STUDY REPORT

Copy No. 1/2

Study Title

IN VITRO ANTI-INFLAMMATORY ACTIVITY AGAINST LPS INDUCED TNF-ALPHA, IL-6 AND NITRIC OXIDE PRODUCTION IN MOUSE MACROPHAGES. _IN VITRO_ COX-2 INHIBITORY ACTIVITY.

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

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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: 12/02/2022

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: 12/02/2022

Management
Dr. Ashok G
C.E.O

DECLARATION

The Study No, RR211182/CB/AI/12-21, entitled "I*n vitro* **anti-inflammatory activity against LPS induced TNF-alpha, IL-6 and Nitric Oxide production in macrophages. COX-2 inhibitory activity in** ***vitro***" 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: 12/02/2022

QA Head
Gopi M

ABBREVIATIONS 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

TPVG : Trypsin Phosphate Versene Glucose Solution

DMEM : Dulbecco's Modified Eagle Medium

DMSO : Dimethyl sulfoxide

CTC_{50} : Cytotoxicity concentration

LIST OF TABLES AND FIGURES

1. STUDY DETAILS

1.1. Study title : *In vitro* anti-inflammatory activity against TNF-alpha, IL-6 and Nitric Oxide in macrophages. COX-2 inhibitory activity *in vitro*.

1.2. Study number : RR211182/CB/ AI/12-21

1.3. Test Substance : Ortho Support

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.

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	:	04/01/2022
Experimental Start Date	:	07/01/2022
Experimental Completion Date	:	10/02/2022
Study Completion Date	:	12/02/2022

1.7. Study Responsibilities

Study Director	:	Dr. Ashok G
Study Co-Ordinator	:	Anuraag Muralidharan

2. OBJECTIVE

The purpose of this study is to assess the anti-inflammatory activity of test substance in Mouse Macrophages (RAW 264.7) cell line by estimating the inflammatory cytokines (TNF-α, COX-2, NO and IL-6) against LPS induced cell damage.

3. 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. Chih-HsiungWu, Ta-Liang Chen,Tyng-Guey Chen, Wei-Pin Ho "Nitric Oxide modulates pro- and anti-inflammatory cytokines in lipopolysaccharide-activated macrophages". *Journal of trauma*, 55(3):540-5.

4. 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.

4. AMENDMENT AND DEVIATION PROCEDURE

No deviation has been observed during the conduct of the experiment

5. MATERIALS

5.1. Test substance information

Test substance/item	:	Ortho Support
Sample Code	:	RR211182
Batch No.	:	ORT202103005
Batch supplied by:	:	M/s. Mallur Flora & Hospitality Pvt.Ltd.
Batch produced on (Date)	:	20 MAR 2021
Expiry date	:	19 MAR 2023
Physical appearance	:	Liquid
Storage conditions	:	RT

5.2. Reference Material/Chemicals

Chemical	Batch / Lot No.	Manufacturer	Expiry Date
MTT	0000307556	Hi-media	-
DMEM-HG	2365585	Gibco	Feb-2024
Fetal Bovine serum	4222743	Gibco	Sep-2026
DPBS	0000474192	Hi-Media	March-2024
Trypsin - EDTA	0000472777	Hi-Media	Mar-2022
Antibiotics	0000493609	Hi-Media	Aug-2023
DMSO	519350205AO	FINAR	--
Nitric oxide assay kit	V31NYASKAP	Elabscience	July- 2022
Mouse TNF-α ELISA Kit	J7B7RJD3QL	Elabscience	July- 2022
Mouse IL-6 ELISA Kit	KZ2Z4XF7DJ	Elabscience	July- 2022
Mouse PTGS2/COX-2 ELISA Kit	4QKLLSMY8Z	Elabscience	July- 2022

5.3 Equipment

S. No.	Name of the Instrument	Make	Instrument ID
1.	Biosafety Cabinet	Ascesension	RRS/INS/CB/01
2.	CO_2 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

6. METHOD

6.1 Outline of the method

The *in vitro* cytotoxicity was performed for the test substances on RAW 264.7 cell line to find toxic concentrations of the test substance and to evaluate the effect of test substance on the levels of LPS induced inflammatory cytokines (TNF-α and IL-6), COX-2 inhibitory activity and NO (nitric oxide) radical scavenging potency.

6.2 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.

6.3 Cell line and Culture medium

Mouse Macrophage cell line (Raw264.7) 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 and all experiments were carried out in 96 well microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

6.4 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, 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° 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 50 µL of MTT is added with DPBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° 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 formazan. The absorbance was measured using a microplate reader at a wave length of 570nm.

6.5 Anti-Inflammatory Activity

6.5.1 *In vitro* TNF-α, IL-6 and Nitric oxide inhibitory activity of test substance

Step I: Induction of TNF-α, IL-6 and Nitric oxide in RAW264.7 cells

Raw264.7 cells will be seeded in to 6 well culture dishes at a cell population 1.5 to $2x10^5$ cells/ml in DMEM with 10% FBS. After 24 h, the cells are treated with known non-toxic concentration of test substance along with 5µg/ml of lipopolysaccharide (LPS) and incubated at 37 °C with 5% CO_2 for 4 h. After incubation, the cell supernatant is collected, centrifuged, separated and stored at -20° C till use.

Step II: Estimation of TNF-α, IL-6 and Nitric oxide in cell supernatant by ELISA and colorimetric assay

All the reagents, standard solutions and samples will be thawed to room temperature before use. 100 µl of samples will be transferred to sample wells and the plates will be incubated for 1.5h at 37 °C as per the manufacturer's instruction. For TNF-α quantification, Mouse TNF-α (Tumour Necrosis Factor Alpha) ELISA kit by Elabscience was employed and for IL-6 estimation, Mouse IL-6 ELISA kit by Elabscience was employed. In case of nitric oxide

estimation, the cells were treated with LPS and test compounds for 24h, followed by collection of supernatant and was quantified using Nitric Oxide Colorimetric Assay Kit (Elabscience).

6.6 Estimation of the effect of test substance on COX-2 enzyme

6.6.1 COX-2 procedure

Cells were treated with different concentrations of the test substance and supernatants were evaluated for the inhibition on COX-2. Then, cells were collected and washed with DPBS for 1-2 times. Centrifuged at 1000g for 5 min and supernatant is preserved in -20^0 C for further detection. All the reagents, standard solutions and samples were thawed to room temperature before use. The assay was performed according to the manufacturer's instructions and the absorbance was measured using microplate reader at 450nm.

7. RESULTS

The test substances showed no cytotoxicity against RAW264.7 cell line. Test doses which exhibited 20% or less cytotoxicity were selected for the anti-inflammatory assay.

Table 1: Cytotoxic properties of test substance against RAW 264.7 cell line

Sample code	Sample number	Concentration (µg/Ml)	% of cytotoxicity	CTC50 (µg/mL)
Ortho Support	RR211182	1000	28.76 ± 0.36	>1000
		500	20.40 ± 0.80	
		250	18.77 ± 0.97	
		125	7.55 ± 0.71	
		62.5	2.35 ± 1.41	
		31.25	3.19 ± 1.51	
		15.625	-0.96 ± 3.06	
		7.8125	-8.09 ± 1.80	

Table 2: Anti-inflammatory effect on TNF-α levels of Test Substance in RAW264.7 cell line

TNF-α estimation on RAW264.7 cells				
Test compound		Concentration tested (µg/mL)	Amount of TNF-α (pg/mL)	% Protection of test compounds over LPS Control
RR211182+ LPS		500 + 5	1100.087 ± 0.001	6.066 ±0.001
RR211182+ LPS		250 + 5	1076.404 ± 0.006	8.088 ± 0.006
Cell control	Normal Control	NT (no-treatment), No LPS	433.025 ± 0.013	-
LPS Control	Positive Control	5 µg/mL	1171.134 ± 0.053	-

Table 3: Anti-inflammatory effect on IL-6 levels of Test Substance in RAW264.7 cell line

IL-6 estimation on RAW264.7 cells				
Test compound		Concentration tested (µg/mL)	Amount of IL-6 (pg/mL)	% Protection of test compounds over LPS Control
RR211181+ LPS		500 + 5	5350.616 ± 0.065	3.451 ± 0.065
RR211181+ LPS		250 + 5	5416.333 ± 0.092	2.265 ± 0.092
Cell control	Normal Control	NT (no-treatment), No LPS	73.336 ± 0.0095	-

LPS Control	Positive Control	5 µg/mL	5541.898 ± 0.0745	-

Table 4: Nitric oxide Inhibition of Test Substances

Nitric oxide estimation on RAW264.7 cells				
Test compound		Concentration tested (µg/mL)	Amount of NO (µmol/L)	% Reduction of Nitric oxide by test compounds over LPS Control
RR211181+ LPS		500 + 5	88.813 ± 0.01	20.766 ± 0.01
RR211181+ LPS		250 + 5	97.932 ± 0.002	12.630 ± 0.002
Cell control	Normal Control	NT (no-treatment), No LPS	52.09±0.0023	-
LPS Control	Positive Control	5 µg/mL	112.09±0.008	-

Table 5: Effect of Test Substance in RAW 264.7cells on Cox-2 levels

Sl. No	Sample		Concentration tested	% Decrease over LPS Control (COX-2)
3.	RR211181+ LPS		500 + 5	0.037 ± 0.009
4.	RR211181+ LPS		250 + 5	0.999 ± 0.005
5.	Cell control	Normal Control	NT (no-treatment), No LPS	-
6.	LPS Control	Positive Control	5 µg/mL	-

8. DISCUSSION AND CONCLUSION

Based on the stock concentration provided, each of the test substances was evaluated for their cytotoxicity with eight different concentrations on RAW264.7 cells. Furthermore, the safest concentration of the test compound that exhibited less than or equal to 20% cytotoxicity was selected for performing the assay. Overall, the test compound assayed did not exhibit any significant anti-inflammatory property against the markers tested. However, in Nitric oxide estimation assay, test substance RR211181 at 500 µg/mL exhibited a mild protective activity. The test substance was also evaluated for its inhibitory property on COX-2 enzyme. However, the test substance did not exhibit COX-2 inhibition in mouse macrophage cells.

9. ARCHIVING

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

10. 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.

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