DRAFT REPORT

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Study Title

Evaluation of moisturizing effect of test substance by *AQP-3* gene expression modulation in Human Keratinocytes

Study Director:

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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: 07/11/2022

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: 07/11/2022

Management
Dr.Ashok G
C.E.O

DECLARATION

The Study No. RR222098/MB/GE/09-22, entitled "**Evaluation of moisturizing effect of test substance by AQP-3 gene expression in Human Keratinocytes**" 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: 07/11/2022

QA, Head
Gopi M

ABBREVIATION USED

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

LIST OF TABLES AND FIGURES

1. STUDY DETAILS

1.1	Study title	:	Evaluation of moisturizing effect of test substance by *AQP-3* gene expression modulation in Human Keratinocytes
1.2	Study number	:	RR222098/MB/GE/09-22
1.3	Test Substance	:	M/s. Tocyen Beauty Cream, Mumbai, India.,
1.4	Test Facility	:	Radiant Research Services Pvt. Ltd No: 99/A, 8th Main, 3rd Phase, Peenya industrial area, Bangalore-560 058
1.5	Test schedule		
	Study Initiation Date	:	26/09/2022
	Experimental Start Date	:	03/10/2022
	Experimental Completion Date	:	26/10/2022
	Study Completion Date	:	31/10/2022
1.6	Study Responsibilities		
	Study Director	:	Dr. Ashok G
	Study coordinator	:	Mr. Gnanesh Rao

2. OBJECTIVE

The purpose of this study is to evaluate test substance for its moisturizing effect in Human Keratinocytes cell line.

3. SUMMARY

The test substance was evaluated for its *in vitro* potency to induce AQP-3 gene expression in Human Keratinocytes cell line (HaCaT), the test substance was first evaluated for its cytotoxicity with different concentrations from 1000 – 7.8 µg/mL. The test product, Tocyen beauty cream (RR222098) exhibited a CTC_{50} value of above 1000 µg/mL on the Human Keratinocytes respectively. Hence non-toxic concentrations were taken for gene expression studies. In the gene expression study, the test substance at tested concentrations showed moderate increase in the level of AQP-3 gene expression as compared to the untreated control in the semi-quantitative RT-PCR procedure.

4. GUIDELINES/REFERENCE

1. Schrader A, Siefken W, Kueper T, Breitenbach U, Gatermann C, Sperling G, Biernoth T, Scherner C, Stäb F, Wenck H, Wittern K, -P, Blatt T: Effects of Glyceryl Glucoside on AQP3 Expression, Barrier Function and Hydration of Human Skin. Skin Pharmacol Physiol 2012;25:192-199.
2. R. D. Barber, D. W. Harmer, R. A. Coleman, B. J. Clark, GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiological genomics 2005, 21 (3), S. 389–395.

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	Common name	Name used in the report	Batch number	Physical appearance	Storage condition
RR22098	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	4222743	Gibco, USA.	Sep-2026
DPBS	0000474192	HiMedia, India.	Mar-2024
DMEM-HG	2365585	Gibco, USA.	Feb-2024
Antibiotics	0000493509	HiMedia, India.	Aug-2023
DMSO	2122353	SRL, India.	Feb-2026
RNA Isoplus	ALZ1011N	Takara, India.	Dec-2023
IPA	DL0F702845	Merck, India.	-
Reverse Transcriptase	64453798	Bio-Rad	June-2023

6.3. Equipments

S. No.	Name of the Instrument	Make
1.	Biosafety Cabinet	Ascesension
2.	CO_2 Incubator	NUAIRE
3.	Inverted tissue culture microscope	Motic China
4.	-20ºC Deep Freezer	Vestfrost
5.	Thermal Cycler	Bio-Rad USA
6.	Gel Electrophoresis Unit	Chromous Biotech India
7.	Gel Documentation System	Syngene Ingenius

7. METHOD

7.1 Outline of the method

The effect of test substance on moisturizing effect by modulation of AQP-3 was estimated by gene expression method, where the level of the expression of AQP-3 in Human Keratinocytes (HaCaT) cell line was determined with respect to untreated HaCaT cells.

7.2 Cell line and Culture medium

HaCaT (Human Keratinocytes cells) was procured from AddexBio, U.S. Stock cells was 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 and all experiments was carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

7.3 RNA isolation and cDNA synthesis

The HaCaT (Human Keratinocytes cell) cells treated with test substance was subjected to cell lysis by treating with Tri-extract reagent. Chloroform was added, to isolate the total RNA from the sample and subjected for centrifugation. Out of the three distinct layers observed, upper layer was collected in fresh tube and equal volume of isopropanol was added and incubated at -20^{0}C for 10mins. After the incubation followed by centrifugation, appropriate volume of ethanol was added to resuspend the pellet. After incubation and centrifugation, the pellet was air dried and appropriate volume of TAE buffer was added. The isolated total RNA was further used for cDNA synthesis. cDNA was synthesized by priming with oligo dT primers followed by reverse transcriptase enzyme treatment according to manufacturer's protocol (Bio-Rad). The cDNA thus synthesized was taken up for PCR for the amplification of AQP-3 and GAPDH (internal control).

7.4 RT-PCR Procedure

The mRNA expression levels of AQP-3 were determined using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). 50µL of the reaction mixture was subjected to PCR for amplification of AQP-3. cDNA using specifically designed primers procured from Eurofins, India and as an internal control GAPDH (Housekeeping gene) was co-amplified with each reaction.

7.5 Amplification conditions for AQP-3 gene

Aquaporin-3: 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing Tm for 30 seconds and extension at 72°C for 45 seconds. This was followed by final extension at 72°C for 10 min.

Primer used:

For I strand synthesis: Oligo dT primer

For II nd strand synthesis:

Forward: 5'- GCTGTCACTTGGGCATCCTG -3'

Reverse: 5'- GCGTCTGTGCCAGGGTGTAG-3'

Product size: 150 bp.

8. OBSERVATION/PARAMETERS FOR EVALUATION

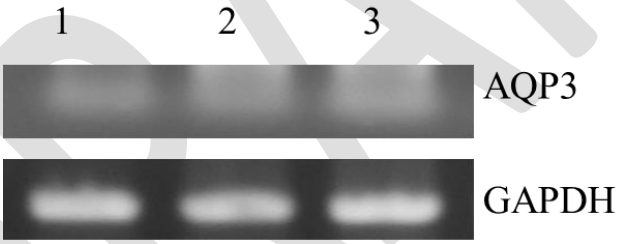

Fig 1: Effect of the Test Substance on AQP-3 transcripts in HaCaT Lane 1: Untreated Control, Lane 2: Test substance Green Tocyen beauty cream (RR222098) at 500 µg/mL, Lane 3: Test substance Tocyen beauty cream (RR222098) at 1000 µg/mL.

9. RESULTS

Table 1: The Quantitative gene expression level of AQP-3 normalized to GAPDH.

Test Sample	Regulation in Terms of Folds
	AQP-3
Cell Control (Untreated)	1.00
Tocyen beauty cream, RR222098 (500 µg/mL)	1.13
Tocyen beauty cream, RR222098 (1000 µg/mL)	1.20

10. DISCUSSION AND CONCLUSION

Test Substance was tested for *in vitro* cytotoxicity studies against HaCaT (Human Keratinocytes cells) by MTT assay exposing the cells to different concentration of test substance.Reverse Transcriptase-PCR experiment was performed by using AQP-3 specific primers. Semi-Quantitative RT-PCR analysis revealed that AQP-3 mRNA was increased moderately in a dose dependent manner over the control value.

The results indicate that test substance Tocyen beauty cream have the potency of moisturizing by increasing the AQP-3 levels in Human Keratinocytes (HaCaT) cell line when treated at non-toxic concentration.

11. ARCHIVING

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

12. 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*****