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# Immunomodulatory Effects of *Ferula assa-foetida* Extract on NF-κB and STAT1 Signaling Pathways in GM-CSF-Differentiated Human Macrophages

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**ABSTRACT**

The immunomodulatory properties of *Ferula assa-foetida*, a perennial herb traditionally used for its anti-inflammatory effects, were investigated in this study, focusing on its influence on NF-κB and STAT1 signaling pathways in GM-CSF-differentiated human macrophages. The extract was prepared using 80% ethanol, and its cytotoxicity was evaluated using the MTT assay, revealing no significant cytotoxic effects at concentrations ranging from 10 to 200 µg/mL. Differentiated macrophages were treated with the extract, and quantitative real-time PCR (qRT-PCR) was employed to assess the expression levels of NF-κB and STAT1 genes. The results demonstrated a dose-dependent downregulation of NF-κB expression by the extract, with significant reductions observed at concentrations of 50 µg/mL and higher. Specifically, NF-κB expression decreased to 0.6719 ± 0.0691-fold at 50 µg/mL ($p = 0.0044$), 0.3959 ± 0.1170-fold at 100 µg/mL ($p = 0.0009$), and 0.1650 ± 0.0509-fold at 200 µg/mL ($p < 0.0001$). In contrast, STAT1 expression remained unaffected across all tested concentrations of the extract, with no statistically significant changes compared to the untreated control group. Lipopolysaccharide (LPS), used as a positive control, significantly upregulated both NF-κB and STAT1 expression, confirming the responsiveness of the macrophages to inflammatory stimuli. These findings suggest that the extract selectively modulates NF-κB-dependent inflammatory pathways without broadly affecting STAT1 signaling. The study highlights the potential of *F. assa-foetida* as a source of bioactive compounds with anti-inflammatory properties. However, further research is needed to elucidate the underlying molecular mechanisms, identify the active compounds, and validate these effects in vivo.

## Introduction

The modulation of immune responses has been recognized as a critical therapeutic strategy for managing a variety of diseases, including chronic inflammation, autoimmune disorders, and infections. The immune system is regulated by intricate signaling pathways, among which the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 1 (STAT1) pathways play pivotal roles. The NF-κB pathway is a well-characterized regulator of inflammation, immune



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responses, and cell survival, while the STAT1 pathway is a key mediator of interferon (IFN)-induced responses, particularly in antiviral and pro-inflammatory signaling (Hayden and Ghosh 2014, Ivashkiv and Donlin 2014). Dysregulation of these pathways has been implicated in the pathogenesis of numerous inflammatory and autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus (Liu, Berthier et al. 2017). As a result, the identification of compounds capable of modulating NF-κB and STAT1 signaling has garnered significant attention in recent years.

Natural products have been extensively investigated for their potential as immunomodulatory agents due to their structural diversity and bioactive properties. Among these, Ferula assa-foetida, a perennial herb belonging to the Apiaceae family, has been traditionally utilized in various medicinal systems for its anti-inflammatory, antimicrobial, and antispasmodic properties (Dehpour, Ebrahimzadeh et al. 2009). The resinous gum extracted from the roots of F. assa-foetida, commonly referred to as "asafoetida," is rich in bioactive compounds such as ferulic acid, sesquiterpene coumarins, and sulfur-containing compounds (Iranshahy and Iranshahi 2011). These phytochemicals have been reported to exhibit a wide range of pharmacological activities, including antioxidant, anti-inflammatory, and anticancer effects (Mahendra and Bisht 2012). Despite its traditional use and pharmacological potential, the molecular mechanisms underlying the immunomodulatory effects of F. assa-foetida remain poorly understood.

The immunomodulatory properties of F. assa-foetida have been suggested by several studies. For instance, the resin has been shown to inhibit the production of pro-inflammatory cytokines such as TNF-α and IL-6 in lipopolysaccharide (LPS)-stimulated macrophages (Mobasheri, Khorashadizadeh et al. 2022). Additionally, the antioxidant activity of F. assa-foetida has been attributed to its ability to scavenge reactive oxygen species (ROS) and reduce oxidative stress, which are closely linked to inflammation (Iranshahy and Iranshahi 2011, Khazdair, Anaeigoudari et al. 2019). Despite these findings, the specific effects of F. assa-foetida on key signaling pathways such as NF-κB and STAT1 remain to be elucidated. The identification of these effects could provide a mechanistic basis for its traditional use in treating inflammatory conditions.

Given the central role of macrophages in regulating inflammatory responses, they represent an ideal model for investigating the effects of immunomodulatory agents like F. assa-foetida on signaling pathways mentioned above. These immune cells are critical mediators of innate and adaptive immunity, performing functions such as pathogen recognition, phagocytosis, and cytokine production. The differentiation of monocytes into macrophages, influenced by growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF), results in a pro-inflammatory phenotype characterized by enhanced production of inflammatory cytokines and chemokines (Mantovani, Sica et al. 2004, Fleetwood, Lawrence et al. 2007). Investigations into the effects of natural products on GM-CSF-differentiated macrophages have provided valuable insights into their potential as therapeutic agents for inflammatory diseases (Al-Shami, Mahanna et al. 1998, Fleetwood, Lawrence et al. 2007, Shapouri-Moghaddam, Mohammadian et al. 2018).

The current study was undertaken to address the gap in knowledge regarding the immunomodulatory effects of F. assa-foetida on NF-κB and STAT1 signaling pathways in human macrophages. The hypothesis that the extract could modulates the expression of the both genes which subsequently alter the expression of other key genes involved in these pathways was tested using GM-CSF-differentiated human macrophages as a model. Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to measure the expression levels of NF-κB and STAT1 genes. The study also aimed to determine whether the extract exerts dose-dependent effects on these gene as the hub genes and to evaluate its potential as an immunomodulatory agent.

## Methods

### *Collection, Processing, and Extraction of Plant Material*

A comprehensive collection of Ferula assa-foetida stem, root, and leaf materials was conducted in the Taftan region of Sistan and Baluchestan Province, Iran, during mid-spring 2023 (Abolhasanzadeh Parizi and Lagzian 2024). The plant parts were precisely washed with distilled water and air-dried in a shaded environment until complete desiccation was achieved. The dried plant material was mechanically ground into a fine powder and extracted using 80% ethanol at a 1:10 (w/v) ratio. Maceration was performed at room temperature for 48 hours with periodic stirring. The extract was subsequently filtered through Whatman No. 1 filter paper, with residues re-extracted to maximize compound yield. The combined filtrate was concentrated under reduced pressure using a rotary evaporator and freeze-dried. The final dried extract was weighed, characterized through phytochemical screening, and stored at -20°C in airtight containers. Preliminary analysis confirmed the presence of characteristic bioactive compounds, including sesquiterpene coumarins, ferulic acid derivatives, and sulfur-containing compounds associated with potential pharmacological activities.

### *Cell Culture and Differentiation*

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood obtained from a healthy male volunteer. The blood collection procedure was performed in accordance with ethical guidelines and with the informed consent of the donor. Whole blood was diluted with an equal volume of phosphate-buffered saline (PBS) to reduce viscosity and facilitate the separation process. The diluted blood was carefully layered over Ficoll-Paque PLUS (density: 1.077 g/mL) in a 1:1 ratio and centrifuged at 400 × g for 30 minutes at room temperature without braking to ensure the formation of distinct cell layers. Following centrifugation, the PBMC layer was carefully collected using a sterile pipette. The collected PBMCs were washed twice with PBS to remove any residual Ficoll and plasma contaminants. Each wash involved centrifugation at 300 × g for 10 minutes at room temperature, followed by resuspension of the cell pellet in fresh PBS. After the final wash, the PBMC pellet was resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2 mM L-glutamine to ensure optimal cell viability and growth. The PBMCs were counted using a hemocytometer, and cell viability was assessed using the trypan blue exclusion method. Cells with a viability of ≥95% were used for further experiments.

To differentiate monocytes into macrophages, the PBMC suspension was seeded in 6-well culture plates at a density of $1\times10^6$ cells/mL and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ for 2 hours to allow monocyte adhesion. Non-adherent cells, including lymphocytes, were gently removed by washing the wells with warm PBS, leaving behind adherent monocytes. To promote differentiation into macrophages, the adherent monocytes were cultured in RPMI-1640 medium supplemented with 10% FBS and 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). The medium was replaced every 3 days to maintain optimal nutrient levels and remove non-adherent cells. Differentiation was allowed to proceed for 7 days, during which the monocytes acquired macrophage-like morphology and characteristics. At the end of the differentiation period, the macrophages were used for subsequent treatments and assays. All procedures involving human blood and PBMCs were conducted under sterile conditions in a biosafety cabinet to minimize contamination risks.

### *Treatment with F. assa-foetida Extract*

The prepared macrophages, derived from peripheral blood mononuclear cells (PBMCs) as described in the previous section, were used for the treatment with the *F. assa-foetida* extract. The extract, which was prepared using 80% ethanol as the solvent and stored at -20°C, was thawed at room temperature prior to use. To prepare working concentrations, the crude extract was diluted in sterile RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The extract was filtered through a 0.22 µm sterile syringe filter to ensure sterility before application to the cells. The macrophages were seeded into 24-well plates at a density of $1 \times 10^6$ cells per well in the above-mentioned growth medium. The cells were allowed to adhere and equilibrate for 24 hours at 37°C in a humidified atmosphere containing 5% $CO_2$. Following equilibration, the culture medium was carefully removed, and the macrophages were treated with five concentrations of the extract from 10 to 200 µg/mL were used for the treatment, as these ranges have been reported in similar studies to evaluate the cytotoxicity of plant extracts (Iranshahy and

Iranshahi 2011, Mahendra and Bisht 2012). Control wells were included in the experimental setup, consisting of macrophages treated with the culture medium alone (negative control) and macrophages treated with lipopolysaccharide (LPS) as a known activator of inflammatory pathways (positive control) at a concentration of 100 ng/mL. The treated cells were incubated at 37°C for 24 hours to allow sufficient interaction between the extract and the cells. At the end of the incubation period, the supernatants were carefully collected and stored at -80°C for future cytokine analysis. The adherent macrophages were washed with warm phosphate-buffered saline (PBS) to remove residual extract and were used for RNA extraction.

### *Cytotoxicity Assays of F. assa-foetida Extract*

The cytotoxicity of the *F. assa-foetida* extract was assessed using the MTT assay. Differentiated macrophages, prepared as described in Section 2.3, were seeded in 96-well plates at a density of $1{\times}10^5$ cells per well and allowed to adhere overnight at 37°C in a humidified atmosphere containing 5% $CO_2$. Following adherence, the macrophages were treated with the *F. assa-foetida* extract at concentrations ranging from 10 to 200 µg/mL and also 100 ng/mL LPS as detailed in Section 2.3. Control wells included cells treated with 0.5% DMSO (vehicle control) to account for any solvent-induced effects on cell viability. After 24 hours of treatment, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated for an additional 4 hours at 37°C. At the end of the incubation period, the medium was carefully aspirated, and the formazan crystals formed by metabolically active cells were dissolved in 100 µL of DMSO. The absorbance was measured at 570 nm using a microplate reader, with a reference wavelength of 630 nm to correct for background absorbance. Cell viability was calculated as a percentage relative to the untreated control group using the formula:

$$(1)\ \boldsymbol{Cell\,Viability}\ (\%) = \left(\frac{\textbf{Absorbance of Control Cells}}{\textbf{Absorbance of Treated Cells}}\right) \times \mathbf{100}$$

Concentrations that maintained cell viability above 90% were considered non-cytotoxic and were selected for subsequent experiments.

### *RNA Extraction and cDNA Synthesis*

Total RNA was extracted from treated and control macrophages using the TRIzol™ reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, the macrophages were washed twice with cold phosphate-buffered saline (PBS) to remove residual media and non-adherent cells. After washing, 1 mL of TRIzol reagent was added directly to each well, and the cells were lysed by pipetting up and down to ensure complete homogenization. The lysates were transferred to microcentrifuge tubes, and 200 µL of chloroform was added to each tube. The samples were vortexed for 15 seconds, incubated at room temperature for 3 minutes, and centrifuged at 12,000 × g for 15 minutes at 4°C. Following centrifugation, the aqueous phase containing RNA was carefully transferred to a new tube, and 500 µL of isopropanol was added to precipitate the RNA. The mixture was incubated at room temperature for 10 minutes and centrifuged at 12,000 × g for 10 minutes at 4°C. The RNA pellet was washed with 1 mL of 75% ethanol, air-dried briefly, and resuspended in nuclease-free water. The concentration and purity of the extracted RNA were determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific). The absorbance ratios at 260/280 nm and 260/230 nm were used to assess RNA purity, with acceptable values ranging between 1.8 and 2.2.

For cDNA synthesis, 1.5 µg of total RNA from each sample was reverse-transcribed into complementary DNA (cDNA) using the AccuPower® RT PreMix kit (Bioneer, Korea) following the manufacturer's instructions. The reaction mixture was prepared by adding the extracted RNA and nuclease-free water to the lyophilized RT PreMix components, which include reverse transcriptase, dNTPs, and oligo(dT) primers. The total reaction volume was adjusted to 20 µL. The reverse transcription reaction was carried out in a thermal cycler under the following conditions: 42°C for 60 minutes, followed by 95°C for 5 minutes to inactivate the enzyme. The synthesized cDNA was stored at -20°C until further use in quantitative real-time PCR (qRT-PCR) analysis.

### *qRT-PCR Analysis*

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess the expression levels of NF-κB and STAT1 genes in the treated macrophages. The reactions were carried out using a real-time PCR system (Bio-Rad CFX96) and SYBR Green-based detection chemistry. The primer sequences for NF-κB, STAT1, and the reference gene β-actin (ACTB), along with their properties, are provided in Table 1. ACTB was used as the housekeeping gene for normalization due to its stable expression in macrophages under the experimental conditions.

qRT-PCR reactions were conducted in a 20 µL volume containing 10 µL of 2× SYBR Green Master Mix (1.5 mM $MgCl_2$), 0.5 µL each of forward and reverse primers (10 µM), 2 µL of cDNA template (equivalent to 100 ng RNA), and 6 µL of nuclease-free water. The cycling conditions

**Table 1. Primer sequences and properties used for qRT-PCR analysis of NF-κB, STAT1, and β-Actin genes.**

| Gene | Size bp | Strand | Sequence (5' → 3') | Length | Tm°C | Location |
|---|---|---|---|---|---|---|
| NF-κB | **146** | **F** | **GGGCCGAAAG ACCTATCCC** | **19** | **61.5** | **216-234** |
| | | **R** | **CAGCTCCGAGC ATTGCTTG** | **19** | **61.5** | **351-333** |
| STAT1 | **186** | **F** | **ATCAGGCTCAG TCGGGGAATA** | **21** | **62.2** | **368-388** |
| | | **R** | **TGGTCTCGTGT TCTCTGTTCT** | **21** | **60.4** | **553-533** |
| β-Actin | **250** | **F** | **CATGTACGTTG CTATCCAGGC** | **21** | **60.8** | **393-413** |
| | | **R** | **CTCCTTAATGT CACGCACGAT** | **22** | **60.2** | **642-622** |

included an initial denaturation at 95°C for 3 minutes, followed by 45 cycles of 95°C for 20 seconds (denaturation), 60°C for 20 seconds (annealing), and 72°C for 20 seconds (extension). A melt curve analysis was performed from 65–95°C with 0.5°C increments to confirm product specificity. All reactions were run in triplicate, and no-template controls (NTCs) were included to monitor for contamination.

Gene expression levels were quantified using the Pfaffl method, which accounts for differences in amplification efficiency between target and reference genes (Pfaffl 2001). The relative expression ratio of the target genes (NF-κB and STAT1) to the reference gene (ACTB) was calculated using the formula:

$$(2)\ \boldsymbol{R} = \frac{\left(\boldsymbol{E_{target}}\right)^{\Delta Cq_{control-sample}}}{\left(\boldsymbol{E_{reference}}\right)^{\Delta Cq_{control-sample}}}$$

Here, Etarget and Ereference represent the amplification efficiencies of the target and reference genes, respectively, which were derived from the slope of the standard curve using the equation E=10(−1/slope). ΔCq denotes the difference in quantification cycle (Cq) values between the control and sample groups. Amplification efficiencies for each primer pair were determined by generating standard curves using 10-fold serial cDNA dilutions, with efficiencies between 90–110% considered acceptable for reliable quantification. Gene expression data were normalized to ACTB expression and presented as fold changes relative to the untreated control group.

### *Data Analysis*

All statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). Cell viability percentages in MTT cytotoxicity assay were calculated based on absorbance readings, and results were expressed as means ± standard error of the mean (SEM). Other data were expressed as mean ± standard deviation (SD) from at least three independent experiments. One-way analysis of variance (ANOVA) was employed to determine significant differences among multiple experimental groups. When ANOVA indicated statistical significance ($p < 0.05$), Tukey's post hoc test was conducted to identify specific group differences. For comparisons between two groups, an unpaired two-tailed Student's t-test was used. The normality of the data was assessed using the Shapiro-Wilk test to confirm the appropriateness of parametric tests. Statistical significance was set at $p < 0.05$ for all analyses.

## Results

### *Differentiation of Monocytes into Macrophages*

Peripheral blood mononuclear cells (PBMCs) were successfully isolated from whole blood using the Ficoll-Paque density gradient centrifugation method. The initial PBMC population consisted of a heterogeneous mixture of mononuclear cells, as observed under an inverted microscope immediately after isolation (Fig.1a). At this stage, the cells appeared round and non-adherent, characteristic of freshly isolated PBMCs.

Following seeding and incubation for 2 hours, non-adherent cells were removed, and adherent monocytes were retained. The adherent monocytes were cultured in RPMI-1640 medium supplemented with 10% FBS and 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) to induce differentiation into macrophages. By day 2 of GM-CSF stimulation, morphological changes were evident, with cells adopting an elongated and spindle-like shape, indicative of early macrophage differentiation (Fig.1b). The cells displayed increased adherence and spreading, which are characteristic of monocyte-to-macrophage transition.

After 7 days of culture with GM-CSF, the differentiated cells exhibited macrophage-like morphology, including an irregular shape with extended cytoplasmic projections and increased cell size (Fig.1c). These morphological features are consistent with fully differentiated macrophages. The differentiation process was further supported by the observation of enhanced adherence and the characteristic appearance of macrophage-like cells under the inverted microscope. These results demonstrate the successful differentiation of monocytes into macrophages, which were subsequently used for downstream experiments.

### *Evaluation of F. assa-foetida Extract Cytotoxicity on Differentiated Macrophages*

The cytotoxicity of the extract on GM-CSF-differentiated macrophages was evaluated using the MTT assay. The results demonstrated that the extract exhibited no significant cytotoxicity at the tested concentrations (10, 25, 50, 100, and 200 µg/mL). Cell viability remained above 90% for all concentrations compared to the untreated control group, indicating that the extract was non-toxic to macrophages within this range (Fig. 2). Specifically, the viability percentages were as follows: 98.3 ± 1.2% at 10 µg/mL, 97.8 ± 1.1% at 25 µg/mL, 96.2 ± 1.2% at 50 µg/mL, 93.8 ± 1.6% at 100 µg/mL, and 90.9 ± 0.8% at 200 µg/mL. In addition to the extract, lipopolysaccharide (LPS), a known activator of inflammatory pathways, was used as a positive control at a concentration of 100 ng/mL.

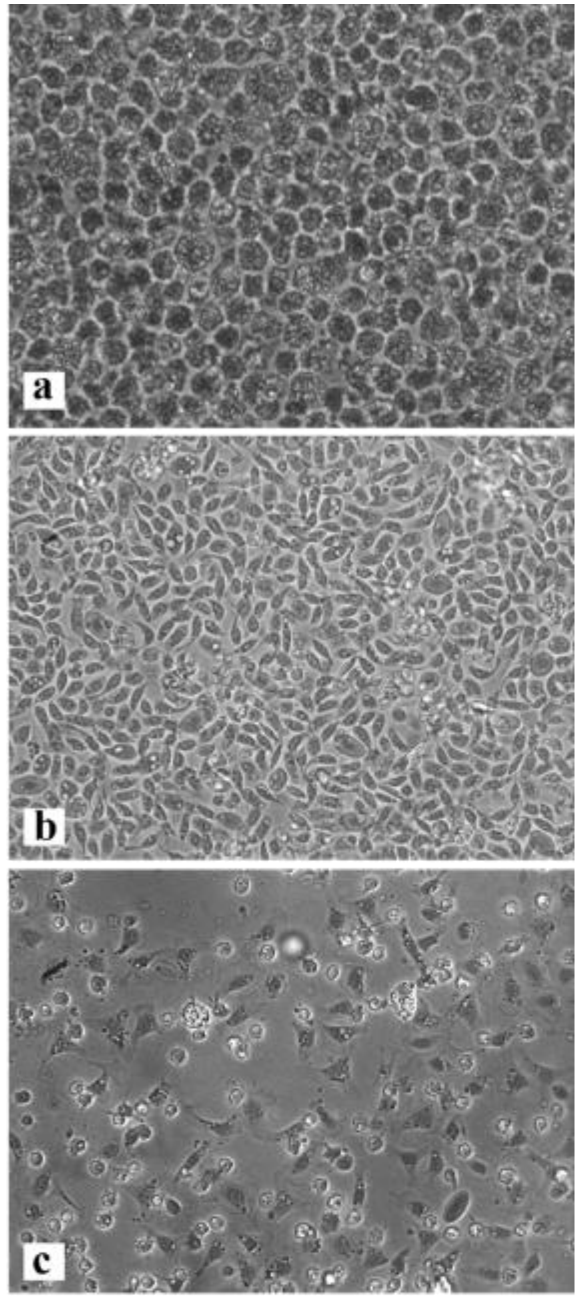


**Fig.1. Morphological changes during monocyte-to-macrophage differentiation. (a) Freshly isolated PBMCs at time zero, showing round, non-adherent cells. (b) Monocytes 2 days after seeding and GM-CSF stimulation, displaying elongated and spindle-like morphology. (c) Fully differentiated macrophages after 7 days, exhibiting irregular shapes and extended cytoplasmic projections. Images were captured using an inverted microscope at 200× magnification.**

The MTT assay results showed that LPS treatment had no apparent cytotoxic effects on macrophages, with cell viability remaining above 95% compared to the untreated control group. The culture medium used to dilute the extract (RPMI-1640 with 10% FBS and 1% penicillin-streptomycin) served as the vehicle control and had no significant impact on cell viability. These findings suggest that the *F. assa-foetida* extract is safe for use in macrophage-based assays within the tested concentration range and is suitable for further investigations into its immunomodulatory properties.

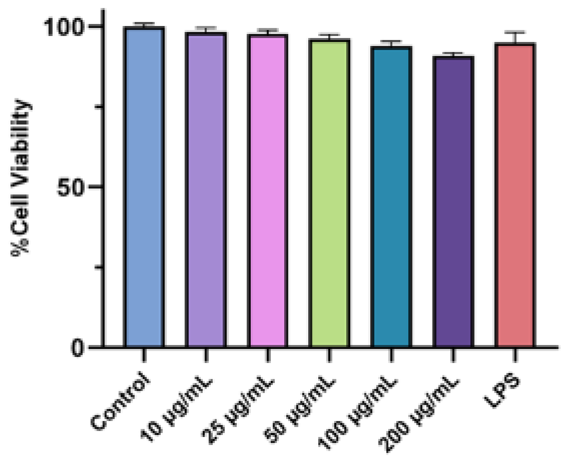


**Fig. 2. Effect of *F. assa-foetida* extract on macrophage viability. GM-CSF-differentiated macrophages were treated with various concentrations of the extract (10, 25, 50, 100, and 200 µg/mL) as well as LPS (100 ng/mL) for 24 hours. Cell viability was assessed using the MTT assay and is expressed as a percentage relative to the untreated control group. Data are presented as mean ± SD from six independent experiments. Statistical analysis showed no significant differences between treated groups and the control group ($p > 0.05$).**

## *RNA Quality, Purity & Specificity of PCR Amplification*

The quality and concentration of RNA extracted from macrophages treated with different concentrations of *F. assa-foetida* extract were assessed using a NanoDrop™ spectrophotometer. The RNA concentrations ranged from 1170 ng/µL to 3512 ng/µL across the control and treated groups, demonstrating sufficient yield for downstream applications (Table 2). The purity of the RNA was evaluated using the absorbance ratios at 260/280 nm and 260/230 nm. The 260/280 ratios for all samples were within the acceptable range of 1.8–2.2, indicating high RNA purity and minimal protein contamination. However, slight variations in the 260/230 ratios were observed, with some samples falling below the optimal range (>2.0), suggesting the presence of minor contaminants such as phenol or other organic compounds. Despite this, the RNA quality was deemed suitable for cDNA synthesis and qRT-PCR analysis.

**Table 2. RNA Sample Concentration and Purity**

| Sample | Conc. (ng/µl) | R260/280 | R260/230 |
|---|---|---|---|
| **Control** | 3123 | 2.10 | 1.64 |
| **10µg/mL** | 3512 | 2.07 | 1.54 |
| **25µg/mL** | 3409 | 2.08 | 1.54 |
| **50µg/mL** | 3245 | 2.08 | 2.06 |
| **100µg/mL** | 1170 | 2.04 | 1.70 |
| **200µg/mL** | 1332 | 2.12 | 2.14 |

The specificity of the PCR amplification for NF-κB, STAT1, and the housekeeping gene β-actin (ACTB) was mainly confirmed through gel electrophoresis of the PCR products which showed distinct bands at the expected sizes for each gene, 146 bp for NF-κB, 186 bp for STAT1, and 250 bp for ACTB (Fig. 3). No bands were observed in the no-template control (NTC) or RT-minus control lanes, confirming the absence of contamination and non-specific amplification such as primer dimer.

To further validate the specificity of the PCR products, melt curve analysis was performed for NF-κB, STAT1, and β-actin. The melt curve analysis showed distinct, sharp peaks for each gene, indicating the amplification of a single, specific product without primer dimer formation or non-specific amplification (Fig. 4).

For β-actin (ACTB) a single peak was observed at a $T_m$ of approximately 92.5°C (red curve in both Fig.4a and b), indicating consistent amplification of the housekeeping gene across all samples. STAT1 is the green curve in Fig.4a showed a single peak at a $T_m$ of approximately 88°C, confirming the specificity of STAT1 amplification. NF-κB is the blue curve in Fig.4b showed a single peak at a Tm of approximately 93.5°C. This higher Tm is consistent with the GC-rich content of the NF-κB gene, which requires higher temperatures for denaturation.

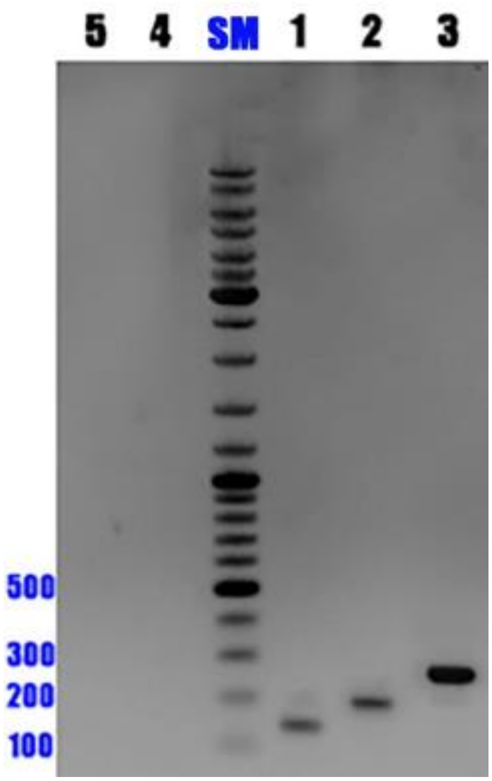


**Fig. 3. Gel Electrophoresis of PCR Products. Lane markers: SM, DNA size marker; 1, NF-κB amplified band (146 bp); 2, STAT1 band (186 bp); 3, β-actin band (250 bp); 4, no-template control (NTC); 5, RT-minus control. In addition to gel electrophoresis, melt curve analysis was performed to further validate the specificity of the PCR products. A single, sharp peak was observed for each gene, indicating the amplification of a single, specific product without primer-dimer formation or non-specific amplification. The melt curve analysis confirmed the high specificity and reliability of the qRT-PCR reactions.**

### *Gene Expression Analysis of NF-κB and STAT1 in Differentiated Macrophages*

Quantitative real-time PCR (qRT-PCR) was performed to evaluate the expression levels of NF-κB and STAT1 genes in GM-CSF-differentiated macrophages treated with *F. assa-foetida* extract. The amplification plots for each gene, including the housekeeping gene β-actin (ACTB), are shown in **Fig.5a** and **b**. The threshold cycle (Ct) values were determined using the red threshold line visible in the amplification plots. The housekeeping gene β-actin exhibited consistent Ct values across all samples, confirming its stable expression and suitability as a reference gene for normalization. The amplification efficiencies of the primers were previously determined using standard curves and were found to be within the acceptable range: 0.98 for β-actin, 0.94 for STAT1, and 0.91 for NF-κB, ensuring reliable and accurate quantification of gene expression. The amplification plots and efficiency data collectively validate the robustness and specificity of the qPCR assay, providing confidence in the reliability of subsequent gene expression analyses.

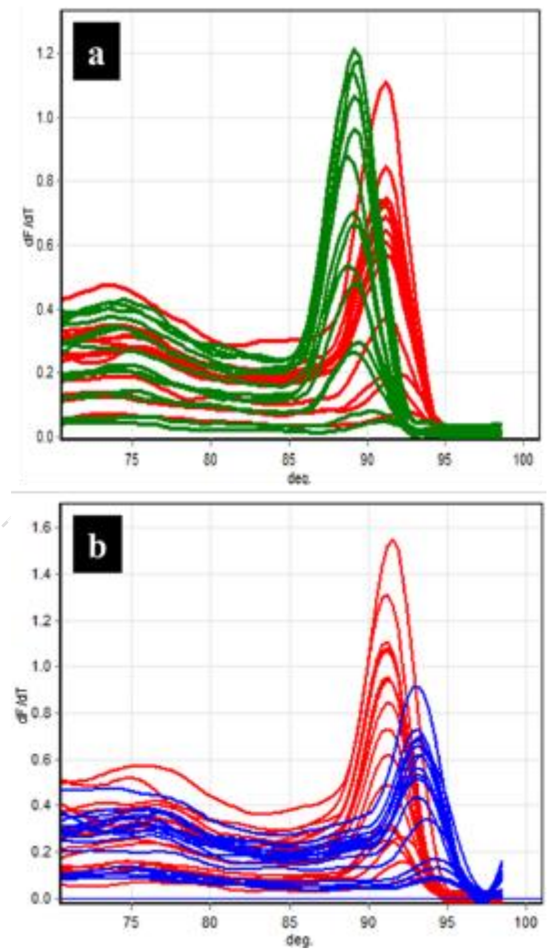


**Fig.4. Representative melt curve analysis from multiple qPCR runs. (a) Melt curve of β-actin (red curve, Tm ~92.5°C) and STAT1 (green curve, Tm ~88°C). (b) Melt curve of β-actin (red curve, Tm ~92.5°C) and NF-κB (blue curve, Tm ~93.5°C). Sharp, single peaks indicate specific amplification of target genes without non-specific products or primer-dimer formation.**

Building on this validated assay, the fold change in NF-κB and STAT1 expression was analyzed using qRT-PCR to evaluate the immunomodulatory effects of *F. assa-foetida* extract. The fold changes were calculated relative to the untreated control group, with β-actin serving as the housekeeping gene for normalization. The results, shown in Fig.6a (NF-κB) and Fig.6b (STAT1), reveal distinct patterns of expression for the two genes in response to the extract and LPS treatment.

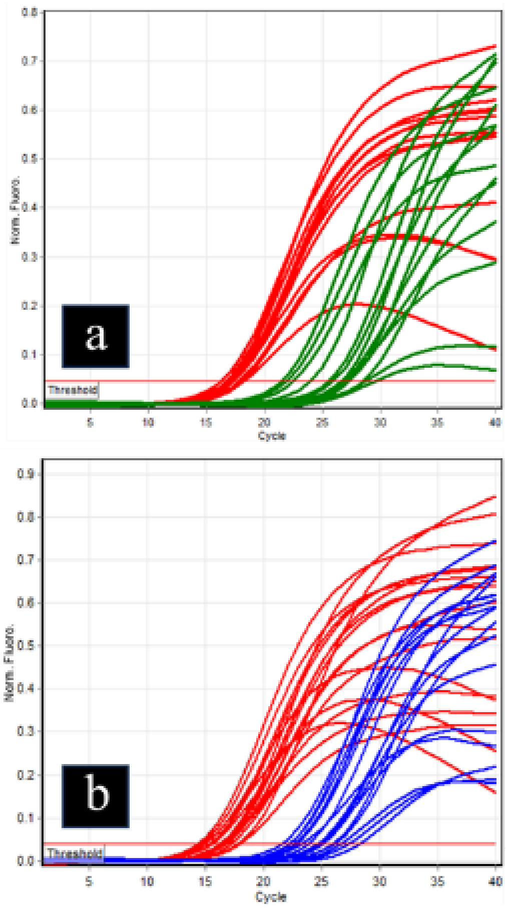


**Fig. 5. Representative amplification plots of qPCR for NF-κB, STAT1, and β-actin (corresponded to the melt curve of Fig. 4). (a) Amplification curves for β-actin (red) and STAT1 (green) in control and treated macrophage samples. (b) Amplification curves for β-actin (red) and NF-κB (blue) in control and treated macrophage samples. The consistent Ct values for β-actin across all samples confirm its stable expression as a housekeeping gene. STAT1 and NF-κB curves demonstrate successful amplification of the target genes with no irregularities.**

The expression of NF-κB was significantly downregulated in a dose-dependent manner in response to the extract. At lower concentrations (10 μg/mL and 25 μg/mL), NF-κB expression showed no statistically significant changes compared to the untreated control group (fold changes: 0.9120 ± 0.1325, p = 0.5194; and 0.7761 ± 0.1042, p = 0.0824, respectively). However, at 50 μg/mL, a significant reduction in NF-κB expression was observed (fold change: 0.6719 ± 0.0691, p = 0.0044). This downregulation became more pronounced at higher concentrations, with fold changes of 0.3959 ± 0.1170 (p = 0.0009) at 100 μg/mL and 0.1650 ± 0.0509 (p < 0.0001) at 200 μg/mL. In contrast, treatment with lipopolysaccharide (LPS, 100 ng/mL), used as a positive control, caused a significant upregulation of NF-κB expression (fold change: 4.7674 ± 0.6335, p = 0.0002). These results demonstrate that *F. assa-foetida* extract exerts a potent inhibitory effect on NF-κB expression, particularly at higher concentrations, suggesting its potential anti-inflammatory properties.

Unlike NF-κB, STAT1 expression remained stable across all concentrations of *F. assa-foetida* extract. As shown in Fig.6b, no statistically significant changes were observed at any concentration compared to the untreated control group. The fold change values were 1.2661 ± 0.1645 (p = 0.6163) at 10 μg/mL, 1.3311 ± 0.3463 (p = 0.6260) at 25 μg/mL, 1.1723 ± 0.2953 (p = 0.9021) at 50 μg/mL, 1.1832 ± 0.2291 (p = 0.8599) at 100 μg/mL, and 1.3306 ± 0.6871 (p = 0.7823) at 200 μg/mL. However, LPS treatment (100 ng/mL) caused a significant upregulation of STAT1 expression (fold change: 3.1758 ± 0.4019, p = 0.0011). This result indicates that while STAT1 is responsive to inflammatory stimuli such as LPS, *F. assa-foetida* extract does not significantly modulate STAT1 expression under the tested conditions.

## Discussion

The present study investigated the immunomodulatory effects of *Ferula assa-foetida* extract on NF-κB and STAT1 signaling pathways in GM-CSF-differentiated human macrophages. The findings revealed that the extract significantly downregulated NF-κB expression in a dose-dependent manner, while STAT1 expression remained unaffected at all tested concentrations. These results provide new insights into the molecular mechanisms underlying the anti-inflammatory properties of *F. assa-foetida* and its potential therapeutic applications in immune-mediated diseases.

### *NF-κB Downregulation and Its Implications*

NF-κB is a key transcription factor involved in the regulation of pro-inflammatory cytokines, chemokines, and other mediators of inflammation. Its activation is central to the pathogenesis of various inflammatory and autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus (Hayden and Ghosh 2014, Liu, Zhang et al. 2017, Mitchell and Carmody 2018). In this study, *F. assa-foetida* extract exhibited a potent inhibitory effect on NF-κB expression, particularly at higher concentrations (≥50 µg/mL). This dose-dependent downregulation suggests that the extract may act by interfering with upstream signaling pathways that activate NF-κB, such as the IκB kinase (IKK) complex, or by directly modulating NF-κB transcriptional activity (Mitchell and Carmody 2018).

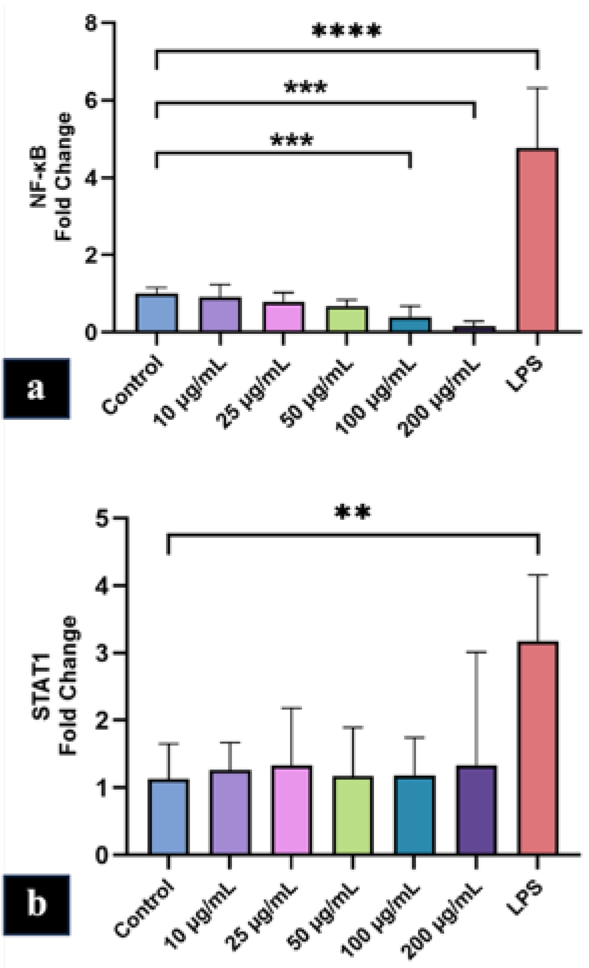


**Fig. 6. Fold change analysis of NF-κB and STAT1 expression in macrophages treated with F. assa foetida extract. (a) Fold change in NF-κB expression relative to the untreated control group. A significant downregulation of NF-κB was observed at 50 µg/mL and higher concentrations ($p < 0.05$). LPS treatment (100 ng/mL) significantly upregulated NF-κB expression, confirming its responsiveness to inflammatory stimuli. (b) Fold change in STAT1 expression relative to the untreated control group. No significant changes in STAT1 expression were observed at any concentration of the extract ($p > 0.05$). LPS treatment (100 ng/mL) significantly upregulated STAT1 expression ($p < 0.05$). Data are presented as mean ± SD from three independent experiments.**

The observed inhibition of NF-κB expression aligns with previous reports on the anti-inflammatory properties of *F. assa-foetida*. For instance, Mahendra group (Mahendra and Bisht 2012) reported that *F. assa-foetida* resin suppresses the production of pro-inflammatory cytokines, such as TNF-α and IL-6. These cytokines are downstream targets of NF-κB, further supporting the hypothesis that the extract exerts its effects by modulating this pathway. Additionally, the bioactive compounds present in the extract, including sesquiterpene coumarins and ferulic acid derivatives, have been reported to exhibit anti-inflammatory and antioxidant activities (Iranshahy and Iranshahi 2011, Khazdair, Anaeigoudari et al. 2019). These compounds may contribute to the observed downregulation of NF-κB by scavenging reactive oxygen species (ROS), which are known to activate NF-κB signaling.
The ability of *F. assa-foetida* extract to inhibit NF-κB expression suggests its potential as a therapeutic agent for conditions characterized by excessive inflammation. However, further studies are needed to elucidate the precise molecular mechanisms involved and to determine whether the extract affects other components of the NF-κB signaling cascade, such as IκBα phosphorylation or nuclear translocation of NF-κB subunits.

### *Lack of Effect on STAT1 Expression*

STAT1 is a critical mediator of interferon (IFN)-induced signaling and plays a central role in antiviral and pro-inflammatory responses (Ivashkiv and Donlin 2014). Unlike NF-κB, STAT1 expression remained unchanged across all concentrations of *F. assa-foetida* extract in this study. This finding suggests that the extract selectively targets specific inflammatory pathways without broadly suppressing immune signaling.

The lack of effect on STAT1 expression may be advantageous in preserving the host's ability to mount an effective defensive response while mitigating excessive inflammation mediated by NF-κB. The selective modulation of NF-κB but not STAT1 may be attributed to the specific bioactive compounds in the extract, for example sesquiterpene coumarins (Mahendra and Bisht 2012). This selectivity underscores the therapeutic potential of *F. assa-foetida* extract as an immunomodulatory agent with minimal off-target effects.

### Comparison with LPS-Induced Responses

The use of LPS as a positive control in this study demonstrated the responsiveness of the macrophages to inflammatory stimuli. LPS treatment significantly upregulated both NF-κB and STAT1 expression, consistent with its well-established role as a potent activator of inflammatory signaling pathways (Guha and Mackman 2001). The contrasting effects of *F. assa-foetida* extract and LPS on NF-κB expression highlight the extract's potential to counteract inflammation without broadly suppressing immune function. Furthermore, the lack of STAT1 modulation by the extract, despite its upregulation by LPS, suggests that the extract may specifically target NF-κB-dependent inflammatory responses.

### Biological and Therapeutic Implications

The findings of this study suggest that *F. assa-foetida* extract may selectively modulate NF-κB-dependent inflammatory responses, which are implicated in various inflammatory conditions. However, it is important to emphasize that these results are based on in vitro experiments and should be interpreted as preliminary evidence of the extract's immunomodulatory potential. Further studies are required to confirm these effects in vivo and to determine their relevance in the context of complex immune responses.

The antioxidant properties of *F. assa-foetida* may also play a role in its observed effects on NF-κB. By scavenging reactive oxygen species (ROS), which are known activators of NF-κB, the extract may provide a dual mechanism of action for mitigating inflammation and oxidative stress (Iranshahy and Iranshahi 2011). However, additional studies are needed to quantify the antioxidant capacity of the extract and to establish a direct link between its antioxidant and anti-inflammatory effects.

### Limitations and Future Directions

While the results of this study provide valuable insights into the immunomodulatory effects of *F. assa-foetida* extract, several limitations should be acknowledged. First, the study focused exclusively on the expression levels of NF-κB and STAT1, without assessing other components of these signaling pathways or downstream targets. Future studies should investigate the effects of the extract on additional inflammatory mediators, such as cytokines and chemokines, as well as the phosphorylation and nuclear translocation of NF-κB and STAT1.

Second, the experiments were conducted in vitro using GM-CSF-differentiated human macrophages. While this model is highly relevant for studying inflammatory responses, the findings may not fully reflect the complex interactions that occur in vivo. Animal studies and clinical trials are needed to validate the therapeutic potential of *F. assa-foetida* extract in the context of inflammatory diseases.

Finally, the bioactive compounds responsible for the observed effects were not specifically identified in this study. Future research should focus on isolating and characterizing these compounds to better understand their mechanisms of action and to facilitate the development of standardized formulations for therapeutic use.

## Conclusion

This study demonstrates that *F. assa-foetida* extract selectively downregulates NF-κB expression in GM-CSF-differentiated human macrophages, with minimal effects on STAT1 expression. These findings highlight the potential of the extract as a selective immunomodulatory agent for the treatment of inflammatory diseases. Further studies are warranted to elucidate the molecular mechanisms underlying these effects and to evaluate the therapeutic efficacy of *F. assa-foetida* in vivo.

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## Conflict of Interest

The authors declare no conflict of interest

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