and resulting CS by IL-6, IL-8, and IFN alpha.[8]This single-stranded RNA virus causes respiratory disease by binding to DPP4 present upon the surface of human Alveolar Epithelial Cells (AECs) via the S1protein and in this way infects the host.[7] With the MERS-CoV virus infection, there are also elevated responses of IFN γ and IL-1β, however, the immune reaction is somewhat delayed.[8] Past scientific literature has revealed that the serum levels of cytokines and chemokines among MERS virus-infected patients depict a positive correlation with the number of monocytes and neutrophils in their Peripheral Blood (PB).[8]

Nymphaea is a group of aquatic plants with an array of traditional medicinal values against several life-threatening diseases along with its edible values. They comprise many distinctive biochemical moieties that can act as leading molecules for treating many human diseases.[9] Ecologically they are found in large numbers along the banks of River Nile, the growth of "sacred blue lotus" (*Nymphaea caerulea*). According to the architectural designs, the Egyptians used to reverence varied parts of lotus, thus the name "sacred blue lotus" was popular in ancient times. In Western Europe, the horticulture of lotus was initiated by Sir Joseph Banks in the year 1787. At present, this plant is also present in many other countries including India.[10]

In the present study, we aimed to explore the anti-viral activity of "sacred blue lotus" (*Nymphaea caerulea*) extract against MERS-CoV spike protein-induced pathogenicity in fertilized egg model of *Gallus gallus domesticus* in terms of homeostasis of cytokine storm and morbid anatomy changes.

MATERIALS AND METHODS

Collection of *Nymphaea caerulea* flower

The flower (Figure 1a) was grown in the horticulture garden of Heritage Institute of Technology, Kolkata by specialized trained person and used after confirmation by a Botanist. Thereafter, the full bloom flower was collected for the research study.

Procurement of protein of MERS-CoV

MERS-CoV recombinant Spike RBD Protein with His tag was produced by the Human Embryonic Kidney (HEK293) cells expression system. The target protein was expressed with sequence (Glu367-Tyr606) of MERS-CoV Spike RBD (Accession#AFS88936.1) fused with a 6 x His tag at the C-terminus. It was procured from Abclonal, USA (Lot number: 9601120201; Catalog number: RP01311).

Extraction of *Nymphaea caerulea* flower

The petals and pollens of freshly collected flowers were separated and cut into small pieces. Extraction was carried out in a 1:10 (w/v) ratio of petals and pollens as one gram and 70% ethanol (Molecular Biology grade) as 10 mL for 72 hr in dark condition. After 72 hr, the supernatant was sieved through Whatman filter paper No. 1 and the filtrate was passed through 0.22 micron syringe filter for storage. The sterile filtrate was stored in an amber-coloured air tight glass bottle at 4°C (Figure 1b).[11]

Eggs collection and inoculation

14th day fertilized chick eggs were purchased from the State Poultry Farm, Kolkata. The surfaces of the eggs were cleaned with distilled water and cotton. The air sacs of the eggs were marked with a marker pen after candling. This process also differentiates the dead ones

Figure 1a: *Nymphaea caerulea* (Blue lotus) flower.

Figure 1b: Ethanol extraction of blue lotus-petals, pollens and calyx.

from the live embryos. Five experimental sets were prepared: Control normally fertilized eggs (3 such); fertilized eggs inoculated with 100 µL MERS virus antigen (3 such); fertilized eggs inoculated with 100 µL blue lotus extract (3 such); pre-treatment set-fertilized eggs inoculated with 100 µL blue lotus extract then challenged by 100 µL MERS virus antigen with one hour gap in between (3 such); Post-treatment set-fertilized eggs inoculated with 100 µL MERS virus antigen then challenged by 100 µL blue lotus extract with one hour gap in between(3 such).

The eggs were incubated at 38ºC with 60-80% humidity for 48 hr.[12]

Harvesting of eggs and allantoic fluid collection

Eggs were kept at 4ºC for 2 hr before harvesting. Harvesting was done with sterile scissors and forceps and 5 mL (approx) of allantoic fluid was collected in a sterile 15 mL falcon tube. The fluids were stored at -80ºC for further molecular biology assay.[12]

Molecular Biology Assay

Total RNA was extracted from the allantoic fluid using RNA isoplus (TAKARA, USA; Cat. No. 9108; Lot No. ALZ1011N). Following extraction, RNA was dissolved in nuclease free water at 56ºC in a water bath and then quantified using A260/A280 ratio via UV-vis spectrophotometer (Carrywin 60 UV-vis, Agilent, Singapore). cDNA was synthesized from isolated RNA with is crypt reverse transcriptase kit following the protocol (Bio-Rad, USA; Cat. No. 1708841; Batch No. 64449565) with T100 conventional PCR (Bio-Rad, USA). 2 µL of cDNA was mixed 18 µL of SYBR green master-mix for RT-PCR analysis (Bio-Rad CFX-96 model, USA; Cat. No. 1725121; Batch No. 64464415). Relative fold change in gene expression analysis was done using $2^{-(\Delta\Delta ct)}$ formula by keeping β-actin as the control house-keeping gene.[12,13]

Morbid Anatomy Study

After harvesting the embryos were studied in detail to note down any morphological changes with comparison to the control set.[13]

UV-Vis spectrophotometer Study

The 70% ethanol extract of *N. caerulea* flower was scanned within the range of 200-800 nm by UV-vis spectrophotometer (Carrywin 60 UV-vis Spectrophotometer, Agilent, Singapore) to study the probable phytochemical constituents. The crude extract was diluted in 1:1 ratio with 70% ethanol before scanning.[14]

Statistical Analysis

The statistical analyses were done using the statistical tool GraphPad Prism version 9.5.3. Two-way ANOVA was carried out on the data to analyze the variance between the column factors and row factors.

RESULTS

Cytokine Study

The findings reveal that there is no such noticeable change in the gene expression of IFN α among the different experimental sets with respect to the control set except the control alcohol set in which it was significantly decreased. There is increased IFN β gene expression in the alcohol (vehicle) control set (~15 times) and in the MERS-CoV antigen control set (~3.5 times) with respect to the control set; whereas in the curative and preventive set, the gene expressions were suppressed in relation to the antigen control. We observed significantly increased gene expression of IFN γ was present in the alcohol control set, but it was markedly decreased in preventive and curative sets in relation to the MERS-CoV antigen set. There is no significant change of IL-6 gene expression among the different experimental sets except the ethanol (vehicle) control set. MERS-CoV antigen increased (~462 times) gene expression of IL-8, however, it was suppressed in the preventive set (~39 times) and also to some extent in the curative set (~276 times). Directly the blue lotus extract control increased IL-8 gene expression ~100 times. We also observed that there was up-regulation of IL-10 up to 3.77 times with MERS-CoV antigen; however, it

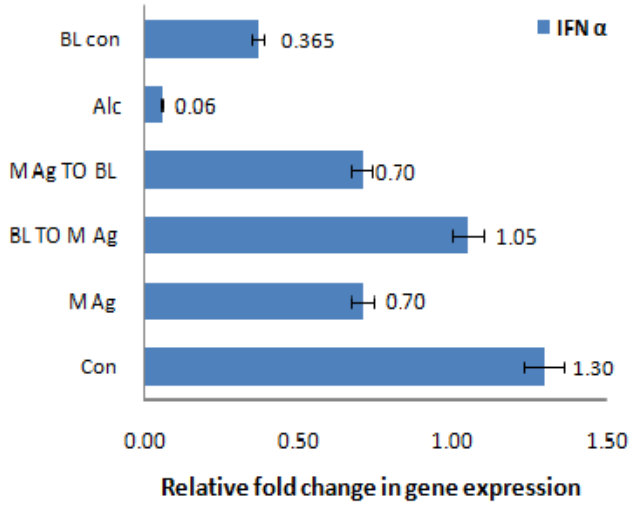


Figure 2a: There is no such noticeable change in the gene expression of Interferon alpha (IFN-) among the different experimental sets with respect to the control set except the control alcohol set in which it was significantly decreased.

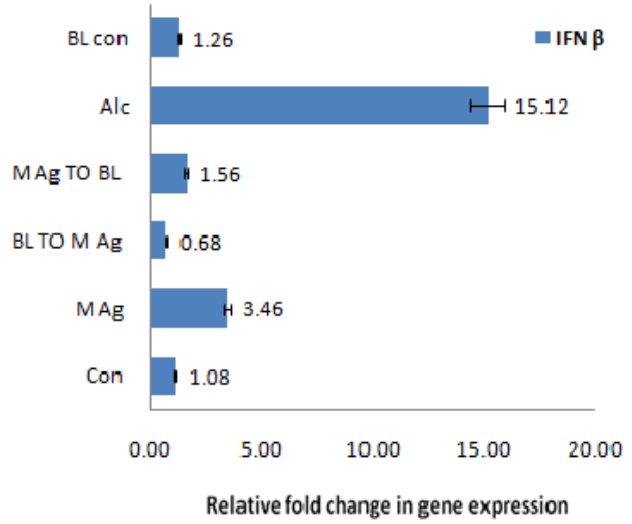


Figure 2b: Increased Interferon β (IFN- β) Gene expression in the alcohol (vehicle) control set (~15 times) and in the MERS-CoV antigen control set (~3.5 times) with respect to the control set; whereas in the curative and preventive set the gene expressions were suppressed in relation to the antigen control.

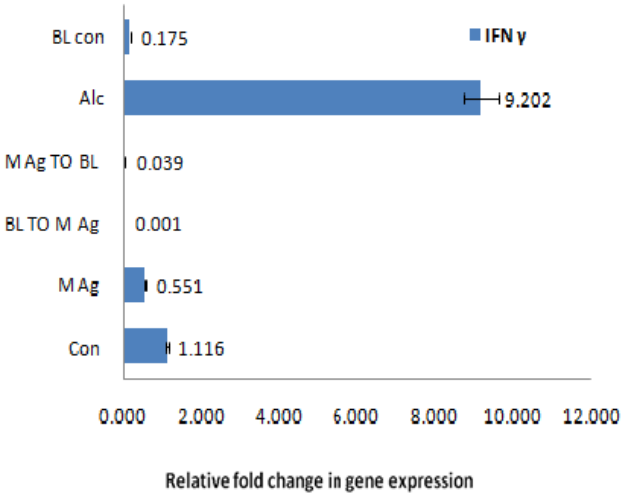


Figure 2c: Significantly increased gene expression of Interferon gamma (IFN-) was present in alcohol control set, but it was markedly decreased in preventive and curative sets in relation to the MERS-CoV antigen set.

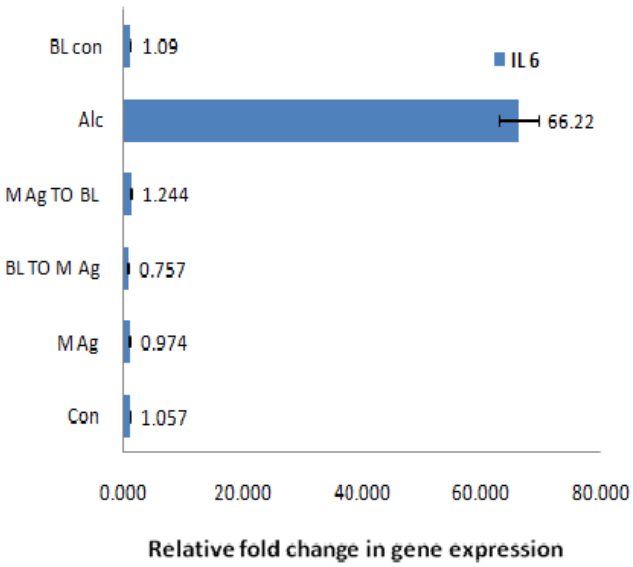


Figure 2d: There is no significant change of Interleukin-6 (IL-6) gene expression among the different experimental sets except ethanol (vehicle) control set.

was decreased both in the preventive and curative sets. In the case of IL-1β the gene expression got up-regulated with MERS-CoV antigen (~96 times), but it was significantly suppressed in the curative set (6.32 times) and in the preventive set (~29 times) (Figure 2a-2g and 3).

Morbidity Study Findings

With MERS-CoV antigen, there was extensive damage to different parts of the embryo including different tissue and organs and due to this the embryo was shrunken in general along with a significantly increased amount of haemorrhagic areas; however, in other experimental sets, no such findings were present. In preventive and curative sets the appearance of the embryo was quite normal (Figure 2h).

UV-vis spectrophotometer

Probable compounds identified by scanning the ethanolic extract of blue lotus were quercetin, rutin, gallic acid, vanillic acid, epi-gallocatechin, and paeoniflorin (Figure 2i and Table 1).[15,16]

Statistical Analysis

The row factors are different experimental sets and the column factors are the cytokine parameters. Both the

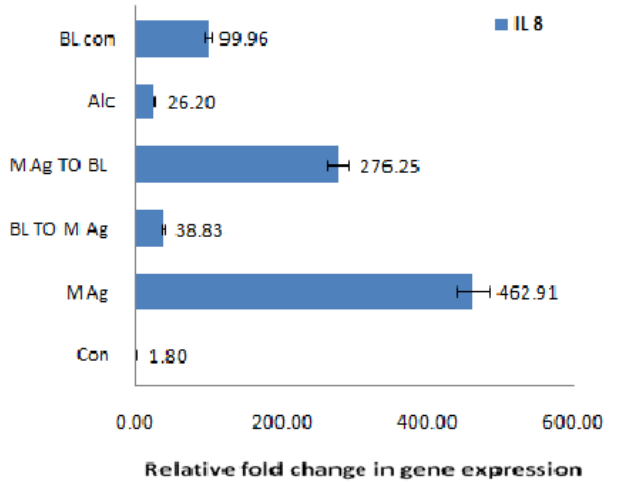


Figure 2e: MERS-CoV antigen increased (~462 times) gene expression of Interleukin-8 (IL-8), however, it was suppressed in the preventive set (~39 times) and also to some extent in the curative set (~276 times). Directly the blue lotus extract control increased IL-8 gene expression ~100 times.

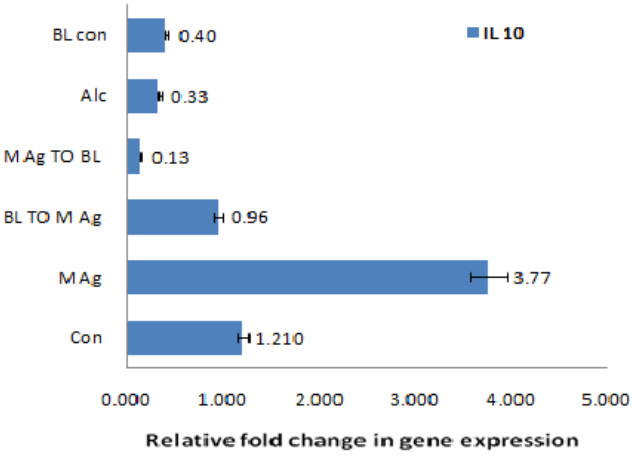


Figure 2f: Up-regulation of Interleukin-10 (IL-10) up-to 3.77 times with MERS-CoV antigen; however, it was decreased both in the preventive and curative sets.

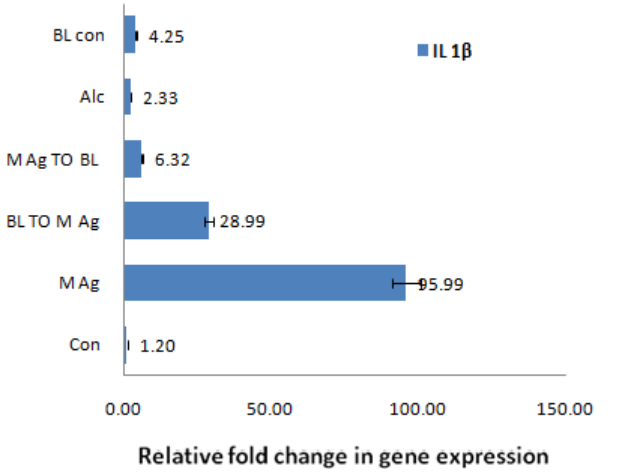
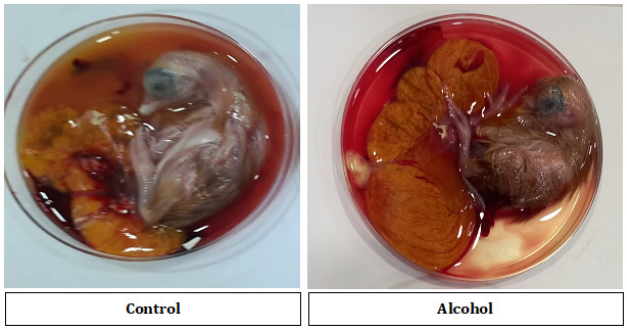
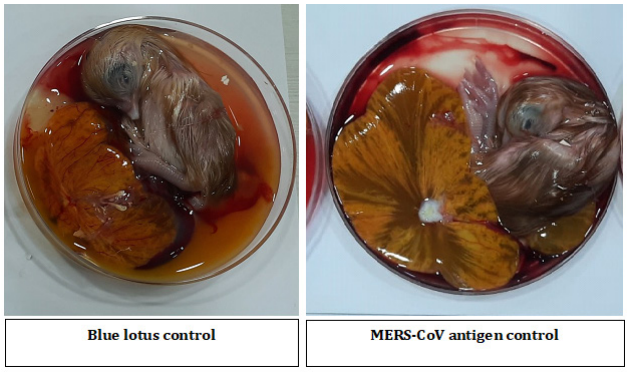


Figure 2g: In case of Interleukin-1β (IL-1β) the gene expression got up-regulated with MERS-CoV antigen (~96 times), but it was significantly suppressed in the curative set (6.32 times) and in the preventive set (~29 times).

Morbid Anatomy Findings





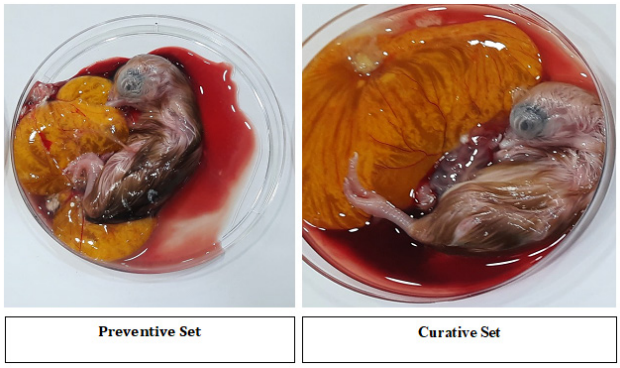


Figure 2h: With MERS-CoV antigen there was extensive damage of different parts of the embryo including different tissue and organs and due to this the embryo was shrunken in general along with significantly increased amount of haemorrhagic areas; however, in other experimental sets no such findings were present. In preventive and curative sets the appearance of the embryo was quite normal.

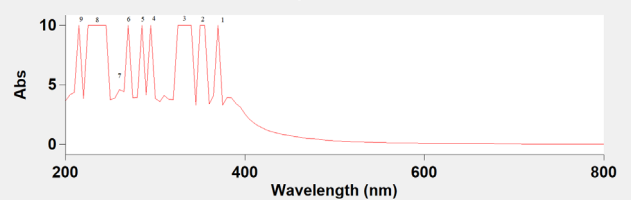


Figure 2i: UV-vis scanning (200-800) nm of the 70% ethanolic extract of *Nymphaea caerulea* flower.

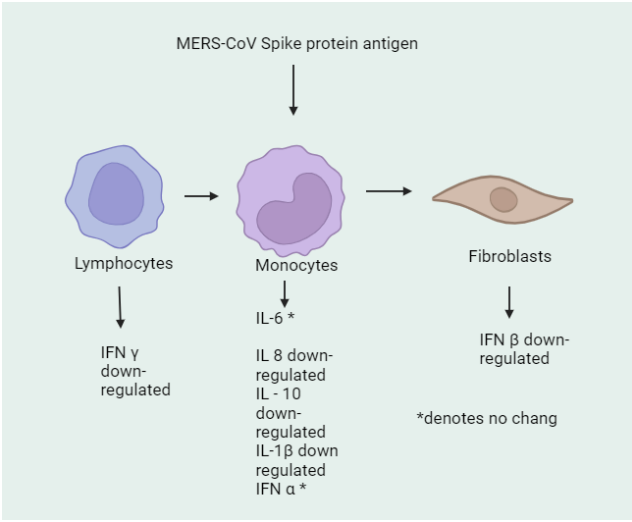


Figure 3: Pictorial representation of effect of blue lotus extract against MERS-CoV spike protein antigen induced pathogenic changes.

Table 1: UV-vis scanning (200-800) nm findings.

Sl. No.	UV-vis spectrum peak (Lambda max nm)	Probable compounds Identified
1	370 (10.00)	Quercetin
2	355 (10.00)	Rutin
3	340 (10.00)	Band B (quercetin)
4	295 (10.00)	Vanillic acid
5	285 (10.00)	Epi-gallocatechin
6	270 (10.00)	Quercetin
7	260 (4.590)	Paeoniflorin
8	245 (10.00)	Band A (quercetin)
9	215 (10.00)	Gallic acid

(Source[15,16])

interaction between the row factors and column factors are found to be statistically significant at *p*-value <0.0001 (Table 2).

DISCUSSION

There is altered cytokine and chemokine profile after infection with MERS-CoV. Scientific studies report that with the infection of MERS-CoV, there is an attenuated Interferon response and no stimulation of inflammatory cytokine at the time of early phase of infection.[8] However, it became a matter of concern for the physician about the rising number of deaths from the infection of MERS-CoV due to the altered cytokine profile associated with it in later phases. Past experimental studies have shown that there is stimulation of IL-6, IL-8 and IL-1β cytokines post 30 hr of infection with MERS-CoV when compared against SARS-CoV in Calu-3 cells. The attenuated IFN β response was also confirmed in the protein measurement of the culture supernatant of Calu-3 cells. Thus, the authors confirmed that there is a delay in the induction of pro-inflammatory cytokines by MERS-CoV infection.[8]

In this experiment we studied cytokines after 48 hr in the chick egg model, within this time IL-10 and IL-1β gene expression changes occur perfectly, however, other gene expressions may not properly gear up within this short time. Thus, in this study, we also observed uniform gene expressions for these cytokines. However, as the immune system of chick embryo is immature the other cytokine gene expressions against the viral antigen may not be properly controlled and we may expect lots of uncontrolled gene expressions in this condition.[17]

Table 2: Statistical Analysis.

Table Analyzed	Data 1				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	**% of total variation**	***p* value**	***p* value summary**	**Significant?**	
Interaction	51.22	<0.0001	****	Yes	
Row Factor	38.05	<0.0001	****	Yes	
Column Factor	10.54	<0.0001	****	Yes	
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	***p* value**
Interaction	439551	30	14652	F (30, 84) = 774.9	*p*<0.0001
Row Factor	326573	6	54429	F (6, 84) = 2879	*p*<0.0001
Column Factor	90456	5	18091	F (5, 84) = 956.8	*p*<0.0001
Residual	1588	84	18.91		
Data summary					
Number of columns (Column Factor)	6				
Number of rows (Row Factor)	7				
Number of values	126				

The surface proteins of the MERS-CoV are present upon the envelope of the virus, Spike protein (S), the Envelope protein (E) and Membrane glycoprotein (M).[4] The special DPP4 receptor not only gives the virus access to the host cellular system but also causes immune suppression of the host which helps the virus to replicate and spread the infection.[6] Dendritic cells are the key players in the development of innate immunity and produce a huge quantity of cytokines and chemokines directly and through activation of other inflammatory cells, they migrate from peripheral to lymphoid tissue and thus activate the T cell population.[18] Therefore, dendritic cells act as a bridge between innate and adaptive immunity. It is very interesting to note that the MERS virus has developed immune dodging strategies and they maneuver the innate immunity to block the production of IFNs which is actual cause behind the high rising fatality by MERS virus infection among the immune compromised patients, in our experiment in immune immature chick embryo.[19,20] TLR 4 identifies the S protein of the virus and causes stimulation of the pro-inflammatory cytokines through the MyD88 signaling pathway which helps in controlling the viral dissemination, though there are certain drawbacks such as pathological damage to tissues.[21]

Interferons (IFN α, β, γ)

As discussed earlier, after infection with MERS-CoV there is an attenuated interferon response and no stimulation of inflammatory cytokine at the time of early phase of infection. They manipulate the innate immunity to block the production of IFNs which is actual cause behind the high rising fatality by MERS virus infection.[8] Our data revealed that there is no such noticeable change in the gene expression of IFN α among the different experimental sets with respect to the control set except the control alcohol set in which it was significantly decreased. There is increased IFN β gene expression in the alcohol (vehicle) control set (~15 times) and in the MERS-CoV antigen control set (~3.5 times) with respect to the control set; whereas in the curative and preventive set, the gene expressions were suppressed in relation to the antigen control. We observed that significantly increased gene expression of IFN γ was present in the alcohol control set, but it was markedly decreased in preventive and curative sets in relation to the MERS-CoV antigen set (Figure 2a-2c; Table 2).

Interleukins (IL-6, IL-8, IL-10, IL-1β)

According to literature reports, there is elevated response of cytokines such as IL-6, IL-8 and IL-1β, however, the immune reaction is delayed. Past scientific literatures have revealed that the serum levels of cytokines and chemokines among MERS virus infected patients depict a positive correlation with the quantity of monocytes and neutrophils in their peripheral blood (PB).[8, 22] Our data revealed that there is no significant change in IL-6 gene expression among the different experimental sets except the ethanol (vehicle) control set. Similar to patients infected with SARS-CoV-2, MERS-CoV is also responsible for elevated production of IL-6 which causes aggravation in lung damage along with viral inflammatory response and death. However, according to another research study, the S protein of the MERS virus does not lead to over production of IL-6 which gets elevated only during the time of active viral replication within the macrophages.[23] Thus here in our data also we could not see any elevated gene expression of IL-6 with spike protein inoculation of the MERS virus and the expression was in control both in the preventive and curative sets when compared with antigen control.

In case of IL-8, it has a direct role in the activation of neutrophil and is responsible for the pathogenesis and disease progression.[24] Our findings showed that with the inoculation of MERS antigen there is ~462 times up-regulation of the gene expression of Interleukin-8 (IL-8), which got immensely suppressed in the preventive set (~39 times) and also to some extent in the curative set (~276 times). Thus, this is an extremely important finding where the damage induced by pro-inflammatory cytokine IL-8 could be immensely suppressed by the blue lotus extract in the preventive set. Therefore, blue lotus extract has a good immune-modulatory action.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine which is observed to be elevated among the COVID-19 patients. The cytokine limits the immune system of the host which prevents damage to the tissues and preserves the homeostasis of the tissues. It works via the negative feedback loop to restrain the inflammation.[25] According to Mahallawi *et al.*, 2018 infection by MERS-CoV enhances the gene expression of IL-10. IL-10 follows the JAK-STAT pathway to restrain the inflammation.[25,26] Our data showed that there was an up-regulation of IL-10 up to 3.77 times with the inoculation of MERS antigen however, it decreased both in the preventive and curative sets with blue lotus extract. As there is no up-regulation of IL-6 among the experimental sets, there is possibly no requirement for IL-10 to balance the inflammatory reaction. Thus, the enhanced level of IL-10 was balanced by blue lotus extract in both the preventive and curative sets.

Interleukin-1β or IL-1β is another pro-inflammatory cytokine and it is activated resulting in inflammation. During the infection by MERS-CoV, there is enhanced level of IL-1β.[27] Here our experimental findings suggest that IL-1β the gene expression got up-regulated with the inoculation of MERS antigen (~96 times) which got highly suppressed in the curative set (6.32 times) and ~29 times suppressed in the preventive set when compared with the control set. Here, the curative effect of blue lotus extract was highlighted (Figure 2d-2g; Table 2).

A study on the anti-viral activity of methanol and acetone extracts of *Nymphaea alba* and also its purified phytochemicals namely Hyperoside, Isoquercetin, Quercetin, Apigenin, Reynoutrin, and Isokaempferide reported to have antiviral activity and might serve as an effective alternative course of therapy against hepatitis C virus (HCV); thus it might be prescribed a synergistic combination with other anti- HCV agents.[28] When studied about the phytochemical constituents of *N. caerulea*, there are seven flavonols reported by Fossen *et. al.,* 1999 among them the novel one is 3-(2"-acetylrhamnosides) of quercetin and myricetin. Some other rare bioactive constituents are quercetin 3-(3"-acetylrhamnoside) and kaempferol 3-(2"-acetylrhamnoside), along with 3-rhamnosides of kaempferol, quercetin with the aid of chromatography, two-dimensional NMR, and electrospray Mass spectrometry. [29] We identified the probable compounds identified by scanning the ethanolic extract of blue lotus were quercetin, rutin, gallic acid, vanillic acid, epigallocatechin, and paeoniflorin which might be responsible for its biological efficacy.

CONCLUSION

Ethanolic extract of *Nymphaea caerulea* flower was observed to have both curative and preventive effects against MERS virus induced pathogenicity. Further detailed study on the bioactive composition of the flower via mass spectrometry is required to target the potential compounds within the extract and exploring the anti-viral activity of the compounds against MERS-CoV. Thus, this is a novel study on the anti-viral activity of ethanolic extract of *Nymphaea caerulea* flower against MERS-CoV to date.

ETHICAL CLEARANCE

As the study was conducted on 14th day old embryonated hen cell eggs and harvesting was completed with 16th day, therefore there is no requirement of ethical clearance from the Institutional Ethics Committee. However, ratification was obtained from the IEC before the initiation of the research study on 22.07.2021.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MERS-CoV: Middle East respiratory syndrome coronavirus; **IFN:** Interferons; **IL:** Interleukins; **SARS-CoV-2:** Severe Acute Respiratory Syndrome Coronavirus 2; **CS:** Cytokine storm; **ARDS:** Acute Respiratory Distress Syndrome; **DPP4:** Dipeptidyl Peptidase-4; **AECs:** Alveolar Epithelial Cells; **RBD Protein:** Recombinant Binding Domain protein; **TLR:** Toll-like receptors; **MyD88:** myeloid differentiation primary response 88; **JAK-STAT:** Janus kinase/signal transducers and activators of transcription; **NMR:** Nuclear Magnetic Resonance.

FUNDING

There was no source of funding.

PATIENT CONSENT

Not Applicable

SUMMARY

From the present study, it can be summarized that ethanol extract of *Nymphaea caerulea* flower possess anti-viral efficacy against MERS-CoV both in the preventive and curative sets in terms of cytokine parameters and morbid anatomy findings. The phytochemical analysis revealed the presence of certain bioactive constituents which might be responsible for the anti-viral activity. Thus, this is a novel work on the anti-viral efficacy of *Nymphaea caerulea* flower against MERS-CoV as no such past scientific confirmations are reported till date.

AUTHOR'S CONTRIBUTION

Author DC conducted the experiment, analyzed the findings and written the first draft of the manuscript. Author BS assisted during the experimental process. Author KP arranged the resources and provided the administrative support to carry out the research study.

Author SD conceptualized the experimental design, interpreted the entire findings and checked the final version of the manuscript.

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