STUDY REPORT

Copy No. 1/2

Study Title

DETERMINATION OF *IN VITRO* HEPATOPROTECTIVE POTENTIAL OF TEST FORMULATION AGAINST ACETAMINOPHEN INDUCED TOXICITY IN HUMAN HEPATOCELLULAR CARCINOMA CELLS (HepG2)

Study Director

Dr. ASHOK GODAVARTHI

Test Facility

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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. RR211181/CB/HPA/12-21, entitled "**Determination of In vitro Hepatoprotective potential of test formulation against Acetaminophen-induced toxicity in Human Hepatocellular Carcinoma 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
CB	: Cell Biology
MB	: Molecular Biology
BC	: Biochemistry
DTL	: Drug Testing Laboratory
PC	: Preclinical
CL	: Clinical
NCCS	: National Centre For Cell Science
FBS	: Fetal bovine serum
PBS	: Phosphate buffer saline
°C	: Degree Centigrade
%	: Percentage
gm	: Gram
h	: Hour
mg	: Milli gram
mL	: Millilitre
nm	: Nano meter
µL	: Micro litre
µg	: Micro gram

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

CTC_{50} : Cytotoxicity concentration

LIST OF TABLES

1. STUDY DETAILS

1.1.	Study title	:	Determination of *in vitro* Hepatoprotective Potential of test formulation against Acetaminophen-induced inflammation Human Hepatocellular Carcinoma cells
1.2.	Study number	:	RR211181/CB/HPA/12-21
1.3.	Test Substance	:	Liver Health 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. Bangalore, 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	:	03/01/2022
	Experimental Start Date	:	05/01/2022
	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 Hepatoprotective property of the test formulation (Liver Health Support) against Acetaminophen induced toxicity in Human Hepatocellular Carcinoma cells.

3. SUMMARY

The test formulation was evaluated for its *In vitro* Hepatoprotective study in Human Hepatocellular Carcinoma cells. Firstly, the test formulation was estimated for its cytotoxicity with different concentrations from 1000 to 31.25 µg/mL. The highest concentration tested (1000 µg/mL) exhibited above 88% cell viability in HepG2 cells; hence, the non-toxic concentrations were chosen were taken for further studies.

Chronic treatment of Human Hepatocellular Carcinoma cells with Acetaminophen significantly caused cell damage as compared to untreated cell control. The test formulation exhibited significant protection against cell damage induced by Acetaminophen in HepG2cells.

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.
- Jannuzzi, A.T., Kara, M. and Alpertunga, B., 2018. Celastrol ameliorates acetaminophen-induced oxidative stress and cytotoxicity in HepG2 cells. *Human & experimental toxicology*, *37*(7), pp.742-751.

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	:	Liver Health Support
Common name	:	Liver Health Support
RR No	:	RR211181
Batch No.	:	LIV202105005
Batch supplied by:	:	M/s. Mallur Flora & Hospitality Pvt.Ltd
Batch produced on (Date)	:	12 MAY 2021
Expiry date	:	11 MAY 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 - 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* Hepatoprotective activity was performed for the test formulation on Human Hepatocellular Carcinoma cells to evaluate the effect of test substance against Acetaminophen-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 sterilization by syringe filtration. Two-fold serial dilutions were prepared from this for carrying out cytotoxic studies.

7.3. Cell Line and Culture medium

Human Hepatocellular Carcinoma cells (HepG2) 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 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 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 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 Acetaminophen 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., DMEM-HG containing 10% FBS. The test formulations were assayed for Hepatoprotective activity against acetaminophen induced toxicity in cells. 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. The cells were treated with acetaminophen (800 µg/mL), followed by addition of the non-toxic concentrations (Table 2) of the test formulation (prepared in medium with 2% FBS). Silymarin was used as the positive control for the experiment at a concentration of 500 µg/mL. 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 540 nm.

8. RESULTS

Table 1: Cytotoxic properties of test drug against HepG2 cell line

Sl. No	Name of Test Sample	Test Conc. (µg/mL)	% Cytotoxicity	CTC_{50} (µg/mL)
1	Liver Health Support	1000 500 250 125 62.5 31.25	10.71±2.68 10.30±2.87 8.84±1.31 6.73±1.81 3.30±0.59 0.07±0.09	>1000

Table 2: Hepatoprotective activity of test substance in HepG2 cells against Acetaminophen-induced toxicity

Sl. No	Samples	Concentration tested	% Protection over positive control
1.	Liver Health Support	1000 µg/mL 500 µg/mL	66.56±2.054 37.19±1.058
3.	Silymarin	500 µg/mL	70.01±2.81

9. DISCUSSION AND CONCLUSION

The test formulation (Liver Health Support) was assayed for *in vitro* cytotoxicity study against HepG2 cell line by MTT assay by exposing the cells to different concentrations of test substances (1000 µg/ml to 31.25 µg/ml). The Liver Health formulation was found to be safe in HEPG2cells in the higher dilutions tested. The CTC_{50} value of Liver Health was above 1000 µg/mL. Hence, the *in vitro* Hepatoprotective activity of test substances was evaluated in Baby Hamster Kidney Fibroblasts cell line at non-toxic concentrations of the test formulation (1000 and 500 µg/mL). When the cells were co-incubated with test substance along with acetaminophen, 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 Liver Health Support could exhibit promising Hepatoprotective effect against Acetaminophen-induced toxicity in HepG2cells.**

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*****