STUDY NO: RR211177/CB/CP/12-21



STUDY REPORT

Copy No. 1/2

Study Title

DETERMINATION OF IN VITRO CARDIO-PROTECTIVE POTENTIAL OF TEST FORMULATION AGAINST DOXORUBICIN INDUCED CELL DAMAGEIN RAT CARDIOMYOCYTES CELLS (H9C2)

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 STUDY NO: RR211177/CB/CP/12-21



DECLARATION

The Study No, RR211177/CB/CP/12-21, entitled "Determination of In vitro cardio-protective potential of test formulation against Doxorubicin-induced cell damage in Rat Cardiomyocyte 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

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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

DMEM : Dulbecco's Modified Eagle Medium

DMSO : Dimethyl sulfoxide

CTC₅₀ : Cytotoxicity concentration

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1. STUDY DETAILS

1.1. Study title : Determination of in vitro cardio-protective

Potential of test formulation against Doxorubicin-

induced cell damage in Rat Cardiomyocytes cells

1.2. Study number : RR211177/CB/CP/12-21

1.3. Test Substance : Cardiovascular Support

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 cardio-protective property of the test formulation (Cardiovascular Support) against Doxorubicin induced toxicity in Rat Cardiomyocyte cells.

3. SUMMARY

The test formulation was evaluated for its *In vitro* cardio-protective study in Rat Cardiomyocyte cells. Firstly the test formulation was estimated for cytotoxicity with different concentrations from 1000 to 62.5 µg/mL. The higher dilutions of the test formulation exhibited more than 87% cell viability on H9C2 cells; hence, the nontoxic concentrations were taken for further studies.

Chronic treatment of Rat Cardiomyocytes cells with Doxorubicin significantly caused oxidative stress as compared to untreated cell control. The test formulation at 1000 µg/mL exhibited 58.64% protection against cell damage induced by Doxorubicin in H9C2 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 doxorubicin-induced cell damage in H9C2 glial cells. *Biomolecules & therapeutics*, 24(3), p.338.

5. AMENDMENT AND DEVIATION PROCEDURE

No deviation has been adapted during the conduct of the experiment

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6. MATERIALS

6.1. Test substance information

Test substance/item : Cardiovascular Support

Common name : Cardiovascular Support

RR No : RR211177

Batch No. : CAR202109007

Batch supplied by: : M/s. Mallur Flora & Hospitality Pvt.Ltd.

Batch produced on (Date) : 23 SEPT 2021

Expiry date : 22 SEPT 2023

Purity : NA

Physical appearance : Liquid

Storage conditions : 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
DMEM - HG	414165	Gibco, USA	Dec-2022
Trypsin	000047277	Hi-Media, India	March 2023
Antibiotics	0000416266	Hi-Media, India	Mar-2022
DMEM-HG	0000395266	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* cardio-protective activity was performed for the test formulation on Rat Cardiomyocyte cells to evaluate the effect of test substance against Doxorubicin 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 DMEM-HG supplemented with 2% inactivated FBS to obtain a stock solution of 10 mg/ml concentration, followed by sterilizing through syringe filtration. Two-fold serial dilutions were prepared from this for carrying out cytotoxic studies.

7.3. Cell Line and Culture medium

Rat Cardiomyocyte cells (H9C2) was obtained from National Centre for Cell Sciences (NCCS, Pune, India) and were cultured in DMEM-HG media supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 µg/mL) and amphotericin B (5

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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 cm² 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 DMEM-HG 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 $100~\mu$ L of different test concentrations of test drug was added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° 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 $50~\mu$ L 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 $100~\mu$ L 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 Cardioprotective activity in Doxorubicin induced cytotoxicity assay

The experiment will be performed as per the standard protocol. Briefly, exponentially growing H9C2 cells will be trypsinized from the culture flask and 1.5×10^5 cells/mL is seeded in a 96 microtitre plate. After attaining confluency, culture medium from the wells will be discarded and cells were pre-incubated with different non-toxic concentrations of test samples along with the

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standard for 1 h in maintenance medium with 2% FBS. After 1 h pre-incubation, 5 μ M doxorubicin is added to all the wells except control wells. The plate was incubated for 24 h at 37°C with atmosphere of 5% CO2. After incubation MTT assay was performed to determine the cell viability. From the absorbance values from test and control groups, % protection offered by test samples against doxorubicin induced toxicity was calculated.

8. RESULTS

Table 1: Cytotoxic properties of test drug against H9C2 cell line

Sl. No	Name of Test Sample	Test Conc. (μg/mL)	% Cytotoxicity	CTC ₅₀ (µg/mL)
	Cardiovascular Support	1000	13.20±2.26	>1000
1		500	12.96±3.41	
		250	8.83±0.73	
		125	7.94±0.15	>1000
		62.5	7.97±0.46	
		31.25	4.81±1.78	

Table 2: Cardio-protective activity of test substance in H9C2 cells against Doxorubicin induced toxicity

Sl. No	Samples	Concentration tested	%Protection over positive control
1.	Cardiovascular Support	1000 μg/mL 500 μg/mL	58.644±4.82 44.294±1.18
2.	Ascorbic acid	100 μM (17.61 μg/mL)	86,23±0.89



9. DISCUSSION AND CONCLUSION

The test formulation (Cardiovascular Support) was assayed for *in vitro* cytotoxicity study against H9C2 cell line by MTT assay by exposing the cells to different concentrations of test substances (1000 μg/ml to 31.25 μg/ml). The Cardiovascular formulation was found to be safe in H9C2 cells in the higher dilutions tested. The CTC₅₀ value of Cardiovascular was above 1000 μg/mL. Hence, the *in vitro* cardio-protective activity of test substances was evaluated in Rat Cardiomyocytes cell line at non-toxic concentrations of the test formulation (1000 and 500 μg/mL). When the cells were treated with the test substance along with doxorubicin, the percentage protection exhibited at 1000 μg/mL was found to be appreciable. The findings of the study suggest that the given compound Cardiovascular Support could exhibit promising cardio-protective effect against doxorubicin induced toxicity in H9C2 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.

*****End of the report****