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Unveiling the Anti-Inflammatory Properties of Ethanolic Extract of *Ruelliatuberosa*: THP1 Cell Line Studies Employing MTT Assay and PGE2 Inhibition

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Abstract

Inflammation plays a pivotal role in various pathological conditions, necessitating the exploration of natural compounds for their anti-inflammatory properties. Medicinal plants have long been recognized as valuable sources of bioactive molecules with therapeutic potential. This study aimed to investigate the anti-inflammatory activity of selected medicinal plants using THP1 cell lines, a widely utilized model for studying inflammatory responses. The experimental setup involved the exposure of THP1 cells to extracts or isolated compounds derived from medicinal plants *Ruelliatuberosa* known for their traditional use in treating inflammatory disorders. Cellular responses were evaluated through assays targeting key inflammatory markers such as cytokine production, NF-κB activation, and expression of inflammatory mediators. Preliminary results demonstrated significant attenuation of inflammatory responses in THP1 cells following treatment with the tested plant extracts or compounds. Suppression of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-alpha (TNF-α) was observed, indicating a potential mechanism of action for the observed anti-inflammatory effects. Furthermore, the downregulation of NF-κB signalling pathway components suggested the involvement of this pathway in mediating the anti-inflammatory activity of the tested plant-derived agents. These findings underscore the therapeutic potential of the medicinal plant *Ruelliatuberosa* in modulating inflammatory responses, offering promising avenues for the development of novel anti-inflammatory agents. Further investigations into the molecular mechanisms underlying their effects and the identification of specific bioactive compounds are warranted to fully exploit their therapeutic potential and facilitate their integration into clinical practice.

Keywords: Anti-inflammatory activity, *Ruelliatuberosa*, THP1 cell lines, cytokines, NF-κB, inflammatory mediators, MTT assay, PEG2 inhibition assay.

Introduction

Many different plants have been utilized medicinally from ancient times. The majority of plant parts have been extracted and may contain antioxidant and anti-inflammatory qualities linked to conditions like cancer, diabetes, atherosclerosis, and neurological illnesses. Furthermore, plant extracts can control the gut microbiota's composition because they are anti-inflammatory agents.The complex phytochemicals found in plants, such as polyphenols, carotenoids, phytosterols, and polysaccharides, are largely responsible for their therapeutic qualities. Numerous natural chemicals found in plant extracts are being studied to determine their mechanism of action through phytochemical and ethnobotanical research.By decreasing the translocation of NF-kB to the nucleus and downregulating pro-inflammatory cytokines like COX, plants can protect against certain illnesses whose aetiology involves immunological malfunction or chronic inflammation. Additionally, the bioactive components of plants have the power to control the formation of reactive oxygen species (ROS) and the antioxidant potential of cell enzymes, thereby mitigating oxidative stress(Jung, et al., 2022).Inflammation is a basic physiologic defence mechanism that the body uses to fend off harmful stimuli. It can cause pain, oedema, or even damage to cells. This mechanism's primary goal is to restore injured tissue to its original state of health. During the early stages of inflammation, the vessels only enlarge around the inflammatory loci (neutrophils, macrophages, and lymphocytes). However, several types of cells reach the neutrophils within 24 hours, followed by the macrophages 48 hours later, and the lymphocytes several days later(Lee and Choi, 2018).

Clinically, discomfort, heat, redness, loss of function, and swelling in the damaged tissue are typical indicators of inflammation(Monnier et al., 2005).Severe fever, leukocytosis, and sepsis are other symptoms. Inflammation can be caused by a variety of things, including external injuries, chemicals or radiation exposure, and pathogens (such as bacteria, viruses, and fungi). There are two types of inflammation: acute inflammation and chronic inflammation. It is believed that the first line of defence against damage is acute inflammation. It happens quickly and is characterized by the emigration of leukocytes such neutrophils as well as the expulsion of fluid and plasma proteins. On the other hand, chronic inflammation is characterized by a protracted duration and is exhibited by the activity of macrophages and lymphocytes, leading to tissue necrosis and fibrosis. From mild ailments to more serious conditions like cancer, inflammation is thought to be one of the most common causes of concern when it comes to diseases(Fuster and Sanz,2007).

Traditional medicine has employed plant-based or herbal remedies to address pain, inflammation, and pain that is mediated by inflammation(Wirth et al., 2005).According to the World Health Organization (WHO), medicinal plants are those that have chemicals that can be utilized therapeutically or that can be used to make valuable pharmaceuticals from their metabolites. The WHO reports that people in poor nations continue to utilize medical plants to cure a variety of illnesses, and the market for these products is expanding, which is a positive indicator of the economic significance of medicinal plants(WHO,2011).

The human leukemia monocytic cell line THP-1 has been widely utilized to investigate the processes, roles, signaling pathways, and drug and nutrition trafficking of monocytes and macrophages. This cell line is now widely used as a model to determine how monocyte and macrophage activity are modulated. The goal of this study is to provide an overview and discussion of recent research on the THP-1 cell model [Mantovani et al., 2022].This cell line has been widely used to study immune responses while cells are not only in the monocyte state but also in the macrophage-like state[Cheng et al., 2012].

Ruelliatuberosa, a tropical perennial plant belonging to the Acanthaceae family, has a long history of traditional use for various medicinal purposes. It has been employed as a remedy for bladder disease, renal disease, bronchitis, gonorrhoea, and syphilis, serving as a diuretic, antipyretic, analgesic, anti-hypertensive, anthelmintic, abortifacient, and emetic (Alam et al., 2014). This plant is native to Southeast Asia, including regions like Thailand and Laos. Many years ago, *Ruelliatuberosa* was introduced to Taiwan, where it gained popularity as a folk medicine due to its diuretic, diabetic, antipyretic, analgesic, and antihypertensive properties (Chen et al.,2006)

Ruelliatuberosa

Materials and methods

Identification and authentication

Whole plants of fresh Ruelliatuberosa were collected in their whole from several locations in Coimbatore, including Marudhamalai Hills, Thondamuthur, and Gobichettipalayam. Agricultural University campus, Botanical Survey of India (Southern Circle), Coimbatore, Tamilnadu, taxonomically identified and authenticated the plant materials (Khalil et al., 2015).

Human ethics committee

The institutional human ethics committee of Avinashilingam Institute for Home Science and Higher Education for Women approves (approval No: AUW/IHEC/BC-22-23/XPD-05)for Antidiabetics and anti-inflammatory activity of selected medicinal plants.

Plant extract preparation

Whole plant powder preparation

The chosen plant collected and shade dried for two weeks at room temperature. The dried flowers were roughly mashed by hand before being blended into a powder with an electric blender. Weighed powdered material was preserved in an airtight container (Danlami et al.,2015)

Ethanolic extract preparation

In a conical flask, 10g of powdered plant are combined with roughly 100ml of ethanol and maintained for about 5 days at room temperature in an open orbital shaker. A Whatman No.1 filter paper was used to filter the mixture, and the extract was collected in a 500ml beaker. The mixture was then suspended in a Rotary evaporator, and following evaporation, a colloidal gel was formed, which was then poured into an Eppendorf tube and kept in the refrigerator for later use (Khalil et al., 2015; Danlami et al., 2015)

Maintenance of cell lines

The THP-1 (Human monocyte/macrophage cell line) was purchased from NCCS, Pune, India. The THP-1 cells were maintained in RPMI-1640 media supplemented with 10 % FBS along with the 1% antibiotic-antimycotic solution and 1% L-Glutamine (200mM) in an atmosphere of 5% CO_2, 18-20% O_2 at 37^0C temperature maintained in the CO_2 incubator and sub-cultured for every 2 days.

MTT assay

MTT assay is a colourimetric assay used for the determination of cell proliferation and cytotoxicity, based on the reduction of the yellow-colouredwater-soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibita purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm(Butler and Spearman 2007).

Individual treatment

200µl cell suspension in a 96-well plate at the required cell density (20,000 cells per well), without the test agent. Allow the cells to grow for about 12 hours. Add appropriate concentrations of the RT extract prepared in complete media.Incubate the plate for 24hrs at 37°C in a 5% CO_2 atmosphere. After the incubation period, takeout the plates from the incubator and centrifuge the plate at 1000rpm/5min at RT remove spent media and add MTT reagent to a final concentration of 0.5mg/ml of total volume.Wrap the plate with aluminium foil to avoid exposure to light. Return the plates to the incubator and incubate for 3 hours.Centrifuge the plate again at 1000rpm/5min at RT remove the MTT reagent and then add 100µl of solubilisation solution (DMSO). Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals, especially in dense cultures. Read the absorbance on an ELISA absorbance plate reader at 570nm wavelength (Wang et al. 2015).

% cell viability is calculated using below formula:

% cell viability=Abs of treated cells/Abs of Untreated cells x 100

Combinational treatment

200µl cell suspension in a 96-well plate at the required cell density (20,000 cells per well), without the test agent. Allow the cells to grow for about 12 hours. Centrifuge the plate at 1000rpm for 5 minutes at RT and stimulate the cells with 1ug/ml of LPS for 2 hours to induce inflammation followed by treating the cells with desired concentrations of RT extract. Incubate the plate for 24 hours. After the incubation period, take out the plates from the incubator and centrifuge the plate at 1000rpm/5min at RT remove spent media and add MTT reagent to a final concentration of 0.5mg/ml of total volume.Wrap the plate with aluminium foil to avoid exposure to light. Return the plates to the incubator and incubate for 3 hours. Centrifuge the plate again at 1000rpm/5min at RT and remove the MTT reagent and then add

100µl of solubilisation solution (DMSO). Gentle stirring in a gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazan crystals, especially in dense cultures. Read the absorbance on an ELISA absorbance plate reader at 570nm wavelength. Used cells and experimental conditions are given in the Table 1.

% cell viability is calculated using below formula:

% cell viability=Abs of treated cells/Abs of Untreated cells x 100

Table 1:Cell line used for MTT assay and concentration

Sl.No	Condition	Cell line	Concentration treated to cells
1	Untreated	THP-1	No treatment
2	LPS	THP-1	1ug/ml
3	Blank	-	Only media without cells
4	RT extract	THP-1	5(12.5, 25,50,100, 200µg/ml)-For individual treatment
5	LPS+RT extract	THP-1	LPS-1ug/ml for 2hours + 5 (12.5, 25, 50, 100,200µg/ml)-For combinational treatment

Prostaglandin E2 (PGE2) is a primary product of arachidonic acid metabolism in many cells. Like most eicosanoids, it does not exist preformed in any cellular reservoir. When cells are activated or exogenous free arachidonate is supplied, PGE2 is synthesized de novo and released into the extracellular space. In vivo, PGE2 is rapidly converted to an inactive metabolite (13,14-dihydro-15-keto PGE2) by the PG 15-dehydrogenase pathway. The half-life of PGE2 in the circulatory system is approximately 30 seconds and normal plasma levels are 3-12 pg/ml.Because of the rapid metabolism of PGE2, the determination of in vivo PGE2 biosynthesis is often best accomplished by the measurement of PGE2 metabolites.

This assay is based on the competition between PGE2 and a PGE2-acetylcholinesterase (AChE) conjugate (PGE2 Tracer) for a limited amount of PGE2 Monoclonal Antibody. Because the concentration of the PGE2 Tracer is held constant while the concentration of PGE2 varies, the amount of PGE2 Tracer that is able to bind to the PGE2 Monoclonal

Antibody will be inversely proportional to the concentration of PGE2 in the well. This antibody-PGE2 complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PGE2 Tracer bound to the well, which is inversely proportional to the amount of free PGE2 present in the well during the incubation.

Treatment of RT extract to LPS induced THP-1 cells

Briefly, 0.5 x 10^6 cells/ml of THP-1 were cultured in a 12 well plate and incubated for 12 hours to reach required cell density. Stimulate the cells with LPS with 1ug/ml for 2hours to induce inflammation followed by treat the cells with required concentrations of RT extract and incubate for 24hours. LPS alone treated cells served as a Positive or disease control and cells without any treatment was considered as a Control. Medium alone considered as a Blank control for all the ELISA studies.

Measurement of PGE2

Prostaglandin E2 (PGE2), Prostaglandin E2 (PGE2) is one of the most typical lipid mediators produced from arachidonic acid (AA) by cyclooxygenase (COX) as the rate-limiting enzyme, and acts on four kinds of receptor subtypes (EP1-EP4) to elicit its diverse actions including pyrexia, pain sensation, and inflammation.

As mentioned previously, after the treatment, the cell culture supernatants were collected immediately following treatment and centrifuged at 1,000 × g for 15 min to remove the particulate matter. PGE2 was determined using an enzyme immunoassay (EIA) kit (catalog no.514010, Cayman Chemical, USA). The assay was performed by following the instruction given in ELISA Kit. After the addition of Ellman's reagent the intensity of the color produced was measured at 405 nm. Concentration details of samples are given Table 2.

Table 2: concentrations details

Sl.No	Culture condition	Cell lines	Concentration treated to cells
1	Untreated	THP-1	No treatment
2	Blank	-	Only Media without cells
3	LPS	THP-1	1ug/ml
4	LPS+RT extract	THP-1	LPS-1ug/ml + 5(12.5, 25, 50, 100, 200ug/ml)

Results and Discussion

Identification and authentication

Ruelliatuberosa L, also called a cracker plant, is commonly used against kidney disorders, bronchitis, and bladder disorders. After screening from 15 medicinal plants, based on Gas chromatography Spectroscopy, *Ruelliatuberosa* L, was used for further studies. The plant was subjected to identification studies. Authentication number BSI/SRC/5/23/2022/Tech/619. Authentication was done by DR.M.U. Sharief (Scientist 'E' & Head of office, Botanical Survey of India, Southern Regional Centre, Coimbatore) whole plant of fresh *Ruelliatuberosa* collected in several locations in Coimbatore, including Marudhamalai hills, Thondamuthur, and Gobichettipalayam. Agricultural University campus, Botanical Survey of India (Southern Circle), Coimbatore (Chen et al., 2006).

Human ethics committee

The institutional human ethics committee of Avinashilingam Institute for Home Science and Higher Education for Women approves (approval No: AUW/IHEC/BC-22-23/XPD-05) for Antidiabetics and anti-inflammatory activity of selected medicinal plant (Proposal No: IHEC/22-23/BC-05, Date:05/01/2023). Members participated from PSG Institute of Medical Science and Research, Coimbatore. THP-1 cell line was collected from Pune and used for MTT assay and PEG 2 Inhibition study.

Plant extract preparation

The chosen plant was collected and shade-dried for two weeks at room temperature. The dried flowers were roughly mashed by hand before being blended into a powder with an electric blender. Weighed powdered material was preserved in an airtight container. Sufficient fine powder of *Ruelliatuberosa with 25g* was filled into the porous cellulose thimble (30 x 80 mm). 250ml of Solvent (100% Ethanol) was added into the Round bottom flask, which was attached to the Soxhlet extractor and Condenser on an isomantle. Further *Ruelliatuberosa* loaded thimble was placed inside the Soxhlet extractor. The side arm is legged with glass wool. The solvent is heated using the isomantle and starts to evaporate moving through the apparatus to the condenser. The condensate further drips into the reservoir containing the thimble. Once the level of solvent reaches the siphon it pours back into the flask and the cycle begins again. The process should run overnight. Once the process has finished, the ethanol should be evaporated using a rotary evaporator, leaving a small yield of extracted plant material (about 2 to 3 ml) in the glass bottom flask which was air-dried to obtain crude extract. The yield of extract obtained from 25g was 240mg which was tightly

sealed and stored at 4°C for future use. *Ruelliatuberosa* ethanolic extract (RTEE) used for further studies (Kader et al., 2012).

The results of anti-inflammatory study were performed by PGE-2 in LPS induced alone and combination of RT extract with different concentrations in LPS pre-treatment model respectively. In LPS alone group, PGE2 cytokine was effectively expressed and gradually inhibited by RT extract treated with different doses respectively. In control group, low concentration of PGE2 cytokine level was observed.

The Cellular pro-inflammatory cytokines expression results suggest us that the given test compound, RT extract showed significant Anti-inflammatory activity by inhibiting the pro-inflammatory cytokines expression viz PGE2 in LPS induced model on dose dependent manner while LPS alone treated cells significantly expressed the PGE2 expression respectively. The obtained values confirmed the satisfactory anti-inflammatory activity of RT extract with different concentrations in relation to the anti-inflammatory effect in LPS induced Human macrophages model by inhibiting the PGE2 cytokine expression.

In summary, overall the observed results concluded that RT extract may have possible effective anti-inflammatory efficacy in LPS induced model and further advanced methods like Flow cytometry or Gene expression studies by RT-qPCR need to be employed to double confirm the molecular mechanism of action behind the anti-inflammatory activity of RT extract. Results are given Table 3,4 &5 and Figure 1&2.

Culture condition	**PGE2±SD (ug/ml)**
Untreated	25.39±3.22
LPS-1ug	187.32±1.18
LPS+RT-12.5ug	177.23±1.91
LPS+RT-25ug	165.02±1.02
LPS+RT-50ug	127.27±2.99
LPS+RT-100ug	53.66±1.98
LPS+RT-200ug	65.37±2.02

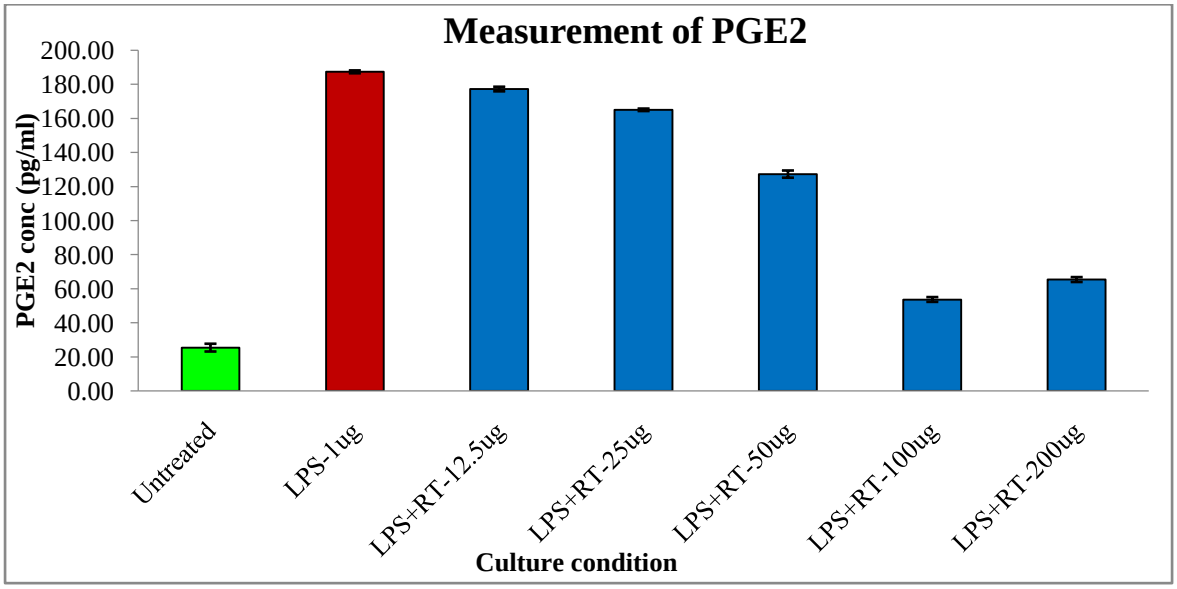
Table-3: Pro-inflammatory cytokine levels observed in different culture conditions of THP-1 cells induced by LPS after the treatment period of 24 hours.

Table 4: Table showed the % cell viability values of THP-1 cells treated with various concentrations of RT extract for the 24hours

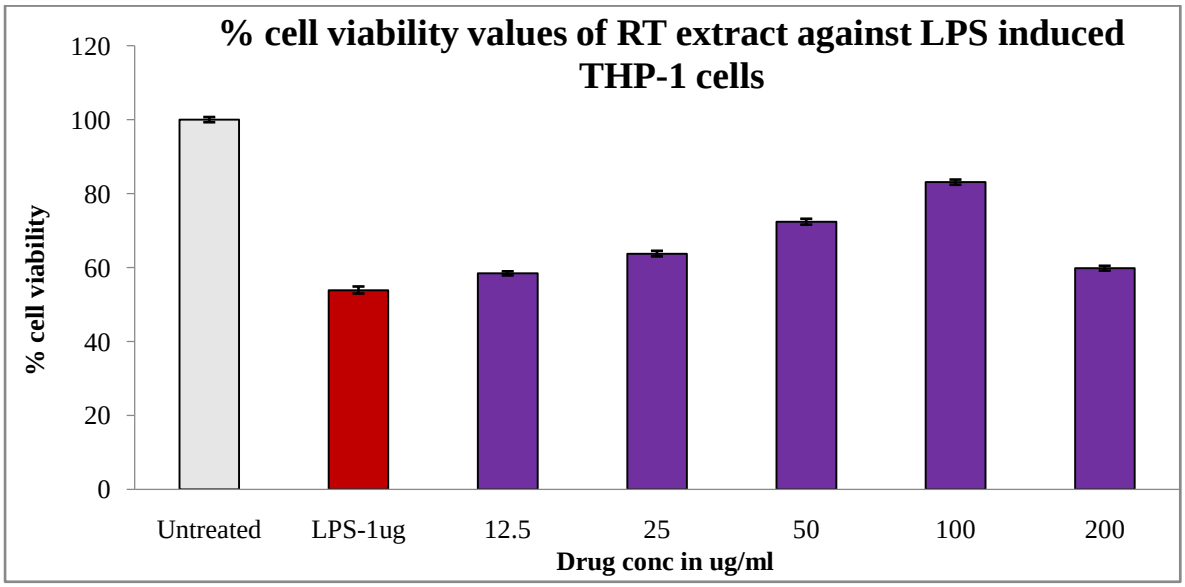
Culture condition (ug/ml)	**% cell viability**	**Max non-toxic conc (ug/ml)**
Untreated	100	**100ug/ml**
LPS-1ug	54.62	
RT extract-12.5ug/ml	99.73	
RT extract-25ug/ml	97.70	
RT extract-50ug/ml	94.92	
RT extract-100ug/ml	91.29	
RT extract-200ug/ml	83.00	

Table 5: Table showed the % cell viability values of LPS induced THP-1 cells treated with various concentrations of RT extract for the 24hours

Culture condition (ug/ml)	**% cell viability**	**Max Protective conc (ug/ml)**
Untreated	100	**100ug/ml**
LPS-1ug	53.91	
LPS+RT extract-12.5ug/ml	58.40	
LPS+RT extract-25ug/ml	63.76	
LPS+RT extract-50ug/ml	72.38	
LPS+RT extract-100ug/ml	83.08	
LPS+RT extract-200ug/ml	59.80	



Graph-1: % cell viability values of THP-1 cells treated with various concentrations of RT extract along with controls used for the study.



Graph-2: % cell viability values of LPS induced THP-1 cells treated with various concentrations of RT extract along with controls used for the study

The observations in statistical data of cell cytotoxicity study by MTT assay suggested that against THP-1 cells, given compound labeled as RT extract showed non-cytotoxic potential properties till the highest concentration of 100ug/ml respectively with greater than 90% cell viability after the 24hours incubation. The combinational treatment of RT extract with LPS on THP-1 cells showed the cytoprotective ability on dose dependent fashion and showed the

better protective ability at 100ug/ml which maximum cell viability values. 200ug/ml concentration showed moderate toxicity on THP-1 cells which decreased cell viability value.The Observed absorbance readings with calculations were enclosed in the separate folder of the report in MS Excel format.Direct microscopic observations of drug treated images of cell lines captured with the magnification of 20x were enclosed in separate folder along with this report.

Conclusion

Given test compound, RT extractwas non-toxic in natureagainst the THP-1 cells withgreater than 90% cell viabilityat highest concentration of 100ug/ml after the treatment of 24hours of incubation at 37^{o}C temperature. Based on the viability values, decided maximum concentration of the compound which showed greater than or equivalent to 90% as a optimum concentration for combinational studies on LPS induced THP-1 cells. Further against the LPS induced THP-1 cells, RT extract inhibited the LPS induced % viability on dose dependent fashion till the maximum dose of 100ug/ml and again moderate toxicity was observed at 200ug/ml. The observations strongly suggested that the RT extractmay have possible therapeutic potential against Macrophages based on the dosage of the drug after the incubation period of 24hours and further studies need to be performed to evaluate the molecular mechanism of action behind the anti-inflammatory potential of the RT extract against the Macrophages in LPS induced model in *invitro* conditions.

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