

Effect of n-Hexane Extract of *Camellia Oleifera* Fruit Hull on Inflammatory Responses in LPS-Induced RAW264.7 Cells

Chuanqi Xie^{1, #}, Shufen Wang^{1, #}, Xinying Lin^{1, 2}, Fan Yang^{1, 3},
Juwu Hu¹, Jing Wu¹, Wei Xiong^{1, *}, Lei Wu^{1, *}

¹Institute of Applied Chemistry, Jiangxi Academy of Sciences, Nanchang, China

²School of Medicine, Xiamen University, Xiamen, China

³School of Food Science and Engineering, Jiangxi Agricultural University, Nanchang, China

[#]These authors contributed equally to this work.

*Corresponding author: xw3886762@126.com, wulei858196@163.com

Received February 20, 2023; Revised March 25, 2023; Accepted April 05, 2023

Abstract *Camellia oleifera* fruit hull has a variety of biological activities such as anti-oxidation, lipid regulation, and anti-cancer etc, however the effect of *C. oleifera* fruit hull on inflammatory responses has not been clearly elucidated. This study aimed to explore the effect of n-hexane extract of *C. oleifera* fruit hull on lipopolysaccharide (LPS)-induced inflammatory responses in RAW264.7 cells using Griess assay, ELISA, western blotting analysis, PCR and immunofluorescence technology. The results demonstrated that n-hexane extract of *C. oleifera* fruit hull ameliorated the secretion of nitric oxide (NO), prostaglandin E2 (PGE2), tumour necrosis factor α (TNF α), interleukin (IL)-1 β , and IL-6, inhibited the protein expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2), downregulated the mRNA level of iNOS, COX2, TNF α , IL-1 β , and IL-6, and improved the cell viability in LPS-induced RAW264.7 cells. Moreover, the phosphorylation of ERK, P38, and JNK and nuclear translocation of nuclear factor-kappa B (NF- κ B) were restrained by the treatment of n-hexane extract of *C. oleifera* fruit hull in LPS-induced RAW264.7 cells. Taken together, these data imply that n-hexane extract of *C. oleifera* fruit hull alleviates the inflammatory response by regulating the activation of mitogen-activated protein kinases (MAPKs) and NF- κ B signaling pathways in LPS-induced RAW264.7 cells.

Keywords: *C. oleifera* fruit hull, n-hexane extract, inflammatory responses, MAPKs, NF- κ B

Cite This Article: Chuanqi Xie, Shufen Wang, Xinying Lin, Fan Yang, Juwu Hu, Jing Wu, Wei Xiong, and Lei Wu, "Effect of n-Hexane Extract of *Camellia Oleifera* Fruit Hull on Inflammatory Responses in LPS-Induced RAW264.7 Cells." *Journal of Food and Nutrition Research*, vol. 11, no.4 (2023): 301-309. doi: 10.12691/jfmr-11-4-4.

1. Introduction

C. oleifera fruit hull, accounting for approximately 60% weight of the single *C. oleifera* fruit, is the byproduct produced by pressing seeds during traditional oil processing, which contains several active components including lignin, cellulose, hemicellulose, polysaccharides, flavonoids, phenols and terpenes [1,2,3]. Several researchers have reported that *C. oleifera* fruit hull has multiple biological activities such as anti-oxidation, lipid regulation, and anti-cancer [1,4]. Xie and co-workers have pointed out that the extract of *C. oleifera* fruit hull could scavenge free radicals, inhibit lipid peroxidation, and reduce the body weight of mice induced by high fat diet [1]. Zhou *et al.* and Zhang and Li have shown that the polysaccharide from *C. oleifera* fruit hull had promising antitumor activity and had strong antioxidant activity *in vitro* and *in vivo* [4,5]. However, the anti-inflammatory effect of *C. oleifera* fruit hull has not been well reported.

Macrophages existing in all tissues play a crucial role in the inflammatory and immune responses to the invasion of pathogenic microorganisms, and other foreign substances by phagocytosis and secreting related molecules such as NO, PGE2, IL-1 β , IL-6, and TNF α [6,7,8]. NO and PGE2 are two inflammatory related factors involved in a variety of physiological and pathological reactions [9,10]. TNF α , IL-1 β , and IL-6 are three proinflammatory mediators regulating inflammatory responses [11,12,13]. LPS, a major component of the cell wall of gram-negative bacteria, continuously induces the activation of macrophages, which was generally used as the cell model for anti-inflammatory researches [8,14,15,16].

MAPKs and NF- κ B are two major signaling pathways related to the activation of macrophages, which regulate the secretion and maturation of several inflammatory cytokines in macrophages [8,17,18]. MAPKs presented in most eukaryotes are serine/threonine protein kinases, which contain three family members including P38, ERK, and JNK, and can be activated by the transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1) [19]. The

activation of MAPKs then promotes the nuclear translocation of activation protein 1 (AP1) from the cytoplasm, which regulates mRNA transcription via binding to the promoter region of related genes and ultimately causes the secretion and maturation of related molecules such as iNOS, COX2, IL-1 β , IL-6 and TNF α [20]. NF- κ B is an important transcription factor that regulates a series of inflammatory responses by promoting the release of related molecules and enhancing the phagocytosis ability of macrophages [21]. NF- κ B in the rest state remains in the cytoplasm by binding to the I κ B inhibitory protein. Once the cells are stimulated by a foreign substance or pathogen, the I κ B inhibitory protein will be phosphorylated by the I κ B kinase (IKK), and dissociated from the NF- κ B complex, then degraded through ubiquitination [22]. The dissociated NF- κ B will be activated and rapidly translocated into nuclear to regulate the transcription of related genes by binding to the mRNA promoter region, which ultimately causes the release of related molecules and activation of macrophages [14].

In this study, we focused on the effect of n-hexane extract of *C. oleifera* fruit hull on inflammatory responses in LPS-induced RAW264.7 cells. The results indicated that n-hexane extract of *C. oleifera* fruit hull ameliorated the secretion of NO, PGE2, IL-1 β , IL-6, and TNF α , inhibited the protein expression of iNOS and COX2, downregulated the mRNA level of iNOS, COX2, IL-1 β , IL-6, and TNF α , and improved the cell viability in LPS-induced RAW264.7 cells. Moreover, the phosphorylation of ERK, P38, and JNK and nuclear translocation of NF- κ B were restrained by n-hexane extract of *C. oleifera* fruit hull in LPS-induced RAW264.7 cells. In conclusion, these data suggested that n-hexane extract of *C. oleifera* fruit hull alleviates inflammatory responses by regulating the activation of MAPKs and NF- κ B signaling pathways in LPS-induced RAW264.7 cells.

2. Materials and Methods

2.1. Reagents

High Glucose DMEM medium was provided by Biological Industries (Israel) and fetal bovine serum (FBS) was obtained from Natocor (Argentina). Penicillin-streptomycin (P/S) solution and RIPA lysis buffer (high) were purchased from Solarbio (Beijing, China). And LPS was from Sigma-Aldrich (USA). ELISA kits for mouse IL-1 β , IL-6, and TNF α were obtained from BOSTER (Wuhan, China), PGE2 kit was from R&D Systems (Minneapolis, USA). BCA Protein Assay Kit was provided by Beyotime (Jiangsu, China). Primary antibodies were supplied by ABclonal (Wuhan, China) or Bioworld Technology Inc (MN, USA). TransZol Up reagent was purchased from TransGen Biotech (Beijing, China) and Primescript™ First-Strand cDNA Synthesis kit was provided by Takara (Beijing, China).

2.2. Preparation of n-Hexane Extract of *C. Oleifera* Fruit Hull

C. oleifera fruit hull purchased from Jiangxi enquan grease Co., Ltd (Shangrao, China) was thoroughly crushed

and passed through 100 mesh sieve. Then the powder of *C. oleifera* fruit hull was immersed in distilled water boiling for 2 h and repeated for 3 times. The supernatants were combined and concentrated after filtration. And the n-hexane extract of *C. oleifera* fruit hull was obtained after extraction with n-hexane solvent, concentration and lyophilization.

2.3. Cell Culture

RAW264.7 cells obtained from Key Laboratory of Pu-er Tea Science, Ministry of Education were purchased from Kunming Institute of Zoology, Chinese Academy of Sciences, which were cultured in High Glucose DMEM medium with 10% FBS and 1% P/S at 37°C and 5% CO₂. The cells were observed every day and the media was changed every 2-3 days.

2.4. Griess Assay

RAW264.7 cells (5×10^4 cells/well) were seeded in a 96-well plate and incubated overnight. The n-hexane extract of *C. oleifera* fruit hull at concentration ranges from 0 to 100 μ g/mL was added for 2 h followed with LPS at 1 μ g/mL for 24 h. 100 μ L of each supernatant medium was transferred into a new 96-well plate and mixed with an equal volume of Griess A and B at room temperature in the dark for 10 min [8]. The absorbance was measured at 540 nm using a microplate reader (Tecan Infinite 200 Pro, Switzerland) immediately, and the concentration of NO was determined through the calibration curve.

2.5. MTT Assay

RAW264.7 cells were treated as in the Griess assay. The remained cells in the 96-well plate with 100 μ L media were exposed to 10 μ L MTT (5 mg/mL) at 37°C for 4 h in the dark. The supernatant was removal from the wells, and 150 μ L DMSO was added for the dissolution of formazan crystals. The absorbance of each well was read at 492 nm through the microplate reader and the proportional cell viability was calculated and normalized to the control group.

2.6. Enzyme-Linked Immunosorbent Assay

RAW264.7 cells (1×10^6 cells/well) were inoculated in a 6-well plate overnight. The n-hexane extract of *C. oleifera* fruit hull at treatment concentrations of 25, 50, 100, and 200 μ g/mL was pretreated for 2 h and LPS at a final concentration of 1 μ g/mL was added for 24 h. The cell culture supernatants were assessed using ELISA kits. Each sample was tested in triplicate.

2.7. Western Blotting

RAW264.7 cells (1×10^6 cells/well) were treated as in the enzyme-linked immunosorbent assay or seeded in dishes with a diameter of 60 mm overnight and pretreated with n-hexane extract of *C. oleifera* fruit hull at 200 μ g/mL for 2 h following treated with LPS at 1 μ g/mL for indicated time (1 h, 2 h and 6 h), respectively. Cells

were harvested and lysed on ice for 30 min with RIPA lysis buffer (high). Protein concentrations were quantified using BCA Protein Assay Kit (Beyotime, Jiangsu, China). Equal amounts of total proteins (40 µg) were separated by 10% SDS-PAGE gel and then transferred onto PVDF membranes (Millipore, CA, USA). The membranes were blocked in 5% skim milk at room temperature for 1 h and then incubated at 4°C overnight with primary antibodies specific for iNOS (A4771, ABclonal), GAPDH (AP0066, Bioworld Technology), COX2 (BS90326, Bioworld Technology), JNK1/2/3 (A4867, ABclonal), p-JNK (BS9939M, Bioworld Technology), P38 (A4771, ABclonal), p-P38 (BS6381, Bioworld Technology), ERK1/2 (A4782, ABclonal) and p-ERK1/2 (AP0485, ABclonal). The membrane was then incubated for an additional 60 min with a secondary antibody (Goat Anti-rabbit IgG/HRP, bs-0295G-HRP, Bioss). Immunoreactive bands were developed using Super ECL plus kit (4A biotech, Beijing, China) and imaging with ChemiScope 3000 mini (Clinx, Shanghai, China).

2.8. RNA Isolation and PCR Analysis

RAW264.7 cells (1×10^6 cells/well) were treated as in the enzyme-linked immunosorbent assay, and the total RNA was isolated using TransZol Up reagent according to the instructions from the manufacturer and quantified with a microplate reader (Tecan Infinite 200 Pro, Switzerland). The cDNA was synthesized using Primescript™ First-Strand cDNA Synthesis kit and amplified by rapid PCR reagent (Vazyme, Nanjing, China). The mRNA levels were detected by agarose gel electrophoresis and the relative expressions of mRNA were normalized to GAPDH using Image J analysis software. The specific primers are shown in Table 1.

Table 1. PCR primer sequences

Primer name	Primer sequences (5'-3')
iNOS-F	CCCTTCCGAAGTTTCTGGCAGCAGC
iNOS-R	GGCTGTCAGAGCCTCGTGGCTTTG
COX2-F	AGAAGGAAATGGCTGCAGAA
COX2-R	GCTCGGCTTCCAGTATTGAG
TNFα-F	TGTCCCTTTCACTCACTGGC
TNFα-R	CATCTTTTGGGGGAGTGCCCT
IL-1β-F	AGCTTCAGGCAGGCAGTATC
IL-1β-R	AAGTGCCACGGGAAAGACAC
IL-6-F	GAGTGGCTAAGGACCAAGACC
IL-6-R	AACGCACTAGGTTTGCCGA
GAPDH-F	CACTCACGGCAAATTCAACGGCA
GAPDH-R	GACTCCACGACATACTCAGCAC

2.9. NF-κB Activation and Nuclear Translocation Assay

RAW264.7 cells (1×10^5 cells/well) seeded in a 6-well plate were pretreated with n-hexane extract of *C. oleifera* fruit hull at 200 µg/mL for 2 h following treated with LPS at 1 µg/mL for 6 h. The supernatants were discarded and the immunofluorescence test was performed according to the instructions from the manufacturer of Cellular NF-κB Translocation Kit (Beyotime, Jiangsu, China). Cells were fixed with the stationary liquid for 15 min and blocked

with blocking solution for 1 h at room temperature. The cells were then incubated overnight at 4°C with the primary antibody NF-κB p65 and future incubated with a Cy3-conjugated secondary antibody for 1 h, and then stained with DAPI for 5 min before determination. The red and blue images were captured using a TS2-FL microscope (Nikon, Tokyo, Japan).

2.10. Statistical Analysis

Three replicates were performed for all experiments, and the results were expressed as mean ± standard deviation. The statistical test was performed using GraphPad Prism, version 8.0.1 (GraphPad Software, San Diego, CA, USA) and the significance analysis was performed by t-test.

3. Results

3.1. Effects of n-Hexane Extract of *C. Oleifera* Fruit Hull on NO Production and Cell Viability in LPS-Induced RAW264.7 Cells

In the study, the effects of n-hexane extract of *C. oleifera* fruit hull on NO production and cell viability in LPS-induced RAW264.7 cells were initially evaluated using Griess assay and MTT method. The results showed that n-hexane extract of *C. oleifera* fruit hull inhibited the production of NO in a concentration-dependent manner (Figure 1a) and improved the cell viability and morphology (Figure 1b and c) in LPS-induced RAW264.7 cells which suggested that n-hexane extract of *C. oleifera* fruit hull may play an inhibition role in LPS-induced inflammatory responses in RAW264.7 cells.

3.2. Effects of n-Hexane Extract of *C. Oleifera* Fruit Hull on the Secretion of Proinflammatory Factors in LPS-Induced RAW264.7 Cells

The release of proinflammatory cytokines including PGE2, TNFα, IL-1β, and IL-6 is one of the significant markers of LPS-induced inflammatory responses in RAW264.7 cells. In this study, the effects of n-hexane extract of *C. oleifera* fruit hull on the secretion of PGE2, TNFα, IL-1β, and IL-6 were detected using ELISA method. The results showed that high concentration of n-hexane extract of *C. oleifera* fruit hull at 100 and 200 µg/mL decreased the release of PGE2 (Figure 2a) and IL-6 (Figure 2c), and the release of IL-1β was presented in a decreasing tendency with the increasing concentration of n-hexane extract of *C. oleifera* fruit hull in LPS-induced RAW264.7 cells (Figure 2d).

In addition, n-hexane extract of *C. oleifera* fruit hull had a slightly reducing effect on TNFα release, although the reducing effect presented significant differences at 100 µg/mL (Figure 2b). In summary, these data indicated that n-hexane extract of *C. oleifera* fruit hull ameliorated the secretion of proinflammatory cytokines to a certain degree.

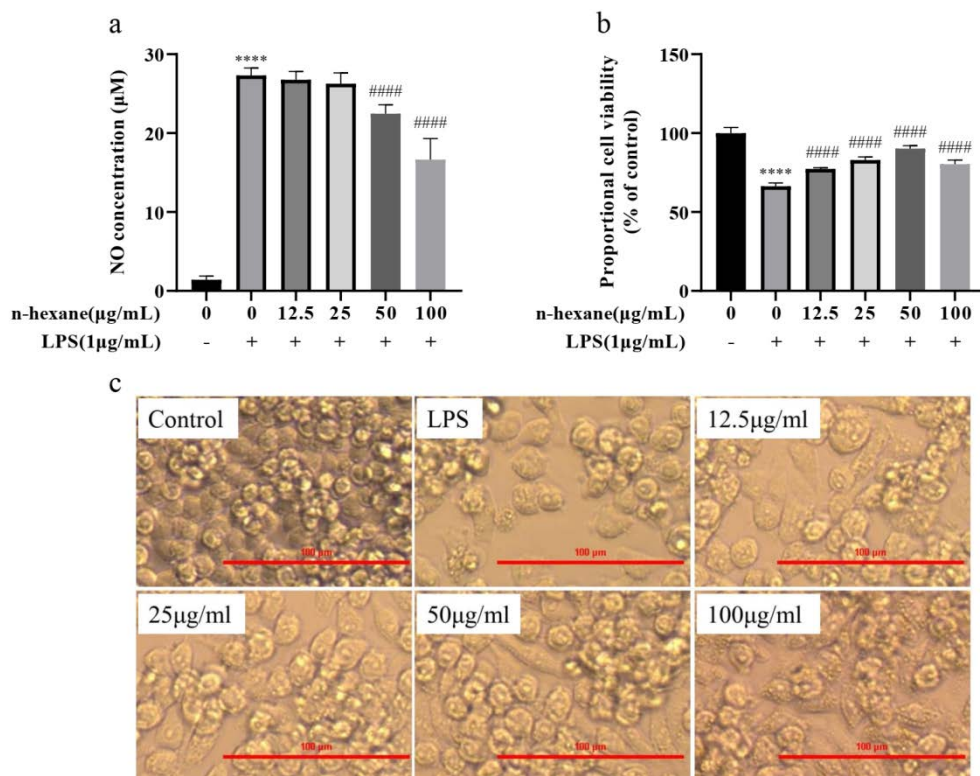


Figure 1. Effects of n-hexane extract of *C. oleifera* fruit hull on NO production and cell viability in LPS-induced RAW264.7 cells. Cells were pretreated with the indicated concentrations of n-hexane extract (0, 12.5, 25, 50, and 100 μg/mL) for 2 h following treatment of LPS (1 μg/mL) for 24 h. Cell supernatants were collected for the detection of NO production by Griess assay (a). Cell viability was evaluated by MTT reagent (b). Cell morphology and density were pictured and identified through microscope (c). The data are presented as means ± SD of three separate experiments. ****p<0.0001 compared with control group, ####p<0.0001 compared with LPS group

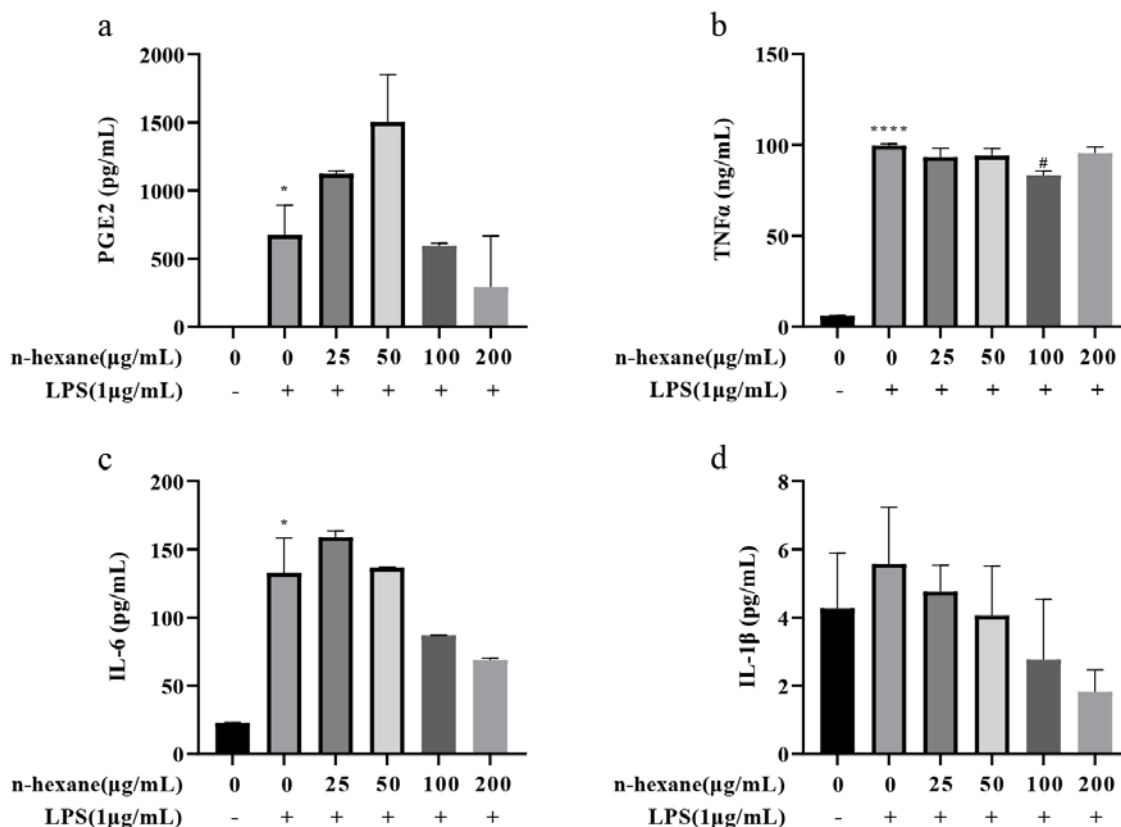


Figure 2. Effects of n-hexane extract of *C. oleifera* fruit hull on the release of proinflammatory factors in LPS-induced RAW264.7 cells. Cells were pretreated with the indicated concentrations of n-hexane extract (0, 25, 50, 100, and 200 μg/mL) for 2 h following treatment of LPS (1 μg/mL) for 24 h. Cell supernatants were collected and the releases of PGE2 (a), TNFα (b), IL-6 (c), and IL-1β (d) were determined using ELISA. The data are presented as means ± SD of three independent experiments. *p<0.05, ****p<0.0001 compared with control group, #p<0.05 compared with LPS group

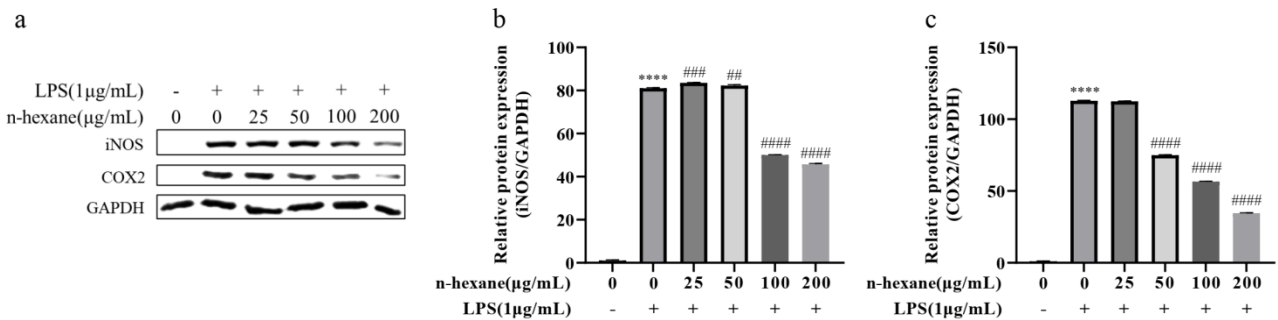


Figure 3. Effects of n-hexane extract of *C. oleifera* fruit hull on the expression of inflammatory proteins in LPS-induced RAW264.7 cells. Cells were pretreated with the indicated concentrations of n-hexane extract (0, 25, 50, 100, and 200 µg/mL) for 2 h following treatment of LPS (1 µg/mL) for 24 h. Cell lysates were obtained and the protein expressions of iNOS, COX2, and GAPDH were analyzed by western blotting. (a). The representative results of western blotting bands. (b-c). The relative protein expressions of iNOS (b) and COX2 (c) were quantified using image J and normalized to GAPDH. The data are presented as means $\pm$ SD of three independent experiments. **** p <0.0001 compared with control group, ## p <0.01, #### p <0.001, ##### p <0.0001 compared with LPS group

3.3. Effects of n-hexane Extract of *C. Oleifera* Fruit Hull on the Expression of Inflammatory Proteins in LPS-Induced RAW264.7 Cells

iNOS and COX2 are two enzymes responsible for the synthesis of NO and PGE2 in LPS-induced RAW264.7 cells. In this study, the effects of n-hexane extract of *C. oleifera* fruit hull on the protein expression of iNOS and COX2 were evaluated by western blotting analysis. The result demonstrated that the protein expression of iNOS and COX2 were at a low level in RAW264.7 cells before treatment, while they were markedly increased after LPS treated in RAW264.7 cells. And pretreatment with n-hexane extract of *C. oleifera* fruit hull suppressed the protein expression of iNOS and COX2 in LPS-induced RAW264.7 cells, especially at high concentrations 100 and 200 µg/mL (Figure 3). This data suggested the anti-inflammatory effect of n-hexane extract of *C. oleifera* fruit hull in LPS-induced RAW264.7 cells.

3.4. Effects of n-Hexane Extract of *C. Oleifera* Fruit Hull on the Mrna Level of Related Inflammatory Molecules in LPS-Induced RAW264.7 Cells

To further explore the anti-inflammatory effect of n-hexane extract of *C. oleifera* fruit hull, the effect of n-hexane extract on the mRNA levels of inflammatory related molecules in LPS-treated RAW264.7 cells were quantified using PCR analysis. In Figure 4, the result showed that n-hexane extract of *C. oleifera* fruit hull dramatically reduced the mRNA level of iNOS (Figure 4b) and IL-1 β (Figure 4e), however the inhibition effect of n-hexane extract of *C. oleifera* fruit hull on the mRNA level of COX2 (Figure 4c) and TNF α (Figure 4d) was relatively slight in LPS-induced RAW264.7 cells. Moreover, n-hexane extract of *C. oleifera* fruit hull played a reducing effect on the mRNA level of IL-6 at concentration ranges from 25 to 100 µg/mL, but increased the mRNA level of IL-6 at 200 µg/mL in LPS-treated RAW264.7 cells (Figure 4f). In conclusion, these data implied the downregulatory effect of n-hexane extract of *C. oleifera* fruit hull on the mRNA level of

proinflammatory molecules in LPS-induced RAW264.7 cells, although the regulating mode may be complicated.

3.5. n-Hexane Extract of *C. Oleifera* Fruit Hull Downregulated the Phosphorylation of Mapks Signaling Pathway in LPS-Induced RAW264.7 Cells

MAPKs signaling pathway played a crucial regulatory role in LPS-stimulated inflammatory responses in RAW264.7 cells. In the study, to evaluate the anti-inflammatory mechanism of n-hexane extract of *C. oleifera* fruit hull, the effect of n-hexane extract on the phosphorylation of MAPKs including ERK, P38 and JNK in LPS-induced RAW264.7 cells were analyzed using western blotting. The results demonstrated that n-hexane extract of *C. oleifera* fruit hull markedly restrained the phosphorylation of ERK and P38 in LPS treated RAW264.7 cells for 2 h, and reduced the phosphorylation level of P38 and JNK in LPS treated RAW264.7 cell for 6 h (Figure 5). These data indicated that the regulatory effect of n-hexane extract of *C. oleifera* fruit hull on the phosphorylation of MAPKs was closely related to the time course and the inhibition effect was marked at a certain time point in LPS-induced RAW264.7 cells.

3.6. n-Hexane Extract of *C. Oleifera* Fruit Hull Prevented the Activation and Nuclear Translocation of NF- κ B in LPS-Induced RAW264.7 Cells

NF- κ B is a classical signaling pathway involved in the activation and inhibition of macrophages. Here, the nuclear translocation and activation of NF- κ B in RAW264.7 cells was examined through immunofluorescence technique to evaluate the anti-inflammatory mechanism of n-hexane extract of *C. oleifera* fruit hull. In Figure 6, NF- κ B remained in the cytoplasm in RAW264.7 macrophages in the resting state, then it was translocated into the nuclear after treated by LPS for 6 h. And the pretreatment of n-hexane extract of *C. oleifera* fruit hull surely prevented the nuclear translocation and activation of NF- κ B in LPS-induced RAW264.7 cells. This data suggested that n-hexane extract of *C. oleifera* fruit hull played a

regulatory effect on the restriction of the activation and nuclear translocation of NF- κ B in LPS-induced RAW264.7

cells, which may be related to anti-inflammatory effect of n-hexane extract of *C. oleifera* fruit hull.

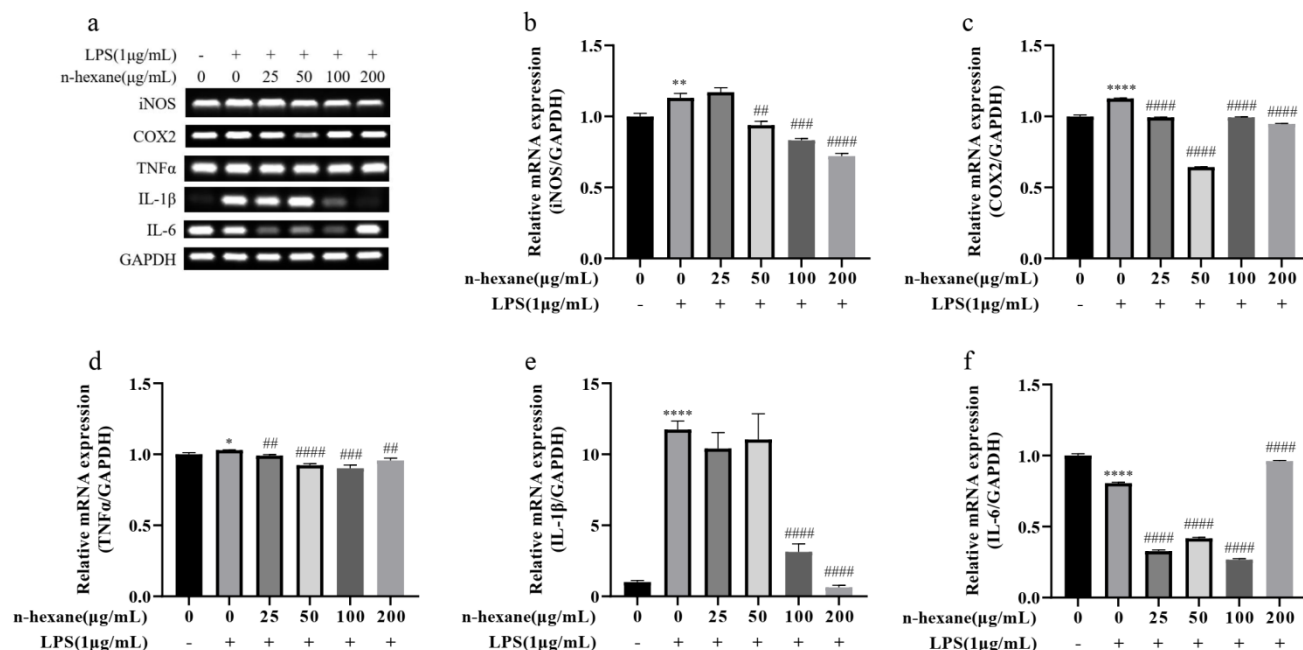


Figure 4. Effects of n-hexane extract of *C. oleifera* fruit hull on the mRNA level of proinflammatory genes in LPS-induced RAW264.7 cells. Cells were pretreated with the indicated concentrations of n-hexane extract (0, 25, 50, 100, and 200 μ g/mL) for 2 h following treatment of LPS (1 μ g/mL) for 24 h. Cell lysates were obtained for RNA extraction and the mRNA levels of iNOS, COX2, TNF α , IL-1 β , IL-6, and GAPDH were analyzed using fast PCR. (a). The representative results of agarose gel electrophoresis bands. (b-f). The relative mRNA levels of iNOS (b), COX2 (c), TNF α (d), IL-1 β (e), and IL-6 (f) were quantified using image J and normalized to GAPDH. The data are presented as means $\pm$ SD of three independent experiments. * p <0.05, ** p <0.01, **** p <0.0001 compared with control group, ## p <0.01, ### p <0.001, #### p <0.0001 compared with LPS group

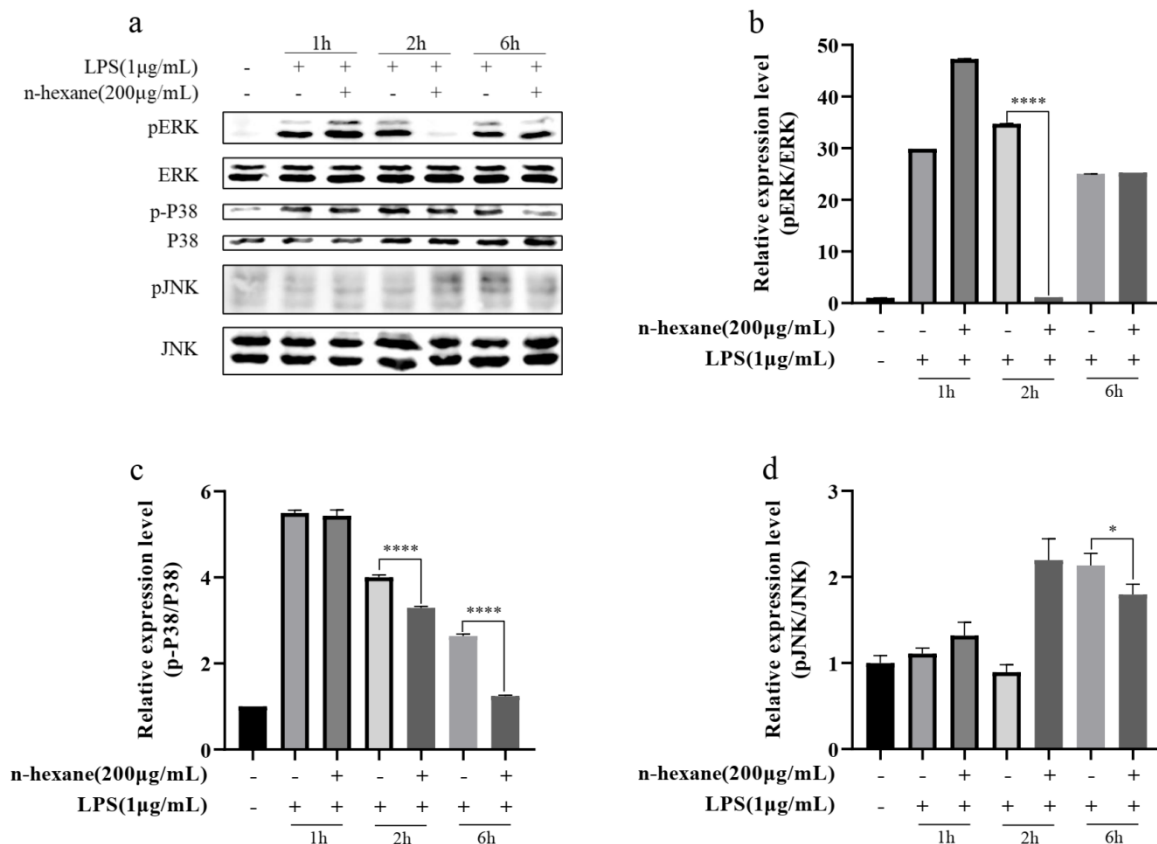


Figure 5. Effects of n-hexane extract of *C. oleifera* fruit hull on the phosphorylation level of MAPKs in LPS-induced RAW264.7 cells. Cells were pretreated with n-hexane extract (200 μ g/mL) for 2 h following treatment of LPS (1 μ g/mL) for the indicated times (1, 2, 6 h). Cell lysates were collected and protein expression and phosphorylation of ERK, P38, and JNK were analyzed using western blotting. (a). The representative results of western blotting bands. (b-d). The relative phosphorylation levels of ERK (b), P38 (c), and JNK (d) were quantified using image J and normalized to that of the unphosphorylated form. The data are presented as means $\pm$ SD of three independent experiments. * p <0.05, **** p <0.0001

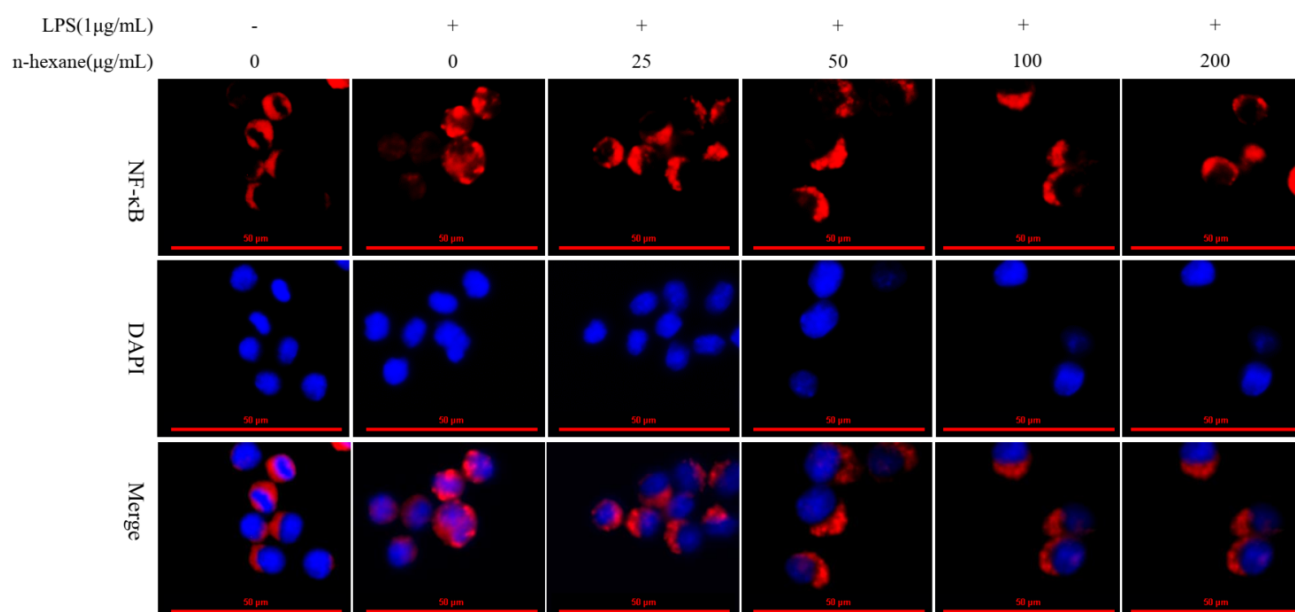


Figure 6. Effects of n-hexane extract of *C. oleifera* fruit hull on the activation and nuclear translocation of NF-κB in LPS-induced RAW264.7 cells. Cells were pretreated with n-hexane extract (200 μg/mL) for 2 h following treatment with LPS (1 μg/mL) for 6 h. Cell supernatants were discarded and cells were used for the detection of NF-κB nuclear translocation via immunofluorescence. The representative graphs of immunofluorescence were presented from three separate experiments

4. Discussion

C. oleifera fruit hull contains several active components including lignin, cellulose, hemicellulose, polysaccharides, flavonoids, phenols, and terpenes, which have various biological activities such as anti-oxidation, lipid regulation and anticancer [1-5,23]. Recent studies indicate that the extract of *C. oleifera* fruit hull had the ability of scavenging free radicals, inhibiting lipid peroxidation, and reducing the body weight of mice induced by high fat diet [1,4,23,24]. However, the anti-inflammatory effect and mechanism of *C. oleifera* fruit hull has rarely been reported. In this study, the effects of n-hexane extract of *C. oleifera* fruit hull on the inflammatory responses in LPS-induced RAW264.7 cells were investigated via Griess assay, ELISA, western blotting analysis, PCR, and immunofluorescence technology, etc.

NO and PGE₂, as the two major inflammatory markers synthesized and secreted by macrophages, were mainly derived from L-arginine and arachidonic acid by the action of iNOS and COX2, respectively [8,20,25,26]. In this study, the production of NO and PGE₂, as well as the protein and mRNA expression of iNOS and COX2 were evaluated. The results showed that n-hexane extract of *C. oleifera* fruit hull suppressed the production of NO and PGE₂, reduced the protein and mRNA levels, and improved the cell viability in LPS-induced RAW264.7 cells, which suggested that the n-hexane extract of *C. oleifera* fruit hull may have the ability of anti-inflammation at protein and mRNA levels.

Proinflammatory cytokines such as TNFα, IL-1β, and IL-6 were released by activated macrophages, which are considered the major mediators in several cell processes including immune reaction, inflammatory response, cell proliferation and cell apoptosis [11,12,13]. In the study, the secretion and mRNA expression of TNFα, IL-1β, and

IL-6 were detected. The results demonstrated that n-hexane extract of *C. oleifera* fruit hull decreased the release of TNFα, IL-1β, and IL-6 in a certain degree and had some inhibition effect on the mRNA expression of TNFα, IL-1β and IL-6 in LPS-induced RAW264.7 cells. These data indicated that the anti-inflammatory activities of n-hexane extract of *C. oleifera* fruit hull may be manifested by inhibiting the production of inflammatory markers and suppressing the release of proinflammatory cytokines at the protein and mRNA levels.

MAPKs and NF-κB signaling pathways have been proved to play a significant role in LPS-induced inflammatory responses by regulating the secretion and maturation of related cytokines in macrophages [8,14,16,18,25,27,28]. Park and colleagues have pointed out scutellarein inhibits LPS-induced inflammation through inactivating NF-κB/MAPKs signaling pathway in RAW264.7 macrophages [14]. Islam *et al.* reported that decursinol angelate inhibits LPS-induced macrophage polarization through modulation of the NF-κB and MAPK signaling pathways [16]. Alam and colleagues proved that cerevisterol alleviates inflammation via suppression of the activation of MAPK/NF-κB/AP-1 and Nrf2/HO-1 signaling Cascades [25]. In the study, the phosphorylation of MAPKs including ERK, JNK, and P38 and the activation and nuclear translocation of NF-κB were investigated. The results indicated that the n-hexane extract of *C. oleifera* fruit hull downregulated the phosphorylation levels of ERK, JNK, and P38 at different time points with different degree and restrained the activation and nuclear translocation of NF-κB in LPS-induced RAW264.7 cells, which implied that n-hexane extract of *C. oleifera* fruit hull may play an anti-inflammation activity via preventing the activation of MAPKs and NF-κB signaling pathways in LPS-induced RAW264.7 cells.

5. Conclusions

In this study, the effect of n-hexane extract of *C. oleifera* fruit hull on inflammatory responses in LPS-induced RAW264.7 cells were explored. The results indicated that n-hexane extract of *C. oleifera* fruit hull alleviates inflammatory responses by regulating the phosphorylation of MAPKs and nuclear translocation of NF- κ B in LPS-induced RAW264.7 cells.

Authors' Contributions

XW and WL conceived and designed the research. XCQ, LXY, and YF conducted experiments. XW and HJW contributed new reagents or analytical tools. WSF and WJ analyzed data. XCQ and WSF wrote the manuscript. All authors read and approved the manuscript.

Ethics Approval

Not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (32260250), the Science and Technology Major Project Foundation of Jiangxi Academy of Sciences (2020-YZD-1 and 2021YSBG10001), the Science Foundation for Young Doctors of Jiangxi Academy of Sciences (2020-YYB-20), and the General Project of Key Research and Development Program of Jiangxi Academy of Sciences (2021YSBG22028).

References

- Xie, Y., Ge, S., Jiang, S., Liu, Z., Chen, L., Wang, L., Chen, J., Qin, L., Peng, W. "Study on biomolecules in extractives of *Camellia oleifera* fruit shell by GC-MS", *Saudi J Biol Sci*, 25(2). 234-236. Feb.2018.
- Zhang, L., Wang, Y., Wu, D., Xu, M., Chen, J. "Microwave-assisted extraction of polyphenols from *Camellia oleifera* fruit hull", *Molecules*, 16(6). 4428-4437. May.2011.
- Quan, W., Wang, A., Gao, C., Li, C. "Applications of Chinese *Camellia oleifera* and its By-Products: A Review", *Front Chem*, 10. 921246. May.2022.
- Zhou, L., Luo, S., Li, J., Zhou, Y., Wang, X., Kong, Q., Chen, T., Feng, S., Yuan, M., Ding, C. "Optimization of the extraction of polysaccharides from the shells of *Camellia oleifera* and evaluation on the antioxidant potential in vitro and in vivo", *Journal of Functional Foods*, 86. 104678. Aug.2021.
- Zhang, S., Li, X.Z. "Inhibition of α -glucosidase by polysaccharides from the fruit hull of *Camellia oleifera* Abel", *Carbohydr Polym*, 115. 38-43. Jan.2015.
- Li, M., Hou, Q., Zhong, L., Zhao, Y., Fu, X. "Macrophage Related Chronic Inflammation in Non-Healing Wounds", *Front Immunol*, 12. 681710. Jun.2021.
- Wynn, T.A., Chawla, A., Pollard, J.W. "Macrophage biology in development, homeostasis and disease", *Nature*, 496(7446). 445-455. Apr.2013.
- Xie, C., Wang, S., Cao, M., Xiong, W., Wu, L. "(E)-9-Octadecenoic Acid Ethyl Ester Derived from Lotus Seedpod Ameliorates Inflammatory Responses by Regulating MAPKs and NF- κ B Signalling Pathways in LPS-Induced RAW264.7 Macrophages", *Evid Based Complement Alternat Med*, 2022. 6731360. Jan.2022.
- Cheng, H., Huang, H., Guo, Z., Chang, Y., Li, Z. "Role of prostaglandin E2 in tissue repair and regeneration" *Theranostics*, 11(18). 8836-8854. Aug.2021.
- Mayer, B., Hemmens, B. "Biosynthesis and action of nitric oxide in mammalian cells", *Trends Biochem Sci*, 22(12). 477-481. Dec.1997.
- Lopez-Castejon, G., Brough, D. "Understanding the mechanism of IL-1 β secretion", *Cytokine Growth Factor Rev*, 22(4). 189-195. Aug. 2011.
- Ng, A., Tam, W.W., Zhang, M.W., Ho, C.S., Husain, S.F., McIntyre, R.S., Ho, R.C. "IL-1 β , IL-6, TNF- α and CRP in Elderly Patients with Depression or Alzheimer's disease: Systematic Review and Meta-Analysis", *Sci Rep*, 8(1). 12050. Aug.2018.
- Yokota, K., Sato, K., Miyazaki, T., Aizaki, Y., Tanaka, S., Sekikawa, M., Kozu, N., Kadono, Y., Oda, H., Mimura, T. "Characterization and Function of Tumor Necrosis Factor and Interleukin-6-Induced Osteoclasts in Rheumatoid Arthritis", *Arthritis Rheumatol*, 73(7). 1145-1154. Jul.2021.
- Park, M.Y., Ha, S.E., Kim, H.H., Bhosale, P.B., Abusaliya, A., Jeong, S.H., Park, J.S., Heo, J.D., Kim, G.S. "Scutellarein Inhibits LPS-Induced Inflammation through NF- κ B/MAPKs Signaling Pathway in RAW264.7 Cells", *Molecules*, 27(12). 3782. Jun.2022.
- Zhang, W., Zhang, Y., He, Y., Wang, X., Fang, Q. "Lipopolysaccharide mediates time-dependent macrophage M1/M2 polarization through the Tim-3/Galectin-9 signalling pathway", *Exp Cell Res*, 376(2). 124-132. Mar.2019.
- Islam, S.U., Lee, J.H., Shehzad, A., Ahn, E.M., Lee, Y.M., Lee, Y.S. "Decursinol Angelate Inhibits LPS-Induced Macrophage Polarization through Modulation of the NF κ B and MAPK Signaling Pathways", *Molecules*, 23(8). 1880. Jul.2018.
- Wancket, L.M., Frazier, W.J., Liu, Y. "Mitogen-activated protein kinase phosphatase (MKP)-1 in immunology, physiology, and disease", *Life Sci*, 90(7-8). 237-248. Feb.2012.
- Jia, X.J., Li, X., Wang, F., Liu, H.Q., Zhang, D.J., Chen, Y. "Berbamine Exerts Anti-Inflammatory Effects via Inhibition of NF- κ B and MAPK Signaling Pathways", *Cell Physiol Biochem*, 41(6). 2307-2318. Apr.2017.
- Xu, Y.R., Lei, C.Q. "TAK1-TABs Complex: A Central Signalingosome in Inflammatory Responses", *Front Immunol*, 11. 608976. Jan.2021.
- Cha, S.M., Cha, J.D., Jang, E.J., Kim, G.U., Lee, K.Y. "Soporaflavanone G prevents *Streptococcus mutans* surface antigen I/II-induced production of NO and PGE2 by inhibiting MAPK-mediated pathways in RAW 264.7 macrophages", *Arch Oral Biol*, 68. 97-104. Aug.2016.
- Hoffmann, A., Baltimore, D. "Circuitry of nuclear factor kappaB signaling", *Immunol Rev*, 210. 171-186. Apr.2006.
- Giridharan, S., Srinivasan, M. "Mechanisms of NF- κ B p65 and strategies for therapeutic manipulation", *J Inflamm Res*, 11. 407-419. Oct.2018.
- Jin, X. "Bioactivities of water-soluble polysaccharides from fruit shell of *Camellia oleifera* Abel: Antitumor and antioxidant activities", *Carbohydrate Polymers*, 87. 2198-2201. Oct.2012.
- Zhang, L.L., Wang, Y.M., Wu, D.M., Xu, M., Chen, J.H. "Comparisons of antioxidant activity and total phenolics of *camellia oleifera* Abel fruit hull from different regions of china", *Journal of Medicinal Plants Research*, 4(14). 1420-1426. Apr.2010.
- Alam, M.B., Chowdhury, N.S., Sohrab, M.H., Rana, M.S., Hasan, C.M., Lee, S.H. "Cerevisterol Alleviates Inflammation via Suppression of MAPK/NF- κ B/AP-1 and Activation of the Nrf2/HO-1 Signaling Cascade", *Biomolecules*, 10(2). 199. Jan.2020.

- [26] Nguyen, H.C.B., Chao, T.N., Cohen, N.A., Mirza, N. "Persistent Inflammation and Nitric Oxide Dysregulation Are Transcriptomic Blueprints of Subglottic Stenosis", *Front Immunol*, 12. 748533. Dec.2021.
- [27] Long, T., Liu, Z., Shang, J., Zhou, X., Yu, S., Tian, H., Bao, Y. "Polygonatum sibiricum polysaccharides play anti-cancer effect through TLR4-MAPK/NF- κ B signaling pathways", *Int J Biol Macromol*, 111. 813-821. May.2018.
- [28] Carter, A.B., Knudtson, K.L., Monick, M.M., Hunninghake, G.W. "The p38 mitogen-activated protein kinase is required for NF-kappaB-dependent gene expression. The role of TATA-binding protein (TBP)", *J Biol Chem*, 274(43). 30858-30863. Oct.1999.



© The Author(s) 2023. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).