Original Article

Antiviral Effect of Doxycycline against Dengue 2, Influenza A (H1N1), Influenza B, Human Rhinovirus 17, Human Adenovirus, and Human Respiratory Syncytial Virus : An *In-vitro* Study

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Objective : The purpose of this study was to determine *in-vitro* antiviral activity of Doxycycline against Dengue 2, Influenza A Virus (H1N1), Influenza B Virus, Human Rhinovirus 17 Virus, Human Adenovirus and Human Respiratory Syncytial Virus in a cell infection model (Prophylactic method and Co-culture method) by the MTT Assay.

Methods : *In-vitro* cell lines culture of Dengue 2, Influenza A Virus (H1N1), Influenza B Virus, Human Rhinovirus 17 Virus, Human Adenovirus and Human Respiratory Syncytial Virus in a cell infection model used (Prophylactic method and Co-culture method) and antiviral activity determined using cytotoxicity test by the MTT Assay.

Result : The *in-vitro* cytotoxicity test was performed for Doxycycline upto 2250µM. The *in-vitro* antiviral activity of Doxycycline against Dengue 2 was performed and the IC_{50} was 135.5 µM in the Prophylactic method and 114.5 µM in the co-culture method, Activity against Influenza A Virus (H1N1) the IC_{50} was 262.3 µM in the Prophylactic method and 184.1 µM in the Co-culture method. Activity against Influenza B Virus was 330.9 µM in the Prophylactic method and 286.4 µM in the Co-culture method, Human rhinovirus 17 and the IC50 was 387.3 µM in the Prophylactic method and 325.9 µM in the Co-culture method. Activity against Human Adenovirus and the IC50 was 146.5 µM in the prophylactic method and 106.6 µM in the co-culture method and activity against Human Respiratory Syncytial Virus the IC50 was 225.5 µM in the Prophylactic method and 165.2 µM in the Co-culture method. Doxycycline exhibits antiviral activity against selected virus in both Prophylactic method and Co-culture methods using the MTT testing was observed.

Conclusion : This study provides valuable insights into the antiviral activity of doxycycline against selected viruses. This antiviral property of Doxycycline will prove beneficial in minimizing secondary infections and complications especially when used as an adjunct.

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Key words **: Doxycycline, Cell Line study, Co-Culture Method, CTT, MTT Assay, Prophylactic Method.**

Viral infections, such as Dengue, Influenza (H1N1), Influenza B, Human Rhinovirus 17, Adenovirus, and Respiratory Syncytial Virus, cause global public health concerns due to their morbidity and mortality rates. Dengue 2 virus is a mosquito-borne pathogen that poses a severe health risk in tropical and subtropical regions[1]. Influenza viruses, including H1N1, result in seasonal outbreaks and pandemics[2]. Human Rhinovirus 17 causes the common cold, while Human Adenovirus leads to respiratory, ocular and gastrointestinal infections as main manifestation[3,4]. Human Respiratory Syncytial Virus mainly affects infants and young children, causing severe respiratory infections[5].

Doxycycline, a tetracycline antibiotic, possesses antimicrobial and anti-inflammatory properties[6]. It is Food and Drug Administration approved antimalarial drug[7]. Besides bacterial infections, doxycycline has

Editor's Comment :

- Doxycycline is a broad-spectrum antibiotic with anti-inflammatory activity, evidence suggest that doxycycline also has antiviral property and was used during COVID-19 pandemic for saving life. This study provides valuable insights into the antiviral activity of doxycycline against viruses causing Flu. This evidence with Doxycycline will prove beneficial in minimizing secondary infections and complications especially when used as an adjunct in patients suffering from viral infections.

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also shown antiviral properties against herpes simplex, dengue and retroviruses[8]. It inhibits the replication of Vesicular Stomatitis Virus and the entry and replication of the Chikungunya virus[9-11]. In silico analysis suggested that doxycycline could potentially act as an inhibitor of the nucleoprotein of the Crimean-Congo hemorrhagic fever virus, a crucial Protein involved in viral replication[12]. These findings highlight the broad-spectrum antiviral potential of Doxycycline across different viral families and its ability to target distinct stages of the viral replication cycle.

Investigating Doxycycline's antiviral effects on various viruses, including Human Rhinovirus 17, Human Adenovirus, Human Respiratory Syncytial Virus, Dengue 2, Influenza A (H1N1), Influenza B and Human Rhinovirus 17, could clarify its potential as a broad-spectrum antiviral therapy. Such research may reduce the global burden of viral illnesses and provide new management approaches.

Materials and Methods

The study utilized Doxycycline, Ribavirin, Fetal Bovine Serum, D-PBS, DMEM, EMEM, MTT Reagent, and DMSO obtained from HiMedia and Sigma. A 96-well plate from Corning was used for cell culture along with viruses and cell lines. The solubility of Doxycycline was by dissolving an adequate quantity of DMSO to produce a Master Stock (MS) solution with a concentration of 225,000 µM. The MS solution was diluted to obtain a series of Working Stock (WS) solutions, as indicated in Table 1 with an assay volume of 200 µl, and the final concentration of DMSO in the assay was maintained at 1%.

Virus Preparation :

A concentration of 100 TCID50/ml or the quantity of virus needed to produce cytopathic effects (CPE) in 80% of infected cells, was obtained by thawing and diluting the frozen viral stock.

Preparation of Cell Line :

A vial of working stock cells was thawed and added to a complete medium, centrifuged, and resuspended in a T-25 flask. The flask was incubated until cell confluency reached around 80%. The cells were then transferred to two T-75 flasks and once cell confluency reached 80-90%, the flask was used for the assay after detaching the cells using trypsin EDTA.

Cytotoxicity Test :

Cells were seeded in a 96-well plate with 200 µl of cell suspension (in complete medium with 10% FBS) without the test agent and allowed to grow for approximately 24 hours. After incubation, spent media in the wells of a 96-well plate was decanted, washed with DPBS and Treatment media (1% pen-strep and 2% FBS) containing appropriate concentrations of Doxycycline were added. The plates were then incubated at 37°C in a 5% CO2 atmosphere for 3 days (72 hours) and an MTT test was performed.

Antiviral activity Test (Prophylactic Method (PM) / Pre-treatment strategy) :

Cells were seeded in a 96-well plate and incubated for 24 hours. After removing the spent media, the cells were treated with the Doxycycline and incubated for an additional period. Then, the virus was added and incubated. After removing the virus, a fresh medium was added and the plates were observed daily for CPE. Once there was 85-90% CPE in the virus control, an MTT assay was performed[13,14].

Antiviral activity Test (Co-culture method (CCM) / During and after the adsorption) :

Cells were seeded with complete media containing 10% FBS in a 96-well plate and allowed to grow for 24 hours. Then, the spent media was removed and 100 TCID50/ml of the virus was added and incubated for 2 hours. After that, a complete medium with a Doxycycline was added and plates were incubated for 72 hours. The plates were checked daily for CPE and if there was no CPE, the incubation period was extended. An MTT assay was performed after there was 85-90% CPE in the virus control[15,16].

Results

Various cell lines were employed to evaluate the efficacy of doxycycline against Dengue 2, Influenza A

Table 1 — *Details of Viruses and Cell Lines*

Name of Viruses	ATCC number of Viruses	Name of cell lines	ATCC number of cell lines	Media
Dengue 2	ATCC® VR-1584™	BHK-21	ATCC® CCL-10™	DMEM
Influenza A Virus (H1N1)	ATCC®VR-219™	MDCK	ATCC® CCL-34™	DMEM
Influenza B Virus	ATCC®VR-1735™	MDCK	ATCC® CCL-34™	DMEM
Human Rhinovirus 17 Virus	ATCC®VR- 1663™	H1HeLa	ATCC® CRL-1958™	EMEM
Human Adenovirus	ATCC®VR-5™	HeLa	ATCC® CCL-2™	EMEM
Human Respiratory Syncytial Virus	ATCC® VR-1540™	HEp-2	ATCC® CCL-23™	EMEM

ATCC: American Type Culture Collection, BHK-21: Baby Hamster Kidney- 21, MDCK: Madin- Darby Canine Kidney, HeLa: Henrietta Lacks, Hep-2: Human Epithelial Type 2 cells, DMEM: Dulbecco's Modified Eagle's Medium, EMEM: Eagle's Minimal Essential Medium

Virus (H1N1), Influenza B Virus, Human Rhinovirus 17 Virus, Human Adenovirus and Human Respiratory Syncytial Virus, employing both the Prophylactic Method (PM) and Co-culture Method (CCM).

Using the PM for Dengue 2 Virus in the BHK-21 cell line, Doxycycline generated an IC_{50} of 135.5 µM, Imax of 85%, and mean inhibition of CPE ranging from 6.5% to 84.9%, whereas, using the CCM, it produced an IC_{50} of 114.5 µM, Imax of 87% and mean CPE of 13.2% to 86.7%. The vehicle control exhibited an Imax of 5.2% and mean CPE of 5.2 ± 0.5% by the CCM, whereas an of Imax of 3.3%, and mean CPE of 3.3 ± 0.5% by the PM for different concentrations.

Doxycycline exhibited a dose-dependent reduction using the PM for Influenza A Virus (H1N1) in the MDCK cell line with an IC_{50} of 262.3 µM, Imax of 75% and mean CPE ranging from 8% to 74.8% whereas, using the CCM, it produced an IC_{50} of 184.1 µM, Imax of 80% and mean CPE of 6.9% to 79.5% The vehicle control produced an Imax of 6.9% and mean CPE of 6.9 ± 0.9% by the CCM and an Imax of 6.8% and mean CPE of 6.8 ± 1.0% by the PM for different concentrations.

Furthermore, when tested against the influenza B Virus in an MDCK Cell Infection Model, Doxycycline generated a mean CPE of 9.2% to 71.0%, IC_{50} of 330.9 µM, and an Imax of 71%, by the PM, whereas it produced a mean CPE of 13.3% to 68.2%, IC_{50} of 286.4 µM and an Imax of 68%, by the CCM. The vehicle control produced a mean CPE of 9.2±0.3% and an Imax of 9.2%, by the PM, whereas it produced a mean CPE of 7.4 ± 1.0%, and an Imax of 7.4%, by the CCM.

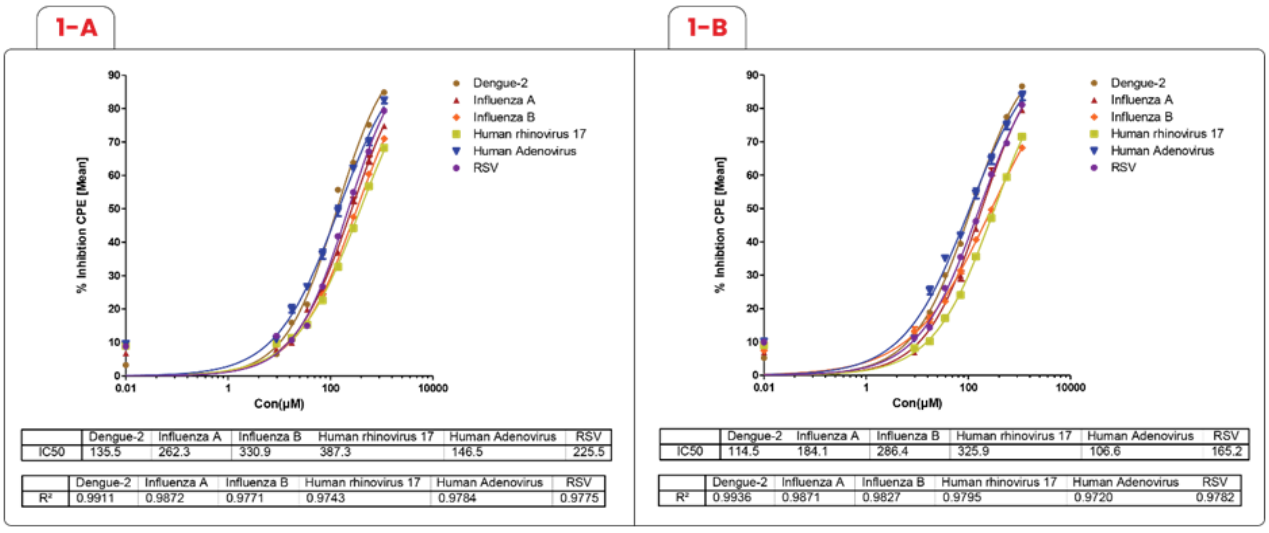
When examined against Human Rhinovirus 17 (HRV-17) in H1HeLa cell lines, Doxycycline generated mean CPE of 9.7% to 68.3%, IC_{50} of 387.3 µM and an Imax of 68.3%, by the PM, whereas it produced mean CPE of 8.2% to 71.6%, IC_{50} of 325.9 µM and an Imax of 71.6%, by the CCM. The vehicle control produced a mean CPE of 9.1 ± 1.3% and an Imax of 9.1%, by the PM, whereas it produced a mean CPE of 9.0 ± 0.3% and an Imax of 9%, by the CCM.

Doxycycline generated a mean CPE of 11.0% to 82.5%, IC_{50} of 146.5 µM and an Imax of 82.5%, by the PM, whereas it produced a mean CPE of 11.1% to 83.8%, IC_{50} of 106.6 µM, and an Imax of 83.8%, by the CCM when observed against Human Adenovirus in the HeLa cell line. On the other hand, vehicle control produced a mean CPE of 9.7±0.5% and an Imax of 9.7%, by the PM, whereas it produced a mean CPE of 10.2 ± 0.6% and an Imax of 10.2%, by the CCM.

Similarly, when examined against the Human Respiratory Syncytial Virus (HRSV) in the HEp-2 cell line, Doxycycline generated mean CPE of 12.0% to 79.3%, IC_{50} of 225.5 µM and an Imax of 79%, by the PM, whereas it produced mean CPE of 11.2% to 81.1%, IC_{50} of 165.2 µM, and an Imax of 81%, by the CCM. The vehicle control produced a mean CPE of 8.8 ± 0.6%, and an Imax of 8.8%, by the PM, whereas it produced a mean CPE of 9.9 ± 0.9% and an Imax of 10.2%, by the CCM.

The CCM generally produced higher mean values for CPE inhibition compared to the PM. These results indicated in Table 2 suggest that Doxycycline may be a more potent drug as it exhibited a dose-dependent reduction than the vehicle control. Also, it did not produce any significant cytotoxicity effect up to 2250 µM. Similarly, the vehicle control had negligible cytotoxic effect.

Fig 1 depicts the antiviral activity of Doxycycline against six different viruses. The IC50 values indicate



	Dengue-2	Influenza A	Influenza B	Human rhinovirus 17	Human Adenovirus	RSV
IC50	135.5	262.3	330.9	387.3	146.5	225.5

	Dengue-2	Influenza A	Influenza B	Human rhinovirus 17	Human Adenovirus	RSV
R²	0.9911	0.9872	0.9771	0.9743	0.9784	0.9775

	Dengue-2	Influenza A	Influenza B	Human rhinovirus 17	Human Adenovirus	RSV
IC50	114.5	184.1	286.4	325.9	106.6	165.2

	Dengue-2	Influenza A	Influenza B	Human rhinovirus 17	Human Adenovirus	RSV
R²	0.9936	0.9871	0.9827	0.9795	0.9720	0.9782

Fig 1 — Antiviral activity of Doxycycline in prophylactic (1-A) and Co-Culture (1-B) methods

the concentration of Doxycycline required to reduce viral replication by 50% *in- vitro*. The lowest IC50value was observed for Human Adenovirus (146.5µM), followed by Dengue-2 (135.5µM), Influenza A (262.3µM), Influenza B (330.9µM) and Human Rhinovirus 17 (387.3µM) by PM (1-A) while Graph 1B depicts the IC50 values for Human Adenovirus (106.6 µM), followed by Dengue-2 (114.5 µM), Influenza A (184.1 µM), Influenza B (286.4 µM) and Human Rhinovirus 17 (325.9 µM) for CCM.

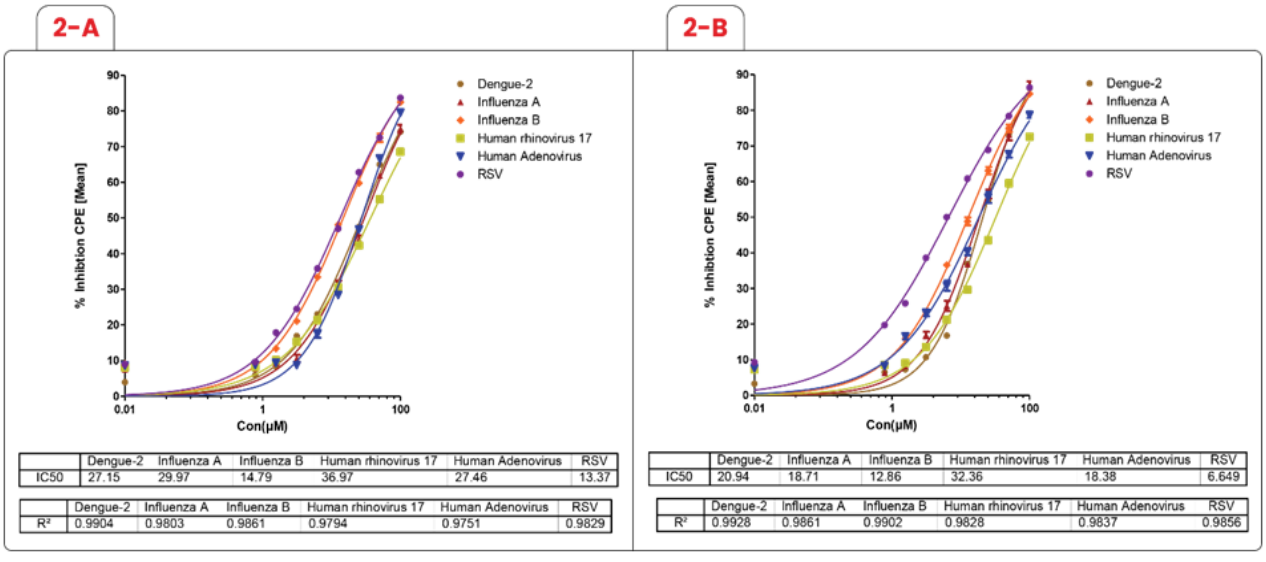
Similarly, Fig 2 depicts the antiviral activity of Ribavirin against the same viruses. The IC50value was observed for Human Adenovirus (27.46 µM), followed by Dengue-2 (27.15 µM), Influenza A (29.97 µM), Influenza B (14.79 µM) and Human Rhinovirus 17 (36.97 µM) by PMin graph 2-A while 2-B graph depicts the IC50 values for Human Adenovirus (18.38 µM), followed by Dengue-2 (20.94 µM), Influenza A (18.71 µM), Influenza B (12.86 µM) and Human Rhinovirus 17 (32.36 µM) for CCM.

Discussion

The study employed two methods, PM and CCM to evaluate the antiviral activity of Doxycycline. In PM, cells were cultured together and allowed to interact directly, while in CCM, different cell types were cultured in the same medium without physical contact, relying on the transfer of soluble materials for interaction. The Co-cultured cells were divided into target cells and helping cells, working together to form functional tissues and perform essential tasks. This co-operative interaction between different cell types in CCM influenced their behavior and activities[17].

The results showed that Doxycycline exhibited antiviral activity against all the tested viruses, although its effectiveness varied depending on the virus and the method used for testing. The PM was involved in treating the cells with the compounds before infecting them with the virus, while the CCM was involved in treating the virus and the cells with the compounds simultaneously. In the PM, Doxycycline exhibited IC50 values ranging from 114.5 to 387.3 µM. The R^2 values were high, indicating a strong correlation between the tested concentrations and the observed antiviral activities.

Ribavirin is an established antiviral agent with a known mechanism of action, specifically inhibiting viral replication (Table 3). It is widely recognized for its broad-spectrum antiviral activity[18]. On the other hand, Doxycycline, although primarily known as an antibiotic, has shown promising antiviral properties in recent studies. While the exact mechanism of Doxycycline's antiviral action is not fully understood, its potential as an adjunct treatment for viral infections is increasingly recognized. By harnessing its antiviral properties, Doxycycline could serve as a valuable therapeutic option to complement existing antiviral agents and minimize complications in patients with viral infections[10-12]. Another recent study observed the pathogenesis involved to attenuate Influenza Virus by inhibiting matrix metalloproteinases within the Neutrophils[19]. The findings of this study suggest that Doxycycline has the potential to be an antiviral drug for the treatment of viral infections and provides valuable insights into its antiviral activity against six different viruses, although its effectiveness varies depending on the virus and the method used.



	Dengue-2	Influenza A	Influenza B	Human rhinovirus 17	Human Adenovirus	RSV
IC50	27.15	29.97	14.79	36.97	27.46	13.37

	Dengue-2	Influenza A	Influenza B	Human rhinovirus 17	Human Adenovirus	RSV
R^2	0.9904	0.9803	0.9861	0.9794	0.9751	0.9829

	Dengue-2	Influenza A	Influenza B	Human rhinovirus 17	Human Adenovirus	RSV
IC50	20.94	18.71	12.86	32.36	18.38	6.649

	Dengue-2	Influenza A	Influenza B	Human rhinovirus 17	Human Adenovirus	RSV
R^2	0.9928	0.9861	0.9902	0.9828	0.9837	0.9856

Fig 2 — Antiviral activity of Ribavirin in prophylactic (2-A) and Co-Culture (2-B) methods

Table 2 — *The antiviral activity of Doxycycline against different viruses and cell lines*

Virus and Cell Lines	1-PM	1-CCM	2-PM	2-CCM	3-PM	3-CCM	4-PM	4-CCM	5-PM	5-CCM	6- PM	+6-CCM
Group (μM)	Mean Inhibition CPE [%] ± SD											
Vehicle control	3.1± 0.5	5.2± 0.5	6.8±1.0	6.9±0.9	9.2±0.3	7.4±1.0	9.1± 1.3	9.0± 0.3	9.7± 0.5	10.2± 0.6	8.8 ± 0.6	9.9 ± 0.9
8.79	6.5±0.8	13.2±0.9	8.0±0.4	6.9±0.8	9.2±0.5	13.3±2.0	9.7±0.6	8.2 ±1.6	11.0±0.6	11.1±1.4	12.0±1.5	11.2±0.5
17.58	16±2.6	18.9±1	9.9±1.0	15.2±0.6	10.9±1.0	16.9±1.7	11.4± 0.7	10.2±1.1	20.1±2.0	25.5±2.1	10.7±0.1	14.3±1.0
35.16	21.4±1.2	30.1±1.5	19.9±0.5	23.1±0.4	15.7±1.3	22.2±1.4	15.4±1.4	17.2±1.3	26.7± 0.4	35.1±1.3	15.0±1.1	26.1±1.5
70.313	36.6±0.9	39.4±1.6	25.6±1.9	29.8±2.6	24.4±1.1	31.4±1.5	22.7±0.8	24.1±0.6	36.4±2.5	41.9±1.1	26.8±2.1	35.5±1.2
140.63	55.7±1.4	54.5±1	36.9±0.5	44.0±0.9	32.8±0.8	40.7±0.3	32.7±1.6	35.6±0.3	49.4± 2.7	54.5± 2.6	41.8±1.0	46.2±2.7
281.3	63.8±1.4	65.1±0.9	52.6±1.6	61.0±1.9	47.6±1.4	49.6±1.3	44.2±1.0	47.2±0.8	62.1±1.1	64.7±2.5	55.0±0.2	60.2±1.8
563	75.2±0.9	77.5±1.5	64.6±2.0	69.8±0.7	60.5±0.6	59.7±0.7	56.8±1.4	59.4±1.1	70.1±1.9	74.9±2.1	67.1±2.4	69.6±1.8
1125	84.9±1.7	86.7±0.7	74.8±0.8	79.5±0.8	71.0±0.5	68.2±0.7	68.3±1.0	71.6±0.7	82.5±1.6	83.8±2.4	79.3±0.3	81.1±1.6
IC50	135.5 μM	114.5 μM	262.3 μM	184.1 μM	330.9 μM	286.4 μM	387.3 μM	325.9 μM	146.5 μM	106.6 μM	225.5 μM	165.2 μM
Imax	85%	87%	75%	80%	71 %	68 %	68.3%	71.6%	82.5 %	83.8 %	79%	81 %

1: Dengue 2 virus in BHK-21cell line; 2: Influenza A Virus (H1N1) in MDCK cell line; 3: Influenza B Virus in a MDCK Cell Infection Model; 4: Human Rhinovirus 17 in H1HeLa cell line; 5: Human Adenovirus in HeLa cell line; 6: Human Respiratory Syncytial Virus in HEp-2 cell line, PM: Prophylactic Method, CCM: Co-culture Method, SD: Standard Deviation, CPE: Cytopathic Effect

Table 2 — *The antiviral activity of Ribavirin against different viruses and cell lines*

Virus and Cell Lines	1-PM	1-CCM	2-PM	2-CCM	3-PM	3-CCM	4-PM	4-CCM	5-PM	5-CCM	6- PM	+6-CCM
Group (μM)	Mean Inhibition CPE [%] ± SD											
Vehicle control	4.0±0.5	3.3±0.5	7.5±0.8	7.3±1.3	9.1±1.0	7.5±1.2	8.1±0.9	7.3±0.8	8.7±0.6	7.7±0.5	9.0±0.4	9.2±1.1
8.79	6.0±0.3	6.5± 0.8	7.7±0.6	6.4±0.9	8.6± 0.4	8.5±0.9	8.2±1.0	8.6±0.6	8.8±0.1	8.3±0.4	9.6±0.7	19.8±1.9
17.58	8.3±1.8	7.3±0.7	9.9±1.9	8.2±0.5	13.4±1.0	15.9±0.7	10.2±0.9	9.1±0.9	9.4±0.1	16.6±1.5	17.8±1.2	25.8±1.9
35.16	17.0±0.8	10.7±1.9	10.6±1.8	16.9±1.6	21.1±1.2	23.4±1.4	15.3±1.1	13.6±0.2	8.7±0.3	23.2±1.7	24.5±2.4	38.6±1.3
70.313	23.1±2.7	16.8±1.4	21.2±1.4	25.1±2.5	33.4±0.2	36.6±0.8	21.4±1.6	13.6±0.2	17.6±1.9	30.8±2.6	35.8±2.7	50.0±0.9
140.63	31.4±1.7	36.9±2.1	31.5±1.7	36.8±1.1	48.1±1.1	48.8±1.9	30.4±1.8	29.7±0.9	28.4±0.5	40.4±1.8	47.0±2.1	60.9±2.2
281.3	47.5±1.9	54.6±1.5	43.9±2.0	56.5±2.2	59.8±1.2	63.1±1.8	42.3±0.7	29.7±0.9	46.8±1.5	55.6±2.7	62.8±0.9	68.9±1.9
563	65.0±0.9	73.2±0.9	61.9±1.4	73.2±2.8	72.5±2.0	75.0±1.9	55.3±1.3	59.5±1.6	66.8±0.4	67.7±1.6	72.5±2.9	78.4±1.2
1125	74.2±0.7	86.3±0.8	75.0±1.9	87.2±1.6	82.5±0.5	84.7 1.2	68.6±1.4	59.5±1.6	79.6±0.9	78.8±1.5	83.7±2.4	86.4±0.7
IC50	27.15 μM	20.94 μM	29.97 μM	18.71 μM	14.79 μM	12.87 μM	36.97 μM	59.5 1.6	27.46 μM	18.38 μM	13.37 μM	6.64 μM
Imax	74%	86%	75%	87%	83 %	85 %	68.6%	72.6%	79.6%	78.8%	84%	86 %

1: Dengue 2 virus in BHK-21cell line; 2: Influenza A Virus (H1N1) in MDCK cell line; 3: Influenza B Virus in a MDCK Cell Infection Model; 4: Human Rhinovirus 17 in H1HeLa cell line; 5: Human Adenovirus in HeLa cell line; 6: Human Respiratory Syncytial Virus in HEp-2 cell line, PM: Prophylactic Method, CCM: Co-culture Method, SD: Standard Deviation, CPE: Cytopathic Effect

The limitations of this study include that the antiviral activity of Doxycycline *in-vitro* may not accurately reflect the Doxycycline's effectiveness in an *in-vivo* or clinical setting. Furthermore, the study was conducted using cell cultures and a limited number of viruses that may not replicate the complexity of viral infections *in-vivo* and clinically. The study did not explore the mechanisms underlying Doxycycline's antiviral activity, which limits our understanding of how the drug works against viruses.

The strengths of the study are that a Doxycycline was examined against a broad range of viruses that provide a comprehensive understanding of its antiviral properties. As this was an *in-vitro* analysis performed in laboratory settings, this approach provided more controlled and consistent results than clinical studies, which can be affected by confounding factors. Since Doxycycline produced effective antiviral activity at higher doses without toxicity against the viruses tested in this study, it could be repurposed as a readily available and cost-effective treatment option for viral infections, particularly in resource-limited settings where access to specific antiviral agents is limited after further analysis and randomized controlled trials.

Conclusion

In conclusion, the findings of this research offer important new information regarding the antiviral activity of Doxycycline against a total of six distinct viruses, however, the degree to which the drug is successful varies not only with the virus but also with the method that was applied. The antiviral impact of Doxycycline was observed when compared to the effects observed with vehicle control.This antiviral property of

Doxycycline will prove beneficial in minimizing secondary infections and complications especially when used as an adjunct.

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Conflict of Interest : All authors declare no Conflict of Interest.

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