JSS ACADEMY OF HIGHER EDUCATION & RESEARCH, MYSURU
(Deemed to be University - Accredited 'A' Grade by NAAC)
JSS College of Pharmacy, Ootacamund
(An ISO 9001:2015 Certified Institute)

Department of Pharmaceutical Biotechnology
Animal Tissue Culture Laboratory
In Vitro Cytotoxicity and Antiviral Studies

Name of the Client: **Dr. L. Ramanathan**
Lead Scientist-GPD

Company: **M/s Apex Laboratories Pvt. Ltd.**
Guindy, Chennai - 600032

Sample(s): **Clevira Tablets – Granules & Syrup**

Cell Line: Vero (African Green Monkey, Kidney)

Assay: Cytotoxicity Studies and Antiviral MTT Assay

Concentration used: 250 – 7.8125 µg/ml

1. In vitro cytotoxicity assay

Determination of mitochondrial synthesis by MTT assay

Principle

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based.

The principle involved is the cleavage of tetrazolium salt MTT (3-(4,5 dimethyl thiazole-2 yl)- 2,5-diphenyl tetrazolium bromide) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The numbers of cells were found to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986).

Procedure

i. The monolayer cell culture was trypsinized and the cell count was adjusted to $1.0x10^5$ cells/ml using DMEM medium containing 10% FBS.
ii. To each well of a 96 well microtitre plate, 100µl of the diluted cell suspension (approximately 10,000 cells/well) was added.
iii. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with medium and 100µl of different test sample concentrations prepared in maintenance media were added per well to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 48 hrs in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded every 24 hours.
iv. After 48 hours, the sample solutions in the wells were discarded and 20 µl of MTT (2mg/ml) in MEM-PR (MEM without phenol red) was added to each well.
v. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO_2 atmosphere.
vi. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan.
vii. The absorbance was measured using a microplate reader at a wavelength of 540nm.

The percentage cell viability was calculated using the following formula and concentration of drug or test samples needed to inhibit cell growth by 50% values were generated from the dose-response curves for each cell line.

$$\left[\% \text{ Cell Viability} = \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}}\right] \times 100$$

Reference:

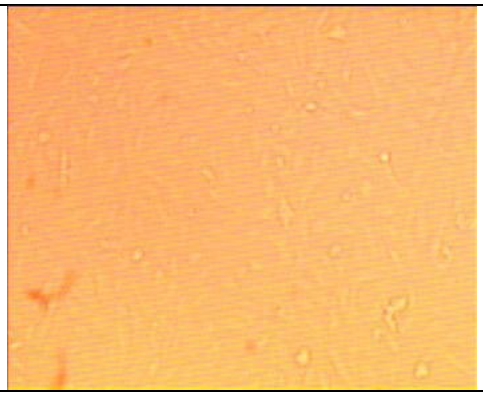
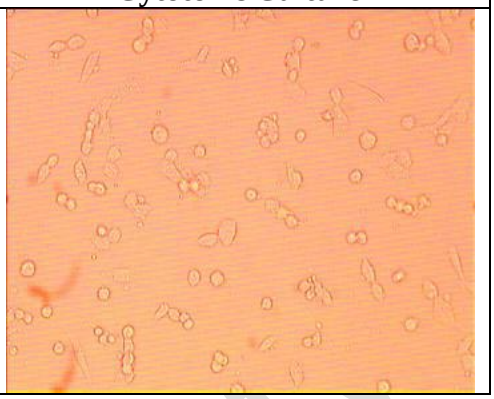
1. Francis D, Rita L. 1986. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods. 89: 271-7.

Table 1: Cytotoxicity Studies

Sample	Cell Line used	CC_{50} µg/ml
Granules	Vero	210.31 ± 7.02
Syrup		197.01 ± 6.69

Values are mean ± standard deviation of three independent experiments.

Figure 1

Cell Line	Normal Culture	Cytotoxic Culture
Vero		

2. *In vitro* antiviral studies – HSV-I

Cell Line: Vero (African Green Monkey, Kidney)

Virus: HSV-1

Assay: MTT antiviral assay

Concentration used: 50, 25, 12.5, 6.25 µg/ml

MTT antiviral assay

A rapid and sensitive procedure to evaluate antiviral compounds *in vitro* is based on spectrophotometrical assessment for viability of virus- infected and mock infected cells via *in situ* reduction of a tetrazolium dye MTT. Mitochondrial enzymes of viable cells convert yellow water soluble dye MTT to a soluble, purple coloured insoluble formazan. The quantitation of the amount of the formazan product present in each well of the microtitre plate is then determined spectrophotometrically at 490/650 nm. While the toxicity of the test compounds to host cells is measured concurrently in the same microtitre plate.
(Kurokawa et al., 2016)

Procedure

Cells (1×10^5 cells/ml) were seeded on 96-well tissue culture plates. After a 24 h period of incubation, the medium was removed and the HSV-1 was added at the dose of 100 $TCID_{50}$ for 2 hrs to ensure the attachment of virus to the cell and after 2 hrs the cells were washed with PBS and replenished with 100 µl of medium containing increasing concentrations of the compounds (serially diluted twofold). As cell control, 100 µl of medium only is added and as virus control 100 µl of 100 $TCID_{50}$ dose was added. After three days of incubation, the

medium was removed and 50 ml of MTT solution (2 mg/ml) was added to each well for 4 h at 37 ºC. Then, 100 µl of DMSO was added to each well in order to dissolve the formazan crystals. After shaking gently the plates for 10 min to dissolve the crystals, the colour reaction was measured in an automated microplate reader at 490 nm. The untreated control was arbitrarily set as 100%. For each compound, the percentage of cell protection/virus inhibition can be calculated as,

$$\frac{(\text{Mean OD of control group} - \text{Mean OD of treated group}) \times 100}{\text{Mean OD of control group}}$$

Reference:

1. Masahiko Kurokawa, Ashish Wadhwani, Hisahiro Kai, Muneaki Hidaka, Hiroki Yoshida, Chihiro Sugita, Wataru Watanabe, Koji Matsuno, and Akinori Hagiwara. Activation of Cellular Immunity in Herpes Simplex Virus Type 1- Infected Mice by the Oral Administration of Aqueous Extract of *Moringa oleifera* Lam. Leaves Short title: Activation of cellular immunity by Moringa oleifera extract. Phytotherapy Research, 2016; 30(5): 797–804.

Results: Antiviral activity

Table 2: Antiviral activity of extracts against HSV-1 virus at 100 $TCID_{50}$ (50% Tissue culture infectivity dose).

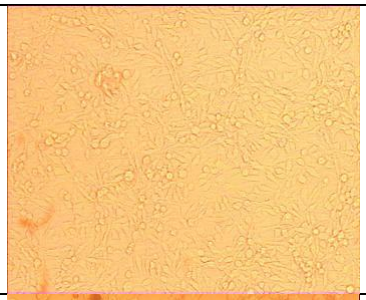
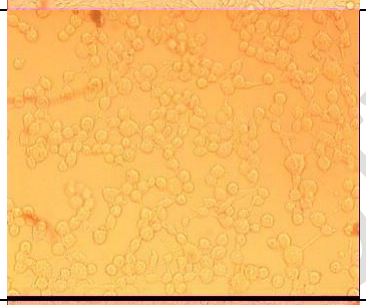
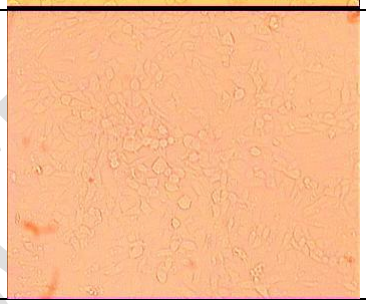
Sample Description	Concentration µg/ml	100 $TCID_{50}$ % Virus Growth
Granules	50	28.45
	25	33.95
	12.5	54.15
	6.25	88.45
Syrup	50	44.4
	25	59.4
	12.5	88.95
	6.25	93.95

Table 3: Anti-HSV-1 activity in Vero cells

Treatment	IC_{50} µg/ml	CC_{50} µg/ml
Granules	18.60 ± 3.05	210.31 ± 7.02
Syrup	41.09 ± 5.13	197.01 ± 6.69

Values are mean ± standard deviation of three independent experiments.

Figure 2

Vero Cell control			
Virus control- HSV1			
Cell protection at 50 µg/ml			

***3. In vitro* antiviral studies – HSV-2**

Cell Line: Vero (African Green Monkey, Kidney)

Virus: HSV-2

Assay: MTT antiviral assay

Concentration used: 50, 25, 12.5, 6.25 µg/ml

MTT antiviral assay

A rapid and sensitive procedure to evaluate antiviral compounds *in vitro* is based on spectrophotometrical assessment for viability of virus- infected and mock infected cells via *in situ* reduction of a tetrazolium dye MTT. Mitochondrial enzymes of viable cells convert yellow water soluble dye MTT to a soluble, purple coloured insoluble formazan. The quantitation of the amount of the formazan product present in each well of the microtitre plate is then determined spectrophotometrically at 490/650 nm. While the toxicity of the test compounds to host cells is measured concurrently in the same microtitre plate.

(Kurokawa et al., 2016)

Procedure

Cells (1×10^5 cells/ml) were seeded on 96-well tissue culture plates. After a 24 h period of incubation, the medium was removed and the HSV-2 was added at the dose of 100 $TCID_{50}$ for 2 hrs to ensure the attachment of virus to the cell and after 2 hrs the cells were washed with PBS and replenished with 100 µl of medium containing increasing concentrations of the compounds (serially diluted twofold). As cell control, 100 µl of medium only is added and as virus control 100 µl of 100 $TCID_{50}$ dose was added. After three days of incubation, the medium was removed and 50 ml of MTT solution (2 mg/ml) was added to each well for 4 h at 37 ºC. Then, 100 µl of DMSO was added to each well in order to dissolve the formazan crystals. After shaking gently the plates for 10 min to dissolve the crystals, the colour reaction was measured in an automated microplate reader at 490 nm. The untreated control was arbitrarily set as 100%. For each compound, the percentage of cell protection/virus inhibition can be calculated as,

$$\frac{(\text{Mean OD of control group} - \text{Mean OD of treated group}) \times 100}{\text{Mean OD of control group}}$$

Reference:

1. Masahiko Kurokawa, Ashish Wadhwani, Hisahiro Kai, Muneaki Hidaka, Hiroki Yoshida, Chihiro Sugita, Wataru Watanabe, Koji Matsuno, and Akinori Hagiwara. Activation of Cellular Immunity in Herpes Simplex Virus Type 1- Infected Mice by the Oral Administration of Aqueous Extract of *Moringa oleifera* Lam. Leaves Short title: Activation of cellular immunity by Moringa oleifera extract. Phytotherapy Research, 2016; 30(5): 797–804.

Results: Antiviral activity

Table 4: Antiviral activity of extracts against HSV-2 virus at 100 $TCID_{50}$ (50% Tissue culture infectivity dose).

Sample Description	Concentration µg/ml	100 $TCID_{50}$ % Virus Growth
Granules	50	21.95
	25	27.45
	12.5	44.45
	6.25	71.95
Syrup	50	54.95
	25	60.95
	12.5	77.45
	6.25	88.95

Table 5: Anti-HSV-1 activity in Vero cells

Treatment	IC_{50} µg/ml	CC_{50} µg/ml
Granules	12.49 ± 2.93	210.31 ± 7.02
Syrup	58.56 ± 3.51	197.01 ± 6.69

Values are mean ± standard deviation of three independent experiments.

Obesrvation:

The antiviral activity of Clevira Tablets – Granules & Syrup was carreid out against HSV-1 and HSV-2 viruses. The 100 $TCID_{50}$ virus concentrtation was used for HSV-1 and HSV-2 viruses. The results indicates that the the granules showed good % of cell protection (71.55%) and selectivity index (CC_{50}/IC_{50}) of 11.66 when compare to syrup (55.6%) and selectivity index of 4.81 against HSV-1 virus. The granules of clevira also showed the activity against HSV-2 virus with % cell protection of 78.05% and selectivity index of 16.81 when compare with syrup (45.05%) and selectivity index of 3.39.

The results clearly indicates that the granules of clevira showed the activity against both the viruses and more specifically with HSV-2 virus when compare to syrup.

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