Dr.D.Y.Patil Medical College, Hospital & Research Centre, Pimpri, Pune– 411018.
Central Research Facility, Interdisciplinary Research
Tissue culture and Cell Biology Laboratory

TEST REPORT

Test requested: ***In vitro* cytotoxicity test-MTT.**

Test material:
Number of test materials: 1
Nature of test material: Herbo mineral formulation
Test material Name: Onconil

Study dates:
Test material received: 21 Jan, 2023
Begin of testing: 07 Feb, 2023
End of testing 25 Mar, 2023
Final report: 10 Apr, 2023

- **Purpose**

The purpose of this test is to determine the potential cytotoxicity of the test material on a mammalian cell culture.

- **Equipment and reagents**

A) Cell lines:

Sr.No	Name of cell line	Tissue origin	Type of cell line
1	Vero	African green monkey kidney	Normal
2	MCF-7	Breast; Mammary gland of human	Adenocarcinoma

B) Reagents
1. Minimum Essential Medium-MEM (Gibco)
2. Fetal Bovine Serum-FBS (HiMedia)
3. Trypsin Versene Phosphate Glucose-TPVG (HiMedia)
4. Phosphate Buffered Saline-PBS (HiMedia)
5. Antibiotic-Antimycotic solution (HiMedia)
6. MTT powder (HiMedia)
7. Dimethyl Sulfoxide-DMSO (HiMedia)
8. Doxorubicin Hydrochloride injection IP (Fresenius Kabi)
9. Liquid Nitrogen (LN_2)

C) Consumables
1. Tissue Culture Flask (T-25) (HiMedia)
2. 96 Well plates (Tarsons)
3. Disposable Serological Pipettes (2,5,10,25 mL) (HiMedia)
4. Cryovials (1.8 mL)
5. Centrifuge tubes (15,50 mL)

6. Syringe and Syringe driven filters (0.22,0.45 µm)

D) Equipments:

1. Biosafety Cabinet (Microfilt)
2. CO_2 Incubator (Remi)
3. Centrifuge (Remi)
4. Inverted Microscope (Olympus)
5. ELISA Reader (Thermo Scientific)
6. Water bath (Equitron)
7. 2-8^oC Refrigerator (Samsung)

- **Experiment design**

Test method

Aseptic procedures were used for handling cell cultures. Vero and MCF-7 cells were cultured in MEM (10% FBS, 1% Penicillin-Streptomycin solution) at 37°C in a 5% CO_2, then digested by 0.25% TPVG to get single cell suspension. Approximately 1.5 x 10^6 cells/ml suspension were obtained by centrifuging (1400 rpm, 5 min) and re-suspending in 1 ml MEM.

The resuspended cells were counted using Neubaeur's chamber and 100 µl of 2 x 10^4 cells per well in 96-well plate was seeded, and cultured in incubator (5% CO_2, 37°C, >90% humidity). Cell morphology was evaluated to verify that the monolayer was satisfactory.

After 24 hours, the spent culture medium was decanted. A stock solution of 10mg/ml of test material was prepared in DMSO. The 96-well plates were then treated with 100 µl of extract of test material at different concentrations, Positive control- Doxorubicin, and Cell control respectively. The 96-well plate was incubated at 37°C and 5% CO_2 in incubator for 24 hours. The test assay was performed in triplicates. The time dependent assay was also performed at 24, 48 & 72 hrs. with repeated doses.

After incubation, the cell morphology was observed and 20 µl MTT (5mg/ml) was added to each well and then incubated at 37°C and 5% CO_2 for 4 hours. The medium in each well was decanted and 100 µl DMSO was added to each well to terminate the assay. The experimental plate was evaluated using spectrophotometer with the measurement wavelength at 570 nm.

- **Calculations and Statistical Analysis**

The concentration at which the OD of treated cells was reduced by approximately 50% with respect to the untreated control was determined to be IC50. The percentage viability of cells in the treatment groups was calculated with the help of the following formula,

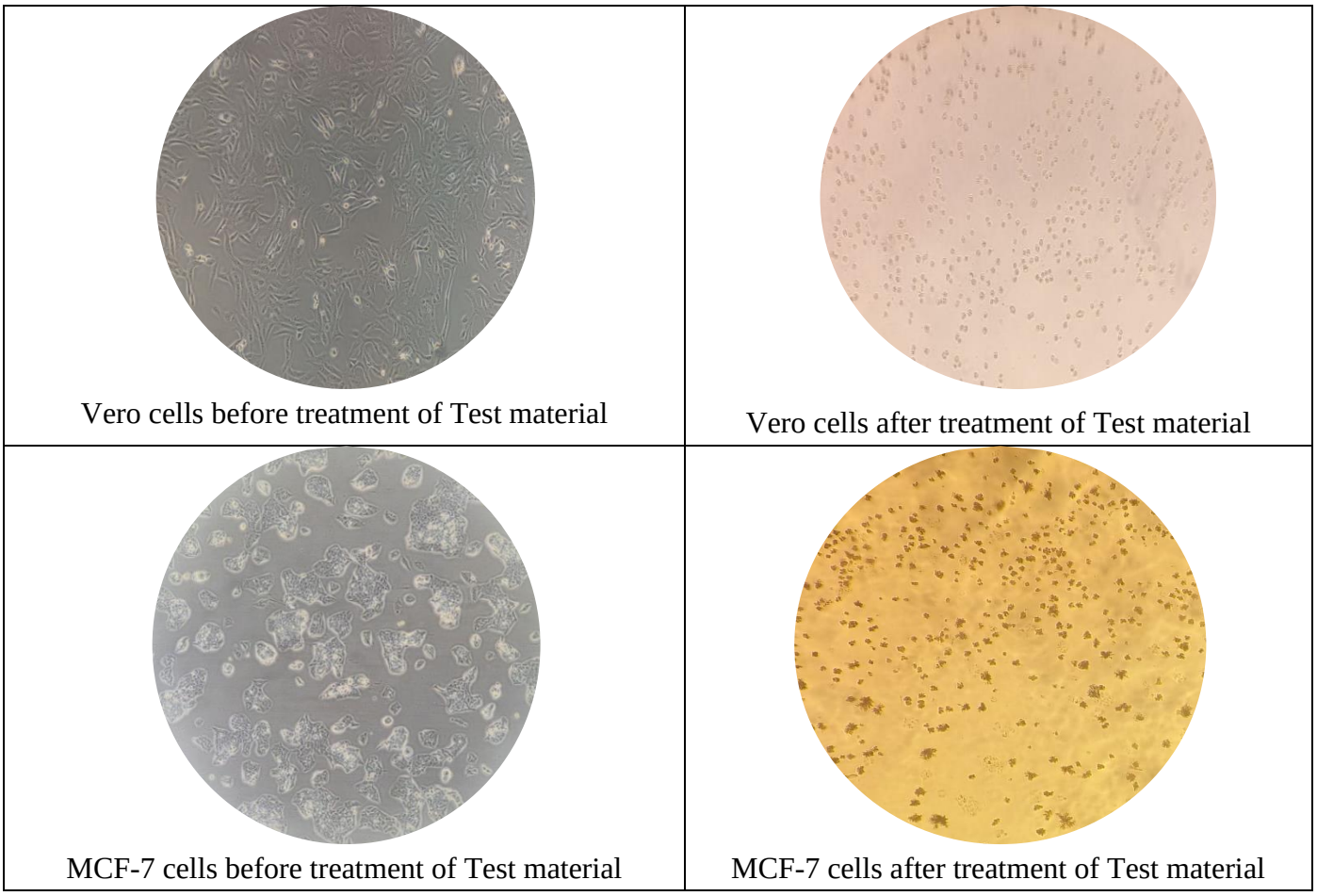
% Cell viability = [average OD of treated cells/average OD of control cells] × 100
Selectivity Index: IC50 of Normal cell line / IC50 of cancer cell line

- **Evaluation criteria**

1. The lower the % Viability value, the higher the cytotoxic potential of the test article is.
2. The higher the Selectivity Index value i.e., >1 theoretically more effective and safer a test substance would be during in-vivo treatment.

- **Results of the test**

1. Results of the cell morphology



Vero cells before treatment of Test material

Vero cells after treatment of Test material

MCF-7 cells before treatment of Test material

MCF-7 cells after treatment of Test material

Figure 1: Morphological observation of cells under inverted microscope (10X)

2. Results of the Cytotoxicity assay

Assay 1:

MCF-7: Onconil			
Conc µg/ml	Average OD	% Viability	% Cell death
5000	0.16	46.78	53.22
1000	0.18	51.20	48.80
500	0.19	54.66	45.34
100	0.25	72.72	27.28
50	0.28	79.83	20.17
10	0.44	125.74	-25.74
5	0.39	113.26	-13.26
IC50	**4330 µg/ml**		

Vero: Onconil			
Conc µg/ml	Average OD	% Viability	% Cell death
5000	0.145	15.66	84.34
1000	0.168	18.14	81.86
500	0.257	27.73	72.27
100	0.461	49.68	50.32
50	0.578	62.32	37.68
10	0.838	90.30	9.70
5	0.895	96.48	3.52
IC50	**167.9 µg/ml**		

Assay 2:

MCF-7: Onconil			
Conc µg/ml	Average OD	% Viability	% Cell death
5000	0.191	33.22	66.78
4000	0.282	49.07	50.93
3000	0.325	56.56	43.44
2000	0.345	60.05	39.95
1000	0.262	45.64	54.36
500	0.434	75.61	24.39
IC50	**3766.25 µg/ml**		

Vero: Onconil			
Conc µg/ml	Average OD	% Viability	% Cell death
500	0.300	37.32	62.68
400	0.307	38.19	61.81
300	0.318	39.60	60.40
200	0.349	43.50	56.50
100	0.415	51.68	48.32
50	0.543	67.58	32.42
IC50	**195.26 µg/ml**		

Assay 3:

MCF-7: Onconil			
Conc µg/ml	Average OD	%Viability	% Cell death
900	0.152	36.80	63.19
600	0.130	31.55	68.44
400	0.102	24.61	75.38
300	0.118	28.49	71.50
200	0.091	21.95	78.04
150	0.114	27.52	72.47
IC50	**1715.88 µg/ml**		

Vero : Onconil			
Conc µg/ml	Average OD	%Viability	% Cell death
900	0.141	24.90	75.10
600	0.166	29.32	70.68
400	0.202	35.81	64.19
300	0.229	40.53	59.47
200	0.250	44.31	55.69
150	0.261	46.14	53.86
IC50	**114.89 µg/ml**		

Assay 4: Time Dependent Assay

MCF-7: Onconil - 24 Hours			
Conc µg/ml	Average OD	% Viability	% Cell death
3000	0.195	43.31	56.69
2000	0.172	38.06	61.94
1000	0.123	27.20	72.80
500	0.077	17.00	83.00
300	0.092	20.40	79.60
150	0.071	15.67	84.33
IC50		**3462 µg/ml**	

Vero: Onconil - 24 Hours			
Conc µg/ml	Average OD	% Viability	% Cell death
3000	0.119	26.39	73.61
2000	0.136	30.08	69.92
1000	0.170	37.62	62.38
500	0.195	43.16	56.84
300	0.210	46.49	53.51
150	0.266	58.91	41.09
IC50		**156.67 µg/ml**	

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MCF-7: Onconil - 48 Hours			
Conc µg/ml	Average OD	% Viability	% Cell death
3000	0.139	24.60	75.40
2000	0.127	22.42	77.58
1000	0.120	21.30	78.70
500	0.118	20.83	79.17
300	0.117	20.65	79.35
150	0.113	19.94	80.06

Vero : Onconil -48 Hours			
Conc µg/ml	Average OD	% Viability	% Cell death
3000	0.110	19.41	80.59
2000	0.108	19.12	80.88
1000	0.106	18.70	81.30
500	0.098	17.40	82.60
300	0.095	16.81	83.19
150	0.088	15.58	84.42

MCF-7: Onconil - 72 Hours			
Conc µg/ml	Average OD	% Viability	% Cell death
3000	0.249	33.88	66.12
2000	0.234	31.93	68.07
1000	0.186	25.34	74.66
500	0.130	17.76	82.24
300	0.128	17.48	82.52
150	0.086	11.67	88.33
IC50		**5081.43 µg/ml**	

Vero: Onconil -72 Hours			
Conc µg/ml	Average OD	% Viability	% Cell death
3000	0.309	42.10	57.90
2000	0.256	34.88	65.12
1000	0.240	32.70	67.30
500	0.160	21.80	78.20
300	0.117	15.94	84.06
150	0.094	12.76	87.24
IC50		**3828.89 µg/ml**	

Results of Positive Control

Vero: Positive Control-Doxorubicin			
Conc µg/ml	Average OD	% Viability	% Cell death
20	0.496	53.41	46.59
15	0.565	60.88	39.12
10	0.531	57.26	42.74
5	0.555	59.81	40.19
2	0.502	54.13	45.87
IC50	**22.1 µg/ml**		

MCF-7: Positive Control-Doxorubicin			
Conc µg/ml	Average OD	% Viability	% Cell death
20	0.291	50.75	49.25
15	0.321	55.87	44.13
10	0.400	69.63	30.37
5	0.420	73.17	26.83
2	0.425	73.98	26.02
IC50	**20.83 µg/ml**		

Cumulative results of 3 assays:

Assay	MCF-7(IC50) µg/ml	Vero (IC50) µg/ml	Selectivity Index
1	4330	167.9	0.04
2	3766.25	195.26	0.05
3	1715.88	114.89	0.07
Average	3270.71	159.35	0.11

- **Conclusion**

1. Average IC50 value of Test material on MCF-7 cell line is **3270.71** µg/ml.
2. Average IC50 value of Test material on Vero cell line is **159.35** µg/ml.
3. IC50 value of Positive control correlates with the literature.
4. The Selectivity Index (SI) of Test material is **0.11** hence it is toxic to normal cells.
5. The test material shows antiproliferative activity on human breast adenocarcinoma cell line-MCF7 and may be evaluated for other cancer cell lines.

- **Authorization:**

Particulars	Name	Designation	Signature
Performed by	Manasi V Chavan	Research Assistant	
	Prachi V Prasad	Research Assistant	
Supervised by	Dr. Vaibhav S Ladke	Research Associate	
Quality Assurance	Avinash A Lendave	Quality Manager	
Approved by	Dr. Chandrashekhar Raut	Laboratory Director	

- **References:**

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