STUDY REPORT

Copy No. 1/2

Study Title

IN VITRO GLUCOSE UPTAKE ASSAY BY NON-RADIO LABELLED ASSAY IN RAT SKELETAL MYOBLAST CELL LINE (L6)

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: 21/02/2022

Study Director
Dr. Ashok G

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: 21/02/2022

Management
Dr.Ashok G
C.E.O

DECLARATION

The Study No.RR211180/CB/GU/12-21, entitled "*In vitro* glucose uptake assay by non-radio labelled assay in Rat Skeletal Myoblast cells (L6)" 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: 21/02/2022

QA, Head
Gopi.M

ABBREVIATION USED

MCR	: Microbiology	℃	: 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

CTC_{50} : Cytotoxicity concentration

LIST OF TABLES

1. STUDY DETAILS

1.1.	Study title	:	*In vitro* glucose uptake assay by non-radio labelled assay in Rat Skeletal Myoblast cells (L6)
1.2.	Study number	:	RR211180/CB/GU/12-21
1.3.	Test Substance	:	Sugar 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. 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	:	05/01/2022
	Experimental Start Date	:	18/01/2022
	Experimental Completion Date	:	18/02/2022
	Study Completion Date	:	21/02/2022
1.7.	Study Responsibilities		
	Study Director	:	Dr. Ashok Godavarthi
	Study Coordinator	:	Anuraag Muralidharan

2. OBJECTIVE

The purpose of this study is to evaluate the anti-diabetic activity of the test substance "Sugar Care" by glucose uptake assay in Rat Skeletal Myoblast cell line (L6).

3. SUMMARY

The test formulation was evaluated for its *In vitro* anti-diabetic activity in Rat Skeletal Myoblast cells. Firstly the test formulation was estimated for cytotoxicity with different concentrations from 1000 to 7.8µg/mL. The higher dilution of the test formulation exhibited more than 20.38% cell viability on L6 cells; hence, the nontoxic concentrations were taken for further studies.

In glucose uptake assay, the test substance at 500µg/ml showed significant activity by glucose uptake in Rat Skeletal Myoblast cells dose dependent increase in glucose uptake was observed.

4. GUIDELINES/REFERENCE

1. 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.

2. Suthar, Manish; Rathore, GarvendraS; Basniwal, PawanK; Jain, Deepti; Gupta, RN; Pareek, Anil (2009). Study of glucose uptake activity of Helicteres isora Linn fruits in L-6 cell lines. International Journal of Diabetes in Developing Countries, 29(4), 170–. doi:10.4103/0973-3930.57349

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	:	Sugar Care
Common name	:	Sugar Care
RR No	:	RR211180
Batch No.	:	SUG202103005
Batch supplied by:	:	M/s. Mallur Flora & Hospitality Pvt.Ltd.
Batch produced on (Date)	:	20 MARCH 2021
Expiry date	:	19 MARCH 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 - EDTA	0000472777	Hi-Media	Mar-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.	CO2 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/12
5.	-20 °C Deep Freezer	Vestfrost	RRS/INS/MB/10

7. METHOD

7.1. Outline of the method

The *In vitro* glucose uptake assay was performed for the test formulation on Rat Skeletal Myoblast cells (L6) to evaluate their modulatory effect of anti-diabetic activity by glucose uptake assay.

7.2. Preparation of test solution

10 mg of test substance was weighed and dissolved in DMEM-HG medium supplemented with 2% inactivated FBS to obtain a stock solution of 10mg/mL. Furthermore, serial-two fold dilutions were prepared from the stock solution to prepare lower concentrations for cytotoxicity testing.

7.3. Cell Line and Culture medium

Rat Skeletal Myoblast cells (L6) 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.

7.4. In-vitro glucose uptake assay

Glucose uptake activity of the test substance was determined in L6 cells. In brief, the 24 h cell cultures with 70-80% confluency in 60mm petri plates maintained in DMEM with 2% FBS. In case of L6 cells, the extent of differentiation was established by observing multinucleation of cells. The cells were serum starved overnight and at the time of experiment cells were washed with HEPES buffered Krebs Ringer Phosphate solution (KRP buffer) once and incubated with KRP buffer with 0.1% BSA for 30min at 37^0C. Cells were treated with different non-toxic concentrations of test and standard drugs for 30 min along with negative controls at 37^0C.

20µl of D-glucose solution was added simultaneously to each well and incubated at 37^0C for 30 min. After incubation, the uptake of the glucose was terminated by aspiration of solutions from wells and washed thrice with ice-cold KRP buffer solution. Cells were lysed with 0.1M NaOH solution and an aliquot of cell lysates were used to measure the cell-associated glucose. The glucose levels in cell lysates were measured using glucose assay kit (ERBA). Two independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls.

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)
1	Sugar Care	1000	20.38±2.24	>1000
		500	19.52±2.11	
		250	17.63±1.55	
		125	11.41±2.75	
		62.5	11.65±2.91	
		31.25	8.18±2.64	
		15.625	9.76±2.76	
		7.8	7.04±2.15	

Table 2: *In vitro* glucose uptake studies for test substance in L6 cell line.

RR No.	Sample ID	Concentration	% Glucose uptake over control
RR 211180	Test Substance	500 µg/ml	159.25
		250 µg/ml	107.4
	Metformin	10mmol	277
	Cell control	-	100

9. DISCUSSION AND CONCLUSION

The test formulation (Sugar Care) was assayed for *in vitro* cytotoxicity study against L6 cell line by MTT assay by exposing the cells to different concentrations of test substances (1000 µg/ml to 7.8 µg/ml). The Sugar Care formulation was found to be safe in L6 cells in the higher dilutions tested. The CTC_{50} value of Cardiovascular was above 1000 µg/mL. Hence, the *in vitro* glucose uptake assay by non-radio labelled assay in Rat Skeletal Myoblast cell line (L6) at non-toxic concentrations of the test formulation (500 and 250 µg/mL). **The findings of the study suggest that the given compound Sugar Care exhibited promising anti-diabetic effect in L6 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*****