TEXAS BIOMEDICAL RESEARCH INSTITUTE

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A. Introduction

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic has become a global threat to public health because of its high rate of infection and high number of deaths. While the majority of SARS-CoV-2 infected patients do not require hospitalization, a minority present with more severe symptoms requiring hospitalization and may experience severe life-threatening complications. For example, patients can develop acute respiratory distress syndrome (ARDS), which may trigger a systemic multi-organ collapse. In addition, an overreaction of the host's immune and inflammatory responses can result in a vast release of cytokines ('cytokine storm'), inducing sepsis and multi-organ damage, which may lead to organ failure. To date, more than 1.5 million patients worldwide have succumbed to the viral coronavirus disease 2019 (COVID-19). In response to the ongoing pandemic, there is a desperate need to identify and test potential therapeutics for this disease.

B. Aims and scope

In the current study, a novel compound (i.e. 3VM-1000 suspension) was evaluated for cytotoxicity and anti-SARS-CoV-2 activity in Vero E6 cells. This study assessed the ability of 3VM-1000 suspension to directly inactivate and inhibit SARS-CoV-2 replication in Vero E6 cells, under *in-vitro* experimental conditions.

C. Experimental Design

C.1. Cell culture

Vero E6 cells obtained from the American Type Culture Collection (ATCC, CRL-1586) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) with penicillin (P; 100 IU/ml), streptomycin (S; 100 µg/ml) and L-glutamine (G; 292 µg/ml)) at 37°C in a 5% CO_2 atmosphere.

C.2. SARS-CoV-2 preparation

SARS-CoV-2, USA-WA1/2020 strain (Gen Bank: MN985325.1), was obtained from The Biological and Emerging Infections Resources Program (BEI Resources NR-52281). A 6th passage (P6) of SARS-CoV-2 was generated by infecting Vero E6 cells obtained from ATCC (CRL-1586) for 72 hours (h). At 72 h post infection, culture supernatants were collected, clarified, and aliquots were stored at -80°C. Viral titers were calculated by conventional plaque assay (protocol in Appendix A).

C.3. 3VM-1000 suspension

3VM-1000 suspension was received in dark glass bottles, from the 3V Medical Research Group Inc. Upon arrival, bottles were stored at room temperature (RT)

in the dark. The 3VM-1000 suspension was shaken very well before making dilutions in culture medium as described below.

C.4. Assessment of cellular toxicity

Cytotoxicity was evaluated by measuring Vero E6 cell viability after exposure to serial dilutions of 3VM-1000 suspension. 3VM-1000 suspension was serially diluted (two-fold dilutions, 100% to 0.09%) in DMEM + 2% FBS + P/S/G medium. Vero E6 cells were seeded into 96- well culture plates in DMEM + 10% FBS + P/S/G medium at a density of approximately 2 x 10^4 cells/well, and incubated at 37°C, 5% CO_2 for 24 h. After 24 h, culture medium was replaced with 100 µl of two-fold serially diluted 3VM-1000 suspension (100% to 0.09%) in DMEM + 2% FBS + P/S/G medium, and the plates were incubated for 1, 6 and 24 h at 37°C in 5% CO_2. Untreated cells were used as a negative control.

To evaluate cytotoxicity by mitochondrial viability parameter, the 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT, Abcam) assay was performed. At 1, 6 and 24 h post addition of 3VM-1000 suspension, culture medium containing the 3VM-1000 suspension was carefully removed and 100 µl of MTT reagent (Abcam) diluted with DMEM (1:1, v/v) was added. Plates were then wrapped in foil paper to prevent dye degradation, and re-incubated for 3 h at 37°C, 5% CO_2 to allow metabolically active cells to reduce MTT to formazan blue crystals. After 3 h incubation period, medium containing MTT reagent was removed and 150 µl of MTT solvent was added to each well, for crystal solubilization. To ensure crystal solubilization, plates were covered with foil and put on an orbital shaker for 15-20 min. Absorbance was read at 590 nm microplate spectrophotometer (M3, Molecular Devices®). Results obtained by these assays were expressed as percentage relative to the mean of the negative control (control cells). Percentages calculated for different sample concentrations were plotted and mean cytotoxic concentration values (CC_{50}) were estimated by non-linear regression analysis.

Percentage cytotoxicity was calculated with following equation using absorbance:

$$\% \text{ Cytotoxicity} = \frac{[100 \text{ X (Control (cells only)- Sample)}]}{\text{Control}}$$

% Viability = 100 - % Cytotoxicity

Note: Untreated cells (triplicate wells) served as controls and were considered 100% viable. Sample wells showing more than 100% viability were considered 100% viable.

C.5. To determine the effect of 3VM-1000 suspension on virus infectivity [Effective Concentration (EC_{50}) determination]

Vero E6 cells (2 x 10^4 cells/well) were seeded in 96- well culture plates in DMEM + 10% FBS + P/S/G and incubated at 37°C, 5% CO_2 for 24 h. 24 h later, 3VM-1000 suspension was two-fold serially diluted (100% to 0.09%) in DMEM +P/S/G medium (50 µl) and incubated with SARS-CoV-2 [Multiplicity of infection (MOI) 0.02; 50 µl]

37°C; 5% CO_2 for 1h. After 1 h incubation, Vero E6 were infected with mixture of 50 µl of serially diluted 3VM-1000 suspension (final concentration 50% to 0.09%) and SARS-CoV-2 (MOI; 0.02) and incubated at 37°C; 5% CO_2 for 1 h. Mock infected cells were treated with 50 µl of DMEM+P/S/G only. After 1 h, the virus was removed and cells were overlaid with medium (DMEM + 2% FBS + P/S/G) containing 1% Avicel (Sigma-Aldrich). Cells infected with SARS-CoV-2 only, cells alone (mock infected cells), and PBS were included as controls. At 24 h post-infection, the overlay media was removed and plates were fixed overnight in 10% neutral buffered formalin (Sigma-Aldrich), followed by 3 washes with PBS. Subsequently, the plaque developing protocol (see Plaque development protocol, Appendix B) was followed in order to develop plaques. Plates were read using a CTL ImmunoSpot plate reader and formed plaques counted by an automated counting software (Cellular Technology Limited, Cleveland, OH, USA).

Percent virus inhibition, was calculated using number of plaques obtained as follows:

$$\% \text{ Virus Inhibition} = \frac{[100X \text{ Control (virus only)} - \text{Sample})]}{\text{Control}}$$

$$\% \text{ Virus Replication} = 100 - \% \text{ Virus Inhibition}$$

A non-linear regression curve fit analysis over the dilution curve was performed using GraphPad Prism vs. 7.0 to calculate effective concentration 50 (EC_{50}) of 3VM-1000 suspension.

C.6*. In-vitro* antiviral activity of 3VM-1000 suspension on viral replication [Effective Concentration (EC_{50}) determination]

Vero E6 cells (2 x 10^4 cells/well) were seeded in 96-well culture plates in DMEM + 10% FBS + P/S/G and incubated at 37°C, 5% CO_2. After 24 h, Vero E6 cells were infected with SARS-CoV-2 (MOI 0.01; 50 µl) for 1 h at 37°C; 5% CO_2. After 1 h of viral adsorption, virus inoculum was removed and post-infection media (DMEM + 2% FBS + P/S/G) containing two-fold serially diluted 3VM-1000 suspension (50% to 0.09%) or Remdesivir (50 µM-0.09 µM) with1% Avicel was added. Remdesivir served as a positive control. Cells infected with SARS-CoV-2 only, cells alone (mock infected cells), and PBS, were included as controls. At 24 h post-infection, the overlay media was removed and plates were fixed overnight in 10% neutral buffered formalin (Sigma-Aldrich), followed by 3 washes with PBS. Subsequently, the plaque developing protocol (see Plaque development protocol, Appendix B) was followed in order to develop plaques. Plates were read using a CTL ImmunoSpot plate reader and formed plaques counted by an automated counting software (Cellular Technology Limited, Cleveland, OH, USA).

Percent virus inhibition, was calculated using number of plaques obtained as follows:

$$\% \text{ Virus Inhibition} = \frac{[100X \text{ Control (virus only)} - \text{Sample})]}{\text{Control}}$$

% Virus Replication = 100 - % Virus Inhibition

A non-linear regression curve fit analysis over the dilution curve was performed using GraphPad Prism vs. 7.0 to calculate effective concentration 50 (EC_{50}) of 3VM-1000 suspension.

D. Results

D.1. Cytotoxicity determination of 3VM-1000 suspension

Cytotoxicity of 3VM-1000 suspension was assessed in the range of 100% to 0.09% concentration at 1, 6 and 24 h post treatment in Vero E6 cells. At all the three time points tested, 100% concentration of 3VM-1000 suspension was found to be highly toxic and showed more than 90% cell death (**Figure 1 A-C**). However, 3VM-1000 suspension at 50% or lower concentration showed minimal or no toxicity at 1 and 6 h post treatment (**Figure 1A-B**). Exact CC_{50} could not be calculated as the other tested concentrations (beginning with 50%) showed minimal or no toxicity at 1 and 6 h post treatment and was reported as ambiguous. For the 24 h exposure, dose dependent cytotoxicity was observed with the CC_{50} value of 38.53% (**Figure 1C**). Untreated cells were considered 100% viable and wells showing more than 100% viability were considered 100% (Raw data provided in in Appendices C to E).

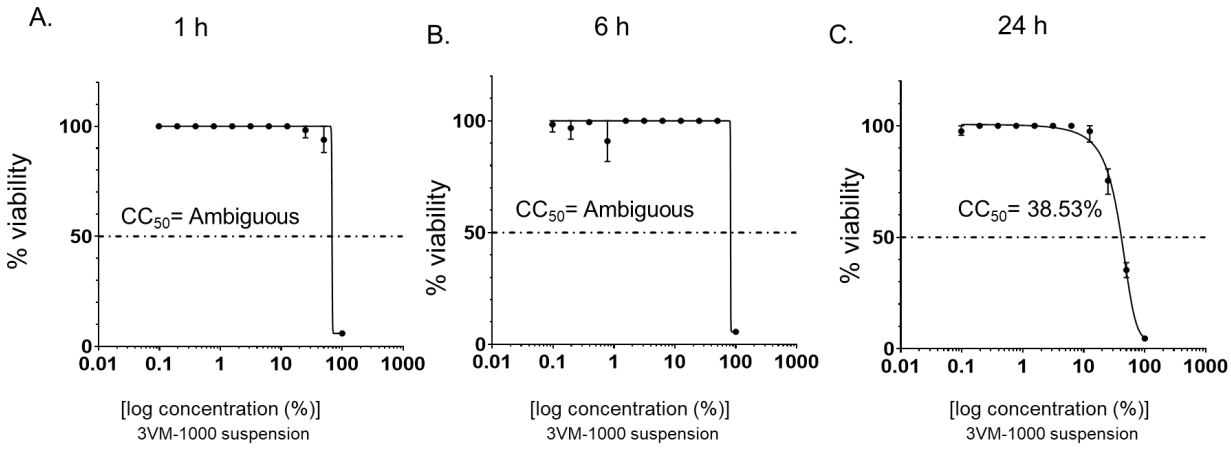

Figure 1: CC_{50} determination by MTT assay of 3VM-1000 suspension at 1h (A) 6 h (B) and 24 h (C), post 3VM-1000 suspension treatment

D.2. To determine the effect of 3VM-1000 suspension on virus infectivity The ability of 3VM-1000 suspension to directly inactivate SARS-CoV-2 was evaluated. 3VM-1000 suspension demonstrated anti-viral activity against SARS-CoV-2, with an EC_{50} of 29.82% (**Figure 2**). As expected, PBS did not show any SARS-CoV-2 inhibition and was comparable to virus control wells. Mean plaque count from four control wells (virus) was 230 and was considered as 100% infection. Wells showing > 230 plaques were considered 100% (Raw data provided in Appendices F and G).

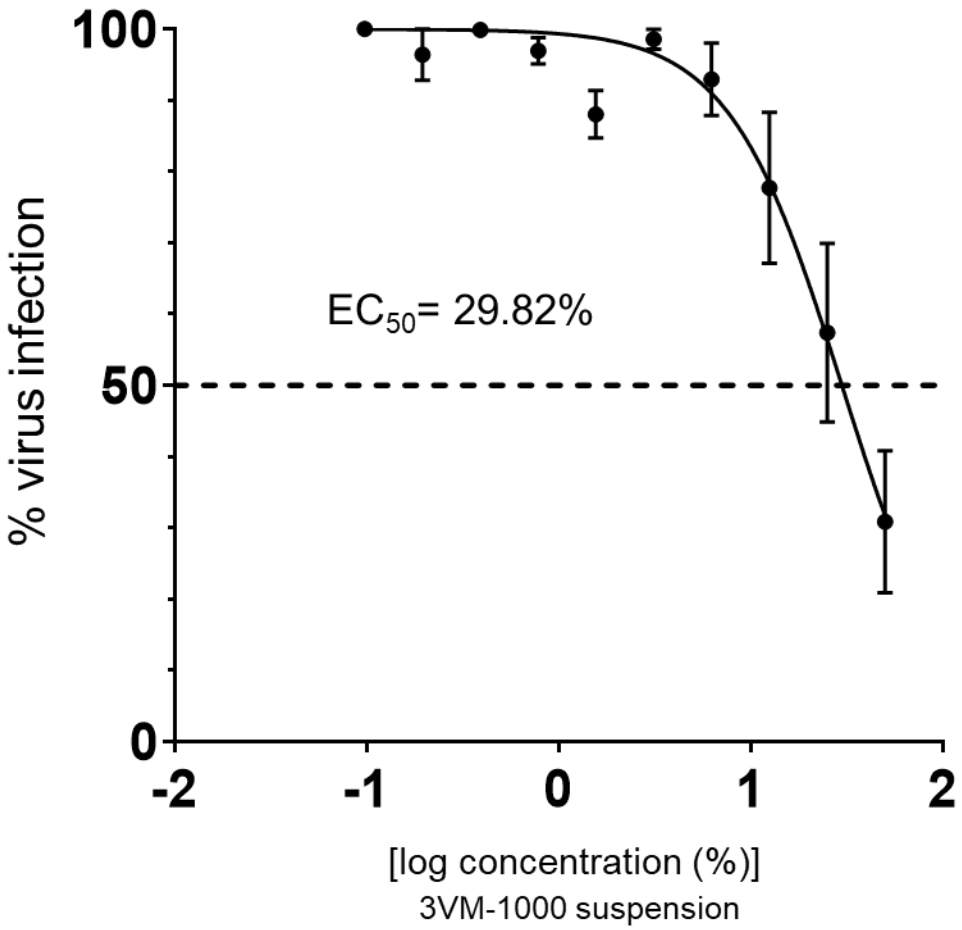

Figure 2: *In-vitro* antiviral activity of 3VM-1000 suspension on SARS-CoV-2 infectivity

D.3. *In-vitro* antiviral activity of 3VM-1000 suspension on viral replication

The ability of 3VM-1000 suspension on SARS-CoV-2 replication was tested on Vero E6 cells in *in-vitro* experimental conditions. The dose–response curve displayed a potent dose-dependent activity of 3VM-1000 suspension on SARS-CoV-2 replication. We observed 98.2% and 90.6% viral inhibition at 12.5% and 6.25% concentration of 3VM-1000 suspension, respectively. The EC_{50} value was calculated as 2.46% (**Figure 3A**). Remdesivir served as a positive control (EC_{50} at 2.3 µM; **Figure 3B**). As expected, PBS did not show any SARS-CoV-2 inhibition and was comparable to virus control wells. Mean plaque count from four positive control wells (virus) was 271 and was considered as 100% infection. Wells showing > 271 plaques were considered 100% (Raw data provided in Appendices H and I).

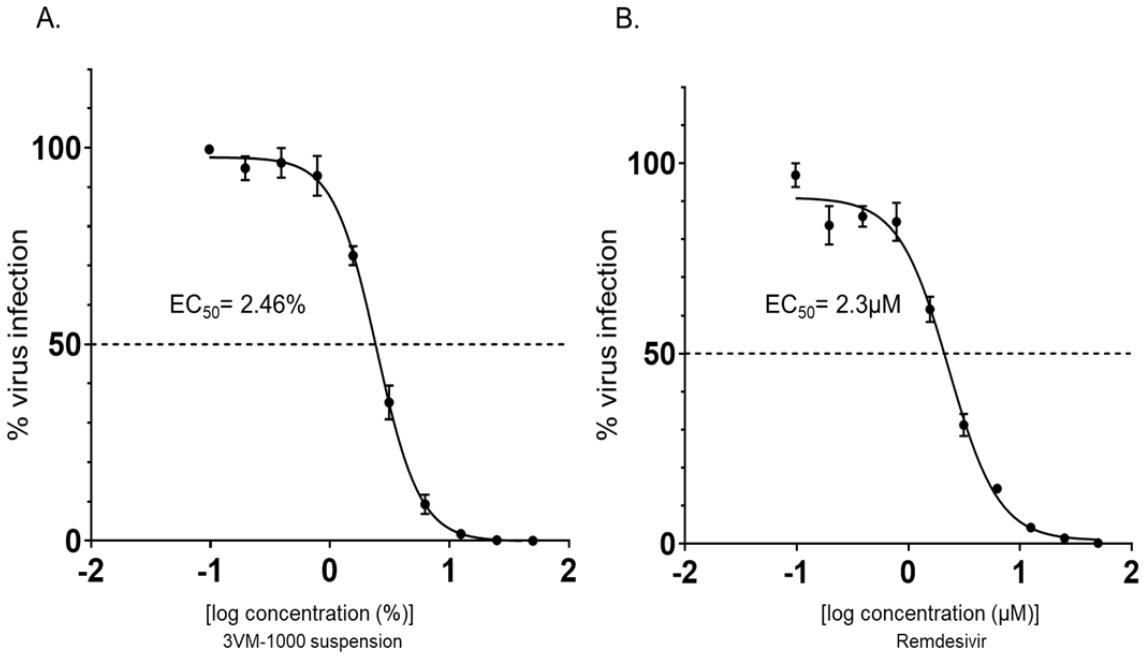

Figure 3: *In-vitro* antiviral activity of 3VM-1000 suspension (A) Remdesivir (B) on SARS-CoV-2 replication

Summary:

The aim of this project was to evaluate the anti-SARS-CoV-2 effect of 3VM-1000 suspension from 3V Medical Research Group Inc. The antiviral activity of 3VM-1000 suspension was assessed in the Vero E6 cell line against SARS-CoV-2 (USA-WA1/2020) strain. First, we evaluated the 3VM-1000 compound's cytotoxic effect in a range from 100% to 0.09% and found that this compound was non-toxic below 50% with CC_{50} value being 38.20% at 24 h post exposure. Further, the 3VM-1000 suspension showed anti-viral efficacy against SARS-CoV-2 infectivity with an EC_{50} of 29.82%, and on SARS-CoV-2 replication with an EC_{50} of 2.46%.

Appendix A: Plaque assay to determine the virus titer

1. Approximately 24 h prior to the assay, use 6-well plates and seed 4 x 10^5 Vero E6 cells/well (in 2 ml, DMEM + 10% FBS + P/S/G).
2. For each sample to be analyzed, make 10-fold serial dilutions in DMEM + Penicillin, Streptomycin, Glutamine (P/S/G) by pipetting up and down several times. Use as negative control DMEM + P/S/G alone.
3. For each compound, plate 400 µl/well of each serial dilution on the Vero E6 cells.
4. Incubate samples for 1 h at 37°C, and rock them manually every 15 min.
5. After 1 h, remove samples and add 3 ml of warmed overlay (DMEM/F-12/Agar mixture (DMEM-F12 with 1% DEAE-Dextran, 2% agar, and 5% $NaHCO_3$ containing 2% Agar) to each well.
6. Incubate plates approximately for 72 h at 37°C with 5% CO_2.
7. After this incubation period, remove the overlay and fix the plates overnight in 10% neutral buffered formalin, followed by one PBS wash.
8. Permeabilize cells with 0.5% Triton-X- 100 for 15-20 minutes at room temperature.
9. Wash three times with PBS, and then add mouse anti-NP SARS-CoV-1/SARS-CoV-2, 1C7C7 antibody at 1 µg/ml in PBS +1% Bovine serum albumin (BSA) for 1 h at 37°C, 5% CO_2.
10. After this incubation period, wash cells with PBS. Prepare and add biotinylated secondary antibody (150 µl) in 10 ml of PBS containing 150 µl of blocking serum for 30 minutes at 37°C, 5% CO_2. (See: Vector laboratories, cat: PK-4000).
11. During incubation, prepare ABC reagent by adding 2 drops (100 µl) of reagent A, 2 drops (100 µl) of reagent B in 10 ml of PBS. (See: Vector laboratories, cat: PK-4000 PK-4000).
12. Post-secondary antibody incubation, add ABC reagent and incubate for 30 minutes at room temperature.
13. Wash plates 3 times with PBS and prepare the substrate solution by adding following reagent in 5 ml of distilled water. If there is a need to add more substrate, add reagents proportionately.
    - 2 drops (84ul) of DAB reagent 1
    - 4 drops of (100ul) of DAB reagent 2
    - 2 drops of (80ul) of DAB reagent 3
    - 2 drops of (80ul) of DAB reagent 4
14. Develop the plates adding Vector DAB substrate as per the manufacturer's instruction (Vector DAB SK-4000).
15. Wash the plates 3 times and count plaques using and calculate the virus titer by the using the formula given below:

Virus (PFU/ml) = number of plaques X dilution at which plaques are being counted X 1/virus inoculum used in ml

Appendix B: Plaque development protocol

1. Post fixation in 10% formalin, the plates were washed 3 times with PBS.
2. Cells were permeabilized with 0.5% Triton-X-100 for 15-20 minutes at room temperature.
3. After washing, cells were immuno-stained with a SARS-CoV nucleocapsid (N) protein cross-reactive monoclonal antibody (mAb, 1C7C7, Cat# ZMS1075, Sigma-Aldrich, Saint Louis, MO) diluted in 1% Bovine Serum Albumin (BSA,1 µg/ml) for 1 h at 37°C.
4. After this incubation, cells were washed with PBS and biotinylated secondary antibody (150 µl) in 10 ml of PBS containing 150 µl of blocking serum was added for 30 minutes at 37°C (See: Vector laboratories, Cat PK-4000).
5. During incubation, ABC reagent was prepared by adding 2 drops (100 µl) of reagent A, 2 drops (100 µl) of reagent B in 10 ml of PBS (See: Vector laboratories, Cat: PK4000).
6. Post- secondary antibody incubation, ABC reagent was added for 30 minutes at room temperature.
7. After 3 washes with PBS, substrate solution was prepared by adding the mixture of reagents below in 5 ml of distilled water and plaques were developed.
   - 2 drops (84 µl) of DAB reagent 1
   - 4 drops of (100 µl) of DAB reagent 2
   - 2 drops of (80 µl) of DAB reagent 3
   - 2 drops of (80 µl) of DAB reagent 4

8. Plates were read using a CTL ImmunoSpot plate reader and formed plaques counted by an automated counting software (Cellular Technology Limited, Cleveland, OH, USA).

   Percent virus inhibition was calculated using number of plaques obtained as follows:

$$\% \text{ Virus Inhibition} = \frac{[100X \text{ Control (virus only)} - \text{Sample})]}{\text{Control}}$$

$$\% \text{ Virus Replication} = 100 - \% \text{ Virus Inhibition}$$

   A non-linear regression curve fit analysis over the dilution curve was performed using GraphPad Prism vs. 7.0 to calculate effective concentration 50 (EC_{50}) of the test compound.

Appendix C: % Cellular viability at 1 h post 3VM-1000 suspension treatment

% viability- MTT assay												
	3VM-1000 suspension (%)	100	50	25	12.5	6.250	3.125	1.563	0.781	0.3906	0.195	0.098
3VM-1000 suspension	Replicate-1	6.01	88.10	177.30	131.03	127.62	134.95	163.48	113.38	151.54	128.06	138.16
	Replicate-2	6.17	93.54	94.76	well error	139.90	151.63	147.99	145.10	137.52	119.13	127.27
	Replicate-3	5.58	135.50	148.63	140.68	117.24	148.16	117.63	146.60	152.10	124.13	133.18

Appendix D: % Cellular viability at 6 h post 3VM-1000 suspension treatment

% viability- MTT assay												
	3VM-1000 suspension (%)	100	50	25	12.5	6.250	3.125	1.563	0.781	0.3906	0.195	0.098
3VM-1000 suspension	Replicate-1	6.29	133.18	109.13	150.04	111.32	107.05	105.78	81.83	137.93	98.61	95.02
	Replicate-2	5.35	115.91	141.90	179.33	162.10	158.08	106.39	90.94	98.21	120.49	144.82
	Replicate-3	5.29	144.19	130.71	117.82	134.35	160.16	168.03	139.41	110.08	91.76	122.85

Appendix E: % Cellular viability at 24 h post 3VM-1000 suspension treatment

% viability- MTT assay												
	3VM-1000 suspension (%)	100	50	25	12.5	6.250	3.125	1.563	0.781	0.3906	0.195	0.098
3VM-1000 suspension	Replicate-1	4.42	31.94	76.29	109.51	136.24	163.06	119.21	165.11	115.81	119.47	96.98
	Replicate-2	4.65	38.66	80.72	92.69	105.64	179.20	108.49	189.09	102.98	115.31	109.87
	Replicate-3	4.77	35.41	69.28	104.32	104.52	167.54	132.63	150.57	109.18	131.53	95.77

Appendix-F: *In-vitro* antiviral activity of 3VM-1000 suspension on virus infectivity (Plaque counts)

	Plaque counts										
	3VM-1000 suspension (%)	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.563**	**0.781**	**0.391**	**0.195**	**0.098**
3VM-1000 suspension (%)	Replicate -1	125	186	231	180	242	196	219	231	277	272
	Replicate -2	90	158	124	215	217	212	213	255	197	279
	Replicate -3	48	132	153	304	266	184	237	234	232	248
	Replicate -4	21	52	208	275	282	218	371	229	299	293
PBS	**PBS**	**PBS**									
	Replicate -1	275									
	Replicate -2	282									
	Replicate -3	218									
	Replicate -4	371									

Appendix-G: *In-vitro* antiviral activity of 3VM-1000 suspension on virus infectivity (% Viral infection)

	% Viral replication										
	3VM-1000 suspension (%)	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.563**	**0.781**	**0.391**	**0.195**	**0.098**
3VM-1000 suspension	Replicate -1	54.35	80.87	100.43	78.26	105.22	85.22	95.22	100.43	120.43	118.26
	Replicate -2	39.13	68.70	53.91	93.48	94.35	92.17	92.61	110.87	85.65	121.30
	Replicate -3	20.87	57.39	66.52	132.17	115.65	80.00	103.04	101.74	100.87	107.83
	Replicate -4	9.13	22.61	90.43	119.57	122.61	94.78	161.30	99.57	130.00	127.39
PBS	**PBS**	**PBS**									
	Replicate -1	119.57									
	Replicate -2	122.61									
	Replicate -3	94.78									
	Replicate -4	161.30									

Appendix-H: *In-vitro* antiviral activity of 3VM-1000 suspension on virus replication (Plaque counts)

Plaque counts											
	3VM-1000 suspension (%)	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.563**	**0.781**	**0.391**	**0.195**	**0.098**
3VM-1000 suspension (%)	Replicate -1	0	0	4	39	112	181	294	346	280	280
	Replicate -2	0	1	4	12	103	191	213	230	283	271
	Replicate -3	0	1	9	34	106	207	252	321	241	294
	Replicate -4	0	0	2	16	61	208	293	274	245	267
Plaque counts											
	Remdesivir (µM)	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.563**	**0.781**	**0.391**	**0.195**	**0.098**
Remdesivir (µM)	Replicate -1	0	4	15	35	107	185	217	221	202	299
	Replicate -2	0	5	15	48	74	164	206	221	243	237
	Replicate -3	1	0	9	37	73	176	226	251	205	282
	Replicate -4	1	7	7	37	84	143	268	239	257	323
	PBS	**PBS**									
PBS	Replicate -1	259									
	Replicate -2	240									
	Replicate -3	267									
	Replicate -4	260									

Appendix-I: *In-vitro* antiviral activity of 3VM-1000 suspension on virus replication (Plaque counts)

% Viral infection											
	3VM-1000 suspension (%)	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.563**	**0.781**	**0.391**	**0.195**	**0.098**
3VM-1000 suspension (%)	Replicate -1	0.00	0.00	1.48	14.39	41.33	66.79	108.49	127.68	103.32	103.32
	Replicate -2	0.00	0.37	1.48	4.43	38.01	70.48	78.60	84.87	104.43	100.00
	Replicate -3	0.00	0.37	3.32	12.55	39.11	76.38	92.99	118.45	88.93	108.49
	Replicate -4	0.00	0.00	0.74	5.90	22.51	76.75	108.12	101.11	90.41	98.52
% Viral infection											
	Remdesivir (µM)	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.563**	**0.781**	**0.391**	**0.195**	**0.098**
Remdesivir (µM)	Replicate -1	0.00	1.48	5.54	12.92	39.48	68.27	80.07	81.55	74.54	110.33
	Replicate -2	0.00	1.85	5.54	17.71	27.31	60.52	76.01	81.55	89.67	87.45
	Replicate -3	0.37	0.00	3.32	13.65	26.94	64.94	83.39	92.62	75.65	104.06
	Replicate -4	0.37	2.58	2.58	13.65	31.00	52.77	98.89	88.19	94.83	119.19
	PBS	**PBS**									
PBS	Replicate -1	95.57									
	Replicate -2	88.56									
	Replicate -3	98.52									
	Replicate -4	95.94									