STUDY REPORT

Copy No. 1/2

Study Title

DETERMINATION OF IN VITRO NEUROPROTECTIVE POTENTIAL OF TEST FORMULATION AGAINST HYDROGEN PEROXIDE INDUCED OXIDATIVE STRESS IN RAT GLIOBLATSOMA CELLS (C6)

Study Director

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COMPLIANCE STATEMENT

The Study Director hereby declares that the work was performed under his supervision and in accordance with the mutually agreed study plan and the in house procedures. It is assured that the reported results represent the raw data obtained during the experimental work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study. The Study Director accepts overall responsibility for the technical conduct of the study as well as the interpretation, documentation and reporting of the results.

Date: 30/01/2022

Study Director
Dr. Ashok Godavarthi

CERTIFICATE OF AFFIRMATION AND CONFIDENTIALITY

The Management hereby attests to the originality, accuracy and authenticity of the study to the best of their knowledge. This report contains confidential and proprietary information of **M/s. Mallur Flora & Hospitality Pvt.Ltd., Sri Venkateshwara Manor,Bengaluru, Karnataka 560032.,** which will not be disclosed to anyone without the expressed or written approval of authorized personnel.

Date: 30/01/2022

Management
Dr.Ashok G
C.E.O

DECLARATION

The Study No, RR211179/CB/NP/12-21, entitled "**Determination of In vitro neuroprotective potential of test formulation against Hydrogen Peroxide-induced oxidative stress in Rat Glioblastoma cells**" has been inspected regularly according to the Standard Operating Procedure of the test facility's Quality Assurance Unit. The report was audited against approved study plan and pertinent raw data and accurately reflects the raw data.

Date: 30/01/2022

QA, Head
Gopi.M

ABBREVIATION USED

MCR	: Microbiology	°C	: Degree Centigrade
CB	: Cell Biology	%	: Percentage
MB	: Molecular Biology	gm	: Gram
BC	: Biochemistry	h	: Hour
DTL	: Drug Testing Laboratory	mg	: Milli gram
PC	: Preclinical	mL	: Millilitre
CL	: Clinical	nm	: Nano meter
NCCS	: National Centre For Cell Science	µL	: Micro litre
FBS	: Fetal bovine serum	µg	: Micro gram

PBS : Phosphate buffer saline

EDTA : Ethylenediaminetetraacetic acid

MTT : 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

TPVG : Trypsin Phosphate Versene Glucose Solution

Ham'S F12 : Medium from Chinese Hamster Ovary with F12 nutrient mixture

DMSO : Dimethyl sulfoxide

CTC_{50} : Cytotoxicity concentration

LIST OF TABLES

1. STUDY DETAILS

1.1.	Study title	:	Determination of *in vitro* neuroprotective potential of test formulation against Hydrogen Peroxide-induced oxidative stress in Rat Glioblastoma cells
1.2.	Study number	:	RR211179/CB/NP/12-21
1.3.	Test Substance	:	Immun Care
1.4.	Sponsor	:	**M/s. Mallur Flora & Hospitality Pvt.Ltd.** Sri Venkateshwara Manor, 490, 3rd Floor, Left Wing, 80 Feet Road, Ravindra Tagore Nagar Main Rd,RT Nagar,Bengaluru, Karnataka 560032.India.
1.5.	Test facility	:	Radiant Research Services Pvt. Ltd No: 99/A, 8th Main, 3rd Phase, Peenya industrial area, Bangalore -560 058, India.
1.6.	Test Schedule		
	Study Initiation Date	:	3/01/2022
	Experimental Start Date	:	5/01/2022
	Experimental Completion Date	:	24/01/2022
	Study Completion Date	:	29/01/2022
1.7.	Study Responsibilities		
	Study Director	:	Dr. Ashok Godavarthi
	Study Coordinator	:	Anuraag Muralidharan

2. OBJECTIVE

The purpose of this study is to evaluate the neuroprotective property of the test formulation (Immun care) against Hydrogen Peroxide induced toxicity in Rat Glioblastoma cells.

3. SUMMARY

The test formulation was evaluated for its *In vitro* neuroprotective study in Rat Glioblastoma cells. Firstly, the test formulation (Immun care) was estimated for cytotoxicity with different concentrations from 1000 to 31.25 µg/mL. The higher dilutions of the test formulation exhibited more than 83% cell viability on C6 cells; hence, the nontoxic concentrations were taken for further studies.

Chronic treatment of Rat Glioblastoma cells with hydrogen peroxide significantly caused oxidative stress as compared to untreated cell control. The test formulation exhibited significant protection against oxidative stress induced by Hydrogen peroxide in C6 cells.

4. GUIDELINES/REFERENCE

- Francis D and Rita L. Rapid "colorometric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability". Journal of Immunological Methods, 1986; 89: 271-277.
- Lee, A.Y., Wu, T.T., Hwang, B.R., Lee, J., Lee, M.H., Lee, S. and Cho, E.J., 2016. The neuro-protective effect of the methanolic formulation of Perilla frutescens var. japonica and rosmarinic acid against H2O2-induced oxidative stress in C6 glial cells. *Biomolecules & therapeutics*, *24*(3), p.338.

5. AMENDMENT AND DEVIATION PROCEDURE

No deviation has been adapted during the conduct of the experiment

6. MATERIALS

6.1. Test substance information

Test substance/item	:	Immun care
Common name	:	Immun care
RR No	:	RR211179
Batch No.	:	IMM202111006
Batch supplied by:	:	M/s. Mallur Flora & Hospitality Pvt.Ltd.
Batch produced on (Date)	:	27 Nov 2021
Expiry date	:	26 Nov 2023
Purity	:	NA
Physical appearance	:	Liquid
Storage condition	:	RT

6.2. Reference Material/Chemicals

Chemical	Batch / Lot No.	Manufacturer	Expiry Date
MTT	0000307556	Hi-media, India	-
Fetal Bovine serum	42F1190K	Gibco, USA	Jan-2024
PBS	0000370943	Hi-Media, India	Jan-2022
Trypsin	000047277	Hi-Media, India	March-2023
Antibiotics	0000416266	Hi-Media, India	Mar-2022
Ham's F-12	0000398781	Hi-Media, India	Jul-2022

6.3. Equipments

S. No.	Name of the Instrument	Make	Instrument ID
1.	Biosafety Cabinet	Ascesension, India	RRS/INS/CB/01
2.	CO_2 Incubator	NUAIRE, USA	RRS/INS/CB/02
3.	Inverted tissue culture microscope	Motic, China	RRS/INS/CB/04
4.	Automated micro plate reader	Biotek, USA	RRS/INS/MB/05
5.	-20 Deep Freezer	vestfrost, Denmark	RRS/INS/MB/01

7. METHOD

7.1. Outline of the method

The *in vitro* neuroprotective activity was performed for the test formulation on Rat Glioblastoma cells to evaluate the effect of test substance against Hydrogen peroxide induced toxicity.

7.2. Preparation of test solution

For studies, 10 mg of test substance was dissolved in DMSO and volume was made up with Ham's F-12 supplemented with 2% inactivated FBS to obtain a stock solution of 10 mg/ml concentration, followed by sterilization by syringe filtration. Two-fold serial dilutions were prepared from this for carrying out cytotoxic studies.

7.3. Cell Line and Culture medium

Rat Glioblastoma cells (C6) was obtained from National Centre for Cell Sciences (NCCS, Pune, India) and were cultured in Ham's F-12 media supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) and amphotericin B (5 µg/mL)

in a humidified atmosphere of 5% CO2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 well microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

7.4. Cytotoxicity studies

The monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using Ham's F-12 containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µL of different test concentrations of test drug was added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^0 C for 1 day in 5% CO2 atmosphere. After 24 h, microscopic examination was carried out and observations were noted. The drug solutions in the wells were discarded and 50 µL of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO2 atmosphere. The supernatant was removed and 100 µL of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated and the concentration of test drug needs to inhibit the cell growth by 50% (CTC50) values were generated from the dose-response curves for each cell line.

7.5 H_2O_2 induced cytotoxicity assay

The monolayer of cells were trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using respective media viz., Ham's F-12 containing 10% FBS. The test formulations were assayed for neuroprotective activity in pre and post H_2O_2 treatment. To each well of the 96 well microtitre plate, 0.1 mL of the diluted cell suspension was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off; the monolayer was washed once with medium. In pre-treatment group, cells were treated with the non-toxic

concentrations (Table 2) of the test substance (prepared in medium with 2% FBS) 3h prior to the addition of H_2O_2. After 3h, 500 µM of H_2O_2 was added to the cells. In post-treatment group, the cells were treated with 500 µM of H_2O_2 and incubated for 3h, followed by addition of non-toxic concentrations of the test substance. Ascorbic acid was used as the positive control for the experiment at a concentration of 100 µM. The plate was then incubated at 37 °C for 24 h in 5% CO_2 atmosphere, and MTT assay was carried out and observations were recorded using a microplate reader at 570 nm.

8. RESULTS

Table 1: Cytotoxic properties of test drug against C6 cell line

Sl. No	Name of Test Sample	Test Conc. (µg/mL)	% Cytotoxicity	CTC_{50} (µg/mL)
1	Immun care	1000	17.08±0.45	>1000
		500	13.68±3.10	
		250	13.26±3.44	
		125	15.21±0.85	
		62.5	9.51±1.49	
		31.25	6.23±1.33	

Table 2: Neuroprotective activity of test substance in C6 cells against Hydrogen peroxide induced toxicity

Sl. No	Samples	Concentration tested	%Protection over positive control	
			Pre-treatment	Post-treatment
1.	Immun care	500 µg/mL	57.31±1.301	56.54±0.598
		250 µg/mL	45.21±1.95	48.05±2.948
2.	Ascorbic acid	100 µM (17.61 µg/mL)	62.87±1.20	66.77±1.85

9. DISCUSSION AND CONCLUSION

The test formulation (Immun care) was assayed for *in vitro* cytotoxicity study against C6 cell line by MTT assay by exposing the cells to different concentrations of test substances (1000 µg/ml to 31.25 µg/ml). The Immun care was found to be safe in C6 cells in the higher dilutions tested. The CTC_{50} value of Immun care was above 1000 µg/mL. Hence, the *in vitro* neuroprotective activity of test substances was evaluated in Rat Glioblastoma cell line at non-toxic concentrations of the test formulation (500 and 250 µg/mL). When the cells were treated with the test substance pre and post-exposure of H_2O_2, the percentage protection exhibited was found to be significant and comparable with (**Table 2**) the standard drug (ascorbic acid). **The findings of the study suggest that the given compound Immun Care could exhibit promising neuroprotective effect against H_2O_2 induced toxicity in C6 cells.**

10. ARCHIVING

- Test Samples will stored for 30 days after the final report submission
- Raw data, documents, report will be archived for 30 days.

11. REPORT DISTRIBUTION

- Sponsor: One signed final report (Copy no. 1/2) in original.
- Archives: One signed final report (Copy no. 2/2) in original along with raw data file.