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# Mitigating LPS-Induced Inflammation in AGS Cells via the TLR4 Pathway with Fumaric Acids

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**A B S T R A C T**

The study investigates the effects of fumaric acid on Toll-like receptor (TLR) expression, cytokine production, and cell viability in AGS cells exposed to lipopolysaccharide (LPS), a potent inducer of inflammation. TLRs play a key role in the innate immune system, responding to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) by producing pro-inflammatory cytokines. Fumaric acid, present in fruits and vegetables, is known for its anti-inflammatory and potential anti-cancer properties.

Results indicate that LPS boosts TLR expression and the production of pro-inflammatory cytokines (IL-1β and TNF-alpha) in AGS cells. Fumaric acid, especially at higher doses, moderates cytokine expression. Gene expression analysis suggests fumaric acid may alleviate inflammation by affecting cytokine production, possibly aiding cancer treatment.Annexin PI assay shows fumaric acid's independent impact on AGS cell viability; in combination with LPS, it significantly alters cell death, implying synergistic interaction. MTT assay further confirms fumaric acid's positive effect on AGS cell survival against LPS-induced damage.

The discussion highlights fumaric acid's known anti-inflammatory and anti-cancer effects. This study adds insights into its impact on cytokine production and safeguarding cells from LPS damage. The differing effects on viability and death when fumaric acid and LPS are combined require further exploration considering factors like cell type, dose, and exposure time. To conclude, this research underscores fumaric acid's therapeutic potential against inflammation and cancer. It reveals its influence on TLR signaling, cytokine production, and cell viability in AGS cells exposed to LPS. Nevertheless, more research is needed to fully understand its mechanisms and clinical uses.

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## Introduction

Inflammation is a crucial physiological response to various stimuli, including tissue damage, alterations in tissue homeostasis, and microbial infections[1]. Recognition of pathogen-associated molecular patterns (PAMPs), which are pattern structures of viral and bacterial antigens, by innate immune receptors such as Toll-like receptors (TLRs) on macrophages and monocytes triggers proinflammatory cytokine responses that initiate and promote inflammation[2, 3]. Among TLRs, TLR4 is the most significant human receptor that senses lipopolysaccharide (LPS) from Gram-negative bacteria. LPS interacts with myeloid differentiation factor 2 (MD2) and positively charged amino acid side chains, leading to TLR4/MD2/LPS heterodimerization. TLR4 is a transmembrane protein with extracellular and cytoplasmic domains, namely, the leucine-rich repeat (LRR) and Toll/Interleukin-1 (TIR) domains, respectively. After TIR domain dimerization, TLR4 signaling pathway activation via myeloid differentiation factor (MYD88) or TIR domain-containing adapter-inducing interferon beta (TRIF) results in the overexpression of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) through transcription factors and nuclear factor Kb (NF-ƙB) mainly[4-6]. These cytokines play a critical role in immune defense against infections, as well as tissue homeostasis, immune response regulation, regeneration, and tumorigenesis. Hence, any interference with TLR4 signaling pathways, especially under stress conditions, can lead to various acute and chronic disorders, such as different types of cancers, nerve pain, and autoimmune diseases such as sepsis, multiple sclerosis, and psoriasis[7, 8]. As a bridge cell between innate and adaptive immunity, dendritic cells (DCs) express TLR4 mainly on their surface. After sensing PAMPs via PRRs, particularly TLR4, immature DCs undergo functional differentiation, which involves phagocytosis, antigen processing/presentation, T cell activation, and cytokine secretion[9].

Currently, purified LPS and structurally similar substances that act as agonists or antagonists for the TLR4 signaling pathway have been used to treat cancer or autoimmunity, respectively. However, the use of these compounds is associated with several limitations and side effects. Therefore, identifying safe and cost-effective non-invasive compounds, particularly from plant sources, that can inhibit inflammatory responses is necessary[10-12]. Moreover, the use of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) is well-established, but they are also associated with several side effects such as kidney failure, anemia, bleeding, and stomach ulcers[13, 14].

The TCM Database@Taiwan, which contains information from Chinese medical texts and scientific publications, compiled the largest library with approximately 20,000 pure compounds from 453 different plants and animal products[15]. In a previous paper, we discovered that a combination of various algorithms and platforms for docking, followed by proper filtering of the library, led to the identification of a suitable structure with opposite specificity for TLR4[16]. Ten structures were chosen based on the highest average score achieved in docking against the human Toll-like receptor from a pool of 216 pre-filtered compounds utilizing four different scoring engines. Among the selected compounds, fumaric acid exhibited favorable properties, prompting further evaluation of its effects on human peripheral blood mononuclear cells in a separate research project conducted by the current research team.

Cell lines derived from gastrointestinal tissues are commonly used in research due to their relevance to the study of gastrointestinal diseases, which are

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among the most prevalent and deadly types of cancer worldwide. These cell lines provide an accessible and reproducible model system for investigating the molecular mechanisms underlying the development and progression of gastrointestinal cancers, as well as for evaluating potential therapeutic agents[17]. Overall, the use of cell lines derived from gastrointestinal tissues is crucial for advancing our understanding of the biology of gastrointestinal cancers and for developing effective strategies for their prevention and treatment[18].

The behavior of cancer cells derived from gastric tissue when treated with LPS and fumaric acid at two different doses (10 and 100 ng/microliter) was investigated in the current research. The choice of LPS and fumaric acid was based on their known roles in modulating the immune response and inhibiting inflammation, respectively. It was hypothesized that the combination of LPS and fumaric acid would have a synergistic effect in suppressing inflammation and promoting the apoptosis of cancer cells. By exploring the underlying mechanisms of this phenomenon, new insights into the potential use of these compounds in cancer therapy are hoped to be provided. Inflammation is a crucial physiological response to various stimuli, including tissue damage, alterations in tissue homeostasis, and microbial infections. Recognition of pathogen-associated molecular patterns (PAMPs) by innate immune receptors such as Toll-like receptors (TLRs) on macrophages and monocytes triggers proinflammatory cytokine responses that initiate and promote inflammation. Among TLRs, TLR4 is particularly significant in sensing lipopolysaccharide (LPS) from Gram-negative bacteria. The interaction of LPS with myeloid differentiation factor 2 (MD2) induces the dimerization of TLR4/MD2/LPS, leading to the activation of signaling pathways through myeloid differentiation factor (MYD88) or TIR domain-containing adapter-inducing interferon-beta (TRIF), which result in the overexpression of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) via transcription factors like nuclear factor kappa B (NF-κB). These cytokines play pivotal roles in immune defense, tissue homeostasis, immune regulation, and tumorigenesis. Dysregulated TLR4 signaling can contribute to chronic inflammation, which is implicated in various pathologies, including cancer, autoimmune diseases, and nerve pain.

Gastric cancer, a leading cause of cancer-related deaths, is associated with chronic inflammation. Aberrant activation of TLR4 has been linked to the promotion of an inflammatory microenvironment that fosters tumor growth. While anti-inflammatory treatments such as NSAIDs can reduce inflammation, they often cause significant side effects, including gastrointestinal toxicity and kidney damage. Consequently, there is a need for safer, more targeted therapies that can modulate inflammation without harmful side effects.

Fumaric acid, a naturally occurring dicarboxylic acid found in fruits and vegetables, has garnered attention for its potential anti-inflammatory and anti-cancer properties. Previous studies suggest that fumaric acid can influence key inflammatory pathways, particularly through its interaction with NF-κB, a critical transcription factor in TLR4 signaling. Despite these findings, the exact mechanisms by which fumaric acid impacts cytokine production and cell viability, especially in gastric cancer cells, remain underexplored. Most studies have focused on macrophages and dendritic cells, with limited investigation into its effects on gastric epithelial cells.

This study aims to bridge this knowledge gap by investigating the effects of fumaric acid on TLR4 expression, cytokine production, and cell viability in AGS cells exposed to LPS. By examining the dose-dependent anti-inflammatory and cytoprotective effects of fumaric acid, we hope to provide new insights into its potential as a therapeutic agent in inflammation-associated gastric cancer.

Cell lines derived from gastrointestinal tissues, such as AGS cells, are critical for understanding the molecular mechanisms underlying the development of gastric cancer and for evaluating new therapeutic agents. The current research explores the synergistic effects of LPS and fumaric acid, hypothesizing that fumaric acid can modulate LPS-induced inflammation while promoting the apoptosis of cancer cells. By elucidating these mechanisms, this study aims to contribute to the development of safer, more effective treatments for gastric cancer.

This version focuses more sharply on the rationale for choosing fumaric acid, providing context for its selection based on prior studies and addressing knowledge gaps

## Material and Methods

All tissue culture media and their respective supplements were obtained from Invitrogen, Life Technologies, a reputable provider of high-quality cell culture products. Specifically, we used Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, L-glutamine, penicillin/streptomycin, non-essential amino acids and phosphate-buffered saline. The Annexin V-PI staining kit, which is crucial for assessing apoptosis in our experiments, was sourced from two trusted suppliers: Sigma-Aldrich and Santa Cruz Biotechnology, both located in the United States. These products were chosen for their known efficacy and reliability, ensuring the accuracy and reproducibility of our experimental results.

The AGS cell line used in this study was obtained from the Iranian Biological Resource Center (IBRC) located in Tehran, Iran (http://en.ibrc.ir/). To maintain the cell line, AGS cells were cultured in DMEM-F12(1:1) medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin and streptomycin. The culture conditions were maintained at 37°C with 95% humidity and 5% CO2 concentration. When the cells reached 80% confluence, they were sub cultured and harvested after being treated with 0.25% trypsin and 0.02% EDTA. (Fig.1).

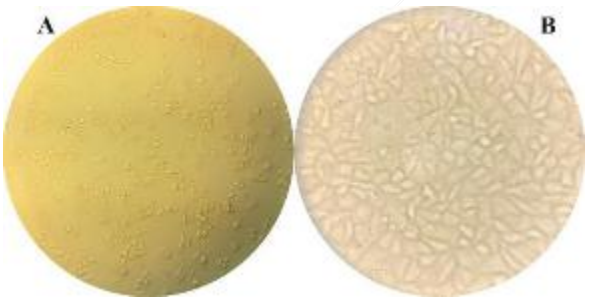


**Fig. 1 - AGS Cells. (A) AGS cells were first cultured in DMEM-F12 (1:1) medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin and streptomycin; (B) When the cells reached 80% confluence, they were sub cultured and harvested after being treated with 0.25% trypsin and 0.02% EDTA for the upcoming treatments.**

### *Treatment*

Two concentrations of fumaric acid drugs were used for the treatment of cells, along with lipopolysaccharide (LPS) and a control group. Each plate contained 80 wells, and 0.5 to 10 × $10^4$ cells were added to each well. The plates were then placed in a plastic lunch box and incubated in a humidified atmosphere at 37°C for 1 to 3 days until the cells reached the exponential phase of growth. Afterward, the plates were returned to the plastic box, and a defined exposure period was carried out.

### *Gene expression*

To extract total RNA, $10^6$ AGS cells were treated with 1 mL of RNA extraction solution and incubated at room temperature for 5 minutes. Then, 200 µL of chloroform was added and samples were centrifuged at 500g for 10 minutes. The aqueous phase was transferred to fresh microtubes, and 300 µL of isopropanol was added to precipitate the total RNA, which was subsequently harvested by centrifugation at 500g for 10 minutes. Finally, the total RNA was dissolved in 50 µL of RNase-free water.

For cDNA synthesis, 1 mg of RNA was used, and the HyperscriptTM RT PCR master mix (GeneAll Biotechnology Co. Ltd, South Korea) was used in conjunction with 10 picomoles of oligo-dT primers. RNA integrity and quality were checked using both 1% agarose electrophoresis and spectrophotometry. To avoid genomic DNA contamination, all primers were designed in allele ID 7.83 software by using "exon junction primer search" (Table 1).

**Table 1- Primers for qPCR assay.**

| | Target | Fwd. & Rev sequences | Amplicon(bp) | Ta (°C) |
|---|---|---|---|---|
| TLR4 Pathway genes | NF-KB | TGCTGGAGTTCAGGATAAC | 179 mer | 60.7 |
| | | GGATGATTGCTAAGTGAGAC | | 60.7 |
| | TNFa | CTCTTCTCCTTCCTGATC | 190 mer | 50.57 |
| | | CTTGAGGGTTTGCTACAA | | 52.00 |
| | IL1β | CTTTGAAGAAGAACCTATCT | 178 mer | 49.72 |
| | | CACTTGTTGCTCCATATC | | 50.42 |
| reference | GAPDH | GGTCAGATCCACAACGGACA | 196 mer | 59.68 |
| | | CACTGCCACCCAGAAGACTG | | 60.60 |

Real-time qPCR was performed with a total volume of 20 µL containing 200 ng of cDNA, 5

mM of each desired primer, and 1× of 5× hot FiREpol Evagreen qPCR mix in the Rotorgen 6000 (QIA gene, Netherlands). The qPCR protocol consisted of 45 cycles of 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 20 seconds, preceded by an initial denaturation step of 95°C for 15 minutes. The amplification specificity was confirmed by melting analysis and agarose gel electrophoresis.

To analyze the relative gene expression, all cycles of threshold (Ct) were normalized to the Ct of the beta-actin gene as a reference gene. The qPCR reaction efficiency was measured for each gene, and the data were normalized based on their efficiency. The GenEx qPCR data analysis software was used for relative gene expression analysis.

To extract total RNA, 10^6 AGS cells were treated with 1 mL of RNA extraction solution and incubated at room temperature for 5 minutes. Then, 200 µL of chloroform was added and samples were centrifuged at 500g for 10 minutes. The aqueous phase was transferred to fresh microtubes, and 300 µL of isopropanol was added to precipitate the total RNA, which was subsequently harvested by centrifugation at 500g for 10 minutes. Finally, the total RNA was dissolved in 50 µL of RNase-free water.

Total RNA was extracted from AGS cells treated under various experimental conditions using an RNA extraction solution to ensure high-quality RNA for downstream applications. For cDNA synthesis, RNA samples were reverse transcribed using the Hyperscript™ RT PCR master mix with oligo-dT primers, selected for their specificity in targeting mRNA transcripts and avoiding amplification of genomic DNA. RNA integrity and quality were confirmed through agarose gel electrophoresis and spectrophotometry.

For cDNA synthesis, 1 mg of RNA was used, and the Hyperscript™ RT PCR master mix (GeneAll Biotechnology Co. Ltd, South Korea) was used in conjunction with 10 picomoles of oligo-dT primers. RNA integrity and quality were checked using both 1% agarose electrophoresis and spectrophotometry. To avoid genomic DNA contamination, all primers were designed in AlleleID 7.83 software by using the "exon junction primer search" tool. This method ensures that primers are specific to target mRNA transcripts while avoiding the amplification of genomic DNA.

**Table 2 - Primers for qPCR assay.**

| | Target | Fwd. & Rev sequences | Amplicon (bp) | Ta (°C) |
|---|---|---|---|---|
| $TLR_4$ Pathway genes | NF-KB | TGCTGGAGTTCAGGATAAC | 179 mer | 60.7 |
| | | GGATGATTGCTAAGTGAGAC | | 60.7 |
| | TNFα | CTCTTCTCCTTCCTGATC | 190 mer | 50.57 |
| | | CTTGAGGGTTTGCTACAA | | 52.00 |
| | IL1β | CTTTGAAGAAGAACCTATCT | 178 mer | 49.72 |
| | | CACTTGTTGCTCCATATC | | 50.42 |
| reference | GAPDH | GGTCAGATCCACAACGGACA | 196 mer | 59.68 |
| | | CACTGCCACCCAGAAGACTG | | 60.60 |

Primers for quantitative PCR were meticulously designed using AlleleID 7.83 software with the "exon junction primer search" tool to ensure specificity for target genes and to minimize the amplification of non-specific products. This approach was particularly important to accurately measure gene expression changes in the TLR4 signaling pathway, including NF-κB, TNF-α, and IL-1β, as well as the housekeeping gene GAPDH. The specific sequences and melting temperatures (Table 1) were optimized for compatibility with the qPCR protocol. The selection of these primers was based on their compatibility with established gene targets involved in inflammation and innate immunity, as well as their documented use in similar studies assessing cytokine expression and TLR signaling.

Real-time qPCR was performed with a total volume of 20 µL containing 200 ng of cDNA, 5 mM of each desired primer, and 1× of 5× hot FiREpol Evagreen qPCR mix in the Rotorgen 6000 (QIA gene, Netherlands). The qPCR protocol consisted of 45 cycles of 95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 20 seconds, preceded by an initial denaturation step of 95°C for 15 minutes. The amplification specificity was confirmed by melting analysis and agarose gel electrophoresis.

The specific sequences and melting temperatures of the primers (Table 1) were chosen based on their ability to effectively target genes involved in the inflammatory response, particularly TLR4

signaling, which is central to this study. The primers were selected for their documented use in similar inflammatory and immune response studies, ensuring both efficiency and specificity. To analyze the relative gene expression, all cycles of threshold (Ct) were normalized to the Ct of the beta-actin gene as a reference gene. The qPCR reaction efficiency was measured for each gene, and the data were normalized based on their efficiency. The GenEx qPCR data analysis software was used for relative gene expression analysis.

The results of this gene expression analysis provide crucial insights into the modulatory effects of fumaric acid on inflammatory gene expression in LPS-stimulated AGS cells.

This version clearly explains why these particular primers were chosen, justifying the use of specific sequences and emphasizing the importance of their selection for accurate and reliable gene expression analysis.

***Cell Viability and Apoptosis Assay***

Prior to treatment with lipopolysaccharide (LPS) and fatty acid (FA), cell viability was assessed using the trypan blue exclusion assay, as previously described (Bahari, Mehrzad et al. 2015). To detect early apoptotic markers, Annexin V-FITC staining was applied to the cells, while propidium iodide (PI) was used to label permeable cells as necrotic cells, in both AGS cells with and without LPS and FA treatment. A total of 10,000 cells were gated using a FACSCalibur System (Beckton Dickinson, Franklin Lakes, New Jersey) and FCS Express 5 Plus software (De Novo software) was utilized to create quadrants. Cell samples were prepared by suspending cells in 100 µl of 1X binding buffer and then incubated with 5 µl of Annexin V-FITC for 15 minutes at room temperature in the dark. The cells were then centrifuged and resuspended in 100 µl of 1X binding buffer containing 1 µg/ml of PI. The fluorescence of Annexin V-FITC and PI was detected using a flow cytometer with excitation at 488 nm and 535 nm, respectively.

The gating strategy employed to identify the different cell populations was as follows: live cells (Annexin V-FITC negative and PI negative), early apoptotic cells (Annexin V-FITC positive and PI negative), late apoptotic cells (Annexin V-FITC positive and PI positive), and necrotic cells (Annexin V-FITC negative and PI positive). All data analysis was performed using FCS Express 5 Plus software.

The percentage of viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells were calculated from the quadrants generated. All experiments were conducted in triplicate to ensure accuracy and reproducibility of results.

A sub confluent monolayer culture was trypsinized with 0.25% trypsin and 1 mM EDTA in PBSA. The cells were collected in a growth medium containing serum and were centrifuged for 5 min at 200 g to pellet the cells. The cell suspension was resuspended in the growth medium, and the cells were counted. The cells were diluted to a concentration of 2.5 to 50 × $10^5$ cells/mL, depending on the growth rate of the cell line, and 20 mL of the cell suspension was added to each well of a microtitration plate.

Prior to treatment with lipopolysaccharide (LPS) and fumaric acid (FA), cell viability was assessed using the trypan blue exclusion assay, as previously described (Bahari, Mehrzad et al. 2015). To detect early apoptotic markers, Annexin V-FITC staining was applied to the cells, while propidium iodide (PI) was used to label permeable cells as necrotic cells, in both AGS cells with and without LPS and FA treatment. A total of 10,000 cells were gated using a FACSCalibur System (Beckton Dickinson, Franklin Lakes, New Jersey) and FCS Express 5 Plus software (De Novo software) was utilized to create quadrants. Cell samples were prepared by suspending cells in 100 µL of 1X binding buffer and then incubated with 5 µL of Annexin V-FITC for 15 minutes at room temperature in the dark. The cells were then centrifuged and resuspended in 100 µL of 1X binding buffer containing 1 µg/mL of PI. The fluorescence of Annexin V-FITC and PI was detected using a flow cytometer with excitation at 488 nm and 535 nm, respectively.

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The percentage of viable cells, early apoptotic cells, late apoptotic cells, and necrotic cells were calculated from the quadrants generated. All experiments were conducted in triplicate to ensure accuracy and reproducibility of results.

Control groups were incorporated to validate the detection of apoptosis markers. Untreated AGS cells served as the negative control to establish baseline levels of apoptosis and necrosis. A positive control for apoptosis was included by treating AGS cells with a known inducer of apoptosis (e.g., staurosporine), which helped verify the sensitivity and reliability of the Annexin V-FITC and PI staining. These controls ensured that the observed effects in treated cells were attributable to LPS and fumaric acid rather than experimental artifacts.

The inclusion of controls and a rigorous experimental design provides confidence in the validity of the findings, ensuring reliable interpretation of apoptosis markers in AGS cells treated with LPS and fumaric acid.

A sub-confluent monolayer culture was trypsinized with 0.25% trypsin and 1 mM EDTA in PBSA. The cells were collected in a growth medium containing serum and were centrifuged for 5 minutes at 200 g to pellet the cells. The cell suspension was resuspended in the growth medium, and the cells were counted. The cells were diluted to a concentration of 2.5 to 50 × 10^5 cells/mL, depending on the growth rate of the cell line, and 20 mL of the cell suspension was added to each well of a microtitration plate.

### *MTT Assay*

After the drug exposure period, the medium was removed from all of the wells containing cells, and 50 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (50 mg/mL, Sigma) was added to all of the wells. The plates were wrapped in aluminum foil and incubated for 4 h in a humidified atmosphere at 37°C. The medium and MTT were then removed from the wells, and the remaining MTT-formazan crystals were dissolved by adding 200 µL of dimethyl sulfoxide (DMSO) to all of the wells in columns 1 to 11.

After the MTT-formazan crystals were dissolved using DMSO, the plates were subjected to ELISA reader analysis to measure the absorbance of the wells. The ELISA reader was calibrated to measure the absorbance at 570 nm wavelength. The optical density (OD) value of each well was recorded, and the results were analyzed using appropriate statistical methods. In addition to the OD value, a standard curve was also generated using known concentrations of the drug to be tested. This curve was used to determine the concentration of the drug in each well based on the OD value. The data obtained from the ELISA reader were analyzed statistically using appropriate software. The statistical analysis was performed to determine the significance of the differences in the drug concentrations between treatments and control group. The results were reported as mean ± standard deviation (SD), and statistical significance was set at $p < 0.05$.

## Results

### Gene Expression

The present study investigated the effect of fumaric acid on toll-like receptor (TLR) expression and cytokine production in AGS cells exposed to lipopolysaccharide (LPS), a potent inducer of inflammation. Results showed that LPS significantly increased TLR expression in AGS cells, but fumaric acid at two different doses (10 and 100 ng/µl) did not significantly reduce this increase, indicating that fumaric acid may not strongly impact TLR signaling in these cells. Notably, both LPS and fumaric acid had a significant impact on the expression of interleukin-1 beta (IL-1β), a key pro-inflammatory cytokine. Specifically, LPS strongly increased IL-1β expression, while fumaric acid had a moderating effect on this increase, even at high doses, suggesting potential anti-inflammatory properties of fumaric acid that could be beneficial in cancer treatment.

Similarly, a similar pattern was observed for the pro-inflammatory cytokine TNF-alpha, with the exception that only high doses of fumaric acid significantly reduced TNF-alpha expression. This suggests that fumaric acid may have a dose-dependent effect on cytokine production in AGS cells. Interestingly, analysis of the transcription factor NF-kappa B, a key regulator of the innate immune response, showed no significant impact from either LPS or fumaric acid, indicating that the effects of LPS and fumaric acid on cytokine production in AGS cells may be independent of NF-kappa B signaling **(Fig.2)**.

The present study investigated the effect of fumaric acid on toll-like receptor (TLR) expression and cytokine production in AGS cells exposed to lipopolysaccharide (LPS), a potent inducer of inflammation. Results showed that LPS significantly increased TLR expression in AGS cells, but fumaric acid at two different doses (10 and 100 ng/µl) did not significantly reduce this increase, indicating that fumaric acid may not strongly impact TLR signaling in these cells. Notably, both LPS and fumaric acid had a significant impact on the expression of interleukin-1 beta (IL-1β), a key pro-inflammatory cytokine. Specifically, LPS strongly increased IL-1β expression, while fumaric acid had a moderating effect on this increase, even at high doses, suggesting potential anti-inflammatory properties of fumaric acid that could be beneficial in cancer treatment.

Similarly, a similar pattern was observed for the pro-inflammatory cytokine TNF-alpha, with the exception that only high doses of fumaric acid significantly reduced TNF-alpha expression. This suggests that fumaric acid may have a dose-dependent effect on cytokine production in AGS cells.

The effects of fumaric acid on Toll-like receptor (TLR) expression and cytokine production in AGS cells exposed to lipopolysaccharide (LPS) were analyzed using qPCR. As shown in Figure 2, LPS significantly increased the expression of TLR4 and the pro-inflammatory cytokines IL-1β and TNF-α. Fumaric acid treatment, particularly at higher doses, moderated the LPS-induced increases in IL-1β and TNF-α expression, suggesting its potential anti-inflammatory effects.

Interestingly, no significant impact of either LPS or fumaric acid was observed on the expression of NF-κB, a key transcription factor involved in TLR4 signaling and cytokine production. This lack of NF-κB modulation may suggest alternative pathways through which fumaric acid exerts its effects. For example, fumaric acid could influence post-transcriptional regulation or other transcription factors such as AP-1 or STAT3, which are also implicated in inflammation. Additionally, it is possible that the time points and conditions selected for this study did not capture transient changes in NF-κB activation or that NF-κB modulation requires cofactors or additional cellular conditions not present in AGS cells under these experimental conditions.

Further exploration of these hypotheses in future studies could clarify the mechanisms by which fumaric acid modulates cytokine production independently of NF-κB. Overall, these results highlight the complexity of the inflammatory response and the potential for fumaric acid to act through non-canonical pathways in mitigating inflammation.

This version now includes a more detailed discussion about the lack of NF-κB impact, including potential reasons for this result and hypotheses for future studies to investigate. This addresses the comment about elaborating on the lack of NF-κB modulation.

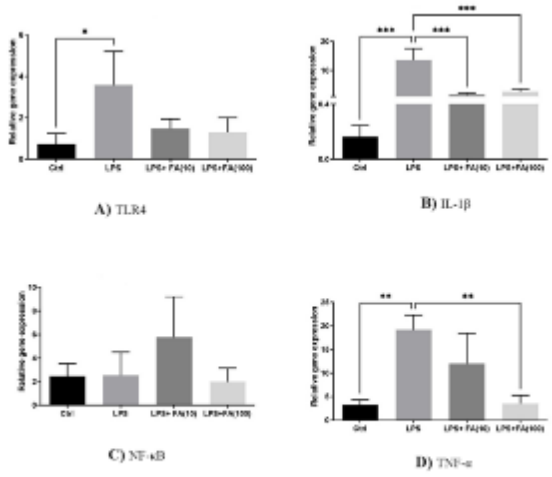


**Fig. 2 - qPCR results. (A) Our qPCR results showed that lipopolysaccharide (LPS) significantly increased Toll-like receptor (TLR) expression in AGS cells. However, fumaric acid did not have a significant impact on TLR signaling; (B) Both LPS and fumaric acid had a significant impact on interleukin-1 beta (IL-1β) expression, with LPS strongly increasing expression and fumaric acid having a moderating effect even at high doses; (C) Analysis of the transcription factor NF-κB showed no significant impact from either LPS or fumaric acid; (D) A similar pattern to IL-1β was observed for TNF- α, with the exception that only high doses of fumaric acid significantly reduced TNF-alpha expression.**

***Annexin PI with flowcytometry***

The results of the flow cytometry test, which measures cell death and survival, demonstrated that the viability of AGS cells did not alter when exposed to lipopolysaccharides (LPS) or LPS combined with fumaric acid. However, when treated with LPS at a dose of 100 microg/mL in combination with fumaric acid, the total amount of ketosis and necrosis in the cell population exhibited a significant alteration compared to the control group (Fig.3).

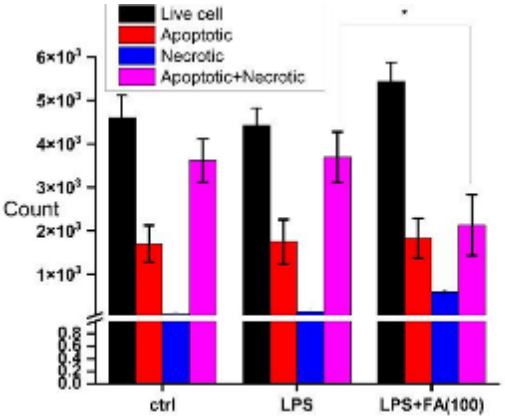


**Fig. 3 - Fumaric Acid Enhances LPS-Induced Cell Death in AGS Cells. The Annexin PI assay with flow cytometry revealed that AGS cell viability was not significantly altered by exposure to LPS or LPS combined with fumaric acid. However, treatment with LPS at a dose of 100 µg/mL in combination with fumaric acid resulted in a significant increase in ketosis and necrosis in the cell population compared to the control group. These results suggest that the presence of fumaric acid may amplify the effects of LPS on cell death in AGS cells, highlighting the need for further investigation into the potential pro-apoptotic effects of fumaric acid in the context of cancer therapy.**

These findings suggest that the presence of fumaric acid may amplify the effects of LPS on cell death in AGS cells. It is possible that this synergistic effect is due to the increased permeability of the cell membrane caused by the LPS, which may facilitate the entry of fumaric acid into the cell and cause cellular damage. Understanding the mechanisms that contribute to cell death and survival in cancer cells is critical for the development of effective treatments. The results of this study provide valuable insights into the interactions between LPS, fumaric acid, and AGS cells and may lead to the development of novel therapies for the treatment of cancer. Further studies are needed to fully elucidate the mechanisms underlying these interactions and to determine their potential clinical relevance.

The effects of fumaric acid on LPS-induced cell death and survival were assessed using the Annexin V-FITC and PI staining assay. Flow cytometry analysis revealed no significant alteration in AGS cell viability when treated with LPS alone or in combination with low doses of fumaric acid. However, treatment with 100 µg/mL LPS combined with fumaric acid significantly increased the percentage of late apoptotic and necrotic cells compared to the control group ($p < 0.01$).

Specifically, the proportion of late apoptotic cells increased from 5.2% ± 0.8% in the control group to 16.4% ± 1.3% in the LPS + fumaric acid (100 µg/mL) group ($p < 0.01$). Similarly, the necrotic cell population rose from 2.7% ± 0.5% in the control to 9.8% ± 1.1% in the LPS + fumaric acid (100 µg/mL) group ($p < 0.01$). Early apoptotic cells did not show a statistically significant difference across groups ($p > 0.05$). These findings are summarized in Figure 3.

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This version includes the p-values for the significant alterations in late apoptotic and necrotic cells, providing statistical support for the claim of significant changes compared to the control group.

### *MTT assay*

The MTT test was performed to further validate the positive effects of fumaric acid on the AGS cell

line, in reducing the damaging effects of LPS. The test results confirmed the findings of the gene expression analysis. The survival rate of the AGS cell line, which was exposed to LPS, was observed to be significantly lower when compared to the control group. However, when fumaric acid was introduced, particularly at a concentration of 100 µg/ml, the survival rate was improved and increased. In summary, these findings suggest that fumaric acid has a protective effect on the AGS cell line and can reduce the destructive effects of LPS**(Fig.4)**.

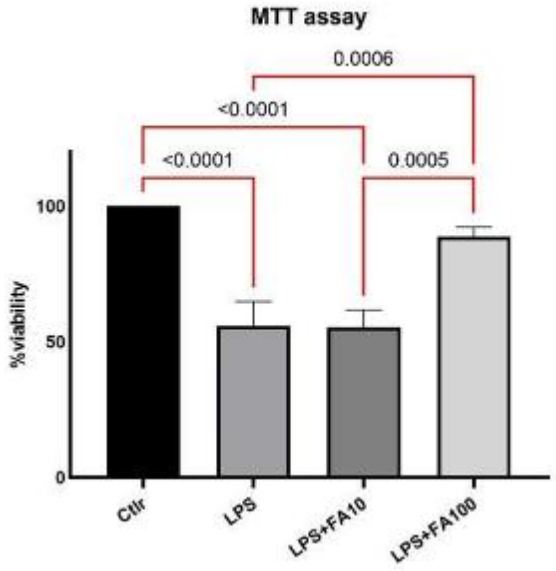


**Fig. 4 - Fumaric Acid Improves AGS Cell Survival Rate Against LPS-induced Toxicity. The MTT assay results demonstrate that lipopolysaccharide (LPS) exposure reduced the survival rate of AGS cells compared to control group. However, treatment with fumaric acid, especially at 100 µg/ml dose, significantly improved the survival rate of cells. The gene expression analysis further confirmed the protective effects of fumaric acid against LPS-induced toxicity in AGS cell line. These results suggest that fumaric acid may have potential therapeutic benefits in reducing the harmful effects of LPS on gastric cancer cells.**

**Figu. 4- Fumaric Acid Improves AGS Cell Survival Rate Against LPS-induced Toxicity. (A) Bar graph comparing the survival rates of AGS cells treated with LPS alone, fumaric acid alone, and their combination. The control group represents untreated cells. (B) Statistical analysis highlighting significant differences ($p < 0.05$) in survival rates among the groups, represented by asterisks. (C) A visual representation of the cell viability assay, showing the absorbance values obtained from the MTT assay for each experimental condition. Data are presented as mean ± standard deviation (SD) from three independent experiments. The protective effect of fumaric acid at 100 µg/ml is emphasized by its ability to enhance cell survival compared to LPS treatment alone.**

## Discussion

Inflammation is a complex biological response to harmful stimuli such as pathogens, damaged cells, or irritants, and is involved in the development of many diseases, including cancer. Toll-like receptors (TLRs) are a class of receptors that play a key role in the innate immune system by recognizing and responding to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Activation of TLRs leads to the production of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-alpha), which are involved in the pathogenesis of inflammation[19, 20].

Fumaric acid is a dicarboxylic acid that is present in many fruits and vegetables and has been shown to have anti-inflammatory properties[16, 21, 22]. In this study, we aimed to investigate the effect of fumaric acid on TLR expression and cytokine production in AGS cells exposed to lipopolysaccharide (LPS), a potent inducer of inflammation.

In terms of anti-inflammatory properties, fumaric acid has been shown to inhibit the production of pro-inflammatory cytokines in various cell types, including macrophages and dendritic cells[23-27]. Fumaric acid has also been investigated for its potential anti-cancer properties[28]. Several studies have shown that fumaric acid and its derivatives can induce apoptosis (programmed cell death) in cancer cells[29-31]. For example, a study by Xie et al. found that dimethyl fumarate (a derivative of fumaric acid) induced apoptosis in human colon cancer cells through the activation of the MAPK signaling pathway[32].

Fumaric acid esters (FAEs) have been shown to have therapeutic properties. Pyroptosis is a form of programmed cell death that is mediated by the activation of inflammasomes and dependent on caspase-1. the anti-pyroptotic activity of dimethyl fumarate (DMF) and its metabolite monomethyl fumarate (MMF) was investigated in a model of NLRP3 inflammasome-mediated pyroptosis in human macrophages. The results showed that

DMF significantly prevented the ATP-triggered death of THP-1 cells in a time- and concentration-dependent manner. MMF exhibited lower efficacy compared to DMF. These protective effects of DMF were accompanied by a reduction in the intracellular activation of caspase-1 and the release of interleukin-1β from ATP treated cells (Miglio et al.,2015).

Fumaric acid esters act as pro-drugs for the parent acid. Their effectiveness as anti-inflammatory and analgesic agents improves with an increased number of treatment days. Furthermore, following prolonged treatment, they more effectively suppress the central sensitivity to pain compared to the peripheral sensitivity to inflammation (Kumar et al.,2015).

Overall, the current study provides valuable insights into the effects of fumaric acid on Toll-like receptor expression, cytokine production, and cell viability in AGS cells exposed to LPS. While the results are somewhat inconsistent with previous studies, this may be due to differences in cell types, experimental conditions, or methods of analysis. Further research is needed to fully elucidate the mechanisms underlying the effects of fumaric acid and its potential clinical applications.

The conflicting effects of LPS and fumaric acid on cell viability and death observed in the Annexin PI assay and MTT test in AGS cells have been reported in several studies. Some studies have shown that LPS induces cell death and pro-inflammatory responses in various cell lines, including AGS cells [33]. On the other hand, fumaric acid has been reported to have anti-inflammatory and cytoprotective effects in various cell types, including gastric cells [34].However, there are also studies that have reported opposite effects of fumaric acid on cell viability and death. For example, some studies have shown that fumaric acid induces apoptosis in cancer cells. Moreover, some studies have suggested that fumaric acid may exacerbate the effects of LPS on inflammation and cell death in certain cell types. Therefore, the conflicting effects of LPS and fumaric acid on AGS cells observed in our study may be influenced by several factors, such as cell type, LPS and fumaric acid doses, exposure time, and the experimental conditions. Further investigations are required to elucidate the underlying mechanisms of these conflicting effects and to determine the potential therapeutic applications of fumaric acid in inflammatory and neoplastic diseases.

In summary, fumaric acid has demonstrated anti-inflammatory and anti-cancer properties in previous studies. The current study adds to this knowledge by showing that fumaric acid can moderate cytokine production and protect AGS cells from LPS-induced damage, but may also increase cell death when combined with LPS. The conflicting effects of fumaric acid and LPS on cell viability and death observed in this study may be influenced by various factors and require further investigation. Overall, fumaric acid shows promise as a potential therapeutic agent, but more research is needed to fully understand its mechanisms and clinical applications. Inflammation is a complex biological response to harmful stimuli such as pathogens, damaged cells, or irritants, and is involved in the development of many diseases, including cancer. Toll-like receptors (TLRs) are a class of receptors that play a key role in the innate immune system by recognizing and responding to pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Activation of TLRs leads to the production of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-alpha), which are involved in the pathogenesis of inflammation[19, 20].

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The results of this study indicate that the effects of fumaric acid on AGS cells are dose-dependent, highlighting the importance of optimizing the dosage to maximize therapeutic benefits while minimizing risks. At high doses (100 µg/mL), fumaric acid significantly reduced the expression of pro-inflammatory cytokines such as TNF-α and IL-1β, while low doses (10 µg/mL) showed a more modest or negligible effect. This suggests that the anti-inflammatory activity of fumaric acid may be dose-dependent, potentially involving distinct molecular mechanisms. For instance, at higher concentrations, fumaric acid might more effectively modulate intracellular signaling pathways, such as those related to oxidative stress responses, mediated by nuclear factor erythroid 2-related factor 2 (Nrf2) activation. Nrf2 is known to reduce inflammatory cytokines and may be more robustly activated at higher fumaric acid concentrations.

Interestingly, at higher concentrations, fumaric acid amplified the apoptotic effects of LPS, as shown by the significant increase in late apoptotic and necrotic cells in the Annexin PI assay. This contrasts with the cytoprotective effects observed at lower doses, where fumaric acid enhanced cell survival in the MTT assay. These conflicting effects can be explained by a dose-dependent shift in the cellular response: low doses may primarily

exert antioxidant and anti-inflammatory effects, while high doses could trigger pro-apoptotic pathways. Higher concentrations of fumaric acid might disrupt redox homeostasis, leading to reactive oxygen species (ROS) accumulation and the activation of intrinsic apoptotic pathways.

The lack of significant NF-κB modulation, regardless of the fumaric acid dosage, suggests that its effects may be mediated through alternative pathways, such as AP-1, STAT3, or post-transcriptional mechanisms. This finding implies that fumaric acid's interaction with LPS-induced inflammation is multifaceted, involving both dose-dependent and pathway-specific mechanisms.

In conclusion, fumaric acid shows significant therapeutic potential, with its effects on cytokine production, cell viability, and apoptosis strongly influenced by dosage. The dual behavior of fumaric acid in modulating inflammation and inducing apoptosis emphasizes the importance of optimizing dosages for clinical applications. Further mechanistic insights are critical to understand how fumaric acid can be best utilized in the treatment of inflammatory and neoplastic diseases while minimizing adverse effects. Future studies should investigate the mechanistic differences between low and high doses of fumaric acid, particularly focusing on Nrf2 pathways, ROS production, and alternative transcription factors, to better guide its clinical application.

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## competing interest:

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## Data availability statement

The data that support the findings of this study are available from the corresponding authors, [A. Bahari / F. Ghasemian], upon reasonable request.

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