D R A F T R E P O R T

Copy No. 1/2

Study Title

Determination of *in vitro* antioxidant potential of test product against hydrogen peroxide induced oxidative stress in Human Keratinocytes (HaCaT) cell line

Study Director

Dr. Ashok G

Test Facility

Radiant Research Services Pvt. Ltd.

#99/A, 8th main, III Phase, Peenya Industrial Area,

Bangalore – 560058

Ph.: +91-8064516699, +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: 31/10/2022

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: 31/10/2022

Management
Dr. Ashok G
C.E.O

DECLARATION

The Study No. RR222098/CB/AO/09-22, entitled "**Determination of *in vitro* antioxidant potential of test product against hydrogen peroxide induced oxidative stress in Human Keratinocytes (HaCaT) 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: 31/10/2022

QA Head
Mr. Gopi M

ABBREVIATION USED

MCR	: Microbiology	%	: Percentage
CB	: Cell Biology	gm	: Gram
MB	: Molecular Biology	hr	: Hour
BC	: Biochemistry	mg	: Milligram
DTL	: Drug Testing Laboratory	mL	: Milliliter
PC	: Preclinical	nm	: Nano meter
CL	: Clinical	µL	: Microliter
PBS	: Phosphate buffer saline	µg	: Microgram
ºC	: Degree Centigrade		
EDTA	: Ethylenediaminetetraacetic acid		
RT-PCR	: Reverse transcription-polymerase chain reaction		
dNTP	: Deoxynucleotide Triphosphate		

LIST OF TABLE

1. STUDY DETAILS

1.1.	Study title	:	Determination of *in vitro* antioxidant potential of test product against Hydrogen Peroxide induced oxidative stress in Human Keratinocytes (HaCaT) cells
1.2.	Study number	:	RR222098/CB/AO/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. # 99/A, 8th Main, 3rd Phase, Peenya industrial area, Bangalore-560058
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	:	29/10/2022
1.7.	Study Responsibilities		
	Study Director	:	Dr. Ashok G
	Study coordinator	:	Mr. Vatsa Kapadia

2. OBJECTIVE

The purpose of this study is to evaluate the antioxidant properties of the test product against Hydrogen Peroxide induced toxicity in Human Keratinocyte (HaCaT) cell line.

3. SUMMARY

The Tocyen Beauty Cream were evaluated for its *in vitro* antioxidant activity in Human Keratinocyte cell line (HaCaT) cell line, the test product was first evaluated for its cytotoxicity with different concentrations from 1000µg/mL – 7.8µg/mL. The test product exhibited a CTC_{50} value above 1000µg/mL on the Human Keratinocyte (HaCaT) cell line. Hence non-toxic concentrations were taken for antioxidant study.

In the antioxidant study, the test product at tested concentrations (1000µg/mL and 500µg/mL) showed cytoprotective activity against oxidative stress induced by Hydrogen peroxide.

4. GUIDELINES/REFERENCE

1) Warinhomhoun, S., Muangnoi, C., Buranasudja, V., Mekboonsonglarp, W., Rojsitthisak, P., Likhitwitayawuid, K., & Sritularak, B. (2021). Antioxidant Activities and Protective Effects of Dendropachol, a New Bisbibenzyl Compound from Dendrobium pachyglossum, on Hydrogen Peroxide-Induced Oxidative Stress in HaCaT Keratinocytes. Antioxidants, 10(2), 252.
2) 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.

5. AMENDMENT AND DEVIATION PROCEDURE

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

6. MATERIALS

6.1. Test product information

Sample code	Sample 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

Chemicals	Batch / Lot No.	Manufacturer	Expiry Date
MTT	0000454015	HiMedia, India	Oct-2024
DMEM-HG	2365585	Gibco, USA	Feb-2024
Fetal Bovine serum	2422662	Gibco, Brazil	Sep-2026
DPBS	0000474192	HiMedia, India	Mar-2024
Trypsin - EDTA	0000472777	HiMedia, India	Mar-2023
Antibiotics	0000493509	HiMedia, India	Aug-2023
DMSO	J19A/0416/0305/13	SDFCL, India	Sep-2024

6.3. Equipments

S. No.	Name of the Instrument	Make	Instrument ID
1.	Biosafety Cabinet	Ascension, India	RRS/INS/CB/01
2.	CO_2 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/05

7. METHOD

7.1. Outline of the method

The *in vitro* antioxidant activity was performed for the test product on Human Keratinocyte (HaCaT) cell line to evaluate the effect of test substances against Hydrogen peroxide induced toxicity.

7.2 Cell line and Culture medium:

HaCaT (Human Keratinocytes cells) was procured from AddexBio, U.S. Stock cells were cultured in DMEM-HG supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100µg/mL) and amphotericin B (5µg/mL) in a 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 and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

7.3 Cytotoxicity studies

The cell culture monolayer was trypsinized and the cell count was adjusted to 1.5 x 10^5 cells for 2 mL using DMEM-HG containing 10% FBS. To each well of the 6 well plates, 2 mL of the diluted cell suspension was added. The cells were incubated for 24hr at 5% CO_2 until it formed a sub confluent monolayer. After 24hr, when a partial monolayer was formed (i.e., confluency 70% to 80%), the growth medium was replaced with 2mL of DMEM media containing test substances in different concentrations with 500µM H_2O_2, positive (Ascorbic acid) and negative control (H_2O_2). All treatments were done in replicates and each well of the 6 well plates were labeled with study number, treatment details, date and replicate number. The cells were kept at 37°C under 5% CO_2 for 24hr for cytotoxicity analysis.

8. RESULTS

Table 1: Antioxidant activity of test product in Human Keratinocytes (HaCaT) cell line against Hydrogen peroxide induced toxicity

Sl. No.	Samples	Concentration tested	% Protection
1.	Tocyen Beauty Cream	1000µg/mL 500µg/mL	125.79 ± 0.02 74.37 ± 0.03

9. DISCUSSION AND CONCLUSION

The test product Tocyen Beauty Cream tested for *in vitro* cytotoxicity studies against Human Keratinocyte cell line by MTT assay exposing the cells to different concentrations. The CTC_{50} value of the test product on HaCaT cell line was above 1000µg/mL. The *in vitro* antioxidant activity of test product was evaluated in Human Keratinocyte (HaCaT) cell line at non-toxic concentrations of the test product. **When the cells were co-treated with the test product and H_2O_2, the percentage protection exhibited by the test product against the control, Tocyen Beauty Cream (RR222098) showed the protection of 125.79 ± 0.02% and 74.37 ± 0.03% at 1000µg/mL and 500µg/mL respectively. The Tocyen Beauty Cream is showing good antioxidant property .**

10. ARCHIVING

- Test Samples will be stored for 3 months 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 original.
- Archives : One signed final report (Copy no. 2/2) in original along with raw data file.

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