STUDY REPORT

Copy No. 1/2

Study Title

IN VITRO GASTROPROTECTIVE PROPERTY OF TEST SUBSTANCE ON HUMAN GASTRIC ADENOCARCINOMA CELL LINE (AGS)

Study Director

Dr. ASHOK G

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: 09/02/2022

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: 09/02/2022

Management
Dr.Ashok G
C.E.O

DECLARATION

The Study No, RR211184/CB/GP/01-22, entitled "**In vitro gastro protective of test substance on Human Gastric Adenocarcinoma 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: 09/02/2022

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 Minimum Eagle Medium

DMSO : Dimethyl sulfoxide

CTC_{50} : Cytotoxicity concentration

LIST OF TABLES

1. STUDY DETAILS

1.1.	Study title	:	*In vitro* Gastroprotective property of test substance on Human Gastric Adenocarcinoma cells
1.2.	Study number	:	RR211184/CB/GP/01-22
1.3.	Test Substance	:	Gastro 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	:	21/01/2022
	Experimental Start Date	:	26/01/2022
	Experimental Completion Date	:	02/02/2022
	Study Completion Date	:	09/02/2022
1.7.	Study Responsibilities		
	Study Director	:	Dr. Ashok Godavarthi
	Study Coordinator	:	Anuraag Muralidharan

2. OBJECTIVE

The purpose of this study is to evaluate the Gastroprotective property of the test formulation (Gastro) against Ethanol induced toxicity in Human Gastric Adenocarcinoma cells.

3. SUMMARY

The test extract was evaluated for its *In vitro* gastroprotective study in Human Gastric Adenocarcinoma cells. Firstly the test extract was estimated for cytotoxicity with different concentrations from 1000 to 7.8 µg/mL. The highest concentration tested (1000 µg/mL) exhibited above 90.18% cell viability in AGS cells; hence, the non-toxic concentrations were chosen were taken for further studies.

Chronic treatment of Human Gastric Adenocarcinoma cells with Ethanol significantly caused cell damage as compared to untreated cell control. The test formulation did not exhibit any protection against toxicity induced by Ethanol in AGS 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.
- Yeo, M., Kim, D.K., Cho, S.W. and Hong, H.D., 2008. Ginseng, the root of Panax ginseng CA Meyer, protects ethanol-induced gastric damages in rat through the induction of cytoprotective heat-shock protein 27. Digestive diseases and sciences, 53(3), pp.606-613.

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	:	Gastro Support
Common name	:	Gastro Support
RR No	:	RR211184
Batch No.	:	GAS202101004
Batch supplied by:	:	M/s. Mallur Flora & Hospitality Pvt.Ltd.
Batch produced on (Date)	:	30 JAN 2021
Expiry date	:	29 JAN 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	-
Fetal Bovine serum	4222743	Gibco	Sep-2026
DPBS	0000474192	Hi-Media	March-2024
DMEM-HG	2365585	Gibco	Feb-2024
Trypsin	000047277	Hi-Media	March 2023
Antibiotics	0000493609	Hi-Media	Aug-2023
DMSO	519350205AO	FINAR	--

6.3. Equipments

S. No.	Name of the Instrument	Make	Instrument ID
1.	Biosafety Cabinet	Ascesension	RRS/INS/CB/01
2.	CO_2 Incubator	NUAIRE	RRS/INS/CB/02
3.	Inverted tissue culture microscope	Nikon	RRS/INS/CB/08
4.	Automated micro plate reader	Biotek	RRS/INS/MB/05
5.	-20 Deep Freezer	Vestfrost	RRS/INS/MB/01

7. METHOD

7.1. Outline of the method

The *in vitro* Gastroprotective activity was performed for the test formulation on Human Gastric Adenocarcinoma cells to evaluate the effect of test substance against Ethanol-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 Gastric Adenocarcinoma Carcinoma cells (AGS) 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 (5µg/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 Ethanol 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 extracts were assayed for Gastroprotective activity against Ethanol 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 ethanol (5%), followed by addition of the non-toxic concentrations (Table 2) of the test formulation (prepared in medium with 2% FBS). Pantoprazole was used as the positive control for the experiment at a concentration of 100 and 50µM. 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 540nm.

8. RESULTS

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

Sl. No	Name of Test Sample	Test Conc. (µg/mL)	% Cytotoxicity	CTC_{50} (µg/mL)
1	Gastro Support	1000	9.82±1.91	>1000
		500	9.64±2.78	
		250	9.23±0.47	
		125	9.17±1.31	
		62.5	8.21±2.68	
		31.25	4.29±1.14	
		15.625	1.61±2.04	
		7.8	-17.20±2.48	

Table 2: Gastroprotective activity of test substance in AGS cells against Ethanol induced toxicity

Sl. No	Samples	Concentration tested	% Protection over positive control
1.	Gastro Support + Ethanol	1000 µg/mL+ 5%	8.55±0.38
		500 µg/mL+ 5%	0.52±0.09
2.	Pantoprazole	100 µM	40.77±2.52
		50 µM	15.31±2.97

9. DISCUSSION AND CONCLUSION

The test formulation (Gastro Support) was assayed for *in vitro* cytotoxicity study against AGS cell line by MTT assay by exposing the cells to different concentrations of test substances (1000 µg/ml to 7.8µg/ml). The Gastro was found to be safe in AGS cells in the higher dilutions tested. The CTC_{50} value of Gastro was above 1000 µg/mL. Hence, the *in vitro* Gastroprotective activity of test substance was evaluated in Human Gastric Adenocarcinoma cell line at non-toxic concentrations of the test formulation (1000 and 500 µg/mL). **The test substance Gastro Support exhibited minimal against ethanol-induced toxicity in AGS cells (Table 2)**. The percentage protection exhibited by the test formulation was insignificant when compared to the standard compound.

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.

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