

DRAFT REPORT

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Study Title

***IN VITRO* ANTI- INFLAMMATORY ACTIVITY EVALUATION OF TEST
SUBSTANCE AGAINST LPS INDUCED TNF- α PRODUCTION INHIBITION IN
MOUSE MACROPHAGE (RAW 264.7)**

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: 08/12/2022

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: 08/12/2022

Management
Dr. Ashok G
C.E.O

DECLARATION

The Study No, RR222098/CB/AI/12-22, entitled “***In vitro* anti- inflammatory activity evaluation of test substance against LPS induced TNF- α production inhibition in mouse macrophage cell line (RAW 264.7)**” 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: 08/12/2022

QA, Head

Gopi Mareedu


ABBREVIATION USED

MCR	: Microbiology	°C	: 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
PBS	: Phosphate buffer saline	RT	: Room Temperature
EDTA	: Ethylenediaminetetraacetic acid		
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide		
RT-PCR	: Reverse transcription-polymerase chain reaction		
TPVG	: Trypsin Phosphate Versene Glucose Solution		
DMEM	: Dulbecco's Modified Eagle Medium		
DMSO	: Dimethyl sulfoxide		
dNTP	: Deoxynucleotide		
CTC ₅₀	: Cytotoxicity concentration		

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DEPARTMENT : CELL BIOLOGY	STUDY NO: RR/222098/CB/ AI/12-22	

1. STUDY DETAILS

- 1.1. Study title : *In vitro* Anti- inflammatory activity of test substance against LPS induced TNF- α production inhibition in mouse macrophage cell line (RAW 264.7)
- 1.2. Study number : RR222098/CB/ AI/12-22
- 1.3. Test Substance : Tocyen beauty cream
- 1.4. Sponsor : M/s.Tocyen Beauty Cream,
Mumbai, India.,
- 1.5 Test Facility : Radiant Research Pvt. Ltd
No: 99/A, 8th Main, 3rd Phase,
Peenya industrial area,
Bangalore-560 058
- 1.6 Test Schedule
- Study Initiation Date : 20/09/2022
- Experimental Start Date : 03/10/2022
- Experimental Completion Date : 05/12/2022
- Study Completion Date : 08/12/2022
- 1.7 Study Responsibilities
- Study Director : Dr. Ashok G
- Study Co-Ordinator : Mr Vatsa Kapadia

2. OBJECTIVE

The purpose of this study is to assess the anti-inflammatory activity of test substance against inflammation induced by LPS on TNF alpha production in mouse macrophage cell line (RAW 264.7).

3. SUMMARY

The test substance was evaluated for its *in vitro* Anti-Inflammatory activity against LPS induced toxicity in mouse macrophage cell line. The test substance was evaluated for cytotoxicity with different concentrations from 1000µg/ml to 7.8µg/ml, which resulted to be >1000µg/ml on RAW 264.7 cell line hence the concentrations 1000µg/ml and 500µg/ml were taken for further studies. The test substance didn't exhibit TNF-alpha inhibition in mouse macrophage cell against LPS induced inflammation.

4. GUIDELINE/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. Varma R S, Ashok G, Vidyashankar S, Patki P and Nandakumar K S. "Anti-inflammatory properties of Septilin in lipopolysaccharide-activated in monocytes and macrophage". *Immunopharmacology and Immunotoxicology*, 2011; 33: 55-63.
3. Tsai Y.-C., Wang S.-L., Wu M.-Y., Liao C.-H., Lin C.-H., Chen J.-J., Fu S.-L. Pilloin "A flavonoid isolated from *Aquilaria sinensis*, exhibits anti-inflammatory activity in vitro and in vivo" *Molecules*. 2018; 23:3177.

5. AMENDMENT AND DEVIATION PROCEDURE

No deviation has been observed during the conduct of the experiment

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DEPARTMENT : CELL BIOLOGY

STUDY NO: RR/222098/CB/ AI/12-22

6. MATERIALS**6.1. Test substance information**

Test substance/item : Tocyen beauty cream

Common name : Tocyen beauty cream

Batch No. : CG6122

Physical appearance : Semi-solid

Storage conditions : RT

6.2. Reference Material/Chemicals

Chemical	Batch / Lot No.	Manufacturer	Expiry Date
MTT	0000454015	HiMedia	Oct-2024
DMEM-HG	2365585	Gibco	Feb-2024
Fetal Bovine serum	4222743	Gibco	Sep-2026
DPBS	0000474192	HiMedia	Mar-2024
Trypsin - EDTA	0000472777	HiMedia	Mar-2023
Antibiotics	0000493509	HiMedia	Aug-2023
DMSO	2122353	SRL	Feb-2026
Mouse TNF- α ELISA kit	FU27HXP6252	Elabscience	April-2023

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
5.	-20°C Deep Freezer	Vestfrost, Denmark	RRS/INS/MB/01

7. METHOD

7.1. Outline of the method

The in vitro cytotoxicity was performed for the test substance on mouse macrophage cell line (RAW 264.7) to find a toxic concentration of the test substance and to evaluate its modulatory effect of anti-inflammatory activity against LPS induced toxicity.

7.2. Preparation of test solution

For studies, 10mg of test substance were separately dissolved and volume was made up with DMEM-HG supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two-fold dilutions were prepared from the stock solution for carrying out cytotoxic studies.

7.3. Cell line and Culture medium:

Mouse macrophage cell line (RAW 264.7) was cultured in DMEM-HG/MEM 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 stock cultures will be grown in 25 cm² culture flasks and all experiments will be carried out in 96 microtitre plates.

7.4. Anti-Inflammatory Activity

7.4.1. *In vitro* TNF- α inhibitory activity of test substance

Step I: Induction of TNF- α in RAW 264.7 cells

Mouse macrophage cell line (RAW 264.7) was seeded into 6 well culture dishes at a cell population 1.5×10^5 cells/ml in DMEM with 10% FBS. After 24 h, the cells was treated with known non-toxic concentration of test substance along with 5µg/ml of lipopolysaccharide (LPS) and incubated at 37 °C with 5% CO₂ for 24 hr.

Step II: Estimation of TNF- α in cell supernatant by bioassay

TNF- α were estimated as per manufacturers protocol.

8. RESULTS

Table 1: Anti-inflammatory effect of Test Substance in RAW 264.7 cells

Sl. No	Samples	Concentration tested (µg/ml)	% TNF- α
1.	LPS + Tocyen beauty cream	5 + 1000	150.00
		5 + 500	136.20
2.	LPS control	5	100.00

9. DISCUSSION AND CONCLUSION

Test substance tested for in vitro cytotoxicity studies against RAW 264.7 cell line by MTT assay exposing the cells to different concentrations of the test substance (1000 µg/ml to 7.8µg/ml) and showed moderate toxicity with CTC₅₀ value >1000µg/ml on RAW 264.7 cell line. Tocyen beauty cream exhibited modulation in TNF-α level at test concentrations tested over control in Mouse macrophage cells.

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

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