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# Isorhamnetin attenuated LPS stimulated inflammatory response in RAW 264.7 macrophages via inhibiting Prostaglandin E2 and Cyclooxygenase 2

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**Abstract**

**Background:** Chronic inflammation is widely recognized as a contributing factor to various diseases, such as cancer, cardiovascular diseases, metabolic syndrome, and neurodegenerative diseases. Current anti-inflammatory medications, like non-steroidal anti-inflammatory drugs (NSAIDs), have limitations due to associated side effects and cost. Isorhamnetin, a significant bioactive flavonoid compound primarily found in the fruits of Hippophae rhamnoides L. and the leaves of Ginkgo biloba L., demonstrates diverse pharmacological effects, including its ability to regulate the immune response and potent antioxidant activity. Therefore, the objective of this study is to investigate the impact of Isorhamnetin on the inflammatory response in lipopolysaccharide-induced RAW 264.7 macrophages.

**Materials and methods**: The effect of isorhamnetin on inflammatory mediators, cytokines, and reactive oxygen species was investigated using the RAW264.7 murine macrophage cell line. Cell viability was determined through the MTT assay, wherein RAW macrophages were exposed to LPS (1μg/ml) and treated with varying concentrations of isorhamnetin for 24 hours. Additionally, the effect of isorhamnetin on the production of nitric oxide and reactive oxygen species (ROS) was examined. Gene expression analysis was conducted using qPCR, and protein levels of pro-inflammatory cytokines were measured through enzyme-linked immunosorbent assay (ELISA).

**Results:** The findings indicated that Isorhamnetin had a protective effect against LPS-induced toxicity, leading to enhanced cell viability at higher concentrations. Moreover, there was a substantial decrease in the production of nitric oxide and reactive oxygen species (ROS). Gene expression analysis demonstrated a significant downregulation of pro-inflammatory cytokines TNF-α, Cox-2, and iNOS in comparison to the LPS-only group following Isorhamnetin treatment. ELISA analysis also showed a marked reduction in protein levels of PGE2 and IL-6 when compared to the LPS-alone group.

**Conclusion:** The results of the current investigation indicate that isorhamnetin exhibits a strong anti-inflammatory effect against with LPS-induced inflammation in RAW 264.7 macrophages.

**Keywords:** Inflammation**,** anti-inflammatory, isorhamnetin, lipopolysaccharide, RAW 264.7, Prostaglandin.

## Introduction

Inflammation, a common defensive response initiated by tissue injury or infection, serves to combat invaders and eliminate damaged host cells [1]. The increasing global prevalence of inflammatory disorders poses a significant public health challenge. Severe inflammatory conditions play a crucial role in the mortality associated with sepsis, shock,

trauma, pneumonia, repeated transfusions, and pancreatitis [2, 3]. The macrophage, a crucial immune cell, plays a central role in host defenses against pathogen infections, particularly during inflammation. The RAW 264.7 cells, derived from a mouse tumor, are a widely used murine macrophage cell line in scientific investigations. Recognized for their applicability in researching immunological responses, inflammation, and various biological processes, these cells provide a practical model for exploring macrophage behavior and functions under different experimental conditions, sharing key characteristics with primary macrophages [4].

Lipopolysaccharide (LPS), a crucial component of Gram-negative bacteria, initiates the inflammatory process, leading to tissue damage, neutrophil infiltration, and cytokine production in various cell types. The pro-inflammatory cytokines induced by LPS contribute to the development of numerous inflammatory diseases [5, 6]. LPS, a well-established potent activator of monocytes/macrophages, has been extensively employed in inflammation research due to the diverse inflammatory responses it triggers through TLR4 signaling. Thus, modulating the functions of monocytes and macrophages proves to be an effective anti-inflammatory approach, including the use of nanotherapies [7, 8]. LPS-induced RAW 264.7 macrophages are cells exposed to lipopolysaccharide (LPS), capable of eliciting a significant immunological response in macrophages. This replicates a bacterial infection and induces the release of inflammatory mediators, such as cytokines and nitric oxide. This model is frequently utilized to study inflammation, immunological responses, and macrophage behavior in the presence of bacterial components [9, 10].

In recent years, there has been a significant surge in the utilization of plant-derived medications in the therapeutic domain. Plants house an extensive array of over 4000 flavonoids, many of which exhibit therapeutic qualities [11, 12, 13]. Isorhamnetin, classified as a flavonoid (specifically a monomethoxy flavanol), is a plant compound present in various fruits and vegetables, including onions, green peppers, berries, and pears. Belonging to the flavanol subgroup, it shares structural links with quercetin and kaempferol. Studies have demonstrated that Isorhamnetin possesses a broad spectrum of pharmacological effects, particularly on cardiovascular disorders [14], different types of cancers [15] as well as the ability to prevent neurodegenerative disorders such as Alzheimer's disease [16]. Furthermore, it demonstrates pharmacodynamic effects in addressing anti hyperuricemia [17] and anti-pulmonary fibrosis [18]. The pharmacological actions of Isorhamnetin are linked to its modulation of the NF-κB, PI3K/AKT, MAPK, and various other signaling pathways, along with their downstream factors [18].

Isorhamnetin's anti-inflammatory properties extend to mitigating inflammatory responses in various conditions, including osteoarthritis and periodontitis. The underlying mechanism of anti-inflammatory potential has shown to be associated with the regulation of inflammatory mediators, cytokines, and reactive oxygen species (ROS). By activating the Nrf2 signaling pathway, Isorhamnetin demonstrates its ability to alleviate LPS-induced inflammation in human gum fibroblasts [19]. Furthermore, Isorhamnetin exhibits anti-arthritic properties, showcasing anti-inflammatory and cartilage-protecting effects in IL-1β stimulated cartilage cells [20]. Its anti-inflammatory prowess extends to inhibiting the NF-κB signaling pathway, consequently reducing the release of inflammatory factors and ROS [21]. In light of the given background, the aim of this study was to assess the potential anti-inflammatory

efficacy of Isorhamnetin against LPS-induced RAW 264.7 macrophages and to elucidate the mechanism of action.

## Materials and Methods

### Chemicals

Lipopolysaccharide (LPS), Phenol-free Dulbecco's modified Eagle medium (DMEM), MTT, Dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), and antibiotic-antimycotic solution (100U penicillin, 100µg streptomycin, and 0.25µg amphotericin B per ml) were acquired from Sigma-Aldrich. Fetal bovine serum was obtained from GIBCO/BRL Invitrogen. Isorhamnetin was procured from TCI Chemicals, India.

### Cell culture

RAW 264.7 cells from NCCS, Pune, at Passage 16, were obtained and cultured in phenol red-free Dulbecco's modified Eagle medium (DMEM). The medium was supplemented with 100units/ml penicillin, 100µg/ml streptomycin, and 10% heat-inactivated fetal bovine serum, and the cells were maintained at 37°C with 5% $CO_2$. On reaching 80-90% confluency, the cells were washed using DMEM and then 0.25% trypsin-EDTA was added. The detached cells were resuspended in DMEM and plated for further experiments at a concentration of 2 x $10^6$ cells/ml.

### Cell proliferation assay or MTT assay

The MTT assay was employed to evaluate the cytotoxicity of Isorhamnetin. Cells were seeded at a density of $5 \times 10^4$ cells/well in a 96-well plate and incubated for 24 hours. Subsequently, the medium was replaced with 100µl solutions containing Isorhamnetin at varying concentrations (5, 10, 20, 40, 80, 160, and 320µM/well) and Lipopolysaccharide (LPS) at a concentration of 1µg/ml. The cells were further incubated for 24 hours, with untreated cells serving as the control group. Following the treatment period, the culture media were removed, and 50µl of MTT solution (5mg/ml in PBS) was added to each well. After a 4-hour incubation at 37°C in a CO2 incubator, the MTT solution was discarded, and the resulting formazan crystals were dissolved in 150µl of DMSO by thorough pipetting. The absorbance of the purple-blue formazan dye was measured at 570nm using an ELISA reader (BIORAD). The optical density of each sample was compared to the control, and graphs were plotted. The $IC_{50}$ for Isorhamnetin was determined through linear regression analysis.

### Estimation Nitric oxide (NO) (Green et al., 1982) [22]

Nitrite, a stable oxidized derivative of nitric oxide (NO), was detected in cell culture media using Griess reagent. Briefly, 50µl of cell culture supernatant was added to 50 µl of 1% (w/v) sulphanilic acid in 5% (v/v) phosphoric acid within a 96-well plate. This mixture was then incubated for 10 minutes at room temperature. Subsequently, 50 µl of 0.1% (w/v) N-1-naphthylethylenediamine HCl in distilled water was added and incubated for an additional 10 minutes at room temperature. The optical density at 540 nm was measured using a microplate reader, and the NO concentration was determined by comparing the results with a $NaNO_2$ (0–100 µM) standard curve. The results were expressed as inhibition of NO production compared to the control using:

$$([\text{nitrite}]_c - [\text{nitrite}]_t) / [\text{nitrite}]_c$$

where $[\text{nitrite}]_c$ and $[\text{nitrite}]_t$ is the nitrite concentration in the control and test sample, respectively.

**RNA Isolation and q - PCR Analysis**

RAW macrophages were treated with 30µM, 60µM and 90µM of Isorhamnetin with 1µg/ml of LPS and incubated for 24h. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and 2µg of RNA was used for complementary DNA synthesis. Quantitative real-time polymerase chain reaction (q-PCR) was performed in an ABI 7500 Real-Time System with SYBR Green PCR Master Mix (Takara). Reactions were initiated with an initial incubation at 50°C for two minutes and 94°C for 10 min, followed by 40 cycles of 94°C for 5s, 60°C for 15s, and 72°C for 10s. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The specific primer sequences used were given below:

INOS: Forward: 5′-ATGTCCGAAGCAAACATCAC-3′
Reverse: 5′-TAATGTCCAGGAAGTAGGTG-3′
COX-2: Forward: 5′-CAGCAAATCCTTGCTGTTCC-3′
Reverse: 5′-TGGGCAAAGAATGCAAACATC-3′
TNF-α: Forward: 5′-ATG AGC ACA GAA AGC ATG ATC-3′
Reverse: 5′-TAC AGG CTT GTC ACT CGA ATT-3′

β - Actin was used as an internal reference gene between different samples.

**Measurement of PGE2 and COX-2 Levels by ELISA analysis**

RAW264.7 cells ($5\times 10^4$ cells/well) were seeded onto 96-well plates and exposed to LPS (1µg/mL) or left untreated. Following a one-hour incubation, cells were subjected to Isorhamnetin treatment at concentrations of 30nM, 60nM, and 90nM for a duration of 24 hours at 37°C. Subsequently, the cultured cells were utilized for the assessment of Prostaglandin E2 (PGE2) and Cyclooxygenase 2 (COX-2) concentrations through ELISA kits, adhering to the provided guidelines from Shanghai Coon Koon Biotech Co Ltd.

**Statistical analysis**

Data obtained from the experiments were expressed as Mean ± SEM. The Statistical analysis of the difference between the groups was evaluated by Dunnett's following one way ANOVA Post Hoc comparisons in Graph pad Prism 7.0 software version. $p<0.001$, $p<0.01$ and $p<0.05$ were considered to be statistically significant.

**Results**

**Effect of Isorhamnetin against LPS induced cytotoxicity**

The cytotoxicity of LPS and the effect of Isorhamnetin was evaluated by MTT assay. The results of the cytotoxicity assay revealed that LPS, a bacterial endotoxin, induces cell toxicity, resulting in only 23% cell viability compared to 100% cell viability in the control group. On the other hand, the Isorhamnetin treated groups maintained the cell viability above 80% at concentrations 160nM and 320nM, attenuating the toxicity induced by LPS. Even at the lowest of 5nM the cell viability was found to be 31.31%. The efficacy of Isorhamnetin was found to be dose-dependent. The $IC_{50}$ of the Isorhamnetin was calculated by linear regression analysis and was found to be 62.67nM.

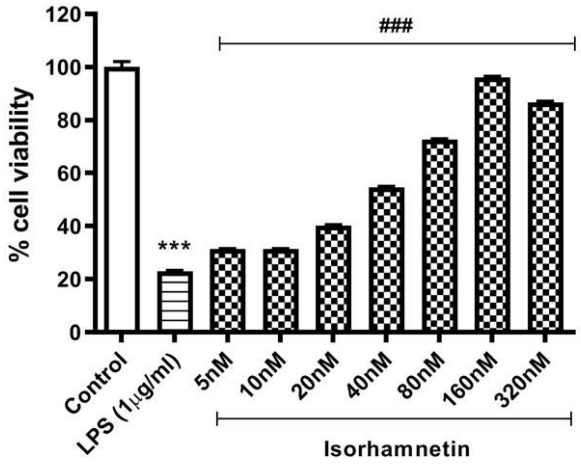


**Figure 1: Effect of Isorhamnetin on RAW264.7 macrophages. Different concentrations of Isorhamnetin (5nM, 10nM, 20nM, 40nM, 80nM, 160nM & 320nM) were treated along with LPS (1µg/ml) stimulation. Bar graph shows percentage cell viability of LPS-stimulated Isorhamnetin treated cells. Data are expressed as Mean ±SEM of triplicate values. ***p <0.001 Vs Control group; ###p<0.001, ##p<0.01, #p<0.05 Vs LPS alone stimulated group.**

**Effect of Isorhamnetin on nitric oxide production and iNOS expression in LPS induced RAW macrophages**

Nitric oxide (NO), a significant contributor to oxidative stress, is generated by inducible nitric oxide synthase (iNOS). Thus, the impact of Isorhamnetin on nitric oxide production was assessed in LPS-stimulated RAW macrophages through the griess assay. Additionally, the influence of Isorhamnetin on the induction of iNOS gene expression was examined. The findings indicate a gradual decrease in nitric oxide production with increasing concentrations of Isorhamnetin. Notably, concentrations of 5nM and 10nM exhibited significant inhibition ($p<0.05$ and $p<0.01$, respectively) of NO production compared to the LPS-induced group alone. Furthermore, concentrations ranging from 20nM to 320nM demonstrated high significance ($p<0.001$) in inhibiting NO when compared to the LPS-alone group. Considering the IC50 of 62.67nM determined through linear regression analysis, three concentrations (30nM, 60nM, and 90nM) were selected for gene expression and ELISA analysis to assess the dose-response anti-inflammatory effect of Isorhamnetin.

Further evaluation of gene expression analysis of iNOS exhibited that in the Isorhamnetin group of all the three concentrations (30nM, 60nM and 90nM), the iNOS gene, responsible for nitric oxide production, was downregulated, while the LPS alone group exhibits **2A** -fold change compared to the control group. This signifies that Isorhamnetin mitigates oxidative stress by suppressing the expression of the iNOS gene.

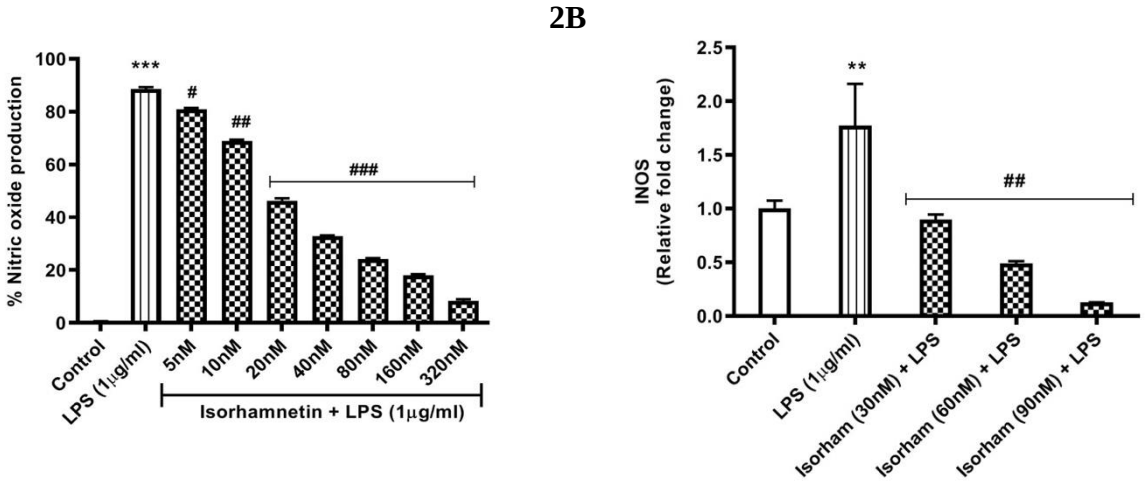


**Figure 2: Effect of Isorhamnetin on (A) nitric oxide production and (B) iNOS gene expression in RAW264.7 macrophages. Different concentrations of Isorhamnetin (5nM, 10nM, 20nM, 40nM, 80nM, 160nM & 320nM) were treated along with LPS (1μg/ml) stimulation. Bar graph 2A shows percentage inhibition of NO production in LPS-stimulated Isorhamnetin treated cells. Bar graph 2B shows mRNA expression of iNOS in LPS-stimulated Isorhamnetin treated cells at concentrations (30nM, 60nM & 90nM). Data are expressed as Mean ±SEM of triplicate values. ***p <0.001 Vs Control group; ###p<0.001, ##p<0.01, #p<0.05 Vs LPS alone stimulated group.**

**Effect of Isorhamnetin on tumour necrosis factor - α and IL-1β gene**

To assess the effect of Isorhamnetin on RAW macrophages stimulated with lipopolysaccharide (LPS), qRT-PCR analysis to examine the expression of pro-inflammatory cytokines, specifically TNF-α and IL-1β was carried out. The gene expression analysis demonstrated a significant ($p<0.001$) 2-fold increase in the mRNA expression of TNF-α and IL-1β following LPS treatment. Interestingly, co-administration of Isorhamnetin at concentrations of 30, 60, and 90nM resulted in a significant ($p<0.001$) reduction in TNF-α and IL-1β levels compared to cells treated with LPS alone (Figure 3). These findings suggest that the flavonoid Isorhamnetin effectively attenuates the synthesis of pro-inflammatory cytokines in activated macrophages.

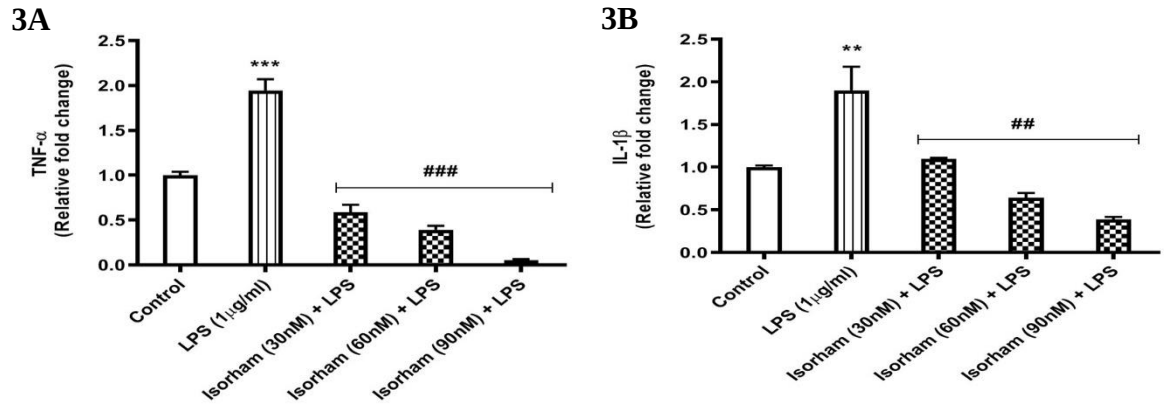


**Figure 3: Effect of Isorhamnetin on (A) TNF-α and (B) IL-1β gene expression in RAW264.7 macrophages. Different concentrations of Isorhamnetin (30nM, 60nM and 90nM) were treated along with LPS (1μg/ml) stimulation. Bar graph 3A shows mRNA expression of TNF-α in LPS-stimulated Isorhamnetin treated cells. Bar graph 3B shows mRNA expression of IL-1β in LPS-stimulated Isorhamnetin treated cells. Data are expressed as Mean ±SEM of triplicate values. ***p <0.001 Vs Control group; ###p<0.001, ##p<0.01, #p<0.05 Vs LPS alone stimulated group.**

**ELISA analysis of PGE2 and COX 2 levels in LPS stimulated RAW macrophages**

In order to further explore the anti-inflammatory activity of isorhamnetin in LPS stimulated macrophages, the levels of PGE2 and COX-2 was evaluated by ELISA analysis. The ELISA analysis results showed LPS stimulation in macrophages has significantly ($p<0.001$) increased the levels of PGE2 and COX2 whereas the treatment with Isorhamnetin at different concentrations of 30nM, 60nM and 90nM has markedly ($p<0.001$) reduced the levels of PGE2 an COX2 in LPS stimulated macrophages.

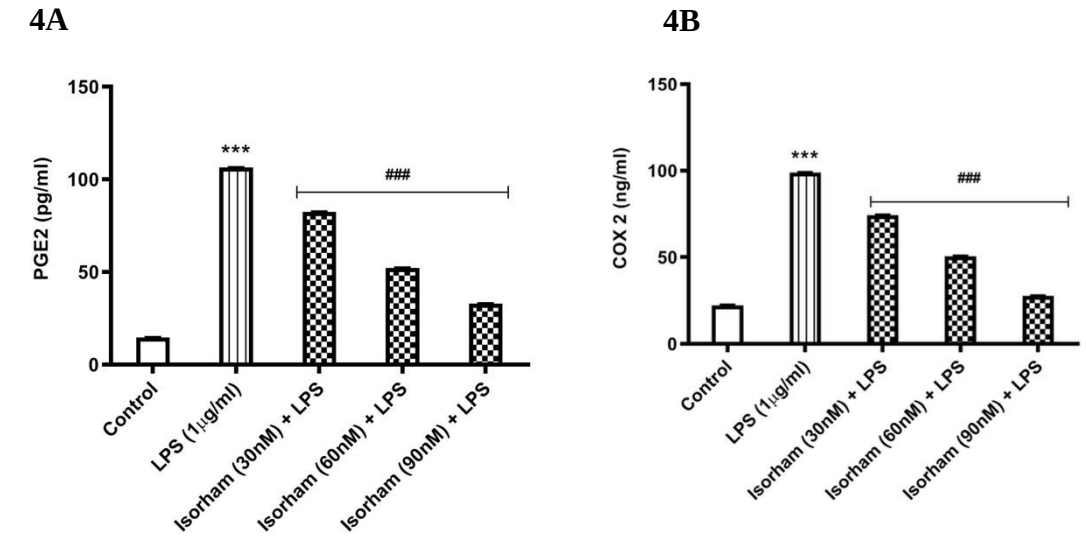


**Figure 4: Effect of Isorhamnetin on (A) PGE2 and (B) COX-2 protein levels in RAW264.7 macrophages. Different concentrations of Isorhamnetin (30nM, 60nM and 90nM) were treated along with LPS (1μg/ml) stimulation. Bar graph 4A shows ELISA analysis of PGE2 in LPS-stimulated Isorhamnetin treated cells. Bar graph 4B shows ELISA analysis of COX-2 in LPS-stimulated Isorhamnetin treated cells. Data are expressed as Mean**

**±SEM of triplicate values. ***p <0.001 Vs Control group; ###p<0.001, ##p<0.01, #p<0.05 Vs LPS alone stimulated group.**

## Discussion

The investigation explored the anti-inflammatory properties of Isorhamnetin, a flavonoid derived from the metabolism of quercetin, using RAW 264.7 macrophages. The findings indicated that Isorhamnetin, in a dose-dependent manner, significantly inhibited the production of nitric oxide, a key player in oxidative stress. In the group treated with Isorhamnetin, there was a noticeable reduction in the expression of the iNOS gene, which is responsible for nitric oxide synthesis, suggesting Isorhamnetin's ability to reduce oxidative stress. Furthermore, the expressions of TNF-α and IL-1β genes were downregulated in the Isorhamnetin-treated groups, showing its potential to lessen LPS-induced inflammation. The protein levels of PGE2 and COX2 were also significantly reduced with Isorhamnetin treatment, highlighting its efficacy in suppressing inflammatory markers and confirming its safety for potential use in anti-inflammatory therapies.

Previous research has shown that chronic inflammation is associated with an increase in inflammatory mediators like iNOS, PGE2, and COX2, as well as pro-inflammatory cytokines such as Interleukin-1β, TNF-α, and Interleukin-6 [23, 24]. Additionally, it has been established that iNOS and COX2 are crucial in raising nitric oxide and Prostaglandin levels, which are significant contributors to inflammatory pathogenesis [25]. Our study observed that LPS induction resulted in the upregulation of iNOS, TNF-α, and IL-1β, aligning with findings by Hwang et al. [26], who also reported an increase in iNOS following LPS induction. Moreover, treatment with Isorhamnetin significantly reduced levels of inflammatory mediators and pro-inflammatory cytokines. Li Y et al. (2016) previously investigated the anti-inflammatory effects of Isorhamnetin in LPS-induced RAW 264.7 cells and an animal model, showing that cytokine levels (IL-6, TNF-α, IL-1β) were measured, and NF-κB protein phosphorylation was examined in an acute lung injury mouse model that interrupted NF-κB signaling [27]. Similarly, *in vitro* treatment with Isorhamnetin, as well as Opuntia ficus-indica (a plant extract from Mexico rich in Isorhamnetin), significantly reduced TNF-α, IL-1β, and IL-6 levels in RAW 264.7 cells and an ear edema model in rodents [28]. Consistent with previous findings, our study also demonstrated that Isorhamnetin decreased NO production and downregulated the mRNA expression of iNOS, TNF-α, and IL-1β. A prior in silico study indicated that polyphenolic compounds such as Stachydrine and Sakuranetin significantly inhibited IL-6 and TNF-α [29]. These results suggest that phytoconstituents like polyphenols and flavonoids have considerable anti-inflammatory potential. Supporting this hypothesis, our study showed that Isorhamnetin could be a promising therapeutic agent for preventing inflammatory diseases.

The present study revealed that Isorhamnetin significantly decreased the protein levels of PGE2 and COX2. Macrophages release endogenous nitric oxide (NO), which stimulates prostaglandin (PG) production. Conversely, cyclooxygenase (COX) activation modulates the L-arginine-NO pathway, while COX inhibition decreases nitric oxide synthase (NOS) activity. These findings indicate a cross-talk between the NO and PG pathways. Anti-inflammatory agents that reduce NO and PG production by simultaneously inhibiting inducible NOS (iNOS) and COX-2 may offer therapeutic benefits in treating inflammatory and infectious diseases [30,

31]. Our study demonstrated that Isorhamnetin inhibited lipopolysaccharide (LPS)-induced NO and PGE2 production in RAW macrophages by reducing the expression of iNOS and COX-2. This suggests that Isorhamnetin may regulate NO and PGE2 production by influencing the transcription of iNOS and COX-2, which are elevated by LPS treatment. Further research is necessary to fully understand the safety profile and medicinal effectiveness of Isorhamnetin. As studies progress, its potential in treating inflammatory disorders may become more evident. However, comprehensive research, including clinical trials, is crucial to establish its safety, optimal dosage, and overall efficacy in managing inflammatory conditions.

**Conclusion**

In essence, the data suggests that isorhamnetin demonstrates anti-inflammatory effects in RAW 264.7 macrophages induced by LPS. Isorhamnetin shows promise in mitigating inflammation by adjusting pro-inflammatory mediators, diminishing NO production, suppressing pro-inflammatory cytokines and COX-2 expression.

**CONFLICT OF INTEREST**

There is no conflict of interest.

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