



Pharmacological Evaluations of Select Herbal Extracts on *TLR7/8*-induced Cytokine and Chemokine Production in Macrophage-like Cells

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ABSTRACT

Inflammation is an innate immune response triggered by harmful stimuli, such as pathogens, tissue injury, or toxins. The purpose is to eliminate the source of infection and initiate the healing process. However, an excessive acute inflammatory response can lead to severe and life-threatening complications, as seen during recent pandemics. In the context of viral infections, the activation of the *TLR7/8* signaling pathway has been implicated in excessive cytokine secretion. In this study, we aimed to replicate the perturbed inflammatory environment resulting from the activation of the *TLR7/8* specific agonists, imiquimod, and resiquimod.

We utilized macrophage-like cells, as macrophages are the first responders during infections and secrete key pro-inflammatory cytokines (*TNF-α*, *IL-6*, and *IL-1β*) to recruit immune cells to the site of infection. Herbal medicines have been traditionally used for centuries to enhance respiratory immune function. In the present study, we employed a prophylactic approach, where macrophage-like THP1 cells, differentiated with PMA, were pre-treated with select herbal extracts/formulations prior to *TLR7/8* activation in the presence of agonists.

Several medicinal plants and formulations known for their therapeutic potential in respiratory ailments were investigated, including *Withania somnifera*, *Tinospora cordifolia*, *Glycyrrhiza glabra*, and AYUSH-64, an herbal formulation. The gene expression and corresponding secreted levels of various inflammatory mediators were measured using RT-PCR and ELISA methods, respectively, in treated and untreated differentiated THP1 cells induced with *TLR7/8* agonists. Comparatively, the gene expression of inflammatory markers was significantly higher in resiquimod-induced cells than in imiquimod-treated cells. Notably, *Withania somnifera* demonstrated pronounced prophylactic efficacy compared to other herbs/formulations, as evidenced by reduction in expression of majority of investigated inflammatory marker genes.

Keywords: Imiquimod; Resiquimod; Aqueous herbal extracts; *Withania somnifera*; Inflammation; THP1 cells

INTRODUCTION

Excessive, acute, and uncontrolled release of pro-inflammatory cytokines into circulation during the recent COVID-19 pandemic resulted in severe life-threatening complications and even death in many cases. Such a storm of cytokines may lead to an increased flux of immune cells (macrophages, neutrophils, and T cells) to the site of infection eventually causing more damage than repair [1]. Herbal therapies have been used in traditional medicine for centuries and are also being explored for their potential benefits in cytokine storm management. While the research on specific herbal treatments for cytokine storm is limited, some herbs have shown

immunomodulatory and anti-inflammatory properties that may help to alleviate the excessive immune response.

Administration of *TLR7/8* agonists, such as imiquimod or resiquimod, has been associated with the development of cytokine storm [2,3]. Among others, *TNF-α*, *IL-6*, and *IL-1β* are the key pro-inflammatory cytokines which drive the excessive secretion of cytokines. Their increased levels are often correlated with the severity of cytokine storm which may eventually lead to tissue damage and organ dysfunction [4-6]. *TNF-α* has been correlated to pro-inflammatory response mediated by *IL-1β* and *IL-6*. It is found to be involved in the regulation of inflammatory responses,

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infectious diseases, and malignant tumors [7]. Higher levels of *TNF- α* were found in severe COVID-19 patients [4]. Among other immune cells, monocytes and macrophages are the main source of these pro-inflammatory cytokines [8]. Human THP1 cells are monocyte-derived macrophage-like cells which mimic the macrophage functions and therefore are routinely used in *in-vitro* cell models in basic research. Such cell-based models were instrumental during the recent pandemic where they not only helped in disease understanding but were also the first line preclinical models to screen a large number of drug candidates for their efficacy and safety. THP1 cells differentiate into macrophage-like cells when incubated in the presence of PMA. *TLR7/8* specific signaling was induced in the presence of specific agonists and measured in terms of gene expression as well as circulating levels of various inflammatory genes.

We have evaluated a few select herbal extracts that have traditionally been used over centuries in therapeutics to cure different infectious as well as immunocompromised conditions. A similar line of investigations has been reported with other herbs or herbal oils using different disease models [9,10]. *Syzygium aromaticum*, *Piper nigrum*, *Cimicifuga racemosa*, *Zanthoxylum asiaticum*, *Camellia sinensis*, *Curcuma longa*, and *Tribulus terrestris* were found to be effective in COVID-19 therapeutics [11]. Similarly, the use of oil formulations such as Anu oil and Til tailya in the management of SARS-CoV-2 was explored where the authors observed a significant reduction in viral load as an effect of treatment with Anu oil [10].

In the present investigation, we have specifically examined the efficacy of *Withania somnifera*, *Tinospora cordifolia*, *Glycyrrhiza glabra*, and AYUSH-64 in mitigating the inflammatory response triggered by *TLR-7/8* induction in the presence of the ligands imiquimod and resiquimod. While the first three are individual herbs, AYUSH-64 is an herbal formulation comprising a combination of four different herbs (*Alstonia scholaris*, *Caesalpinia crista*, *Picrorhiza kurroa* and *Swertia chinata*), each contributing to the overall therapeutic benefits. To assess the prophylactic effects of these herbs/formulations, we employed a macrophage-like cell line model. Our objective was to identify the herb or formulation that exhibited the highest efficacy in either suppressing the *TLR7/8*-induced inflammatory response by promoting an anti-inflammatory environment. We evaluated various inflammatory gene markers, including cytokines and chemokines, both at the gene expression level and their secreted levels in the supernatant. To analyse gene expression, we extracted RNA from the cell lysates and employed the RT-PCR method. Simultaneously, we measured the secreted cytokine levels in the culture supernatant using the ELISA method. These analyses enabled us to determine the impact of the tested herbs/formulations on the expression of inflammatory genes and the corresponding secretion of cytokines.

METHODOLOGY

Cell culture

THP1 is a spontaneously immortalized monocyte-like cell line, derived from the peripheral blood of a child suffering from acute monocytic leukemia [12]. THP1 cell line was purchased from ATCC (ATCC#TFB-202) and cultured as per ATCC recommendations. Briefly, RPMI 1640 (Gibco) supplemented with 10% FCS (Hyclone) and 1X penicillin (stock concentration=100 U/mL), and streptomycin (stock concentration=100 μ g/mL) were used to culture the cell line. Cells were maintained in complete

media at 37°C in humidified conditions with 5% CO₂. Trypan blue was used to confirm cell viability. THP1 cells were seeded in the presence of PMA (5 ng/mL) and allowed to differentiate for 48 hours and subsequently used.

Preparation of the herbal extracts/formulations

Powdered form of well-characterized herbal extracts- *W. somnifera*, *T. cordifolia*, *G. glabra* and A64, prepared under the GMP conditions, were obtained from the National Medicinal Plants Board (NMPB), Ministry of AYUSH, Government of India. A stock solution of 4 mg/mL was prepared in the RPMI medium [13]. RPMI medium (1 mL) was added to 4 mg of each extract/formulation powder and kept in dark for overnight shaking at room temperature. Subsequently, the extract solutions were centrifuged at 10,000 rpm for 10 minutes and the supernatant was passed through a 0.22 μ m syringe filter in sterile environment. This supernatant was assumed to be an absolute concentrate of the herbal extract and was used for investigations in this study. All stocks were prepared fresh for each experiment and the filtered stocks were saved at 4°C in dark.

Cell viability assay

Cell viability in the presence and absence of herbal extracts/formulations was evaluated by MTT assay. Differentiated THP1 cells were treated with various concentrations of herbal extracts (100 μ g/mL, 300 μ g/mL, and 1000 μ g/mL). After 24 hours of treatment, cells were incubated in dark with 0.5 mg/mL MTT (Sigma#M2128) for 4 hours at 37°C. The culture supernatant was gently removed and replaced with 100 μ L DMSO per well (Sigma#276855). The cells were allowed to incubate in dark at RT with constant shaking for 30 minutes. Subsequently, the absorbance was measured with a microplate reader (Molecular Devices, Spectramax M2) at 570 nm wavelength. Dexamethasone (Selleck Chemicals#S1322) (1 μ M) was used as an internal reference. Latter is a steroidal drug with powerful anti-inflammatory properties.

qPCR to study gene expression of inflammatory mediators

Differentiated THP1 cells (1 million cells/well), seeded in a 6-well plate, were pre-treated for 60 minutes with herbal extracts/formulations at different concentrations (3 μ g/mL, 10 μ g/mL, 30 μ g/mL, 100 μ g/mL, and 300 μ g/mL). Subsequently, *TLR7/8* agonists (Imiquimod (Tocris#3700/50)/Resiquimod (Sigma#SML1096), respectively) were added to induce TLR signaling. Post induction, the cells, and culture supernatants were collected separately. Total RNA was extracted using a Trizol reagent (Sigma#T9424). Qualitative and quantitative RNA analysis was done on a Multiskan GO plate reader (Thermo Scientific). Reverse transcription of 250 ng RNA was done using the high-capacity cDNA reverse transcription kit (Applied Biosystems#4368814). For the real-time quantitative PCR, the cDNA was amplified using Power up SYBR Green mix (Applied Biosystems#A25742) following the standard protocol for the dye on QuantStudio 6 Real-Time PCR System (Applied Biosystems). Cycling conditions were UDG activation at 50°C for 2 minutes, Hold Dual-Lock™ DNA polymerase at 95°C for 2 minutes, and 40 cycles of Denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute each. For each reaction 300 nM of forward and reverse primer mix was used (Table 1). The relative mRNA expression data were analysed using the $\Delta\Delta$ Ct method and the values were normalized to beta-actin mRNA levels. The data was expressed with respect to the fold change as compared to the untreated control.

Table 1: Human specific sequences of Forward and Reverse primers for various inflammatory genes

S.No.	Primer	Sequence
1	<i>bActin</i>	Forward: 5'-AGAGCTAGGAGCTGCCTGAC-3' Reverse: 5'-AGCACTGTGTTGGCGTACAG-3'
2	<i>TNF-α</i>	Forward: 5'-TGGGATCATTGCCCTCTTGAG-3' Reverse: 5'-TCTAAGCTTGGGTTCCGACC-3'
3	<i>MCP1</i>	Forward: 5'-ACTGCACTCCTGGTTGTCCT-3' Reverse: 5'-CGGCACAGATCTCCTTATCC-3'
4	<i>IL-6</i>	Forward: 5'-TACCCCCAGGAGAAGATTCC-3' Reverse: 5'-TTTTCTGCCAGTGCCTCTTT-3'
5	<i>IL-8</i>	Forward: 5'-TCTGCAGCTCTGTGTGAAGG-3' Reverse: 5'-AATTTCTGTGTTGGCGCAGT-3'
6	<i>CCL5</i>	Forward: 5'-CGCTGTCATCCTCATTGCTA-3' Reverse: 5'-GAGCACTTGCCACTGGTGTA-3'
7	<i>IL-10</i>	Forward: 5'-TGCCTTCAGCAGAGTGAAGA-3' Reverse: 5'-GGTCTTGGTTCTCAGCTTGG-3'
8	<i>IL-1β</i>	Forward: 5'-GGGCCTCAAGGAAAAGAATC-3' Reverse: 5'-TTCTGCTTGAGAGGTGCTGA-3'
9	<i>CXCL-10</i>	Forward: 5'-CTGTACGCTGTACCTGCATCA-3' Reverse: 5'-TTCTTGATGGCCTTCGATT-3'

Cytokine measurement in the culture supernatants

Inflammatory cytokines/chemokines levels were determined in the culture supernatant using ELISA kits as per the manufacturer's instructions (Invitrogen, ThermoFisher Scientific). Sample dilutions were calibrated for respective analyte separately prior to analysing test samples. The reaction fluorescence was measured at 450 nm and 570 nm wavelength (Molecular devices, Spectramax M2).

Statistical analysis

All experiments were carried out independently in triplicate. The statistical analysis was done by one-way ANOVA with the Tukey test for multiple comparisons and a two-tailed unpaired Student's t-test for comparisons between two groups on GraphPad Prism (Version 9). Data are expressed as mean ± SEM. A p-value of less than 0.05 between the compared groups was considered statistically significant.

RESULTS

Given the pressing need for immunosuppressants derived from plants, which offer favorable safety profiles and the ability to regulate the inflammatory response without compromising overall immune function, our study aims to evaluate the efficacy of three herbal extracts (*W. somnifera*, *T. cordifolia*, *G. glabra*) and an herbal formulation (A-64) in restoring the dysregulated inflammatory environment within a *TLR7/8*-induced macrophage cell model. As a reference compound, dexamethasone was included to assess the sensitivity of our model. In our experimental approach, macrophage-like cells were differentiated and pre-treated with aqueous extracts of the selected herbs at various concentrations (ranging from 3 µg/mL to 300 µg/mL) prior to TLR induction using either IMQ or RSQ. The induced cells exhibited a significant increase in the expression of inflammatory markers. Conversely, the cells treated with dexamethasone demonstrated improvement in the expression of all the investigated inflammatory genes. This investigation

aims to shed light on the potential of these herbal extracts and formulation to modulate the dysregulated inflammatory response observed in our *TLR7/8*-induced macrophage cell model. By assessing their impact on the expression of inflammatory markers, we can gain insights into their efficacy as immunosuppressants. Notably, our study highlights the promising role of plant-derived compounds in restoring the balance of the inflammatory milieu without compromising the immune system's ability to mount an appropriate response.

Titration of agonist concentration and treatment duration

To determine the appropriate dose and induction duration for herbal evaluation, inflammatory genes (*TNF-α*, *MCP1*, *IL-6*, *IL-1β*) were examined in cells and culture supernatants treated with TLR agonists (IMQ and RSQ). Various concentrations (1 µg/mL, 5 µg/mL, and 10 µg/mL) of agonists were tested in differentiated macrophage-like cells for 8, 16, 24, and 36 hours. IMQ induced *TLR7/8* signaling after 8 hours, while RSQ showed induction after 24 hours. IMQ had maximum induction at 5 µg/mL, whereas RSQ induced signaling at 1 µg/mL. Thus, separate induction profiles were designed for herbals: 8 hours with 5 µg/mL IMQ and 24 hours with 1 µg/mL RSQ. *TNF-α*, *MCP1*, and *IL-1β* showed significant induction with 5 µg/mL IMQ after 8 hours, while *IL-6* showed induction after 16 hours (Supplementary Figures 1 and 2).

Before studying the effect of herbals on TLR signaling, their impact on cell viability was assessed using MTT assay, both in the absence and presence of *TLR7/8* agonists. Even at a high concentration of 1000 µg/mL, none of the herbs showed any harmful effects on cell viability (Supplementary Figure 3). Subsequently, the efficacy of selected herbal extracts in mitigating IMQ/RSQ-induced inflammatory responses in macrophage cells was investigated. The study design involved inducing TLR signaling with agonists, resulting in increased expression/circulating levels of inflammatory markers, and assessing the effect of the herbals on these markers.

Prophylactic effect of *W. somnifera* on *TLR7/8*-induced inflammatory markers

W. somnifera, commonly known as Ashwagandha, is an Indian shrub used in Ayurvedic medicine for holistic health benefits, stress and anxiety management, reproductive health support, physical endurance enhancement, and immunomodulation [9,14,15]. *W. somnifera* showed suppression of pro-inflammatory cytokine-induced Th1, Th2, and Th17 differentiation. It created an immunosuppressive environment in hamster and hACE2 transgenic mice models of COVID-19, suggesting potential efficacy against acute viral infections. *W. somnifera* had a more pronounced prophylactic effect in RSQ-induced cells compared to IMQ-induced cells (Figure 1). Increasing *W. somnifera* concentration significantly corrected the expression levels of inflammatory genes (Figure 1B). *W. somnifera* effectively mitigated *TNF-α*, *CXCL10*, and *IL-1β* expression in both IMQ and RSQ-induced macrophage-like cells (Figure 1A). Interestingly, *W. somnifera* treatment decreased *IL-10* levels in RSQ-induced cells but had no such effect after IMQ induction. *W. somnifera* also significantly reduced *MCP1* and *IL-6* levels in RSQ-induced cells (Figure 1B). ELISA results showed significant reduction of *MCP1* levels in culture supernatants of *W. somnifera*-treated IMQ-induced cells across all concentrations (Figure 2A). Treatment with 300 µg/mL *W. somnifera* mitigated the effect of RSQ induction on *IL6* and *IL10* (Figure 2B). No correction in levels of *TNF-α* was observed in the supernatant of *W. somnifera*-treated cells induced with either agonist.

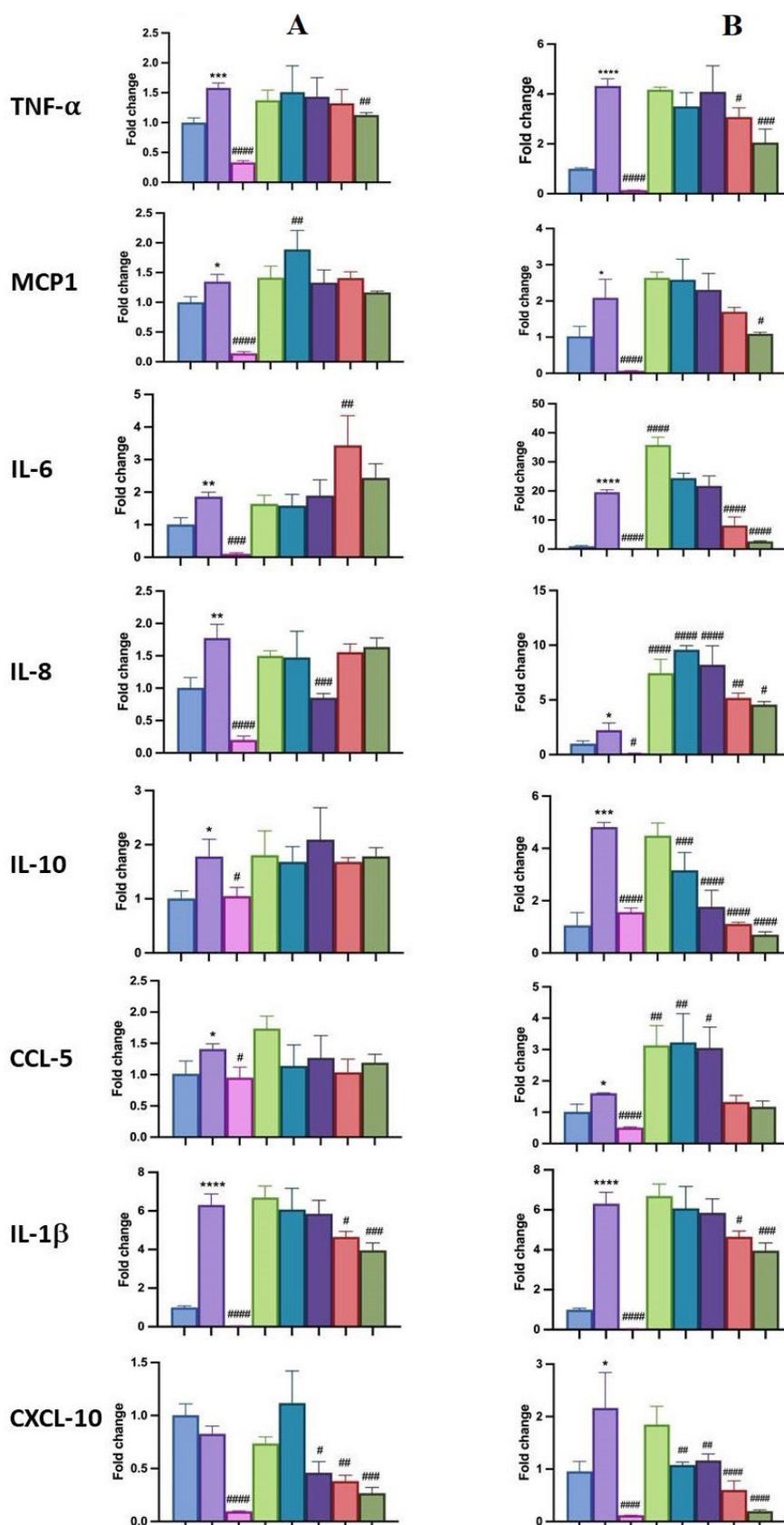


Figure 1: Concentration-dependent prophylactic effect of *W. somnifera* on the TLR7/8-induced gene expression of inflammatory markers. The cells were pretreated with *W. somnifera* for at least 30 minutes at 300 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 3 $\mu\text{g/mL}$ concentrations and subsequently induced with IMQ **Note:** (A) Group A: Control (■); IMQ 5 $\mu\text{g/mL}$ (■); Dex 30 nM (■); / RSQ (■). Dexamethasone (30 nM) was used as a reference control for the assay. All p-values were determined with respect to IMQ/RSQ-induced untreated group as well as the uninduced untreated group. All data are expressed as mean \pm SEM. **Note:** (B) Group B; Control (■); RSQ 1 $\mu\text{g/mL}$ (■); Dex 30 nM (■); *W. somnifera* 3 $\mu\text{g/mL}$ (■); *W. somnifera* 10 $\mu\text{g/mL}$ (■); *W. somnifera* 30 $\mu\text{g/mL}$ (■); *W. somnifera* 100 $\mu\text{g/mL}$ (■); *W. somnifera* 300 $\mu\text{g/mL}$ (■)

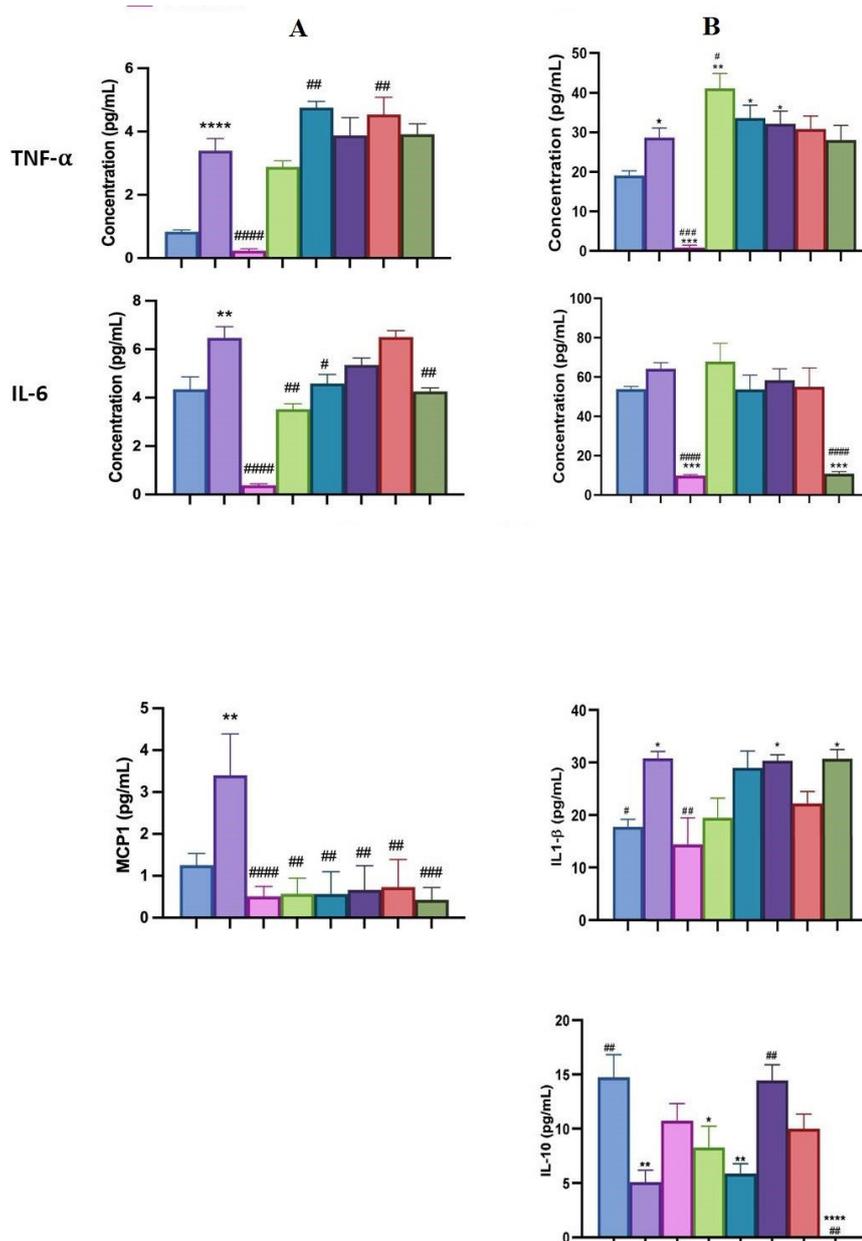


Figure 2: Concentration-dependent effect of WS on TLR7/8 induced secreted levels of cytokines/chemokines. The effect of *W. somnifera* treatment is portrayed on secreted levels of inflammatory markers in IMQ-induced Note: (A) Group A: Control (■); IMQ 5 $\mu\text{g/mL}$ (■); Dex 30 nM (■); and RSQ-induced (B) macrophage-like cells. All p-values were calculated with respect to IMQ/RSQ-induced untreated group as well as the uninduced untreated group. Dexamethasone (30 nM) was used as a reference control. Samples were diluted 50 times for *TNF- α* and *MCP1* cytokines. All data are expressed as mean \pm SEM. Group B; Control (■); RSQ 1 $\mu\text{g/mL}$ (■); Dex 30 nM (■); *W. somnifera* 3 $\mu\text{g/mL}$ (■); *W. somnifera* 10 $\mu\text{g/mL}$ (■); *W. somnifera* 30 $\mu\text{g/mL}$ (■); *W. somnifera* 100 $\mu\text{g/mL}$ (■); *W. somnifera* 300 $\mu\text{g/mL}$ (■)

TLR7/8-induced inflammatory markers in *T. cordifolia* pretreated cells

T. cordifolia, also known as Giloy or Guduchi, is a perennial shrub native to tropical regions of India and other neighboring countries. In Ayurveda, the stem, leaves, and roots of *T. cordifolia* have long been used for therapeutic purposes. It exhibits immunomodulatory properties, supporting the body's defense against infection, and possesses antioxidant activity to reduce stress. Additionally, it offers anti-inflammatory, antipyretic, and hepatoprotective effects [16]. In silico studies suggest that its active phytoconstituents bind to RNA-dependent RNA polymerase [17].

The prophylactic effect of *T. cordifolia* on IMQ/RSQ-induced

macrophage-like cells showed significant impact on inflammatory markers (Figures 3A and 3B). After 24 hours of treatment with 300 $\mu\text{g/mL}$ *T. cordifolia*, differentiated macrophages exhibited a noteworthy reduction in RSQ-induced expression of *TNF- α* , *MCP1*, *IL-6*, *IL-10*, *CCL-5*, and *CXCL-10* (Figure 3B). Similar to *W. somnifera*, *T. cordifolia* demonstrated a dose-response relationship with increasing *T. cordifolia* concentrations leading to significant reductions in *IL-10* and *CXCL-10* expression. Notably, ELISA results of *T. cordifolia*-treated cell culture supernatants revealed interesting findings (Figures 4A and 4B). While RSQ-induced *IL-6* levels were mitigated at all investigated *T. cordifolia* doses, the maximum decrease was observed at a minimal *T. cordifolia* dose of 3 $\mu\text{g/mL}$ (Figure 4B).

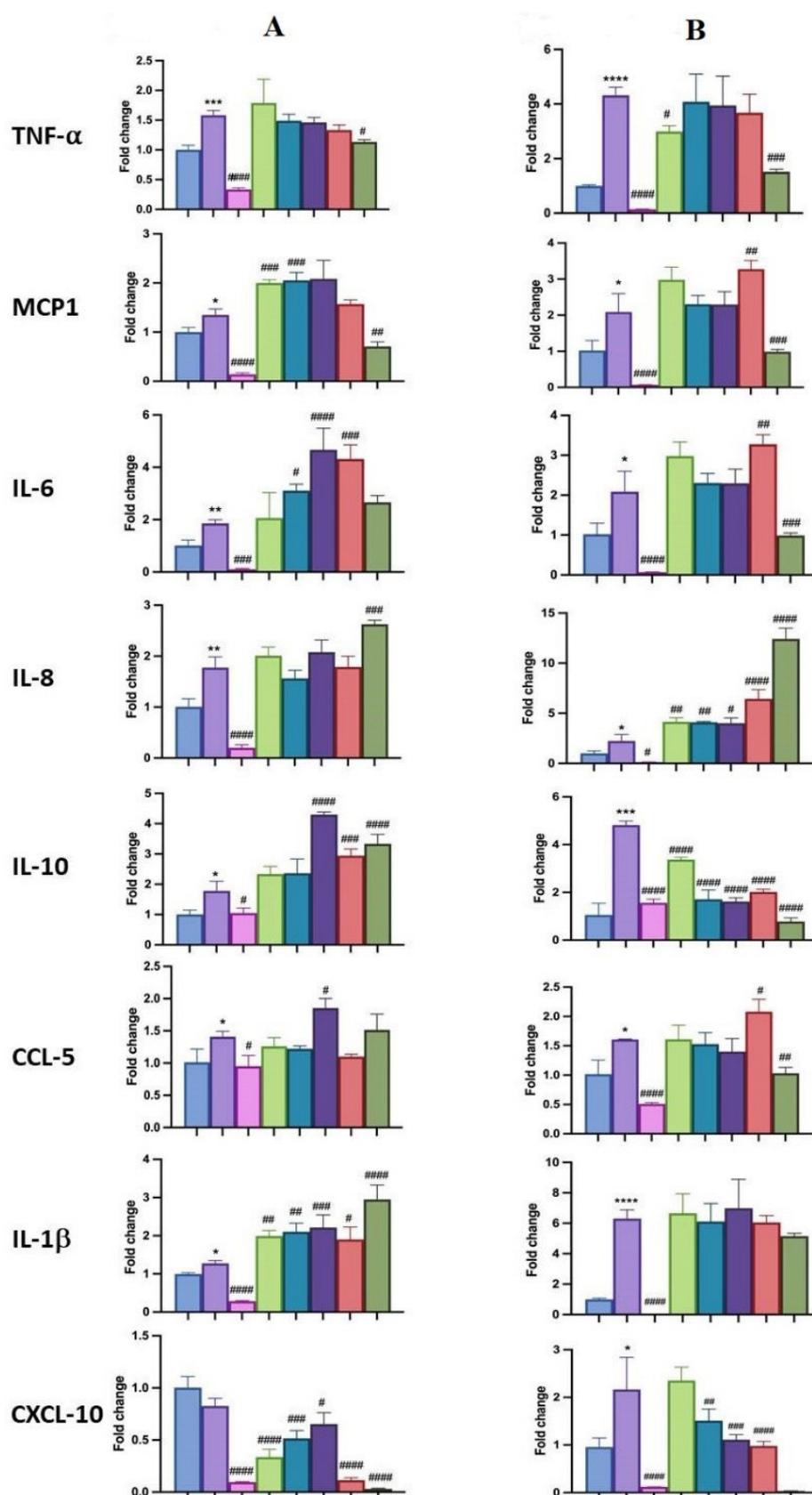


Figure 3: Concentration-dependent prophylactic effect of *T. cordifolia* on the TLR7/8-induced gene expression of inflammatory markers. The cells were pre-treated for at least 30 minutes in the presence of *T. cordifolia* at 300 μg/mL, 100 μg/mL, 30 μg/mL, 10 μg/mL, and 3 μg/mL concentrations and subsequently induced with either IMQ. Note: (A) Group A: Control (■); IMQ 5 μg/mL (●); Dex 30 nM (●); or RSQ (■). (B) Group B: control (■); RSQ 1 μg/mL (■); Dex 30 nM (■); *T. cordifolia* 3 μg/mL (■); *T. cordifolia* 10 μg/mL (■); *T. cordifolia* 30 μg/mL (■); *T. cordifolia* 100 μg/mL (■); *T. cordifolia* 300 μg/mL (■). All p-values were determined relative to IMQ/RSQ-induced untreated group as well as the uninduced untreated group. All data are expressed as mean ± SEM.

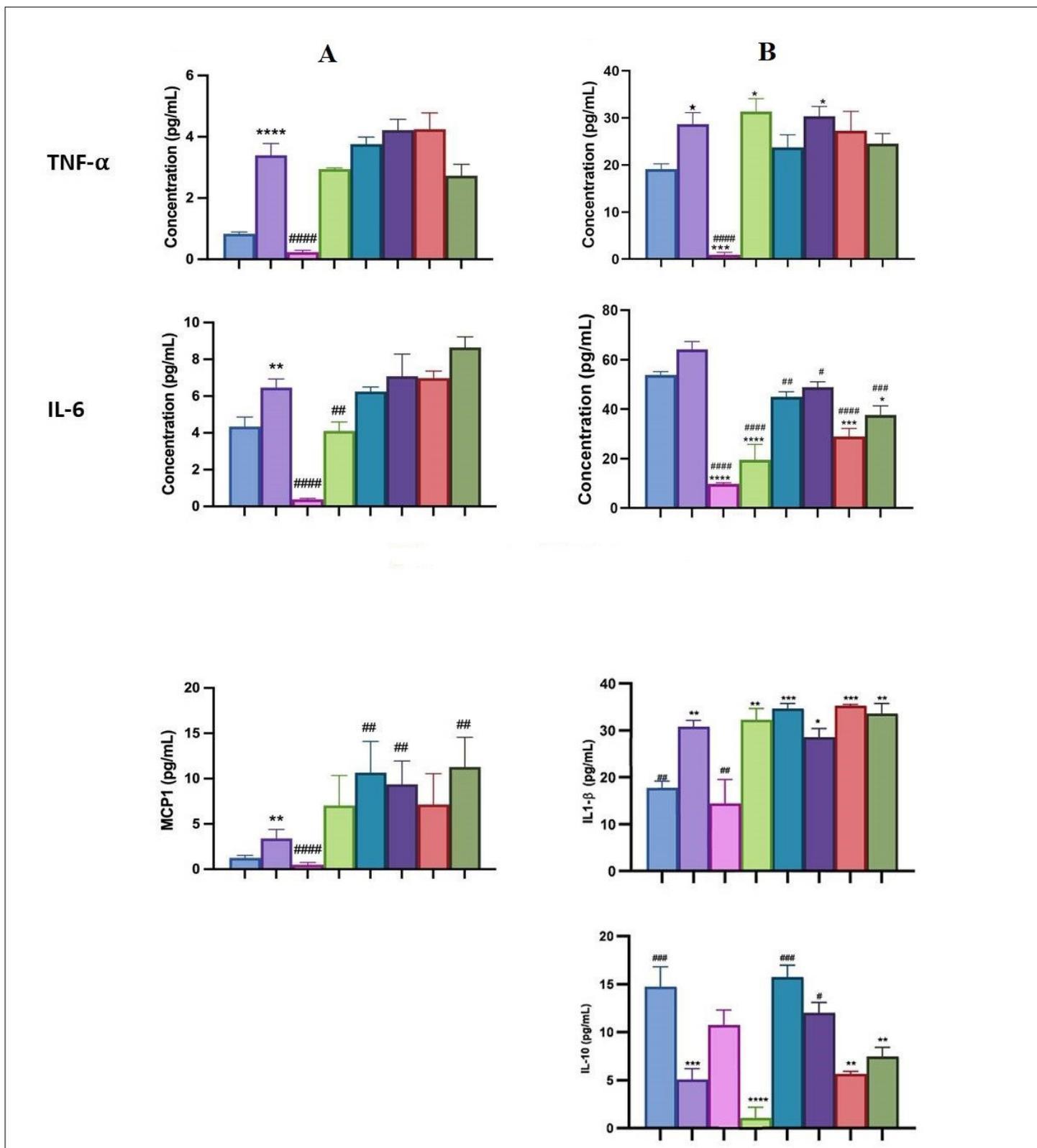


Figure 4: ELISA results of concentration-dependent prophylactic effect of *T. cordifolia* on TLR7/8 induced secreted levels of inflammatory markers. The bar graph depicts the effect of *T. cordifolia* treatment on secreted levels of different cytokines in IMQ-induced Note: (A) Group A: Control (■); IMQ 5 µg/mL (■); Dex 30 nM (■); and RSQ-induced (B) Macrophage-like cells. All p-values were calculated with respect to IMQ/RSQ-induced untreated group as well as the uninduced untreated group. Dexamethasone was used as a reference control. Sample dilution was optimized to 50-fold to ensure optimal measurement of TNF-α and MCP1 cytokines. All data are expressed as mean ± SEM. Note: (B) Group B; control (■); RSQ 1 µg/mL (■); Dex 30 nM(■); *T. cordifolia* 3 µg/mL (■); *T. cordifolia* 10 µg/mL(■); *T. cordifolia* 30 µg/mL(■); *T. cordifolia* 100 µg/mL(■); *T. cordifolia* 300 µg/mL(■).

Effect of *G. glabra* pretreatment on TLR7/8-induced inflammatory markers

Popularly known as liquorice or mulaithi, *G. glabra* is an ancient

Ayurvedic herb widely used for its medicinal properties. *G. glabra* roots contain active compounds like glycyrrhizin, flavonoids, coumarins, and triterpenoids, contributing to its anti-inflammatory effects in arthritis, expectorant properties for respiratory congestion,

gastric and digestive benefits, antimicrobial properties, and immune regulation during infections [18]. *G. glabra* administration in hamster models of COVID-19 demonstrated significant downregulation of pro-inflammatory cytokines *TNF- α* and *IL-6* [19,20]. In our study, *G. glabra* treatment led to a notable reduction in *TNF- α* and *IL-6* expression and levels in macrophage cells induced with *TLR7/8* agonists. *IL6* gene expression significantly decreased at 3 $\mu\text{g}/\text{mL}$ *G. glabra* concentration but increased with higher *G. glabra* concentrations. *IL-8* gene expression was inhibited at 300 $\mu\text{g}/\text{mL}$, 30 $\mu\text{g}/\text{mL}$, and 10 $\mu\text{g}/\text{mL}$, while *CXCL10* gene expression decreased in a concentration-dependent manner in IMQ-induced cells, with the maximum reduction observed at 300 $\mu\text{g}/\text{mL}$ *G. glabra* (Figure 5A). *G. glabra* treatment significantly

reduced *TNF- α* expression at all investigated concentrations. Similarly, *G. glabra* treatment corrected *IL-10* expression in RSQ-induced cells (Figure 5B). *IL8* depletion was observed at 10 $\mu\text{g}/\text{mL}$ *G. glabra*, while *CCL5* gene expression was significantly corrected at lower *G. glabra* concentrations (3 $\mu\text{g}/\text{mL}$ and 10 $\mu\text{g}/\text{mL}$) in RSQ-induced cells. Surprisingly, *IL1- β* gene expression increased with increasing *G. glabra* concentration in IMQ-induced cells. *MCP1* gene expression showed an increasing trend in *G. glabra*-treated cells induced with both *TLR7/8* agonists. Likewise, circulating *MCP1* protein levels increased in culture supernatants with higher *G. glabra* concentrations (Figure 6A). However, *IL6* levels were significantly reversed at most *G. glabra* concentrations investigated in RSQ-induced cells (Figure 6B).

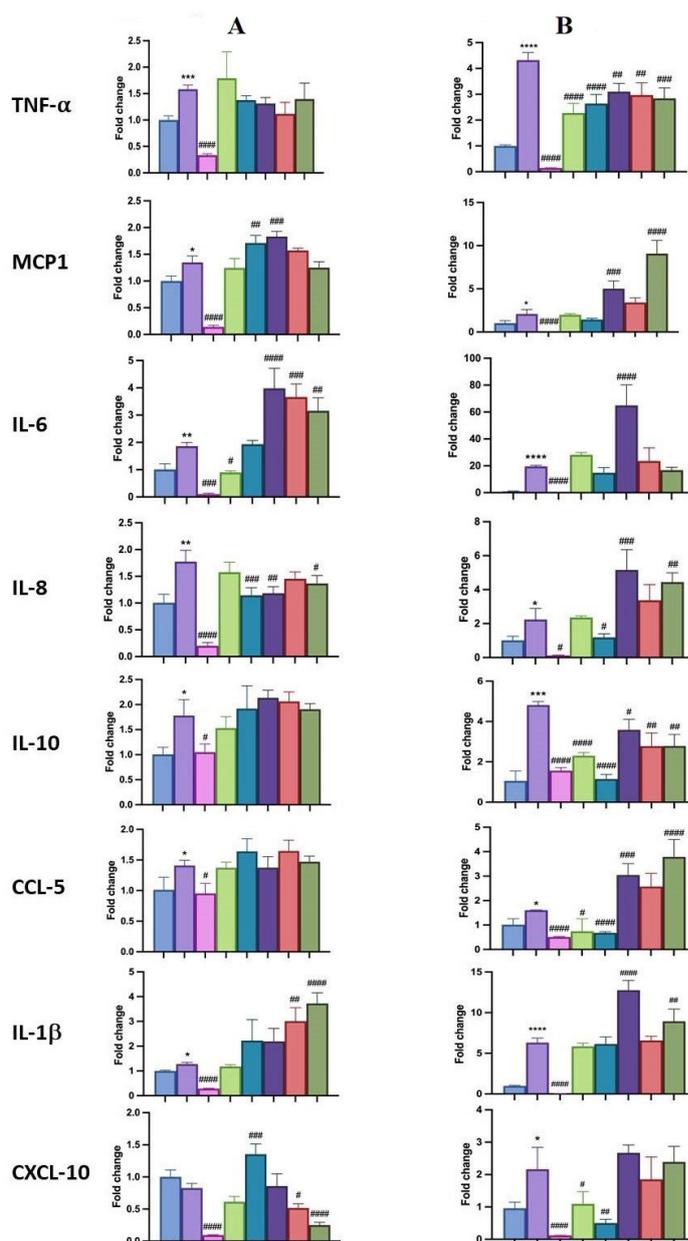


Figure 5: Concentration-dependent prophylactic effect of *G. glabra* treatment on the *TLR7/8* induced gene expression of inflammatory markers. The cells were pretreated with *G. glabra* at 300 $\mu\text{g}/\text{mL}$, 100 $\mu\text{g}/\text{mL}$, 30 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$, and 3 $\mu\text{g}/\text{mL}$ concentrations and thereafter induced with IMQ (A) Note: (A) Group A: Control (■); IMQ 5 $\mu\text{g}/\text{mL}$ (■); Dex 30 nM (●); RSQ (B). Dexamethasone (30nM) was used as a reference control. All p-values were determined with respect to IMQ/RSQ-induced untreated group as well as the uninduced untreated group. All data are expressed as mean \pm SEM. Note: (B) Group B; control (■); RSQ 1 $\mu\text{g}/\text{mL}$ (■); Dex 30 nM (●); *G. glabra* 3 $\mu\text{g}/\text{mL}$ (■); *G. glabra* 10 $\mu\text{g}/\text{mL}$ (■); *G. glabra* 30 $\mu\text{g}/\text{mL}$ (■); *G. glabra* 100 $\mu\text{g}/\text{mL}$ (■); *G. glabra* 300 $\mu\text{g}/\text{mL}$ (■).

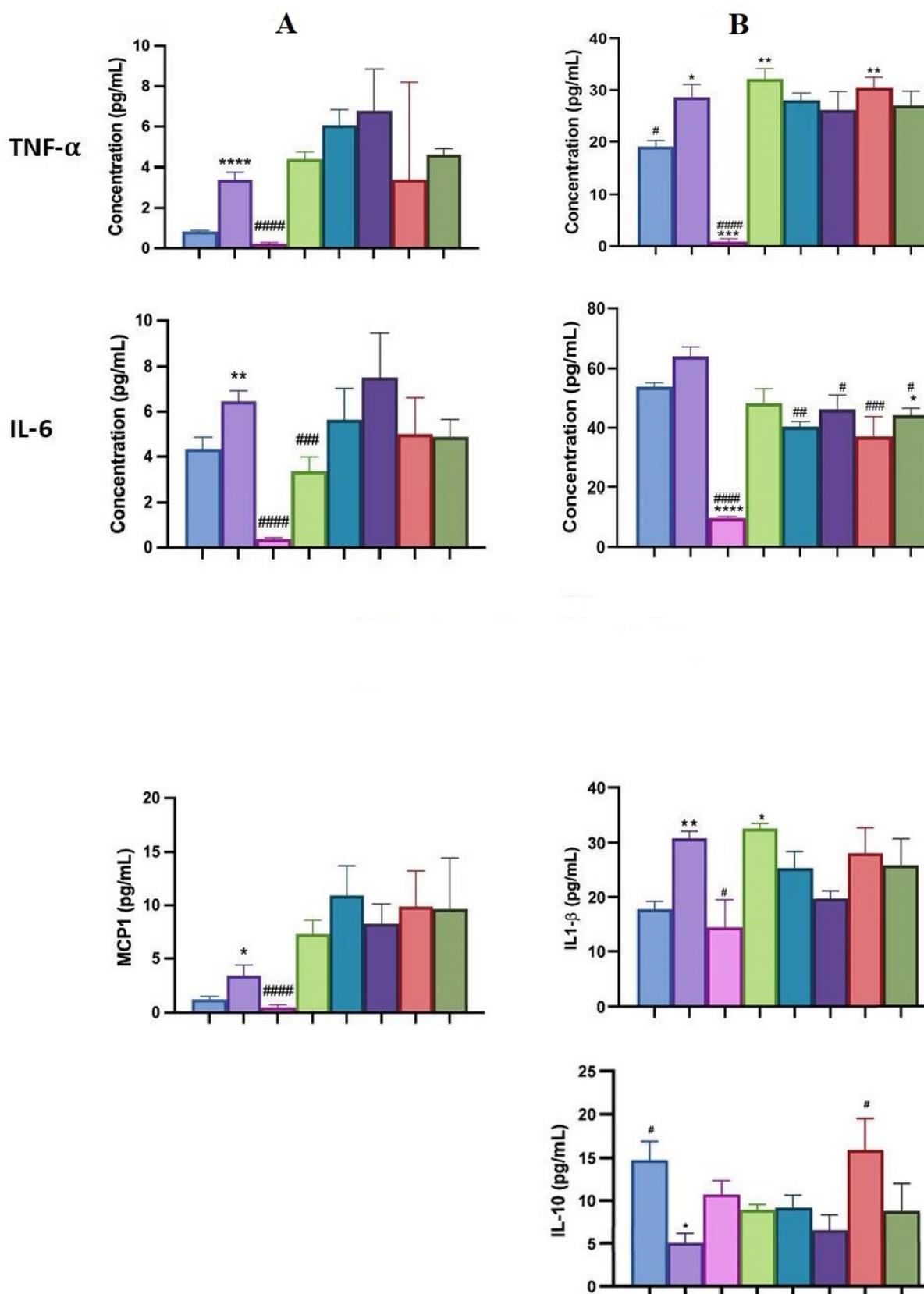


Figure 6: Concentration-dependent prophylactic effect of *G. glabra* treatment on TLR7/8 induced secreted levels of inflammatory markers. The bar graph presents changes in secreted levels of inflammatory markers after treatment with a range of *G. glabra* concentrations in IMQ-induced Note: (A) Group A: Control (■); IMQ 5 µg/mL (■); Dex 30 nM(●); and RSQ-induced (B) macrophage-like cells. All p-values were calculated with respect to IMQ/RSQ-induced untreated group as well as the uninduced untreated group. Dexamethasone was used as a reference control. Sample dilution was calibrated to 50-fold for optimal measurement of *TNF-α* and *MCP1* cytokines. All data are expressed as mean ± SEM. Note: (B) Group B; control (■); RSQ 1 µg/mL (■); Dex 30 nM(●); *G. glabra* 3 µg/mL (■); *G. glabra* 10 µg/mL(■); *G. glabra* 30 µg/mL(■); *G. glabra* 100 µg/mL(■); *G. glabra* 300 µg/mL(■).

Effect of the pretreatment of cells with AYUSH-64 (A-64) on the *TLR7/8* induced expression and release of inflammatory markers

AYUSH-64 is an Ayurvedic formulation with immunomodulatory and anti-inflammatory properties. It contains herbs such as *Alstonia scholaris*, *Caesalpinia crista*, *Picrorhiza kurroa*, and *Swertia chirata* [21]. Safety studies have confirmed its tolerance up to 500 mg/kg body weight in rodents. A clinical trial conducted by the Ministry of AYUSH, India, and the Council of Scientific and Industrial Research, India, demonstrated its efficacy in managing mild to moderate COVID-19 patients, with a significant decrease in *IL-6*

levels [22-24]. In our study, we evaluated the effect of A-64 on the expression of inflammatory genes in the presence of *TLR7/8* inducers (Figures 7A and 7B). We observed a significant reduction in *TNF- α* expression and levels in the supernatant of A-64 pre-treated cells. While, *IL6* levels showed no overall change in expression or in the release in the supernatant, *IL-1 β* levels decreased significantly with increasing A-64 concentration, indicating a concentration dependent-response relationship (Figures 8A and 8B). Moreover, *IL-10* and *CXCL10* expression were mitigated in a concentration-dependent manner, decreasing with increasing AYUSH-64 concentrations.

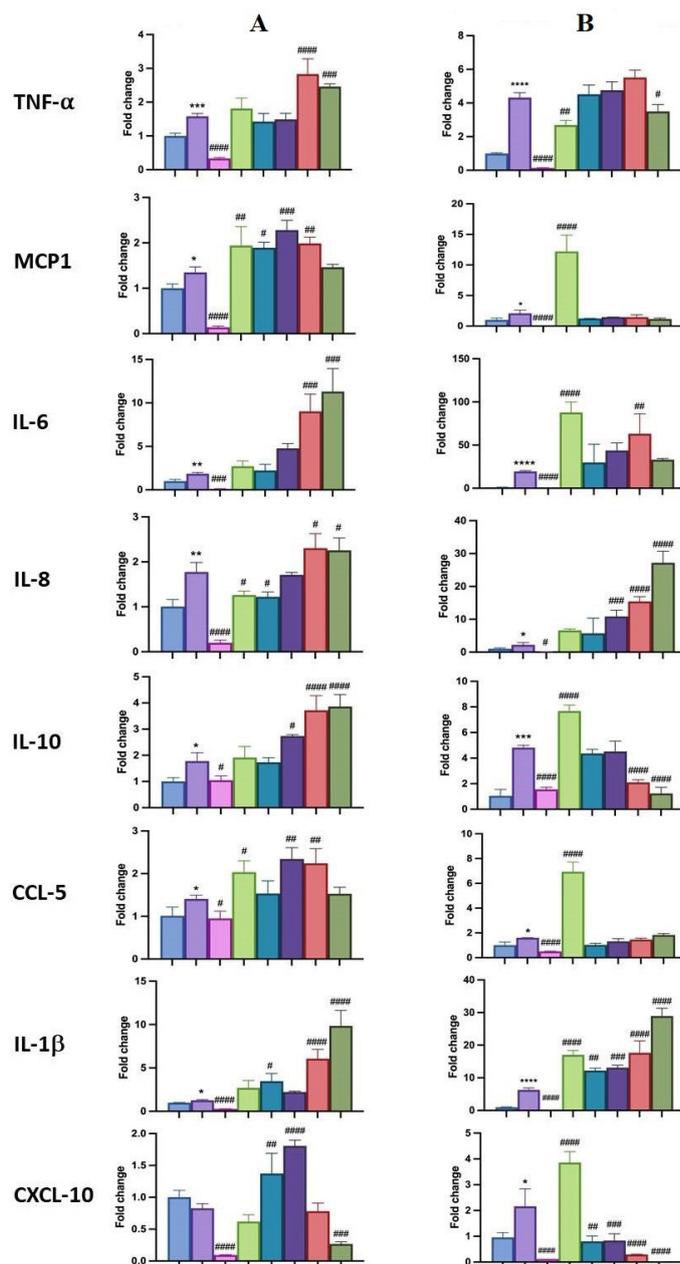


Figure 7: Concentration-dependent prophylactic effect of A-64 treatment on the *TLR7/8* induced gene expression of inflammatory markers. The cells were pretreated for at least 30 minutes with A-64 at 300 μ g/mL, 100 μ g/mL, 30 μ g/mL, 10 μ g/mL, and 3 μ g/mL concentrations and later induced with IMQ Note: (A) Group A: Control (■); IMQ 5 μ g/mL (■); Dex 30 Nm(■); / RSQ (B). Dexamethasone (30nM) was used as a reference control for the alleviation of *TLR7/8* response. All p-values were determined with respect to IMQ/ RSQ-induced untreated group as well as the uninduced untreated group. All data are expressed as mean \pm SEM. Note: (B) Group B; control (■); RSQ 1 μ g/mL (■); Dex 30 nM(■); A-64 3 μ g/mL (■); A-64 10 μ g/mL(■); A-64 30 μ g/mL(■); A-64 100 μ g/mL(■); A-64 300 μ g/mL(■).

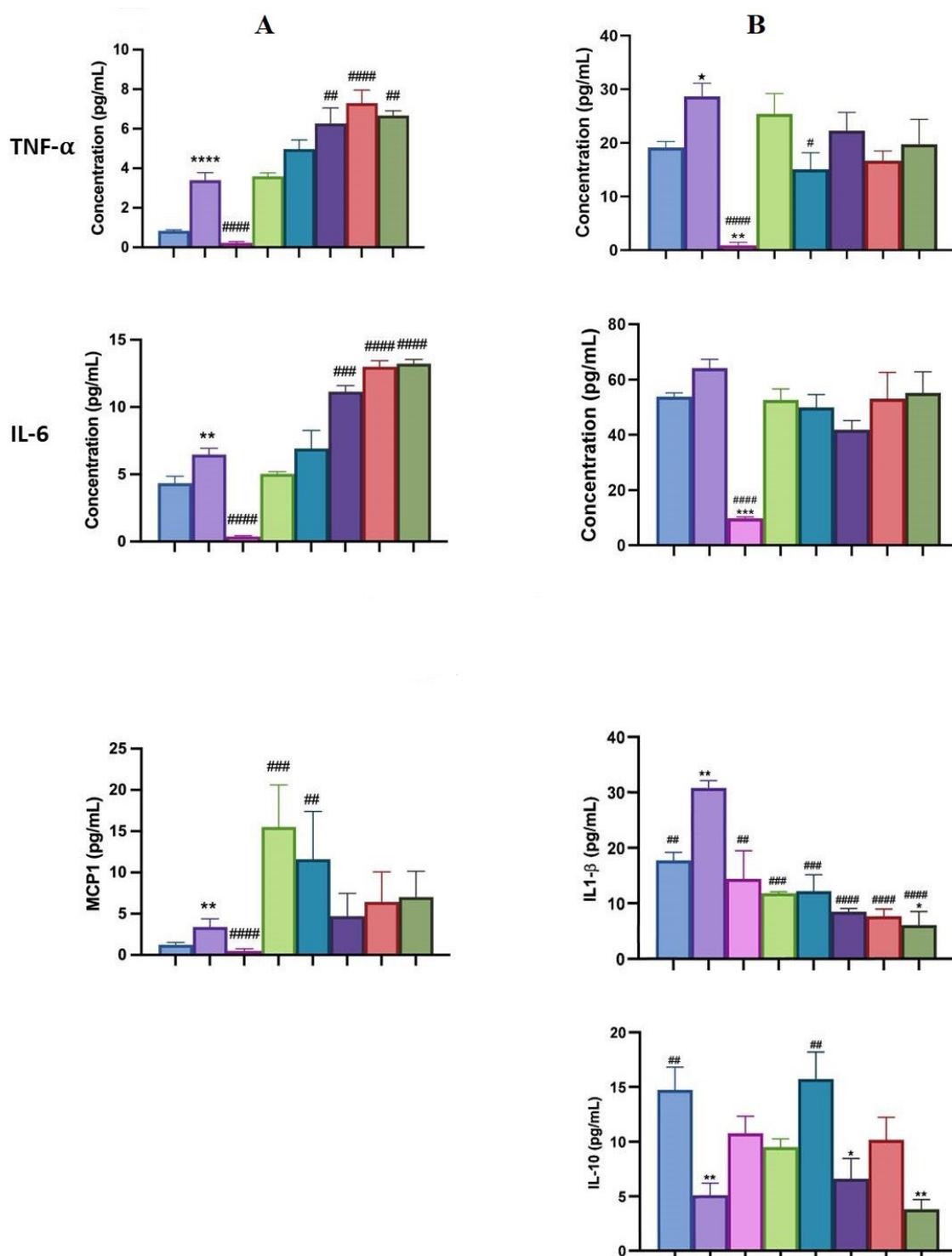


Figure 8: Prophylactic effect of A-64 treatment on *TLR7/8* induced secreted levels on inflammatory markers. The bar graph depicts changes in secreted levels of different inflammatory markers as a result of treatment with AYUSH-64 herbal formulation in IMQ-induced Note: (A) Group A: control (■); IMQ 5 μg/mL (■); Dex 30 nM (■) and RSQ-induced (B) macrophage-like cells. All p-values were calculated with respect to IMQ/RSQ-induced untreated group as well as the uninduced untreated group. Dexamethasone (30nM) was used as a reference control. Sample dilution was calibrated to 50-fold for the optimal determination of both *TNF-α* and *MCP1* cytokines. All data are expressed as mean ± SEM. Note: (B) Group B: control (■); RSQ 1 μg/mL (■); Dex 30 nM (■); A-64 3 μg/mL (■); A-64 10 μg/mL (■); A-64 30 μg/mL (■); A-64 100 μg/mL (■); A-64 300 μg/mL (■).

Overall, our findings contribute to the growing body of research on plant-based therapeutics, particularly in the context of immunomodulation. The use of herbal extracts and formulations holds great potential in the development of effective treatments for inflammatory disorders, offering a safe and well-tolerated

alternative to conventional immunosuppressive agents.

DISCUSSION

The dysregulated inflammatory response has emerged as a prominent pathology during the recent COVID-19 pandemic.

To gain a comprehensive understanding of the disease, cell-based models have played a crucial role in elucidating the mechanisms behind various candidate molecules in COVID-19 therapeutics. Specifically, the activation of Toll-like receptor 7/8 (*TLR7/8*) signaling pathway in response to the presence of viral RNA within host cells has been implicated in the release of pro-inflammatory cytokines, essential for mediating the anti-viral immune response. In our study, we employed macrophage-like differentiated THP1 cells as a model system to mimic *TLR7/8* induction. This was achieved by treating the cells with imidazoquinolinamines, namely imiquimod (IMQ) and resiquimod (RSQ). IMQ and RSQ are well-known *TLR7/8* agonists that have demonstrated potent ability to induce the production of *Interferon- α* (IFN- α) and other cytokines [2,25].

By utilizing this experimental setup, we aimed to gain mechanistic insights into the *TLR7/8*-mediated inflammatory response and its potential implications in immune-compromised pathologies including COVID-19. The use of cell-based models has proven to be invaluable in unravelling the complex interplay between viral components and the host immune system, paving the way for the development of novel therapeutic strategies. By elucidating the specific mechanisms underlying herbal modulation of *TLR7/8* signaling and subsequent cytokine release, our study contributes to a better understanding of the dysregulated inflammatory response observed during COVID-19. These findings may have implications for the development of targeted therapeutics aimed at modulating the *TLR7/8* pathway to mitigate the excessive inflammation associated with the disease.

Excessive and dysregulated release of pro-inflammatory cytokines, known as cytokine storm, can occur during severe infections and autoimmune disorders [26]. This uncontrolled cytokine release was observed in the recent COVID-19 pandemic, leading to life-threatening systemic inflammation. Immunomodulatory therapies targeting specific cytokine pathways, such as *TNF- α* , *IL-6*, and *IL-1 β* , are used to manage cytokine storm [27]. Tocilizumab, an *IL-6* receptor inhibitor, has shown efficacy in this regard [28]. *IL-1 β* , on the other hand, induces *IL-6* and *TNF- α* production and activates immune cells [29]. Elevated levels of *TNF- α* , *IL-6*, and *IL-1 β* are associated with severe symptoms and tissue damage. Blocking *IL-1* signaling with antagonists like anakinra has potential in managing the inflammatory response [30]. However, synthetic immunomodulatory agents often come with adverse side effects [31]. In contrast, the herbs investigated in this study (*W. somnifera*, *T. cordifolia*, *G. glabra*, A-64) have a long history of traditional use. Their anti-inflammatory and immunomodulatory properties offer a potential approach to manage the hyper inflammatory response without toxic side effects [32,33]. These botanical sources of immunomodulatory agents provide a novel avenue to moderate the exaggerated cytokine response and restore immune balance [32,33].

TLR7/8 activation plays a crucial role in recruiting immune cells to the site of viral infection by triggering pro-inflammatory cytokine production [34,2,3]. However, the administration of *TLR7/8* agonists like imiquimod or resiquimod can lead to cytokine storms in certain clinical scenarios. Macrophage-like THP1 cells, which differentiate from monocytes when exposed to PMA, provide a relevant model to study the dysregulated cytokine response induced by *TLR7/8* agonists and evaluate the prophylactic effect of herbal extracts. The study model was optimized for agonist dosage and treatment duration to achieve optimal TLR signaling induction. RSQ showed a higher induction rate of cytokine expression

compared to IMQ, and the concentration of RSQ required for *TLR7/8* induction (1 $\mu\text{g/mL}$) was five times lower than that of IMQ (5 $\mu\text{g/mL}$). Similar observations were reported by another research group using a similar model [8].

Induction of *TLR7/8* signaling with agonists increased *TNF- α* expression, which was moderated in presence of dexamethasone. Treatment with herbal extracts significantly improved *TNF- α* gene expression in macrophage-like cells, with expression generally decreasing as extract concentration increased. Among the extracts, *W. somnifera* and *T. cordifolia* showed significant correction of *IL-6* gene expression, while all treatment groups exhibited perturbed levels of *IL-6* in the culture supernatant. *W. somnifera* treatment also corrected *TLR7/8*-induced *IL-1 β* levels, whereas other extracts had no effect on *IL-1 β* gene levels. The interplay between *IL-1 β* and *IL-6* regulation is crucial for understanding inflammatory processes and developing therapeutic strategies [35]. A-64 inhibition of *IL-1 β* resulted in suppressed *IL-6* release. Additionally, the selected herbs significantly corrected *CXCL-10* expression, an immune cell trafficking and activation chemokine. *CXCL-10* levels are associated with disease severity and can serve as a biomarker in inflammatory conditions [36].

Among all the investigated herbal extracts, *W. somnifera* showed remarkable efficacy in mitigating the increase in expression of inflammatory markers induced by *TLR7/8* activation. *W. somnifera* exhibited its effects by attenuating excessive cytokine release and regulating the production and activity of pro-inflammatory cytokines. *W. somnifera* contains bioactive compounds, including withanolides, known for their anti-inflammatory and immunomodulatory properties. Previous studies have demonstrated the ability of *W. somnifera* to suppress *TNF- α* , *IL-6*, and *IL-1 β* cytokines in various inflammatory conditions, such as LPS-induced neuroinflammation [37]. Furthermore, *W. somnifera* has shown a protective effect against COVID-19 by modulating the inflammatory response and inhibiting pro-inflammatory cytokine-induced differentiation of Th1, Th2 and Th17 cells [38].

It is essential to acknowledge that although these botanical drugs have demonstrated promising results in our preclinical model, further research is warranted to comprehensively understand their mechanisms of action. Additionally, optimizing dosages and conducting thorough evaluations of their safety and efficacy in animal models are crucial steps to advance their potential as therapeutic interventions. While our study model exhibits sensitivity and robustness, rigorous clinical validation is imperative to establish the viability and effectiveness of these botanical drugs as potential treatment options.

CONCLUSION

Based on our study, it is evident that herbal therapeutics hold promise in regulating dysregulated inflammatory responses. Resiquimod exhibited better efficacy as an agonist for activating the *TLR7/8* signaling pathway in our experimental model. Among the evaluated herbs/formulations, *Withania somnifera* demonstrated significant prophylactic efficacy by effectively mitigating the upregulation of numerous inflammatory genes in the PMA-differentiated THP1 cells activated *via* the *TLR7/8* signaling pathway. These findings provide a basis for exploring the potential of herbal interventions in modulating inflammatory cascades, but additional research is required to fully understand their mechanisms of action and therapeutic applications.

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AUTHOR'S CONTRIBUTIONS

Manisha Dagar and Kamala Priya performed the *in vitro* cell-based experiments, writing, and interpretation. Ajay Kumar and Madhu Dikshit conceptualized the overall study design, interpretations, writing, and editing of the manuscript. All authors have read and accepted the manuscript.

Manisha Dagar and Kamala Priya contributed equally and are therefore sharing first authorship.

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AVAILABILITY AND DATA MATERIALS

All data are included within the article. Any additional specific information may be made available if desired.

DECLARATIONS

Ethics approval and consent to participate

Ethical approval was obtained from Institutional Biosafety Committee (IBSC) at THSTI (IBSC Approval No 221/2020) after a brief explanation about the study objectives and protocols. All experimental protocols were approved and carried out in accordance with relevant guidelines and regulations. Consent to participate is not applicable in this study.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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