STUDY REPORT

Copy No. 1/2

Study Title

In vitro antioxidant study on GSH modulation in Human Hepatocytes

Study Director:

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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: 29/01/2022

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: 29/01/2022

Management
Dr. Ashok G
C.E.O

DECLARATION

The study no, RR/211183/CB/GSH/12-21, entitled "**In vitro antioxidant study on GSH modulation in Human Hepatocytes**" 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:29/01/2022

QA, Head
Gopi Mareedu

ABBREVIATION USED

MCR	: Microbiology	°C	: Degree Centigrade
CB	: Cell Biology	%	: Percentage
MB	: Molecular Biology	gm	: Gram
BC	: Biochemistry	hr	: Hour
DTL	: Drug Testing Laboratory	mg	:Milligram
PC	: Preclinical	mL	:Millilitre
CL	: Clinical	nm	:Nanometer
NCCS	: National Centre For Cell Science	µl	:Microlitre
FBS	: Fetal bovine serum	µg	: Microgram
PBS	: Phosphate buffer saline	RT	: Room Temperature

EDTA : Ethylenediaminetetraacetic acid

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

RT-PCR : Reverse transcription-polymerase chain reaction

TPVG :Trypsin Phosphate Versene Glucose Solution

DMEM :Dulbecco's Modified Eagle Medium

DMSO :Dimethyl sulfoxide

dNTP : Deoxynucleotide

CTC_{50} : Cytotoxicity concentration

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1. STUDY DETAILS

1.1. Study title	:	In vitro antioxidant study on GSH modulation in Human Hepatocytes
1.2. Study number	:	RR/211183/CB/GSH/12-21
1.3. Test Substance	:	Anti Ageing 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

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 Co-Ordinator	:	Mr. Anuraag Muralidharan

2. OBJECTIVE

The purpose of this study is to evaluate the modulatory property of the test formulation (Anti-Ageing Support) on GSH against Hydrogen peroxide induced toxicity in Human Hepatocyte cells.

3. SUMMARY

The test formulation was screened for its *In vitro* modulatory property on GSH in Human Hepatocyte cells. Test substance was first evaluated for its cytotoxicity with different concentrations from 1000 – 7.8 µg/mL. The test substance exhibited a CTC_{50} value above 1000 µg/mL in HepG2 cells. Hence, the higher dilutions were chosen for the antioxidant assay.

Chronic treatment of Human Hepatocyte cells with Hydrogen peroxide significantly decreased the levels of GSH compared to untreated cell control. The test formulation exhibited significant increase in the level of GSH compared to H_2O_2 control.

4. GUIDELINE/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.

Ethyl Acetate Fraction of Abelmoschus manihot (L.) Medic Flowers Exerts Inhibitory Effects Against Oxidative Stress in H2O2-Induced HepG2 Cells and D-Galactose-Induced Aging Mice

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	:	Anti Ageing Support
Common name	:	Anti Ageing Support
RR No	:	RR211183
Batch No.	:	ANT202012005
Batch supplied by:	:	**M/s. Mallur Flora & Hospitality Pvt.Ltd.**
Batch produced on (Date)	:	23 DEC 2020
Expiry date	:	22 DEC 2022
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
Trypsin	0000472777	Hi-Media, India	March-2023
Antibiotics	0000416266	Hi-Media, India	Mar-2022
DMEM-HG	0000395266	Hi-Media, India	Jul-2022
GSH assay kit	855AK17GB1	Elabscience, China	July-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* modulatory activity on GSH was performed for the test formulation in Human hepatocytes cells to evaluate the modulatory effect of test formulation on GSH against Hydrogen peroxide induced oxidative stress.

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 Hepatocytes 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% CO_2 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% CO_2 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 2.0 x 10^5 cells/ml using respective media viz., DMEM-HG containing 10% FBS. The test formulations were assayed for GSH modulatory activity post H_2O_2 treatment. To each well of the 12 well plates, 1.0 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 H_2O_2 (500 µM) and incubated for 3h, followed by addition of the non-toxic concentrations (Table 2) of the test formulation (prepared in medium with 2% FBS). Ascorbic acid was used as the positive control for the experiment at a concentration of 100 µM.

7.6 GSH modulation assay

The cell culture supernatant was collected from different treatment wells, centrifuged for 20 min at 1000Xg at 2-8°C. The samples were analysed to estimate the levels of GSH using Elabscience GSH ELISA kit by following the manufacturer's instruction.

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	Anti Ageing Support	1000	11.91±0.71	>1000
		500	11.04±0.91	
		250	10.88±0.82	
		125	10.76±0.54	
		62.5	9.46±0.69	
		31.25	8.83±0.73	
		15.625	7.94±0.15	
		7.8125	6.97±0.46	

Table 2: Modulatory activity on GSH by test substance in HepG2 cells against Hydrogen peroxide induced oxidative stress

Sl. No	Samples	Concentration tested	% Increase in GSH level over H_2O_2 control
1.	Anti Ageing Support	1000 µg/mL 500 µg/mL	106.37±0.53 97.15±0.74
2.	Ascorbic acid	100 µM (17.61 µg/mL)	141.96±0.14

9. DISCUSSION AND CONCLUSION

The test formulation (Anti- Ageing support) was assayed for its *in vitro* cytotoxicity in HepG2 cell line by MTT assay. The cells were exposed to different concentrations of test substances (1000 µg/ml to 7.8 µg/ml) to determine the CTC_{50} value. The Anti-Ageing formulation was found to be safe in HepG2 cells even at the highest concentration tested (1000 µg/mL). The CTC_{50} value of Anti-Ageing was found to be above 1000 µg/mL. Hence, the test concentrations for *in vitro* modulatory activity were chosen as 1000 and 500 µg/mL. The test formulation at 1000 and 500 µg/mL exhibited a remarkable increase in the levels of GSH over hydrogen peroxide control (Table 2). **The findings of the study suggest that the given compound Anti Ageing Support could exhibit promising modulatory effect on GSH levels against hydrogen peroxide induced toxicity in HepG2 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****