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RESEARCH ARTICLE

Antiviral Effect of Bee Venom on Foot and Mouth Disease Virus (An *in-vitro* Study)

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ABSTRACT

Foot-and-Mouth Disease (FMD) is the most important contagious disease of cloven-hooved animals. The disease is known to causes huge economic losses to livestock production and trade; therefore, requiring the development of effective preventive and therapeutic interventions. Recently, Bee venom (BV) has been reported to exhibit antiviral activities against many enveloped and non-enveloped viruses. The antiviral properties of BV against Foot-and-mouth disease virus (FMDV) have not been enough investigated. Hence, in this work, we evaluated the inhibitory effects of BV against FMDV using cell-based virus inhibition assay, real time PCR and electron microscopy. Treatment of FMDV with BV caused significant (25.7%) reduction in virus titers suggesting a virucidal activity of BV. BV also caused a 20.8% reduction in virus titers when cells were treated with BV before infection suggesting antiviral state induction in cells. This finding was supported by increased interferon-gamma (IFNγ) levels in BV treated cells. These findings suggest BV could be used as a preventive or therapeutic agent.

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INTRODUCTION

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals. The disease is caused by the *foot-and-mouth disease virus (FMDV)*, which is a member of the family *Picornaviridae,* genus *Aphthovirus* (Grubman and Baxt, 2004). FMDV is a spherical (20-30 nm), non-enveloped, single-stranded, positive-sense RNA virus (Domingo *et al.,* 2003). The 8.4 kb single-strand RNA is enclosed within a protein capsid, both synthesized in the cytoplasm of the infected cells. The viral genome includes three parts, i.e. 5′ untranslated region (5′ UTR), middle coding region and 3′ UTR. The middle coding region is divisible into P1, P2 and P3 regions. The P1 region encodes leader proteinase (Lpro) and structural proteins VP4, VP2, VP3, and VP1. The P2 and P3 regions encode non–structural proteins (Mason *et al.,* 2003). The virus capsid is composed of 60 identical protomers, and each protomer contains four structural proteins VP1, VP2, VP3 and VP4 that are arranged into an icosahedral lattice (Logan *et al.,* 1993). FMDV is classified into seven serotypes i.e., A, C, O, Asia 1, SAT 1, SAT 2 and SAT 3, and each serotype may have multiple subtypes (Carrillo *et al.,* 2005).

FMDV is highly transmissible, spreads by aerosol. It is one of the most economically important diseases of livestock worldwide. FMD affects the livestock industry both directly and indirectly. Direct losses caused by FMD include loss of milk production, loss of weight and deaths among young animals, while indirect losses resulting from the additional costs of diagnostic tests, vaccines, movement control as well as international trade (Rweyemamu and Astudillo, 2002). Therefore, both control and preventive measures are needed. Traditionally, vaccines for FMD have been used as a preventive measure. However, infection/ vaccination with one serotype does not provide cross-protection against other serotypes, and sometimes very poor cross-reactivity is observed among subtypes within individual serotypes. Therefore, vaccinations inherit some drawbacks, mainly the matching of prevalent serotypes as well as the time needed to trigger the immune responses and type of adjuvant present in the vaccine (Jamal *et al.,* 2012). Since vaccination alone is not enough for the control or prevention of FMD, therefore, rapid and effective

control of FMD can be complemented by the use of antiviral compounds. Both *in vitro* and *in vivo* studies, though few, suggest that livestock could be protected against FMD within 24 hours (h) following antiviral treatment. Such prophylactic/therapeutic antiviral drugs could complement emergency vaccination and be applied to treat livestock in endemic and previously disease-free regions (Goris *et al*., 2008).

BV has been reported to have antiviral and anti-inflammatory effects due to a variety of compounds present in it, that include peptides such as melittin (MLT), adolapin, apamin, and mast cell degranulating peptide; enzymes such as phospholipase A2 (PLA2), biologically active amines such as histamine and epinephrine and non-peptide components such as carbohydrates, lipids, and free amino acids (Son *et al.*, 2007). *In vitro* and *in vivo* studies, though very few, have proven the antiviral properties of BV. These studies showed that co-incubation of non-cytotoxic amounts of BV with enveloped viruses (*Influenza A virus, Vesicular Stomatitis Virus, Respiratory Syncytial Virus,* and *Herpes Simplex Virus) as well as non-enveloped viruses (Enterovirus-71* and *Coxsackie Virus)* inhibited their replication Such antiviral properties were mainly explained by the virucidal mechanism (Uddin *et al.*, 2016). BV and its components have been shown to stimulate type I interferon (IFN), and therefore suppress viral replication in the host cell (Bachis *et al.*, 2010). PLA2 in BV has been shown to inhibit the replication of *vesicular stomatitis virus (VSV), coxsackie virus (H3), enterovirus-71 (EV-71), herpes simplex virus (HSV)* and *Adenovirus (AdV)* by blocking the attachment of the virus to cells (Hewawaduge *et al.*, 2016).

Many reports confirm that FMD is endemic in Egypt with three strains i.e. A, O and SAT 2. Keeping in view the economic impact of FMD in Egypt, we evaluated the *in vitro* antiviral activity of BV against FMDV. In this study, we used serotype O since it is one of the most prevalent serotypes in Egypt (El-Rhman, 2020). Additionally, we evaluated the interaction between BV and FMDV by using the Transmission Electron Microscopy. We have studied that BV could be used as a potential antiviral drug against FMDV.

MATERIALS AND METHODS

Virus, cell line and bee venom: *Foot-and-Mouth Disease Virus (FMDV- O Pan-Asia 2 Strain)* was obtained from the Veterinary Serum & Vaccine Research Institute, Egypt. Baby Hamster Kidney-21 (BHK-21) cells line was obtained from American Type Culture Collection (ATCC) and maintained in Minimal Essential Medium-Hanks (MEM-H) (Biowest, France) supplemented with 10% FBS and antibiotics. Bee venom of *Apis mellifera lamarckii* was obtained from Bee Keeping Department, Agriculture Research Center, Egypt. A stock solution of BV was prepared in sterile distilled water at 0.1 % and sterilized by filtration through 0.2 µ pore-size filter as described previously (Kamal, 2016).

***In vitro* cytotoxicity (CC_{50}) and effective concentration (EC_{50}) of BV:** The cytotoxicity of BV for BHK-21 cells was determined by quantifying the cell viability using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described previously (Berridge *et al.*, 2005). Briefly, a two-fold serial dilution of BV (100 µg/ml) was prepared in MEM-H (supplemented with 2% FBS) and added to confluent BHK-21 monolayers and incubated at 37°C for 24 h. The dilution medium without BV served as a negative control. Then cytotoxicity of BV was determined using the MTT assay kit (Sigma-Aldrich, USA) according to the manufacturer recommendations. The absorbance was measured at 570 nm wavelength using a microplate reader (Biotek-Elx-800, USA). The cytotoxicity values were calculated using the Masterplex-2010 software.

MTT assay also used to determine the EC_{50} of BV as described previously (Andrighetti-Frohner *et al.*, 2003). Briefly, a two-fold serial dilution of BV (100 µg/ml) was prepared in MEM-H (supplemented with 2% FBS), and 100 µl of each BV dilution was mixed with 100 µl of FMDV (6.74 log $TCID_{50}$/0.1ml) and incubated for 1 hour at 37°C. Then, 100 µl of the mixtures were added to BHK-21 monolayers in 96-well cell culture plate and incubated for 24 h at 37°C. Then, MTT assay was used as described previously for CC_{50} assay.

Virucidal and Antiviral assay of BV against FMDV: The assay was conducted as described previously (Uddin *et al.*, 2016). The virucidal activity was determined by co-incubation method. Briefly, 100 µl/well of BV (2.0 µg/ml) was mixed with 100 µl/ well of FMDV (6.74 log $TCID_{50}$/0.1ml) and incubated for 1 hour at 37°C. Then, the mixture (100µl/well) was added to BHK-21 monolayers (10^5 cells/ml) prepared in 96-well cell culture plates.

The antiviral activity was determined by pre-treatment method. Briefly, confluent BHK-21 monolayers were treated with BV at a concentration of 3.0 µg/ml (100 µl/well) for 24 h at 37°C. Then, BV was discarded and FMDV (6.74 log$TCID_{50}$/0.1ml) was added to the cells.

At 24 h and 48 h post-incubation, cells supernatants were collected and virus titers were determined. Un-treated, FMDV infected cells served as a positive control, while untreated-uninfected cells served as a negative control.

Detection of interferon-gamma (IFNγ) mRNA levels: Interferon-gamma (IFNγ) transcription levels were measured by real-time RT-PCR (Bio-Rad SYBR® Green Master Mix) in different treatment groups after 48 h incubation, i.e. BHK cells treated with BV only (3.0 µg/ml), BHK cells infected with BV (2.0 µg/ml) treated FMDV (6.74 logTCID50/0.1ml) (co-incubation method), and BHK cells pretreated with BV (3.0 µg/ml) and then infected with FMDV (6.74 logTCID50/0.1ml) (pre-treatment method). Untreated cells served as negative control while FMDV infected cells served as a positive control.

Briefly, the total RNA was extracted from the BHK-21 cells using Qiagen kit. One µg of extracted RNA was reverse transcribed to cDNA using PrimeScript™ RT Reagent Kit with gDNA Eraser following the manufacturer's instructions. Briefly, the reaction mixture was prepared by mixing 1 µL cDNA, 10 µL primers (Table 1) and 12.5 µL of SYBR® Green Master Mix to a final volume of 25 µL. The reaction was performed at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 56°C for 30 s,

and 72°C for 30s. The specificity of the reaction was analyzed by dissociation curve analysis. The expression levels of IFNγ mRNA genes were normalized against those of porcine β-actin mRNA, which served as an internal control. The relative gene expression was determined using the 2^ ΔΔCT method.

Examination of the interaction between BV and FMDV by Electron Microscopy: The BV and FMDV were examined by transmission electron microscopy (TEM) as described previously (Hayat, 1981). A drop of BV solution (0.1%), a drop of FMDV (6.74 $LogTCID_{50}$/0.1ml) and a drop of the BV and FMDV mixture were applied separately to copper grids (Cu-300-PELCO®) and left to air dry. Then a drop of 1.5% phosphotungstic acid was applied to the grids, incubated for 5 minutes and excess fluid was removed. The ultrastructure of BV and FMDV and the interaction between them were visualized by transmission electron microscope (JEOL JEM-1400 TEM, Korea). Images were captured by Aptronics camera.

We also evaluated the blocking effect of BV against FMDV by TEM. BHK-21 were pretreated with BV then infected with FMDV as mentioned in the pre-treatment method. The uninfected cells served as a negative control and the FMDV infected cells served as a positive control. BHK-21 cells were processed for transmission electron microscopy as described previously (Monaghan *et al.*, 2004). Cells were washed with PBS, then resuspended using Trypsin (Bio Basic Canada Inc. - Canada) and pelleted by centrifugation at 4°C. The cells in the pellet were fixated by glutaraldehyde and osmium tetroxide, dehydrated in alcohol and embedded in epoxy resin. Ultra-thin sections (75-90 nm thick) were stained with uranyl acetate and lead acetate, then examined by TEM and images were captured as stated earlier. The TEM work was done in TEM Lab in Faculty of Agriculture Research Park (FARP) – Cairo University.

RESULTS

CC_{50}, and EC_{50} of BV: Different concentrations of BV were tested on BHK-21 cells to determine the CC_{50} value, the concentration of BV at which 50% of cells remain viable. The CC_{50} was 6.044±0.37 µg/ml. Then we determined the EC_{50} of BV, the concentration at which FMDV treatment with BV caused 50% reduction in virus titers compared to the control (infected cells without BV) The EC_{50} was 0.698±0.04 µg/ml. Based on the EC_{50} and CC_{50}, max. 3 µg/ml of BV was used in *in vitro* experiments.

The selectivity index (SI) of the individual compound is defined by the ratio of CC_{50} over EC_{50}. The higher the SI ratio, the more effective and safer a drug would be during in treatment for a viral infection. The results showed that the SI value of BV was 8.66.

Virucidal effect and antiviral activity of BV against FMDV: To assess the virucidal activity of BV, FMDV was treated with BV and then the viral titers were determined and compared with un-treated viral titers. BV caused 10.7% and 25.7% reduction in FMDV titers at 24 h and 48 h, respectively compared to the control as shown in Table (2). These results suggested that BV reduced the infectivity of the FMD virus particles.

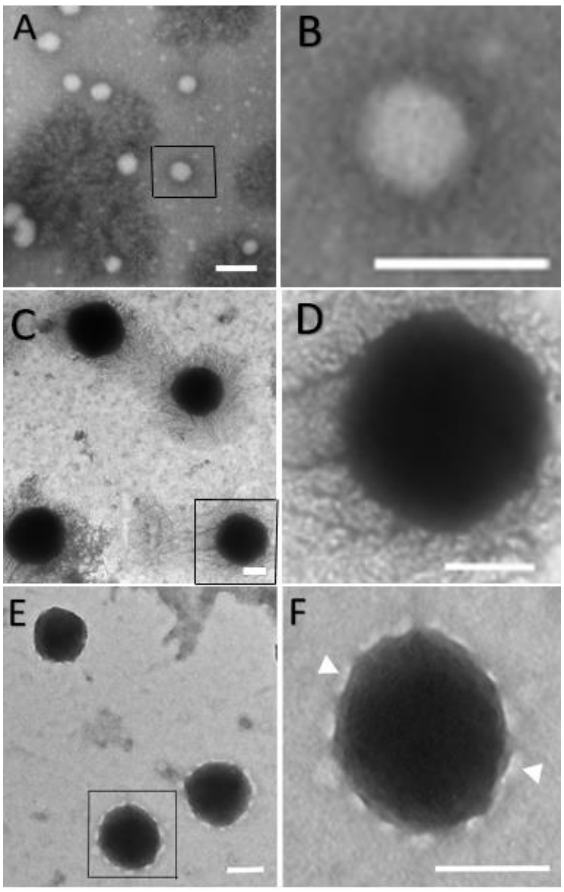

Fig. 1: Transmission electron microscopy (negative staining) of the interaction between BV and FMDV. (A) Image of FMDV particles. (B) Enlarged view of the squared area in "A" showing FMDV pentameric capsid. (C) Image of MLT particles in BV appearing as large, electron-dense molecules. (D) Enlarged view of the squared area in "C". (E) Image showing the interaction of BV with FMDV. (F) Enlarged view of the squared area in "E". The arrowheads showing the FMDV particles attached on the surface of MLT. (Scale Bar = 100 nm).

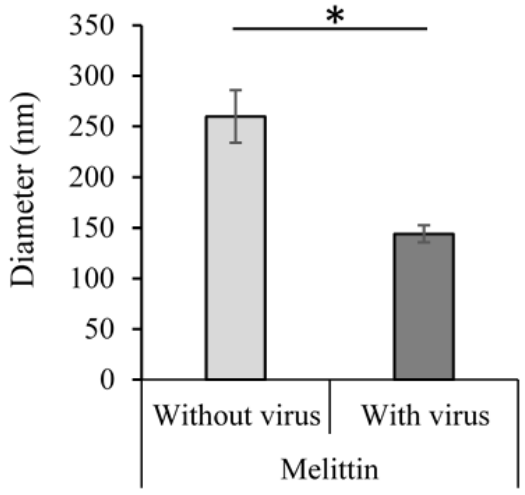

Fig. 2: Comparison of the size of naïve and FMDV treated melittin particles. The size of melittin alone or the melittin mixed with FMDV was measured and compared. The interaction between MLT particles and FMDV particles caused a significant reduction in MLT size, P<0.05.

To assess the antiviral activity of BV, cells were first treated with BV and then infected with FMDV. Interestingly, BV caused 11% and 20.8% reduction in virus titers at 24 h and 48 h respectively compared to the virus control as shown in Table 2.

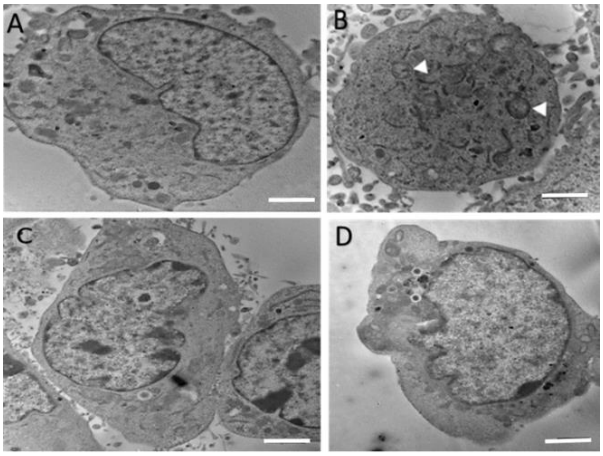

Fig. 3: Transmission electron microscopy of the BHK21 cells infected with FMDV in the presence or absence of pre-treatment with BV. (A) Uninfected BHK-21 cells (control cells). (B) BHK-21 cells infected with FMDV exhibiting CPE in the form of rounding of the cells and single and double membrane inclusion blebs (arrowheads) inside the cytoplasm. (C, D) BHK-21 cells pretreated with BV then infected with FMDV; the cells show minimal cytoplasmic changes. (Scale Bar = 1 μm).

Table 1: primers used in real-time PCR

Primer name	Primer sequence
IFNγ-F	5`-CTAATTATTCGGTAACTGACTTGA-3`
IFNγ-R	5`-ACAGTTCAGCCATCACTTGGA-3`
β-actin-F	5`-GTGACATCCACACCCAGAGG-3`
β-actin-R	5`-ACAGGATGTCAAAACTGCCC-3`

Table 2: Virucidal and antiviral activity of BV against FMDV at 24 and 48 hours post-infection

Treatment		viral titers (log 10)		%
		Mock*	BV treated	Reduction
Virucidal activity	24 h.p.i.	6.16±0.19	5.13±0.09	10.72
(Co-incubation)	48 h.p.i.	6.37±0.52	4.98±0.51	25.70
Antiviral activity	24 h.p.i.	6.16±0.19	5.89±0.80	11
(Pre-treatment)	48 h.p.i.	6.37±0.52	5.53 ±1.10	20.8

*un-treated, infected cells.

Table 3: INFγ mRNA levels in BHK-21 cells

Mode of Action	INFγ Fold Change
Negative control	1
Positive control	0.95
BHK cells treated with BV only*	2.76
Virucidal activity (Co-incubation)**	4.33
Antiviral activity (Pre-treatment)***	1.74

*3.0 μg/ml of BV: **FMDV (6.74 $logTCID_{50}$/0.1ml) mixed with BV at a concentration of 2.0 μg/ml, then BHK cells infected (co-incubation method): ***BHK cells first treated with BV at a concentration of 3.0 μg/ml, then infected with FMDV (6.74 $logTCID_{50}$/0.1ml) (pre-treatment method).

BV induces the IFNγ production: We also evaluated the effect of BV on the induction of IFNγ in BHK-21 cells by measuring INFγ mRNA levels. As shown in Table 3, BV caused more than 2.5 fold induction in the INFγ mRNA levels compared to untreated cells. This suggested that BV could induce antiviral state by stimulating the IFNγ production. The FMDV virus did not seem to suppress or induce INFγ. Interestingly, a 4.3 fold increase in IFNγ mRNA level was observed when BV-treated FMDV was applied to the cells. In contrast, only a 1.7 fold increase in INFγ levels was observed in cells pre-treated with BV and then infected with FMDV.

BV directly interact with the FMDV particles: To understand the nature of the interaction between BV and FMDV, both were mixed as stated in materials and methods, and electron micrographs were taken. Close examination of BV mixed with FMDV revealed virus-like particles bound to the surface of MLT particles (Fig. 1). Then, the size of MLT particles alone or mixed with FMDV were measured. The MLT particles appeared as large negatively stained molecules with an average diameter of 260 ±25 nm. Interestingly, about a 40% reduction in the size of MLT particles was observed when mixed with FMDV (Fig. 2).

The blocking effect of BV against FMDV in BHK Cells: Electron microscopy of the BHK-21 cells infected with FMDV 24 hours post-infection (h.p.i.) revealed that the cells exhibited CPE in the form of rounding of the cells and single and double membrane inclusion blebs inside the cytoplasm. (Fig. 3.B). Interestingly, BHK-21 cells treated with BV then infected with FMDV 24 h.p.i. showed minimal cytoplasmic changes (Fig. 3C & D). The blocking effect of BV may be due to the inhibition of the replication of FMDV by inhibiting the release of the viruses or blocking their attachment to the cell surface.

DISCUSSION

Recently, the potential of Bee Venom as a therapeutic or prophylactic agent has got the attention of researchers around the globe. Studies have shown the efficacy of BV against different types of cancer as well as anti-viral activity including human immunodeficiency virus (HIV) (Wehbe *et al.*, 2019). Since FMD is known to cause huge economic losses to the livestock industry, and no data regarding the therapeutic potential of BV against FMD is available, we explored the possible antiviral and virucidal activity of BV against FMD in this study.

The use of a candidate agent for therapeutic purposes relies on its safety for the host tissues. One way to assess the safety of such an agent is *in-vitro* cytotoxicity assay. The cytotoxicity of BV seems to vary with the type of cell line used. In one study, the CC_{50} of BV was 6.25 & 8.98 μg/ml for Hep2 and HeLa cell lines, respectively; and BV at a concentration of 2.0-3.0 μg/mL caused inhibition of different RNA and DNA viruses (Uddin *et al.*, 2016). On the contrary, another study reported 0.5 μg/mL of BV as a safe dose in Hep2 and MCF7 cell lines (Kamal, 2016). In the present study, CC_{50} of BV for BHK21 cell line was in agreement with these earlier reports. The cytotoxicity (CC_{50}) of the BV was calculated at 6.044 μg/ml for BHK-21 at 24 h post-treatment, whereas, the concentration for BV used in our experiments were 2.0 and 3.0 μg/ml. Therefore, it is reasonable to believe that reduction in virus titers was not a result of cytotoxicity of the BV. The SI value is reported to give better information on the safety of a test compound since CC_{50} and EC_{50} values might vary between different cell lines (Flamand *et al.*, 2014). In this study, the SI value was 8.66 that is better than the SI value reported previously for different cell lines.

Previous studies reported that the virucidal activity of BV varies depending on the type of virus. Treatment of Herpes simplex virus-1 with BV for 24 h was ineffective whereas treatment of Adenovirus type-7 with BV for 6 h caused a significant reduction in virus titers. Evaluation of the virucidal activity of BV against an RNA model virus, the West Nile virus (WNV), revealed a significant reduction in WNV infectivity 48 hours post-treatment (Ramadan *et al.*, 2009). Other studies also reported virucidal

activity of BV against both enveloped and non-enveloped viruses including rift valley fever virus, herpes simplex virus and a picornavirus (Enterovirus71)(Hassan *et al.,* 2015; Uddin *et al.*, 2016). In agreement with previous studies, we observed a 25% reduction in virus infectivity by BV.

An antiviral drug may exert its inhibitory effect by interfering with the virus replication process inside the host cell at any stage or by directly interacting with the virus particle causing structural damage/alterations. We found that pre-treatment of BHK-21 cells with BV caused a 20% reduction in virus titers. This could be due to the induction of an antiviral state in the cells. The formation of cytoplasmic blebs in FMDV-infected BHK-21 cells has been reported. We also found cytoplasmic blebs in FMDV infected cells. However, very few cells treated with BV then infected with FMDV showing cyto-plasmic blebs suggesting that virus replication /or infection rate is reduced. However, further studies are required.

Previously, it was shown that treatment of HEK293T cells with BV induced Type I IFN (Uddin *et al.*, 2016). In the present study, we also observed 2.5 fold induction in IFNγ levels in BV-BHK-21 cells. Interestingly, a strong induction in IFNγ levels was observed when cells were infected with BV-treated FMDV compared to un-treated FMDV. Previous studies have shown that UV-inactivation increased the IFN induction capacity of avian influenza virus particles (Marcus *et al*., 2005). The infectivity results in the present study suggest that BV at 2-3 µg/ml concentration caused inactivation of the virus and this inactivated virus caused increased production of IFNγ.

We also investigated the interaction between BV and FMDV by the electron microscopy. BV mainly consists of MLT, Phospholipase A2, biologically active amines (apamin, adolapin), diverse peptides and non-peptide components. MLT constitutes 40-50% of dry BV (Lariviere and Melzack, 1996; Moreno and Giralt, 2015). Kamal (2018) showed using electron microscopy that camelpox virus was bound to the MLT particles or was internalized into the MLT particles, with some structural destabilization of virus particles. Uddin *et al.* (2016) also reported that influenza A virus particles associated with MLT particles using sedimentation velocity ultracentrifugation. In our study, we also observed virus particles associated with MLT particles. Previous and our findings suggest that virus-MLT interaction makes the virus biologically non-available for host cell entry. Such interaction also seems to destabilize the virus structure and requires further investigation.

In summary, BV inhibited FMDV in two ways. First, it induced antiviral state in cells as indicated by increased IFNγ level. Secondly, MLT in BV inactivated FMDV by directly binding with the virus particles i.e. virucidal activity. Additionally, MLT-bound FMDV particles induced more IFNγ compared to BV or FMDV alone. Based on these results, the applications of BV in the field of treatment and prevention of FMDV infection can add a new control measure of the disease.

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Authors contribution: NA, IE and AE conceived and designed the project. MM, NA and AE executed the experiments and contributed reagents/materials/analysis tools. MM and RM wrote the paper. RM analyzed the data. NA, IE and RM critically reviewed the manuscript for important intellectual contents and approved the final version.

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