

Antioxidant and Anti-Inflammatory Impact of Jujuboside B on LPS-Stimulated RAW 264.7 Cells

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ABSTRACT

Aim: *Zizyphus jujuba* Mill, a well-known traditional Eastern medicine, has been documented to display a variety of biological system-related actions. *Zizyphus jujuba* fruits contain a natural saponin called jujuboside B (JB), which is one of their active ingredients. **Materials and Methods:** In the current work, the viability of the RAW-264.7 cells was assessed by treating them with varying concentrations of JB. Also, measurement of nitric oxide and intracellular ROS generation. We examined the antioxidant enzymes and anti-inflammatory activities of jujuboside B on RAW-264.7 cells stimulated with Lipopolysaccharide (LPS). **Results:** At every treatment concentration, the cells remained viable. Antioxidant enzyme levels were downregulated after LPS treatment, although NO, ROS, and pro-inflammatory cytokines levels were significantly elevated. This suggests that cells exposed to LPS display signs of inflammation and oxidative damage. Following JB therapy, antioxidant enzyme levels increased due to a considerable reduction in NO, ROS, and pro-inflammatory cytokine levels. This demonstrates JB's anti-inflammatory and antioxidant properties. **Conclusion:** Our findings provide scientific justification for the utilization of JB for treating inflammatory illnesses by indicating that it modulates the major inflammatory processes and reduces the inflammatory responses stimulated by LPS in RAW 264.7 cells.

Keywords: Jujuboside B, LPS, Antioxidant, Anti-inflammatory, RAW-264.7 cells.

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INTRODUCTION

In reaction to harmful attacks, including microbial infection, tissue damage, and other toxic situations, higher species have evolved the defensive mechanism of inflammation. It is a vital immunological reaction by the body's immune system that permits eliminating detrimental stimuli and restoring damaged tissue.^[1] Any organism with a functioning innate immune system often experiences inflammation within minutes of being stimulated by an appropriate agent, such as a microbial infection, foreign invaders, or any exterior or interior irritation. The intended target tissues of inflammatory pathways differ greatly depending on the type of stimulants used. When a bacterial infection occurs, immune cells recognize pathogens right away thanks to certain receptors. Activation of pathogen-specific receptors results in the generation of inflammatory mediators, which include chemokines and cytokines associated with inflammation like Tumor Necrosis Factor (TNF), Interleukin-1 (IL-1), and Interleukin-6 (IL-6).^[2]

According to conventional definitions, ROS is strongly oxidizing partly reduced metabolites of oxygen. At high quantities, they are harmful to cells; nevertheless, at low doses, they perform intricate signaling tasks. The other main source, where ROS are not generated as by-products, is NADPH oxidases, which are found in different types of cells, such as endothelium and professional phagocyte cells, which are crucial to the development of the inflammatory response.^[3] LPS is a bipolar molecule made up of a lipid A moiety and a polysaccharide component. It is an integral feature of the Gram-negative bacteria's exterior membrane. Both the mouse and human systems are used to study how LPS causes monocytic cells to produce cytokines. The cytokine generation of monocytic cells as a consequence of LPS is studied using a variety of monocyte cell types, including the murine macrophage cell lines such as RAW 264.7.^[4] LPS-induced macrophage activation results in the generation of iNOS, pro-inflammatory cytokines such as IL-1 β , TNF- α & IL-6, and NO. Subsequently, these cytokines activate nearby cells and more macrophages.

However, the pathogenesis of numerous inflammatory disorders, such as rheumatoid arthritis, atherosclerosis, pulmonary fibrosis, and inflammatory brain disease, has been linked to their overproduction by activated macrophages. Thus, LPS-stimulated macrophages provide an excellent model for investigating inflammation and the workings of putative anti-inflammatory mediators.^[5] The development of natural substances that can



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inhibit the inflammatory reflex with fewer adverse effects is crucial to creating innovative anti-inflammatory therapy approaches, as inflammation-related disorders are prevalent and serious.^[6] This research focuses on the therapeutic potential of Jujuboside B, a saponin derived from *Zizyphus jujuba*, which has been shown to modulate inflammatory pathways. The study specifically examines the effects of JB on LPS-stimulated RAW264.7 cells, highlighting its specific impact on liver inflammation and its potential to regulate inflammatory mediators. The findings may encourage further exploration into Jujuboside B's therapeutic applications, particularly in liver-related diseases and inflammatory conditions, contributing to the broader field of saponin research and highlighting their specific roles in human health.^[7,8]

From a medicinal perspective, triterpenoid saponins are the most intriguing since they are made up of triterpenoid aglycones, which have a pentacyclic C30 structure. Many different types of medicinal plants have been found to contain triterpenoid saponins. Plant-derived saponins have a range of biological properties, including cytotoxic, anticancer, immunomodulatory, anti-inflammatory, anti-mutagenic, anti-hepatotoxic, anti-diabetic, hemolytic, antiviral, antibacterial, and molluscicidal effects.^[9] The existence of many complex chemical components with varying compositions, including secondary metabolites like alkaloids, flavonoids, terpenoids, saponins, and phenolic compounds, scattered throughout the plant, is what gives medicinal plants their therapeutic capabilities. Members of the Rhamnaceae family, including *Zizyphus jujuba* Mill, also go by the name Bor. Jujubosides A, A1, B, B1, and C, protojujubosides A, B, and B1 and acetyljujuboside B, are among the saponins that were extracted from *Z. jujuba* seeds. They are traditionally used as tonics and aphrodisiacs, and they can also have hypnotic-sedative and anxiolytic effects, as well as antifungal, cognitive, anticancer, cardiostimulant, anti-inflammatory, antiulcer, antifertility, antispastic, antibacterial, immunostimulant, antinephritic and hypotensive, antioxidant, and wound curative potent activities.^[10] To confirm JB's pharmacological activities, we evaluated its anti-inflammation potential and inherent mechanisms in LPS-triggered RAW-264.7 cells.

This study evaluates JB's effects on RAW 264.7 macrophages, focusing on its ability to reduce pro-inflammatory cytokine production, modulate oxidative stress, and regulate key signaling pathways. This work offers a strong foundation for the investigation and development of JB as an anti-inflammation medication.

MATERIALS AND METHODS

Reagents and chemicals

Kits, chemicals, reagents, and jujuboside B were acquired from commercial suppliers and These were all of the analytical variety.

Cell Culture

The macrophage RAW-264.7 cells were acquired from the cell bank. The RAW-264.7 cells were grown up at room temperature in an incubator with 5% CO₂ and complemented with 1% penicillin, fetal bovine serum (10%), and streptomycin (1%). The cultured RAW-264.7 cells were used for the experiments.

Analysis of Cell Viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) methodology was employed to assess the viability of JB on the RAW264.7 cells after treatment with LPS and varying dosages of JB. Macrophages (1 × 10⁴ cells per well) were seeded in a 48-well plate. The cells were induced for 30 min with 0, 2.5, 5, 10, 15 and 20 μM/mL JB after 24 hr. This was followed by a 24-hr incubation phase at 37°C with CO₂ (5%). After the growth medium was withdrawn, the cells were nurtured for two hours with the MTT solution (1 mg/mL). Following that, the formazan crystals that had formed were suspended by the addition of DMSO (200 μL) after the MTT solution had been removed. The absorbance was estimated using a microplate reader at 570 nm.

Estimation of nitric oxide generation

The Griess reagent (Cat No: MAK367-1KT, Sigma Aldrich) was used to calculate the amount of nitrite oxide in the culture media. The cells were stimulated with JB and Dex at dosages after being seeded into 96-well plates at a density of 1*10⁴ cells/well. For a duration of 24 hr, the cells were activated at room temperature with media containing 1 μg/mL of LPS. After combining Griess reagent (100 μL) with culture supernatant (100 μL), the combination was left undisturbed for ten minutes. The mixture's absorption was assessed at 540 nm using a microplate reader.

Estimation of intracellular ROS generation

The ROS-sensitive fluorescent indicator DCFH-DA was utilized to quantify the levels of intracellular ROS. In summary, 96-well plates were seeded with the cells under study at a density of 1*10⁴ cells per well, previously treated for one hour with purified with JB and Dex at dosages then triggered for three hours with 1 μg/mL of LPS. The cells were then treated with 10 μM of the DCFH-DA detection reagent and rinsed thrice with 1X washing buffer. Following a duration of 30-min dark incubation period at room temperature, the cells were three times rinsed using 1 × washing buffer. A fluorescence reader fitted with a 488/525 nm excitation/emission filter was used to evaluate the DCF fluorescent.

Determination of antioxidant enzymes

In a 96-well plate, 1*10⁴ cells/well were pre-incubated for 2 hr with JB and Dex, followed by a further 20 hr of incubation with LPS. After removing the media, the cells underwent two PBS washes. After the cells were scraped off, 1 mL of 50 mmol per L potassium phosphate buffer, containing 1 mmol per L EDTA, was administered with a pH of 7.0. The cell suspensions underwent

three 5-sec sonications on ice, followed by 20-min centrifugation at $10,000 \times g$ at a temperature of 4°C . Enzyme activity measurements for antioxidants were conducted using cell supernatants. The determining protein concentration employed BSA as the reference standard.^[11] Superoxide Dismutase (SOD) was assessed by observing pyrogallol auto-oxidation. The quantity of enzyme that slowed down the rate at which pyrogallol was oxidized was called a unit of SOD activity. Catalase activity was determined using analysis of the reduction in H_2O_2 absorbance.^[12] For two minutes, the absorbance at 240 nm decreased. The technique was utilized to evaluate the activity of Glutathione peroxidase (GSH-px). One unit of GSH-px is the quantity of enzyme needed to oxidize one nanomol of NADPH used per 60 sec.^[13]

Determination of pro-inflammatory cytokine

ELISA kits were employed to examine JB and Dex's potential to prevent the production of pro-inflammatory cytokines. In summary, 96-well plates were seeded with 1×10^4 cells/well of RAW-264.7 cells, and the plates were then incubated for 12 hr at 37°C with 5% CO_2 . The cells were rinsed thrice in PBS after the entire media was discarded, and then they were revived in a DMEM medium. After that, cells received a one-hour pre-treatment with JB and Dex fixed concentrations. Following varying LPS ($1 \mu\text{g}/\text{mL}$) treatments, the culture media was subjected to centrifugation for 5 min at 1500 rpm. The amount of cytokines (IL- 1β , TNF- α , IL-6, and MCP-1) produced in the collected supernatant was measured with ELISA kits. Optical density at 450 nm was then measured using a microplate.

Statistical Analysis

The data are presented as the mean and standard deviation for determinations made in triplicate. To find significant distinctions between the samples ($p < 0.01$ & $p < 0.05$), Analysis of Variance (ANOVA) and Tukey's test were used by SPSS v 20.

RESULTS

Potential activity of JB on cell viability

Figure 1, depicts the potential activity of JB on the viability of RAW-264.7 cells. The results of the cell viability experiment indicated that JB did not significantly ($p < 0.01$) cytotoxically affect RAW-264.7 at doses of 0, 2.5, 5, 10, 15, and 20 $\mu\text{M}/\text{mL}$. Consequently, a concentration range of 0-20 $\mu\text{M}/\text{mL}$ was employed for JB following functional research.

Effect of JB on NO production

Figure 2, shows the effect of JB on the generation of NO in the RAW-264.7 cells. As seen in Figure 2, cells treated with LPS alone released much more NO than cells that were not treated. On treatment with JB (15 μM) and Positive control Dexamethasone (Dex) 10 μM the concentration of NO release in the cells was pointedly reduced in comparison to the LPS alone treated.

Effect of JB on ROS production

Figure 3, illustrates the effect of JB on ROS production in the RAW-264.7 cells. The ROS levels in the LPS alone treated cells were considerably high in comparison to the control cells. On

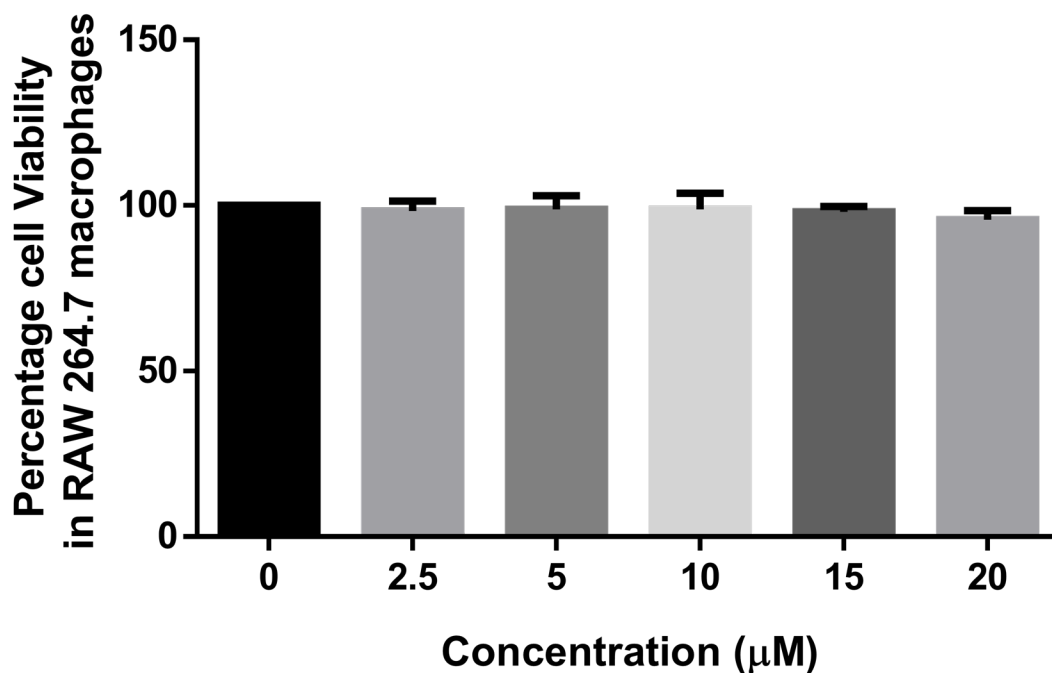


Figure 1: Effects of JB on the viability of Raw264.7 macrophages. An MTT assay was used to assess cell viability after 24 hr of treatment; the values represent the average of three independent experiments.

treatment with JB (15 μ M), the level of ROS drastically reduced when compared to the LPS control group. The cells treated with JB exhibit a similar pattern to the Dex 10 μ M treated cells (positive control).

Effect of JB on Antioxidant Enzymes

The potential outcome of JB on the antioxidant enzyme is shown in Figure 4. The assessment of oxidative stress encompassed tests of SOD, GSH-Px, and CAT, which are the principal constituents of the natural antioxidant defense system. The LPS-induced group exhibited a considerable reduction in the activities of SOD, GSH-Px, and CAT levels in comparison to the control groups. The treatment of JB considerably elevated the CAT, SOD, and

GSH-Px levels in comparison to the LPS group. The cells treated with JB exhibit a similar pattern to the Dex 10 μ M treated cells (positive control).

Potential activity of JB on pro-inflammatory cytokines

Figure 5, depicts the potential of JB on cytokine levels generated by inflammation in LPS-triggered cells. The LPS-triggered cells exhibited notably higher levels of IL-1 β , IL-6, TNF- α , and MCP-1 expression in comparison to the control group. On the other hand, cells treated with JB pointedly reduced MCP-1, TNF- α , IL-6, and IL-1 β expression in comparison to the LPS-treated

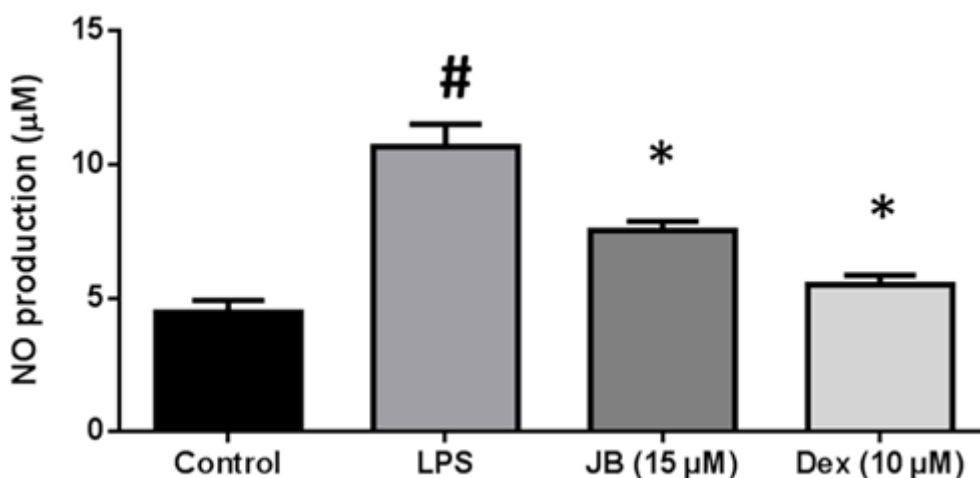


Figure 2: Measurement of Nitric Oxide production (NO). The values are presented as means SD of three independent experiments. # $p < 0.01$, compared to the control group. * $p < 0.05$ compared to the LPS-treated group.

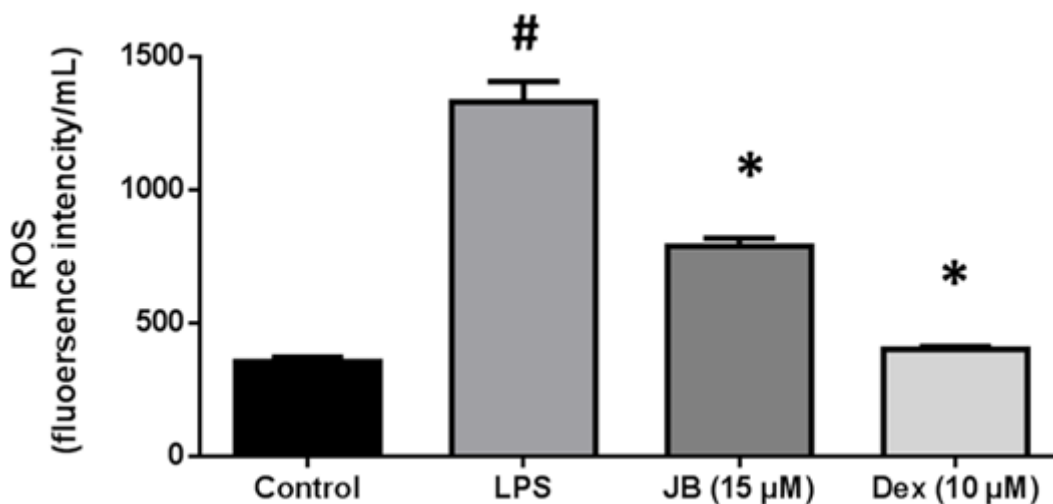


Figure 3: Measurement of Reactive Oxygen Species production (ROS). The values are presented as means SD of three independent experiments. # $p < 0.01$, compared to the control group. * $p < 0.05$ compared to the LPS-treated group.

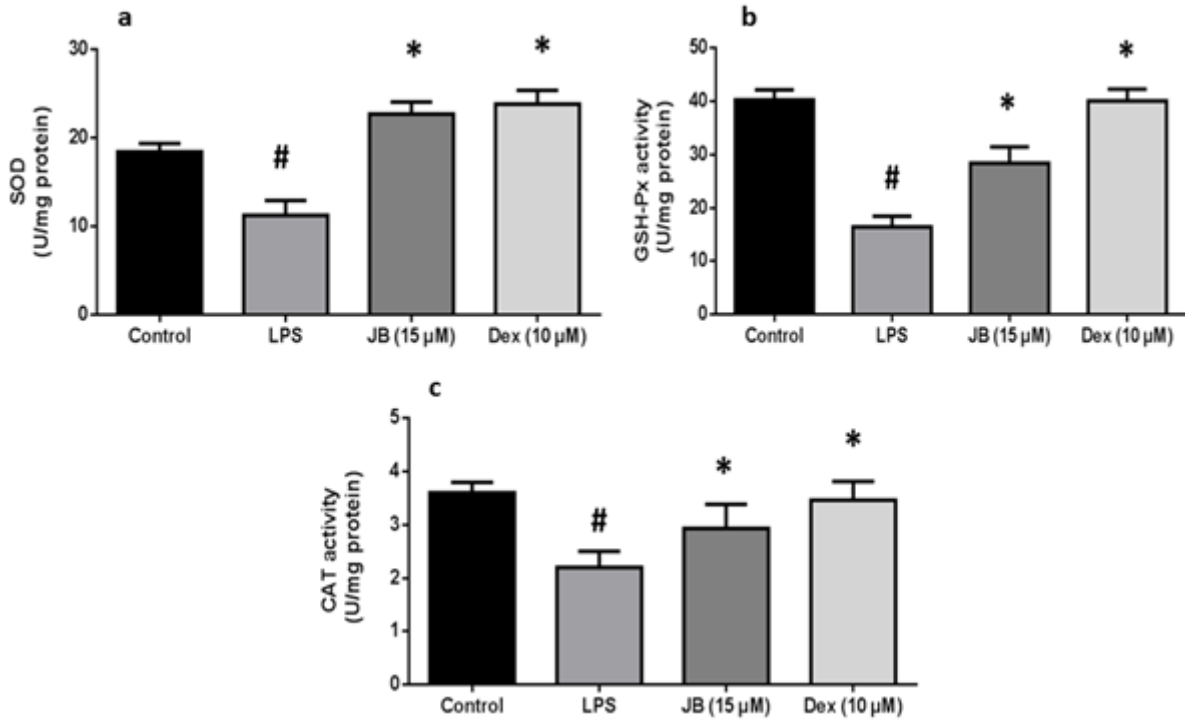


Figure 4: Determination of (a) SOD, (b) GSH-Px, and (c) CAT Activities. The values are presented as means SD of three independent experiments. # $p < 0.01$, compared to the control group. * $p < 0.05$ compared to the LPS-treated group.

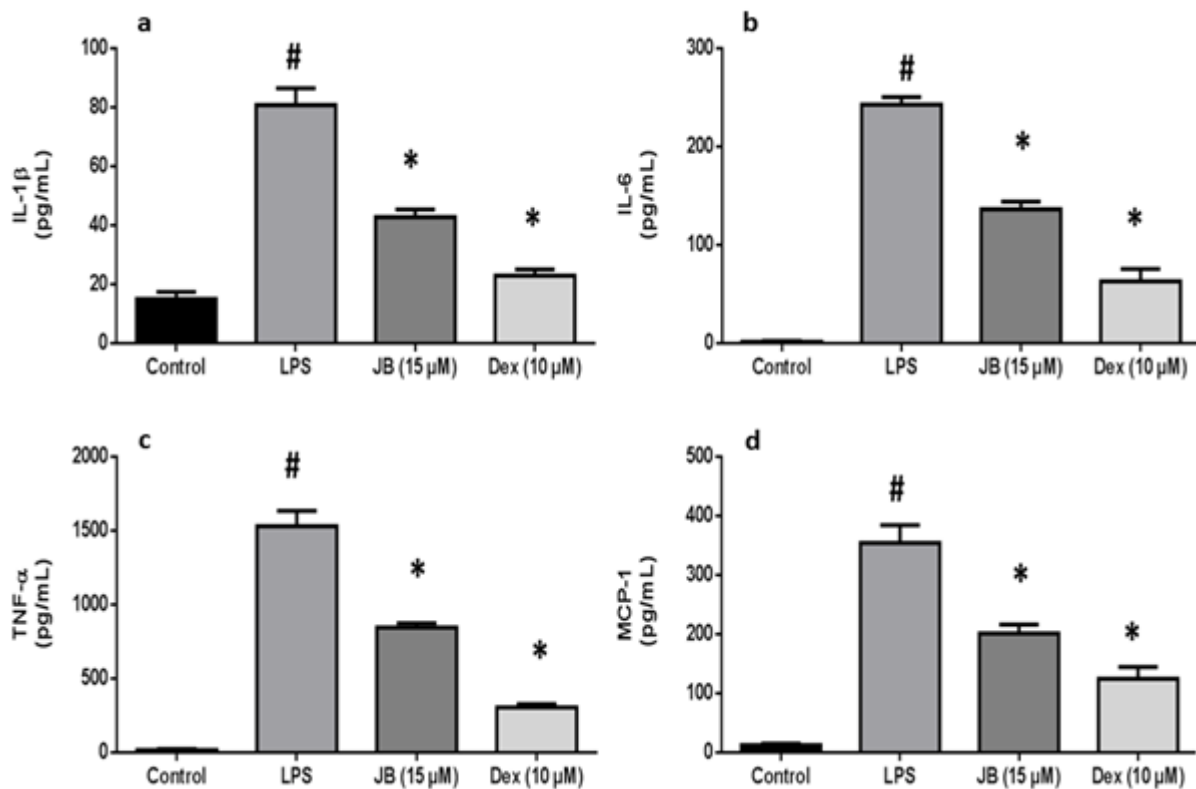


Figure 5: Inhibitory effect of JB on pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells. IL-1 β (a), IL-6 (b), TNF- α (c), & MCP-1 (d). The values are presented as means SD of three independent experiments. # $p < 0.01$, compared to the control group. * $p < 0.05$ compared to the LPS-treated group.

cells. The cells treated with JB exhibit a similar pattern to the Dex 10 μ M treated cells (positive control).

DISCUSSION

The common factor in the onset, development, and complications of illnesses is a persistent pro-inflammatory state marked by excessive generation of ROS. Particularly, ROS are the principal root of cellular injury, and the mitochondria are where they are mostly produced. Under normal circumstances, several enzymes such as catalase, Glutathione peroxidase (GPx), and Superoxide Dismutase (SOD) detoxify these reactive species. Oxidative damage arises and builds up in mitochondria when these enzymes are unable to convert ROS quickly enough, changing the metabolism and functioning of the organelles.^[14] Numerous phytochemicals have been demonstrated to possess strong anti-inflammatory properties; they are especially found in culinary and medicinal plants. Alkaloids, phenolics, terpenoids, and tannins are among the main groups of phytochemicals that can avert illness as well as function as anti-cancerous, anti-inflammatory, anti-microbial, immunity-promoting, detoxifying, anti-oxidant, and neuropharmacological agents. Chemically speaking, triterpenoids, steroid alkaloids, and glycosylated steroids are all considered to be saponins.^[15] The concept that plants may serve as a resource of antioxidants has gained greater traction in recent years since oxidative stress is now recognized as a key contributing factor to the majority of human illnesses, and human antioxidant defense systems are often insufficient to combat the body's level of free radicals.^[16] Potent antioxidant and free radical scavenging properties, together with an anti-inflammatory effect, are often possessed by antioxidant phytochemicals; these properties provide the foundation of further bioactivities and health advantages.^[17]

The present investigation demonstrates that Lipopolysaccharide (LPS) can cause macrophages to become excessively activated, which in turn can cause inflammatory outbursts and oxidative stress. Based on experiments, JB can prevent LPS-induced macrophage inflammation and oxidative damage. In the current investigation, treating the RAW 264.7 cells with JB did not exert any effect on the cell lines. The dosage for further treatments was identified from the experiment. Numerous detrimental events, including infections, damaged cells, and irritants, might cause NO to be activated.^[18] Macrophages are important in the process of inflammatory reactions because they regulate the formation of NO, a chemical that promotes inflammation. Inflammatory disorders may arise from an overabundance of NO production. Consequently, lowering NO generation and expression as well as other NO-related parameters can have a significant effect on a variety of illnesses and inflammation.^[19] Prior research has indicated that jujuboside B limits the generation of NO by LPS-induced cells.^[20] Similarly in our present investigation, the LPS-treated cells exhibited a high level of NO in comparison

to the control cells. On treatment with JB, the level of NO was reduced. This indicates the anti-inflammatory activity of JB.

Oxidative stress is caused by an imbalance in redox resulting from ROS build-up resulting from the endogenous antioxidant response. Inflammation is one of the many additional clinical disorders that is closely linked to increased ROS generation. Intracellular ROS generation is accelerated by the entrance of macrophages, which is facilitated by inflammatory mediators and cytokines. Furthermore, ROS function as auxiliary messengers and engage in the inflammatory signaling cascade.^[21] The ROS level was high in the LPS-only induced cells indicating inflammation and oxidative stress conditions in the cells. On treatment with JB, the level of ROS was significantly ($p < 0.01$) reduced indicating the antioxidant and anti-inflammatory action of JB.

The initial phase of resistance against superoxide radicals, the primary harmful species generated from oxygen, which create Hydrogen Peroxide (H_2O_2) and O_2 (Oxygen), and hence against oxidative stress, is formed by the enzyme superoxide dismutase. By eliminating H_2O_2 produced by the oxidation of organic materials and the auto-oxidation of lipids, catalase mediates its activity.^[22] Other enzymes, including GSH-Px, employ GSH's thiol-reducing ability to mitigate protein and oxidized lipids.^[23] In the current study, the level of all the antioxidant enzymes was significantly ($p < 0.01$) low in LPS-treated cells representing oxidative stress in the cells. When the cells were treated with JB, the level of antioxidant enzymes was considerably enhanced and combated the oxidative stress in the cells.

The study highlights the biological relevance of Reactive Oxygen Species (ROS) and Nitric Oxide (NO) in inflammation, which are key mediators in the pathogenesis of inflammatory diseases like atherosclerosis, arthritis, Chronic Obstructive Pulmonary Disease (COPD), and neurodegenerative diseases like Alzheimer's. Jujuboside B, a compound that significantly reduces ROS and NO levels in LPS-stimulated macrophages, has the potential to alleviate oxidative stress and potentially prevent secondary inflammatory responses. Its upregulation of antioxidant enzymes, such as Superoxide Dismutase (SOD), Glutathione peroxidase (GPx), and catalase, can enhance the cell's intrinsic antioxidant defense mechanisms, reducing oxidative stress and conferring cytoprotective effects.^[24] The findings have broad implications, including therapeutic potential in chronic diseases, neuroprotection, inflammation resolution, and tissue repair.^[25] Jujuboside B could serve as a complementary therapy alongside standard treatments to further reduce oxidative damage and promote better outcomes. The study underscores the importance of understanding the role of ROS and NO in inflammation and the potential therapeutic implications of Jujuboside B.^[26]

The study reveals that Jujuboside B (JB) significantly reduces ROS levels in LPS-stimulated RAW 264.7 macrophages, suggesting it

acts as a potent antioxidant. This reduction is biologically relevant because it can limit oxidative damage and protect cellular structures from harm, breaking the cycle of oxidative stress-induced inflammation. JB helps reinforce the cell's antioxidant defense system, protecting cells from the harmful effects of excessive ROS.^[27] Nitric Oxide (NO) can become problematic in inflammation when overproduced, especially via inducible Nitric Oxide Synthase (iNOS). JB helps mitigate nitrate stress and inflammation by down-regulating iNOS expression, reducing the formation of peroxynitrite, which would otherwise lead to protein and lipid damage, further perpetuating inflammatory processes.^[28] JB enhances the levels or activity of antioxidant enzymes, suggesting it helps restore the cell's redox balance and strengthens the cell's intrinsic defense mechanisms. The Nrf2/HO-1 pathway is a master regulator of antioxidant responses, leading to increased synthesis of detoxifying and antioxidant enzymes. Oxidative stress and inflammation are intimately linked, with ROS activating pro-inflammatory signaling pathways and promoting the production of inflammatory cytokines.^[29]

The foundational component of the human immune system is macrophages. Together with other significant inflammatory mediators, macrophages produce pro-inflammatory cytokines including PGE2 and TNF- α during inflammation.^[30] Cytokines such as TNF- α , IL-1 β , and IL-6 at high concentrations are essential for the onset and spread of the inflammatory cascade.^[31] MCP-1, also termed monocyte chemoattractant protein-1, is a pro-inflammatory cytokine that can control immunological responses. MCP-1 in particular can draw immune cells to inflamed areas. Lipopolysaccharide (LPS) stimulation triggers the signal transduction pathways of macrophages, which in turn increases the production of cytokines and other components connected to inflammation, leading to a variety of illnesses.^[32]

Previous studies reported that JB treatment reduces both ROS and pro-inflammatory cytokine levels, interrupting the ROS-inflammation cycle. JB modulates the production of TNF- α , IL-1 β , and IL-6 through its multifaceted effects on the NF- κ B, MAPK, and Nrf2/HO-1 signaling pathways. By inhibiting key pro-inflammatory pathways and enhancing antioxidant defenses, JB effectively reduces cytokine-driven inflammation. These mechanisms underline the therapeutic potential of JB in diseases where chronic inflammation and oxidative stress play a critical role.^[33] Several limitations exist, including the reliance on a single cell line, the lack of *in vivo* validation, limited mechanistic exploration, and the absence of long-term exposure analysis. Addressing these limitations in future research will help solidify JB's potential as a therapeutic agent and provide a more complete understanding of its effects across different biological systems.

The present study revealed that JB significantly reduced the level of pro-inflammatory cytokine and chemokines of which the level of elevated due to the trigger of LPS in the cells. This reveals the anti-inflammation action of JB. JB has a favorable prospect for

becoming a drug candidate due to the findings of your study due to its powerful anti-inflammatory and antioxidative properties. Its dual facultative capacity towards oxidative stress and inflammation makes it applicable to a variety of diseases posed chronically. However, there is still a long way to go before JB can be actualized as a primary therapy; increasing its bioavailability, post-*in vivo* studies and logistics of preclinical and clinical variants remains work to be done. Due to the potential positive results of JB, it can come in handy when the present-day anti-inflammatory and anti-oxidant treatment options are ineffective.

CONCLUSION

According to our research, JB exhibited antioxidant and anti-inflammation effects on RAW-264.7 cells stimulated by LPS. Cell viability was not affected by the increased concentration of JB. On treatment with JB the level of ROS, NO, and pro-inflammatory cytokines were significantly downregulated. The level of antioxidant enzymes was considerably elevated on treatment with JB. We may thus draw the conclusion that JB can prevent oxidative-stress and inflammation in RAW-264.7 cells and may be proven to be a potent anti-inflammatory and antioxidant drug.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

SOD: Superoxide dismutases; **ELISA:** Enzyme-Linked Immunosorbent Assay; **LPS:** Lipopolysaccharide; **NO:** Nitric oxide; **IL:** Interleukin; **JB:** Jujuboside B; **ROS:** Reactive oxygen species; **MCP-1:** Monocyte chemoattractant protein-1; **TNF:** Tumor necrosis factor; **GSH-Px:** Glutathione peroxidase; **DCFH-DA:** Diacetyldichlorofluorescein diacetate; **DMEM:** Dulbecco's Modified Eagle's Medium; **iNOS:** Inducible nitric oxide synthase; **CO₂:** Carbon dioxide; **BSA:** Bovine Serum Albumin; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

SUMMARY

The ameliorative action of JB against LPS-triggered RAW 264.7 cells was thoroughly examined in the present work. In the course of the study, it decreases the activity of ROS, NO, and pro-inflammatory cytokines, indicating the anti-inflammatory and anti-oxidative properties of JB. Moreover, there was a substantial enhancement in the level of antioxidant enzymes demonstrating JB antioxidant properties. Thus, it is anticipated

that JB will be utilized as a therapeutic drug to reduce inflammatory illness.

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