DRAFT REPORT

Copy No. 1/2

Study Title

Evaluation of *in vitro* Cytoprotective effect of test substance against UV induced melanin synthesis in Mouse Skin Melanoma (B16-F10) cell line

Study Director

Dr. Ashok G

Test Facility
Radiant Research Services Pvt. Ltd
99/A, 8 main, III Phase, Peenya Industrial Area
Bangalore – 560 058
Ph: +91-80-50516699, +91-99640 27999
Email: info@radiantresearch.in www.radiantresearch.in

Table of Contents

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: 05/11/2022

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. Tocyen Beauty Cream, Mumbai, India**.,which will not be disclosed to anyone without the expressed or written approval of authorized personnel.

Date: 05/11/2022

Management
Dr. Ashok G
C.E.O

DECLARATION

The Study No: RR222098/CB/UV/09-22, entitled "**Evaluation of *in vitro* Cytoprotective effect of test substance against UV induced melanin synthesis in Mouse Skin Melanoma (B16-F10) cell line**" 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: 05/11/2022

QA Head
Mr. Gopi M

ABBREVIATIONS USED

MCR	: Microbiology	ºC	: 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

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

TPVG : Trypsin Phosphate Versene Glucose Solution

DMEM-HG : Dulbecco's Minimum Essential Media- High Glucose

DMSO : Dimethyl sulfoxide

CTC_{50} : Cytotoxicity concentration

EDTA : Ethylenediaminetetraacetic acid

IU : International Unit

LIST OF TABLE

1. STUDY DETAILS

1.1.	Study Title	: Evaluation of *in vitro* Cytoprotective effect of test substance against UV induced melanin synthesis in Mouse Skin Melanoma (B16-F10) cell line
1.2.	Study Number	: RR222098/CB/UV/09-22
1.3.	Test Product	: Tocyen beauty cream
1.4.	Sponsor	: M/s. Tocyen Beauty Cream, Mumbai, 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	: 14/09/2022
Experimental Start Date	: 16/09/2022
Experimental Completion Date	: 26/10/2022
Study Completion Date	: 05/11/2022

1.7. Study responsibilities

Study Director	: Dr. Ashok
Study Coordinator	: Mr. Vatsa Kapadia

2. OBJECTIVE

The purpose of this study is to evaluate test substance for its *in* vitro melanin inhibitory properties against UV induced melanin synthesis in B16-F10 cell line.

3. SUMMARY

In-vitro cytotoxicity of the test product (Tocyen beauty cream) was tested on Mouse Skin Melanoma (B16-F10) cell line by MTT assay. Cells were exposed to different concentrations ranging from 1000µg/mL to 7.8µg/mL in order to determine the percentage growth inhibition on Mouse Skin Melanoma (B16-F10) cell line. The test product, Tocyen beauty cream has exhibited a CTC_{50} value which is greater than 1000µg/mL on Mouse Skin Melanoma (B16-F10) cell line. The test product showed melanin inhibitory properties against UV induced in Mouse Skin Melanoma (B16-F10) cell line. The percentage of UV induced Melanin inhibition is 22.53 ± 0.0333 and 4.21 ± 0.0339% with the test product, Tocyen beauty cream.

4. GUIDELINES/REFERENCE

1. Francis D and Rita L. “Rapid colorimetric 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. Caporarello, Nunzia & Olivieri, Melania & Cristaldi, Martina & Rusciano, Dario & Lupo, Gabriella & Anfuso, Daniela. (2017). Melanogenesis in uveal melanoma cells: Effect of argan oil. International Journal of Molecular Medicine. 40. 10.3892/ijmm.2017.3104.

5. AMENDMENT AND DEVIATION PROCEDURE

No deviation has been adapted during the conduct of the experiment.

6. MATERIALS

6.1. Test product information

Test Substance	Common name	Name used in the report	Batch number	Physical appearance	Storage condition
RR222098	Tocyen beauty cream	RR222098 / Tocyen beauty cream	CG6122	Semi-solid	RT

6.2. Reference Material/Chemicals

Chemical	**Batch / Lot No.**	**Manufacturer**	**Expiry Date**
MTT	0000454015	HiMedia, India	Oct-2024
Fetal Bovine serum	2422662	Gibco, Brazil	Sep-2026
DPBS	0000474192	HiMedia, India	Mar-2024
DMEM-HG	2457194	Gibco, USA	Dec-2024
Sodium Hydroxide	2718590418	Fisher Scientific, USA	Apr-2023
Trypsin - EDTA	0000472777	HiMedia, India	Mar-2023
Antibiotics	0000493609	HiMedia, India	Aug-2023
DMSO	519350205	FINAR, India	Feb-2026

6.3. Equipments

S. No.	Name of the Instrument	Make	Instrument ID
1.	Biosafety Cabinet	Ascension, India	RRS/INS/CB/01
2.	CO2 Incubator	NUAIRE, USA	RRS/INS/CB/02
3.	Inverted tissue culture microscope	Nikon, Japan	RRS/INS/CB/08
4.	Automated micro plate reader	Biotek, USA	RRS/INS/MB/12
5.	-20 °C Deep Freezer	Vestfrost, Denmark	RRS/INS/MB/10

7. METHOD

7.1. Outline of the method

The *in vitro* cytotoxicity was performed for test product (Tocyen beauty cream) on Mouse Skin Melanoma (B16-F10) cell line to find a toxic concentration of the test product by MTT assay. The percentage inhibition of melanin content was examined in Mouse Skin Melanoma (B16-F10) cell line.

7.2. Preparation of test solution

10mg of test product 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.

10mg of test product was weighed and dissolved in DMEM-HG medium supplemented with 2% inactivated FBS to obtain a stock solution of 10mg/mL. Furthermore, non-toxic concentrations were prepared from the stock solution for anti-pigmentation studies.

7.3. Cell line and culture medium

Mouse Skin Melanoma cell line (B16-F10) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM-HG supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/mL) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 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. Cytotoxicity study was carried out in 96 well microtitre plate and anti-pigmentation studies was carried out in 6 well microtitre plate (Tarsons India Pvt. Ltd., Kolkata, India).

7.4. Cytotoxicity study by MTT assay

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, the monolayer washed once with medium and different test concentrations were added on to the partial monolayer in the microtitre plates. The untreated cells were maintained as cell control for comparison. The plates were then incubated at 37^o C for 24 h in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted after 24h, the test solutions in the wells were discarded and 100 µL of MTT is added with DPBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o 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 wave length of 490nm.

7.5 *In vitro* Melanin inhibition activity

Initially, the cytotoxicity studies in B16-F10 cells will be carried out by standard methods to determine CTC50 value. 1.5 x 10^5 cells will be seeded in 6 well plate after reaching 70-80% confluency, the study will be initiated. The supernatants will be aspirated from wells, cultures will be washed with DPBS and 2mL of DPBS will be added to each well. To perform the Irradiation part of the assay, the cells will be irradiated at room temperature for 50 minutes through the lid with 1.7mW/cm² (5 J/cm²). Non Irradiated control will be kept at room temperature in a dark box for approximately 50 minutes. After irradiation, DPBS will be decanted and the cells will be washed once with DPBS. The DPBS will be replaced with culture medium containing test substances and the plates will be incubated at 37º ± 1ºC for 24 hours. The plates will be then incubated at 37º C for 24 h in 5% CO2 atmosphere, and microscopic examination will be carried out and observations will be noted after 24h, the supernatants will be aspirated from wells and cultures will be washed twice with DPBS. The cells will be harvested by trypsinization and cell suspension will be centrifuged at 2500 rpm for 15min. The cell pellet will be washed with DPBS, centrifuged and cells will be lysed in 0.1N NaOH containing 10% DMSO. Samples will be heated at a temperature of 60 °C for 1h and cooled, the absorbance of the cell lysates will be measured at 490nm.

8. RESULTS

Table 1: Melanin modulation of test product (Tocyen Beauty Cream) against Mouse Skin Melanoma (B16-F10) cell line

Sl. No	Name of Test sample	Test Conc.	% Melanin Inhibition
1.	RR222098	1000µg/mL	22.53 ± 0.0333
2.	RR222098	500µg/mL	4.21 ± 0.0339

9. DISCUSSION AND CONCLUSION

The test product RR222098 (Tocyen beauty cream) was tested for their *in vitro* cytotoxicity studies on Mouse Skin Melanoma (B16-F10) cell line by MTT assay, where the test product was exposed to different concentrations ranging from 1000µg/mL to 7.8µg/mL in order to determine the percentage growth inhibition on Mouse Skin Melanoma (B16-F10) cell line. The test product RR222098 has exhibited a CTC_{50} value which is >1000µg/ml on Mouse Skin Melanoma (B16-F10) cell line. Further studies were carried out for both the test product at the non-toxic concentration of 1000µg/mL and 500µg/mL. The Percentage of UV induced-Melanin Inhibition was observed to be 22.53 ± 0.0333 and 4.21 ± 0.0339% with test product, Tocyen beauty cream (RR222098).

10. ARCHIVING

- Test Samples will be stored for 30 days after the final report submission.
- Raw data, documents report will be archived for 3 years.

11. REPORT DISTRIBUTION

- Sponsor: One signed final report (Copy no. 1/2) in the original.
- Archives: One signed final report (Copy no. 2/2) in original along with raw data file.

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