ATATURK UNIVERSITY PUBLICATIONS

Research Article

DOI: 10.5152/Pharmata.2023.23010

Effect of Tryptanthrin Compound on Lipopolysaccharide-Induced Inflammation Model in L929 Cell Line

Mehmet YILMAZ
Zerrin KUTLU

Department of Biochemistry, Atatürk University, Faculty of Pharmacy, Erzurum, Turkey

ABSTRACT

Objective: Our aim is to evaluate the effects of the tryptanthrin compound, with antimicrobial and anti-inflammatory effects, on the lipopolysaccharide inflammation model created in the L929 cell line.

Methods: L929 cell lines were placed in a 5% CO_2 incubator at 37°C, using standard cell culture procedures, and lipopolysaccharide was applied with Prn pure substance with a final concentration of 2 µL and 1 µL after 1 hour. Following the lipopolysaccharide application, the (3- [4,5- dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide protocol, a colorimetric method, was applied to determine cell viability at the end of the required 24th, 48th, and 72nd hours incubation times.

Results: IC_{50} values were calculated as 0.3 µg/mL for tryptanthrin and 1 µg/mL for lipopolysaccharide . Application of lipopolysaccharide to L929 cell lines caused a significant decrease in cellular index depending on time. Also, in the tryptanthrin + lipopolysaccharide groups, it was found that the decreased cell index significantly increased even closer to the control compared to the lipopolysaccharide applied group. It was found that the inflammation and cell damage caused by lipopolysaccharide applied to the L929 cell line improved after tryptanthrin application.

Conclusion: When we look at the results of our study as a whole, it was determined that the viability rates of the cells were above 85% as a result of the tryptanthrin application and it was significantly above the 70% viable cell ratio recommended by ISO 10993-5: 2009 Dın en ISO (2009) for cytotoxicity.

Keywords: Inflammation, L929, LPS, MTT, Tryptanthrin

INTRODUCTION

Tryptanthrin (TRP) is indeed a natural compound found in indigo plants, and it belongs to the class of indole quinazoline alkaloids. Researchers have been studying the effects of TRP due to its various biological and pharmacological activities. One notable area of investigation is the antimicrobial activity of TRP. Studies have shown that TRP exhibits antimicrobial properties against a range of microorganisms, including bacteria, fungi, and parasites. It has demonstrated efficacy against both Gram-positive and Gram-negative bacteria, making it a potentially valuable compound for the development of new antimicrobial agents. Tryptanthrin has also been found to possess anti-inflammatory properties. Inflammation is a natural response of the immune system, but excessive or chronic inflammation can lead to various diseases. Tryptanthrin has been shown to inhibit the production of pro-inflammatory molecules and suppress the activation of inflammatory pathways, thus potentially providing a therapeutic benefit in conditions characterized by inflammation. Additionally, TRP has shown antiallergic activity. Allergic reactions occur when the immune system overreacts to harmless substances. Tryptanthrin has been found to inhibit the release of histamine, a key mediator of allergic reactions, from mast cells. This action may help alleviate allergic symptoms and provide relief for individuals with allergies. It is worth noting that while TRP shows promise in these areas, further research is still needed to fully understand its mechanisms of action and explore its potential applications in medicine. Researchers are also interested in investigating the synergistic effects of TRP with other compounds found in plants, as well as its potential side effects and safety profile.[1]

Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria, and it is commonly used to induce an inflammatory response in research studies. Lipopolysaccharide has the ability to activate macrophages, which are immune cells involved in the innate immune response. When macrophages are exposed to LPS, it triggers a series of signaling pathways within these cells. Three important pathways that are activated in response to LPS are the NF-κB (nuclear factor kappa-ligh

Received: 26.06.2023
Accepted: 04.07.2023
Publication Date: 31.07.2023

Corresponding Author:
Zerrin KUTLU
E-mail: kutluzerrin@atauni.edu.tr

Cite this article as: Yılmaz M, Kutlu Z. Effect of tryptantrhin compound on lipopolysaccharideinduced inflammation model in L929 cell line. *Pharmata*, 3(3), 54-58.

t-chain-enhancer of activated B cells), MAPK (mitogen-activated protein kinase), and JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathways. The NF-κB pathway is a key regulator of immune and inflammatory responses. Activation of this pathway leads to the production of pro-inflammatory cytokines, chemokines, and other molecules that promote inflammation. The MAPK pathway is involved in cellular processes such as cell proliferation, differentiation, and inflammation. Lipopolysaccharide activates MAPK signaling, which can lead to the production of pro-inflammatory mediators and the regulation of immune responses. Macrophages secrete growth factors, including transforming growth factor-beta (TGF-β), which can stimulate fibroblast proliferation and activation. This interaction between macrophages and fibroblasts is crucial for tissue repair and wound-healing processes. Overall, the activation of macrophages by LPS and their subsequent signaling pathways play a vital role in the inflammatory response and immune regulation. Understanding these processes can help in the development of therapeutic strategies for various inflammatory and immune-related conditions.[2]

Inflammation is a vital defense mechanism of the immune system. It is a complex biological response that can be triggered by a variety of factors, including damaged cells, toxic compounds, and invading pathogens. The primary purpose of inflammation is to protect the body from harmful stimuli and initiate the healing process. When tissues are damaged or infected, the immune system recognizes these threats and responds by mobilizing immune cells and releasing signaling molecules.

Inflammation is typically characterized by the classic signs of redness, heat, swelling, pain, and sometimes loss of function in the affected area. These symptoms arise due to increased blood flow, increased permeability of blood vessels, and the influx of immune cells and fluid into the tissues. While acute inflammation is an important and necessary response for maintaining health, chronic inflammation can be harmful. Prolonged or unresolved inflammation can lead to tissue damage and contribute to the development of various chronic conditions, including autoimmune disorders, cardiovascular diseases, and certain cancers. Therefore, maintaining a balanced inflammatory response is crucial for overall health.[3]

It is important to note that the specific details of the inflammatory response can vary depending on the type of tissue involved and the nature of the stimulus. However, the fundamental steps you mentioned—recognition of stimuli, activation of pathways, release of mediators, and activation of inflammatory cells—are indeed central to the inflammatory response in all tissues (Figure 1).[4]

Phagocytes, including neutrophils, macrophages, and dendritic cells, are key players in the inflammatory response and have pathogen recognition receptors (PRRs) on their surfaces. These PRRs recognize specific molecular motifs on pathogens, known as pathogen-associated molecular patterns (PAMPs). Cytokines, such as interleukins and tumor necrosis factor-alpha (TNF-α), serve as signaling molecules that mediate and amplify the immune response. In addition to cytokine release, the activation of intracellular signaling pathways is a crucial step in the inflammatory response. The JAK-STAT pathway is involved in cytokine signaling and regulates gene expression. Activation of this pathway can lead to the production of specific cytokines and the modulation of immune cell functions. These signaling pathways work in coordination to amplify the inflammatory response

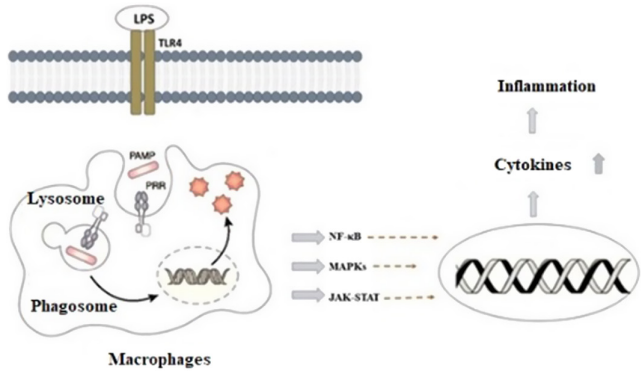

Figure 1. The general mechanism of LPS-induced inflammation

and regulate the expression of genes involved in immune and inflammatory processes. Understanding the activation of these pathways and the release of proinflammatory molecules is crucial for unraveling the complex mechanisms underlying inflammation and designing targeted interventions for inflammatory diseases.[5]

In the process of inflammation, various plasma proteases from systems such as the kinin-kallikrein, complement, coagulation, and fibrinolytic systems are activated. Activation of these proteases leads to the generation of specific products that contribute to the inflammatory response. Neutrophils are the first responders and play a crucial role in combating pathogens. They are attracted to the inflammatory site and migrate across the blood vessel walls to reach the site of infection or tissue damage. Macrophages, as you mentioned, are another important cell type involved in inflammation. Macrophages are versatile immune cells that reside in tissues, including inflamed tissues. They play a vital role in protecting the body against pathogens through various mechanisms, one of which is phagocytosis. Macrophages recognize and engulf pathogens through phagocytosis, internalizing them into specialized compartments where they can be destroyed. Furthermore, macrophages contribute to the inflammatory response by releasing additional inflammatory mediators and cytokines, amplifying the immune response and coordinating the activities of other immune cells. Together, the activation of plasma proteases, the resulting products, changes in vascular permeability, recruitment of neutrophils, and the role of macrophages all contribute to the complex process of inflammation and help the body combat pathogens and initiate the healing process.[6] Interleukin-1β (IL-1β), interleukin-6 (IL-6), and TNF-α are key cytokines produced by activated macrophages. Interleukin-1β, IL-6, and TNF-α have various roles in the inflammatory response. The release of lipid-based mediators and cytokines by activated macrophages allows them to communicate with other immune cells and coordinate the inflammatory response. Therefore, understanding the regulation of these molecules and their functions is crucial for developing targeted therapeutic approaches for inflammatory conditions.[7]

It is fascinating to see your interest in investigating the effects of TRP in the LPS-induced inflammation model using the L929 cell line. Indeed, fibroblasts play a crucial role in the inflammatory process, and their activation and interaction with other cells contribute to the overall inflammatory response and tissue repair. As you mentioned, fibroblasts act as sentinel-like cells in tissues and are among the first to respond to damage signals. They contribute to maintaining the structural integrity of connective

tissues by promoting immune cell adhesion and secreting components of the extracellular matrix that support collagen protein and adjacent cells.[2] Given the potential therapeutic benefits of plant-derived compounds and their anti-inflammatory properties, exploring the effects of TRP in the LPS-induced inflammation model is a valuable approach. The LPS-induced inflammation model is widely used because it triggers macrophage activation and subsequent activation of multiple inflammatory pathways.[7]

Using the L929 cell line, you can investigate the effects of TRP in this model and assess its impact on the inflammatory response. This approach can provide insights into the anti-inflammatory potential of TRP and its mechanisms of action, shedding light on its therapeutic potential. By examining the effects of TRP in the LPS-induced inflammation model, you can contribute to the understanding of plant-based compounds as potential therapeutic agents for inflammation-related conditions. It is an exciting area of research that can pave the way for the development of new treatments or interventions targeting inflammation. Remember to carefully design your experiments, control for variables, and analyze your results rigorously to draw meaningful conclusions.

METHODS

Cell Lines

In this study, the L929 cell line was used to evaluate the effects of the TRP compound on LPS-induced inflammation-induced cell damage in the L929 (ATCC® CRL-2091) cell line. The L929 cell line was purchased from the American Type Culture Collection (ATCC, USA) and used by reproducing from existing stocks available in the Cell Culture Laboratory, affiliated with the Department of Medical Pharmacology, Ataturk University School of Medicine.

Experimental Protocol and Details

This study was carried out in the Cell Culture Laboratory of the Department of Medical Pharmacology, Faculty of Medicine, Ataturk University. First, different concentrations of doses were determined to calculate the IC_{50} value of the TRP compound and LPS (Table 1).

After the cells preserved in Cryotube in the liquid nitrogen tank were dissolved, they were planted in flasks of T75 cm^2 (Gibco®, UK) with filtered cap. After the cells were determined with the help of Inverted (Leica®, Germany) light microscope (20×-40×), where 85%-90% of them became confluent (20×-40×), 5×10^3 cells were sown in each well of 96-well microplates. After the TRP and LPS applications, the colorimetric method (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) protocol) was applied.

Preparation of Tryptanthrin and Lipopolysaccharide

Since the TRP compound is insoluble in water, 1% dimethyl sulfoxide was added to dissolve the substance after vortexing. Different concentrations (20 µg/mL; 15 µg/mL; 10 µg/mL; 7.5 µg/mL; 5 µg/mL; 2.5 µg/mL; 1 µg/mL) were prepared after passing through the sterile filter (CAS: 13220-57-0, 97%, MW:248.24, BLDpharm, UK) with a sol pore diameter of 0.2 µm . Lipopolysaccharide (*E. Coli* O55: B5, Sigma-Aldrich®, St. Louis, USA) in lyophilized form was dissolved in pure water to prepare different concentrations.

Table 1. Doses Used to Calculate IC_{50} Values of Lipopolysaccharide and Tryptanthrin

LPS	TRP
Concentrations	
20 µg/mL	20 µg/mL
15 µg/mL	15 µg/mL
10 µg/mL	10 µg/mL
7.5 µg/mL	7,5 µg/mL
5 µg/mL	5 µg/mL
2.5 µg/mL	2,5 µg/mL
1 µg/mL	1 µg/mL

LPS, lipopolysaccharide; TRP, tryptanthrin.

Cell Culture Studies

According to cell culture studies, the L929 cell line is cultured in a medium consisting of 89% DMEM (Dulbecco's modified eagle medium), 10% FBS (fetal bovine serum), 1% PSA (penicillin–streptomycin–amphotericin B), and 2% L-glutamine. These components provide the necessary nutrients, growth factors, and antibiotics to support cell growth and maintain cell viability.

The cells are proliferated using standard cell culture procedures in a 5% CO_2 incubator at 37°. This temperature and CO_2 concentration create an optimal environment for cell growth and maintenance. To determine cell density and viability, the cells are examined using an inverted light microscope, such as the Leica microscope. Once the cells reach a confluent state with 85%-90% density, the trypan blue exclusion method is used to assess cell viability.

In this method, a mixture of 100 µL trypan blue, 90 µL PBS, and 10 µL medium containing cells is prepared in a microcentrifuge tube. A small volume (10 µL) of this mixture is then transferred to a cell counting device, such as the INNOVATIS device, which allows for accurate cell counting and viability determination. After performing the necessary calculations, the cells are seeded into the experimental wells. It is stated that 5×10^3 cells are seeded in each well, with the number of groups multiplied by 5×10^3 to ensure consistent cell seeding across all groups. These details provide a clear understanding of the cell culture conditions and procedures used for the L929 cell line. Following proper cell culture techniques and accurately determining cell density and viability are crucial for reliable experimental outcomes.

Evaluation of Cell Viability with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide Method

To summarize the experimental procedure:

1. L929 cell lines were cultivated in 3 microplates, with 5×10^3 cells (5000 cells) seeded in each well, using a standard cell culture medium.
2. The microplates were placed in a 5% CO_2 incubator at 37° for 24 hours to allow the cells to adhere and grow.
3. After the 24-hour incubation, the TRP compound was applied at the predetermined doses. The final concentration of the TRP compound was 2 µL.
4. Following the TRP application, the LPS was applied after 1 hour at a final concentration of 1 µL.
5. After the application of LPS, the cells were further incubated for 24, 48, and 72 hours to allow for the desired incubation times.
6. At the end of each incubation time (24th, 48th, and 72nd hours), the cytotoxic effects were assessed using the MTT assay, a colorimetric method. The MTT assay measures the metabolic activity of cells, which is indicative of cell viability.
7. The % viable cell ratio was calculated using the following equation:
   - % viable cell ratio = (absorbance of treated sample/absorbance of control) × 100
   - The absorbance values were obtained using a spectrophotometer or a microplate reader, and the control sample represents the untreated cells.

- By applying the MTT assay, determine the cytotoxic effects of the TRP compound and LPS on the L929 cells at different time points can be determined. This assay provides information on cell viability and can help assess the impact of the compounds on cell health.
- The MTT assay protocol provided by the manufacturer (Roche, Cat. No. 11 465 007 001) should be followed for accurate and reliable results. This protocol typically involves the addition of MTT reagent, formazan crystal dissolution, and absorbance measurement.
- By analyzing the % viable cell ratio, the potential cytotoxic or protective effects of the TRP compound and LPS on the L929 cells over time can be evaluated. This information can contribute to understanding the cellular response to these substances and their potential therapeutic implications.

$$\%\,\text{Percentage viability} = \frac{(\text{Compound} - \text{treated cell absorbance}) - (\text{absorbance of the empty well})}{(\text{control cell absorbance} - \text{absorbance of the empty well})} \times 100$$

Statistical Analysis

The data were assessed statistically utilizing Statistical Package for Social Sciences (SPSS) software 22.00 (IBM, USA). It was conducted utilizing a Tukey test, which comprised one-way analysis variance and post hoc multiple comparative assays.

RESULTS

IC_{50} Values of Lipopolysaccharide and Tryptanthrin

To determine the most effective dose on the L929 cell line, TRP and LPS different concentrations were applied. IC_{50} values that inhibit the proliferation of 50% of different concentrations of TRP and LPS on the L929 cell line in vitro were determined (Table 1).

IC_{50} values were calculated as 0.3 µg/mL for TRP and IC_{50} for LPS as 1 µg/mL using the logarithmic transformation of TRP and LPS concentration and nonlinear regression sigmoidal dose–response analysis (GraphPad 8.0 Prism, La Jolla, Calif, USA).

Results of Cell Viability

Based on the results obtained, it appears that the TRP compound has a protective effect on L929 cells against inflammation-induced damage caused by LPS. The LPS group showed a significant decrease in live-cell ratio compared to the control group, indicating that LPS-induced cytotoxicity in the L929 cells. Treatment with different doses of TRP pure substance (ranging from 20 µg/mL to 1 µg/mL) resulted in increased cell proliferation, with the highest live-cell ratio observed at a dose of 5 µg/mL.

At the 24th hour, the LPS group exhibited a live-cell ratio of 61.29%. The TRP group showed a significantly higher live-cell ratio of 90.81%, and the TRP+LPS group had a live-cell ratio of 83.01%. (Table 2)

At the 48th hour, the live-cell ratio in the TRP group decreased to 43.75%. However, in the LPS group, the live-cell ratio was significantly lower at 27.38%. The TRP+LPS group showed a higher live-cell ratio of 84.40%.

At the 72nd hour, the live-cell ratio in the TRP group increased to 92.10%. The TRP+LPS group had a slightly lower live-cell ratio of 88.21%, but it was still significantly higher than the LPS group (12.91% live cells). (Table 2)

Overall, the TRP compound was found to prevent inflammation-related cell damage in the L929 cell line induced by LPS. The cell viability rate in the TRP+LPS group was significantly higher than in the LPS group at all time points (24th, 48th, and 72nd hours) (Table 2).

The findings suggest that TRP promotes cell proliferation and helps protect L929 cells from inflammation-induced damage. The cell viability rates observed in all time periods (above 92%) exceed the recommended 70% rate for cytotoxicity according to ISO 10993-5:2009 guidelines.

These results support the potential use of TRP as a therapeutic agent for mitigating inflammation-induced cell damage and promoting cell viability in the L929 cell line. Further studies may be warranted to explore the underlying mechanisms of TRP's protective effects and its potential applications in inflammatory conditions.

DISCUSSION

The study conducted investigated the effects of the TRP compound, isolated from the root of Ferulago pauciradiata plant, on LPS-induced inflammation-induced cell injury in the L929 cell line. The results of the study support the hypothesis that the TRP compound can prevent inflammation and reduce cell damage. Throughout the study, it was observed that the TRP compound had a protective effect on the L929 cells. At 24th hours, 48th hours, and 72nd hours, the TRP compound prevented inflammation-induced cell injury and increased cell proliferation. These effects brought the cell viability closer to that of the control group, indicating a positive impact of the TRP compound on cell health. The results obtained from this study are consistent with the hypothesis that the TRP compound can reduce inflammatory cell damage. This suggests that the TRP compound derived from the root of *Ferulago pauciradiata* plant may have the potential as a therapeutic agent for mitigating inflammation and promoting cell viability. Further research and investigations may be needed to better understand the mechanisms of action and potential applications of the TRP compound in treating inflammation-related conditions.

The inflammatory response is the natural defense system of the host against pathogens in general, and inflammatory cells and macrophages are activated in this process.[9] Lipopolysaccharide, which is a component of the cell wall of Gram-negative bacteria, is often used in the inflammatory response due to its ability to activate macrophages.[10] The induced macrophages strengthen the inflammatory response by destroying the pathogen with the various juices they secrete and reshaping damaged tissue structures. Pharmacological reduction of LPS-induced inflammatory mediators is considered one of the attractive therapeutic strategies for acute and chronic inflammatory diseases.[8] In the inflammatory process, food components with anti-inflammatory properties may attract attention, provided they can be preserved

Table 2. Results of Cell Viability According to Groups and Hours

Hours	LPS	TRP	TRP+LPS
Groups			
24th	61.29%	90.81%	83.01%
48th	27.38%	43.74%	84.40%
72nd	12.91%	92.10%	88.21%

LPS, lipopolysaccharide; TRP, tryptanthrin.

in the digestive process.[11] In recent years, natural products and their synthetic derivatives have been gaining increasing popularity as potential drugs for the treatment of various diseases affecting humans.[12] Also, it is known that many plants show anti-inflammatory activity based on folk medicine knowledge. Therefore, researchers turn to plants for new therapeutic agents.

Tryptanthrin which is present in indigo plants is an indole quinazoline alkaloid. Many studies have revealed that TRP has many biological and pharmacological activities such as antimicrobial, anti-inflammatory, and antiallergic ones.[1] In accordance with the results of the studies conducted, while the effects of TRP, which is a coumarin derivative, on L929 cells at the 24th, 48th, and 72nd hours did not show any toxic effect compared to the control group, it was determined that TRP application increased the amount of decreased cell index in LPS groups.

As a result, studies[12-15] found that coumarins have a proliferative effect. When LPS was applied 1 hour after applying different doses of the TRP compound it was found that as the concentration of the TRP pure substance increased, the cell proliferation increased and the highest viable cell ratio was observed at a dose of 8 µg/mL.

When we look at the results of our study as a whole, it was determined that the viability rates of the cells were above 85% as a result of the TRP application and it was significantly above the 70% viable cell ratio recommended by ISO 10993-5: 2009 Dın en ISO (2009) for cytotoxicity.

Ethics Committee Approval: There is no 'Ethics committee approval because this is a cell culture study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – M.Y., Z.K.; Design – M.Y., Z.K.; Supervision – M.Y., Z.K.; Resources – M.Y., Z.K.; Materials – M.Y., Z.K.; Data Collection and/or Processing – M.Y., Z.K.; Analysis and/or Interpretation – M.Y., Z.K.; Literature Search – M.Y., Z.K.; Writing Manuscript – M.Y., Z.K.; Critical Review – M.Y., Z.K.

Declaration of Interests: The authors declare that they have no competing interest.

Funding: The authors declared that this study has received no financial support.

REFERENCES

1. Popov A, Klimovich A, Styshova O, et al. Design, synthesis and biomedical evaluation of mostotrin, a new water soluble tryptanthrin derivative. *Int J Mol Med*. 2020;46(4):1335-1346. [CrossRef]
2. Moeed A, Michael M, Emma GM. Fibroblast activation and inflammation in frozen shoulder. *Plus One*. 2019:1-16.
3. Chen L, Deng H, Cui H, et al. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*. 2018;9(6):7204-7218. [CrossRef]
4. Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy--from molecular mechanisms to therapeutic benefits. *Biochim Biophys Acta*. 2005;1754(1-2):253-262. [CrossRef]
5. Netea MG, Balkwill F, Chonchol M, et al. A guiding map for inflammation. *Nat Immunol*. 2017;18(8):826-831. [CrossRef]
6. Xu J, Zhao Y, Aisa HA. Anti-inflammatory effect of pomegranate flower in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. *Pharmaceutical Biology*. 2017;55(1):2095-2101. [CrossRef]
7. Shao J, Li Y, Wang Z. A novel naphthalimide derivative, exhibited anti-inflammatory effects via targeted-inhibiting TAK1 following down-regulation of ERK1/2- and p38 MAPK-mediated activation of NF-kB in LPS-stimulated RAW264.7 macrophages. *Int Immunupharmacol*. 2013;17:216-228.
8. Zhang S, Ma J, Sheng L, et al. Total Coumarins from Hydrangea paniculata show renal protective effects in lipopolysaccharide-induced acute kidney injury via anti-inflammatory and antioxidant activities. *Front Pharmacol*. 2017;8:872. [CrossRef]
9. Vysakh A, Prasad G, Kuriakose J. Rotula aquatica Lour attenuates secretion of proinflammatory mediators and cytokines in lipopolysa ccharide-induced inflammatory responses in murine RAW 264.7 macrophages. *Inflammopharmacology*. 2018;26:29-38.
10. Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal*. 2001;13(2):85-94. [CrossRef]
11. Bistrian B. Systemic response to inflammation. *Nutr Rev*. 2007;65(12 Pt 2):S170-S172. [CrossRef]
12. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev*. 2009;22(2):240-273. [CrossRef]
13. Neumann U, Louis S, Gille A, et al. Anti-inflammatory effects of Phaeodactylum tricornutum extracts on human blood mononuclear cells and murine macrophages. *J Appl Phycol*. 2018;30(5):2837-2846. [CrossRef]
14. Mustafa YF, Najem MA, Tawffiq ZS. Coumarins from creston apple seeds: isolation, chemical modification, and cytotoxicity study. *J Appl Pharm Sci*. 2018;8(08):49-56.
15. Kasumbwe K., Kabange N., Venugopala N. 2017 Synthetic Mono/dihalogenated Coumarin Derivatives and Their Anticancer Properties. Anti-Cancer Agents in Medicinal Chemistry, 17(2),276-85.