**RESEARCH ARTICLE**

# *In-vitro* and *In-vivo* Studies of Anti-inflammatory Potential of Compounds Derived from *Piper mullesua*

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**ABSTRACT**

The identification and development of new anti-inflammatory drugs continue to benefit greatly from the use of natural substances and their synthetic counterparts. Studies have been conducted on a compounds obtained from the *Piper mullesua* plant. The current work aims to use *in-vitro* and *in-vivo* experiments to investigate these substances' anti-inflammatory properties. Our investigation's findings showed that various substances had varying percentages of cell viability on RAW 264.7 cells. These substances were also tested for suppression of nitric oxide. Novel approaches to the creation of pharmaceuticals have demonstrated the potential of natural product derivatives. This study set out to investigate PM208-209's potential anti-inflammatory properties. The impact of PM208-209 on inhibiting tumor necrosis factor-alpha (TNF-α) and IL-6 in LPS-induced RAW 264.7 cells was examined using enzyme-linked immunosorbent assay (ELISA). Additionally, PM208-209 demonstrated anti-inflammatory effects in mice, as evidenced by the suppression of vascular permeability and migration of leukocytes. The information gathered suggested that PM208-209 should be investigated further in terms of pharmacological research and could be regarded as a possible therapeutic anti-inflammatory option.

**Keywords:** Inflammation, *Piper mullesua*, Inhibition, TNF-α and IL6.

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## INTRODUCTION

The idea of returning to the "roots" of medicine is gaining traction in these modern times. Finding new therapeutic agents in natural sources and conducting interventional studies on them has been a major focus of biomedical research in the last few years.[1] In therapeutics, several molecules derived from plants have demonstrated promising results. The Piperaceae family of pepper plants, which have been studied extensively, is one that has shown a great deal of promise. For commercial, economic, and medicinal purposes, piper species are very significant[2]. *Piper mullesua*, also known as Pahari peepal, is a rare plant with anti-arthritis activity among the *Piper* family. It is native to India and is widely distributed in the Himalayas at an altitude of approximately 600 to 1500 mSO. It has been studied to treat arthritis in many piper species.[3] In our recent study, the plant is collected from the chemistry division of IIIM, Institute, Jammu, India.

While innate immune mechanisms are the basis for inflammation, they may also trigger adaptive immunity. Most of the time, it spreads quickly, stays contained, and disappears when the pathogens are destroyed. On the other hand, inflammation can sometimes result in harmful, even fatal outcomes (such as severe infection or trauma, defective regulation).[4]

There are two distinct types of inflammation that an individual could experience, namely acute inflammation and chronic inflammation. Basically, acute inflammation refers to the initiation of the immediate reaction of the immune system against infections and injuries that affect the integrity of cells. It is regulated by the vasoactive amines and eicosanoids, promoting the migration of leukocytes and plasma into the affected site[5]. Historically, acute inflammation has been associated with manifestations such as erythema, heat, pain, edema, and impaired functionality[6].

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Sensitive skin affects every part of the body, and many skin diseases cause mild pain. For example, chronic inflammation of artery walls can lead to atherosclerosis, which leads to blockage of the arteries, plaque buildup, and eventually heart disease. Furthermore, chronic pain significantly contributes to other health issues, including sleep disturbances, physical impairment and obesity.[7]

Rheumatoid arthritis (RA) and the consequences that are linked with it have become a major worry for the health of people all over the world. This inflammatory autoimmune condition predominantly impacts multiple systems. In the United States of America alone, there are around 46 million individuals who are afflicted, and it is anticipated that this number will increase to 67 million by the year 2030.[8] Joint inflammation, often known as RA, is an inflammatory disease that causes the immune system's response to assault joint tissues and possibly other organs or areas of the body. Consequently, RA induces pain, and inflammation and ultimately leads to joint deformity and damage. Beyond causing symmetric joint damage that results in pain on both sides of the body, RA can instigate feelings of malaise, fatigue, and fever in affected individuals.[9]

Chronic cellular activation within joints and other organs initiates autoimmunity and the formation of immune complexes, marking the onset of RA. Immune cells infiltrating the synovial fluid result in increased swelling, congestion, and inflammation of the synovial membrane. The progression of RA unfolds through three phases: initiation, characterized by non-specific inflammation; amplification, where cytokines namely IL-1, IL-6 and TNF-α induce tissue damage during chronic inflammation brought on by T-cell activation; and chronic inflammation, marked by persistent cellular activation. The objectives of RA treatment encompass reducing joint pain and inflammation, improving joint function, and preventing deformity and destruction.

Treatment strategies involve a combination of medications, weight-bearing exercise, disease education, and periods of rest. The development of individualized treatment plans is a pivotal aspect of comprehensive healthcare. These plans are meticulously tailored, taking into consideration various factors that contribute to the uniqueness of each patient's situation such as the course of the disease, affected joints, age, general health, employment, adherence, and knowledge of the condition.[10] The management of chronic inflammatory disorders, such as RA, cartilage degeneration, and IBD, includes the utilization of various medications. The different categories of the synthetic anti-rheumatic drugs are available for the management of the RA.[11]

On the other hand, it is of the utmost importance to realize that these treatments have the potential to cause toxic effects, including gastrointestinal, hepatotoxic, heart disease, and pneumonitis, in addition to an elevated probability of illnesses that might potentially be fatal.[12] In our study, the analytical method employed was ELISA to investigate the impact of PM208-209 on inflammatory markers, specifically TNF-α and IL-6. In addition, the *in-vivo* efficacy of PM208-209s was assessed by inducing inflammation in Balb/c mice using LPS.

## MATERIAL AND METHODS

### Establishment of Cell Culture

In this investigation, the murine macrophage cell line, namely RAW 264.7 cells, were cultivated under monitored conditions at 37°C. The seeded cells were incubated in a hydrated incubator containing 5% carbon dioxide. The Dulbecco's modified eagle's medium (DMEM) media was used for the growth of cell culture. In addition, the media was amplified by adding 10% fetal bovine serum (FBS). Antibacterial drugs such as penicillin and streptomycin were added to the mixture for the control of bacteria.

### Determination of Cell Vitality

The MTT technique was used to determine whether or not RAW 264.7 macrophages that had been treated with PM 208-209, PM 241-250, and PM 301-310 were able to maintain their vitality. About 96-well culture plates were utilized for planting the cells at a density of $1 \times 10^5$ cells per mL at a certain concentration. After adding PM 208-209, PM 241-250, and PM 301-310 in varying quantities, the resulting mixture was then incubated for a period of 48 hours during the process. The MTT solution was added after the period of incubation, and the cultivated cells were then incubated for an additional four hours at 37°C. After that, 150 µL of DMSO was introduced, and the absorption values at a wavelength of 570 nm was analyzed with the assistance of a Synergy Mx plate reader.

$$\text{cell viability} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100$$

### Quantification of Nitrite using the Griess Reaction

The concentration of nitrite was determined by treating the seeded RAW 264.7 cells with PM 208-209, PM 241-250, and PM 301-310 for a period of twenty-four hours. This treatment was conducted by adding or omitting 1-µg/mL of lipopolysaccharide (LPS). The Griess reaction plays an important role in investigating inorganic nitric oxide (NO) concentration that developed in the cell culture fluid.

In brief, 100 µL of supernatant was collected using a centrifuge set at 1000 rpm. The collected supernatant was then subjected to a 10-minute incubation at room temperature. During this interval of incubation, an equivalent volume of Griess reagent was added to the mixture. The absorbance was determined at a specified 540 nm using a Synergy Mx plate reader.

The quantity of nitrite was estimated through the utilization of a reference curve that was established with $NaNO_2$. The amount of nitrate in the group that LPS produced was used as the control for the calculation of the NO inhibition (%), which was derived by the formula:

$$\text{NO inhibition (\%)} = \frac{[\text{NO}]\ \text{OD control} - [\text{NO}]\text{OD sample}}{[\text{NO}]\ \text{OD control}} \times 100$$

### Cell Culture and *In-vitro* Cytokine Measurement

According to the protocol, a specified density of RAW 264.7 cells was meticulously placed into a 96-well plate. In this case, the density chosen was $1 \times 10^5$ cells per well. This step ensures a consistent and controlled number of cells for each well, facilitating standardized conditions across the experiment. Following the cell seeding, the plate was placed in an incubator and allowed to incubate overnight. Subsequently, the cells were treated for one hour with either MTX or BA-25 alone. The cells were stimulated throughout the day with 1-µg/mL LPS. Following the duration of the stimulation period, the excess fluid were taken out and the quantities of IL-6 and TNF-α were measured by utilizing ELISA Kits. Every experiment was conducted in triplicate to assure the consistency and reliability of the results.

### Estimation of Antioxidant Activity

The use of the fluorescent probe DCFH-DA allowed for the evaluation of the accumulation of reactive oxygen species (ROS) inside the cells. Following the conclusion of the investigation, the cells were subjected to treatments using PBS. Subsequently, they were incubated in the dark at room temperature for a duration of 30 minutes with a concentration of 10 µM DCFH-DA dye. The fluorescence produced during the experiment was analyzed using the BD FACS Calibur. The levels of fluorescence also acted as an indicator of the levels of ROS that were present inside the cells.

### *In-vivo* Investigation

*Anti-inflammatory activity*

Six-week-old male Balb/c mice were acclimated for two weeks and subsequently divided into six groups: control, LPS only, LPS+PM208-209 (10 mg/kg), LPS+PM208-209 (20 mg/kg), LPS+PM208-209 (30 mg/kg), and LPS+DEXA (10 mg/kg). Intraperitoneal injection of LPS (2 mg/kg) was administered four hours after the initial dose of up to 30 mg/kg PM 208-209 or another chemical, followed by a subsequent injection 2 to 3 hours later.

Blood serum was obtained by centrifuging 0.6 mL of blood from the retro-orbital plexus (ROP) for 20 minutes at 600 g. For assessing the TNF-α and IL-6 in serum for each cytokine, an ELISA kit were utilized.

## RESULTS

### Estimation of Cell Viability

Cell viability and morphological screening were conducted for PM 208-209, PM 241-250, and PM 301-310 at concentrations of 10, 1, and 0.1 µM on cultured cell of RAW 264.7. The bioactivity on the RAW 264.7 cell line following treatment with all analogues at different concentrations was assessed using the MTT assay (Figure 1). Figure 1 provides evidence of the effect of *Piper mullesua* compounds on the survival of RAW 264.7 cells at a range of concentration factors. The findings make it quite clear that PM 208-209, in all concentrations, demonstrates the least amount of toxicity.

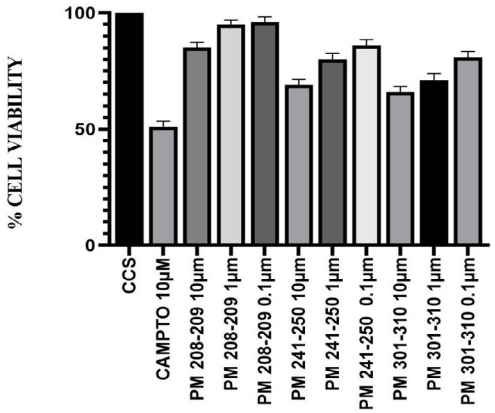


**Figure 1:** *Piper mullesua* compounds effects on RAW 264.7 cell lines

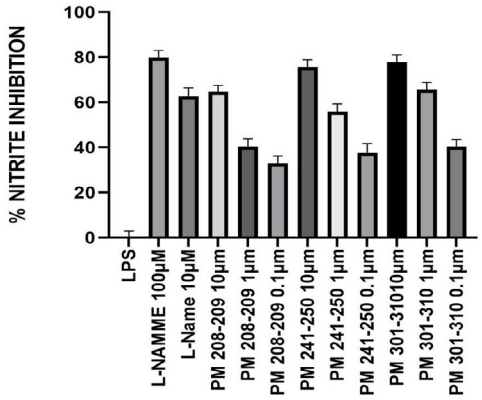


**Figure 2:** Findings of nitric oxide inhibition

### Evaluation of Nitric Oxide Inhibition

For this investigation, one lakh RAW 264.7 cells have been planted in each well of the plate. Figure 2 demonstrated nitric oxide inhibition on RAW 264.7 cell line. The findings exhibited that PM 208-209 is most effective in NO inhibition, although other compounds are inhibiting more but that is because of the cytotoxicity. So, we took PM 208-209 for further experiments.

### Potency to Inhibit TNF-α and IL-6

This involved analyzing the culture supernatants to determine the generation of these pro-inflammatory cytokines. Overall, the findings showed that PM 208–209 was having an increasing dose-dependent inhibitory effect on pro-inflammatory cytokines. Figure 3 depicts the impact of PM 208-209 at concentrations of 10, 1, and 0.1 on the levels of TNF-α in LPS-induced RAW-264.7 cells, as determined by ELISA. Figure 4 illustrates the influence of PM 208-209 at concentrations of 10, 1, and 0.1 µM on the levels of IL-6 in LPS-induced RAW-264.7 cells, as determined by ELISA

### Detection of ROS

Treatment with varying concentrations of PM 208-209 (10, 1, and 0.1 µM) reduced ROS formation, according to the results (Figure 5). Figure 5 demonstrates the impact of PM 208-209 at

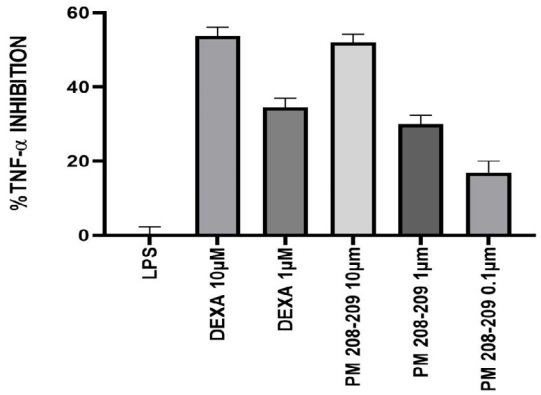


**Figure 3:** Effect of PM 208-209 on TNFα

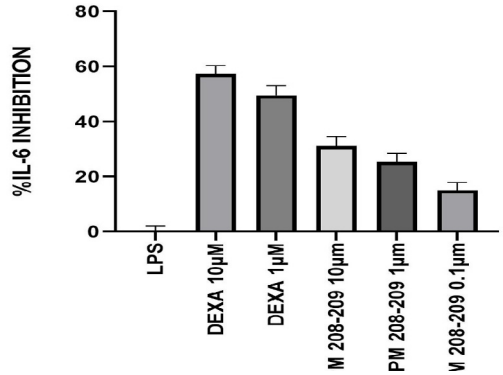


**Figure 4:** The influence of PM 208-209 on IL-6

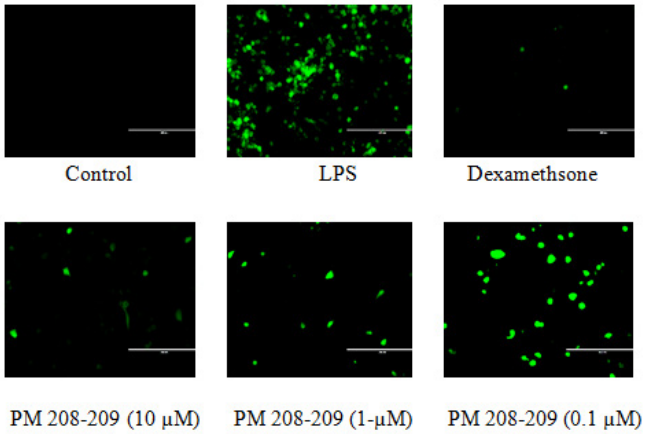


**Figure 5:** Impact of PM 208-209 on the inhibition of ROS

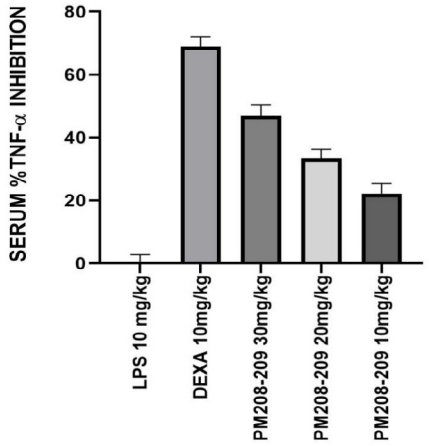


**Figure 6:** Effect on TNF-α inhibition

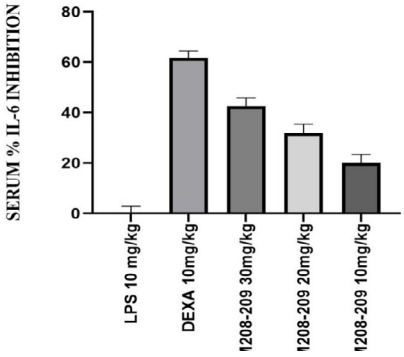


**Figure 7:** Effect on IL-6 inhibition

concentrations of 10, 1, and 0.1 µM on the inhibition of ROS in LPS-induced RAW-264.7 cells. Overexposure to ROS damages cells' antioxidant defense mechanisms and results in oxidative stress. When comparing the fluorescence levels of the groups treated at 1 and 0.1 µM to those treated at 10 µM, there was a significant decrease in the former.

### *In-vivo* Experimentation

In order to validate the anti-inflammatory properties of the compound PM 208-209 in vivo, an experiment was conducted. Figures 6 and 7 displays the percentage inhibition of TNF-α and IL-6, respectively. The findings demonstrated PM 208-209 significantly inhibited the growth of pro-inflammatory cytokines in LPS-treated mice. The percentage inhibition has shown. PM 208-209 at concentrations 30, 20, 10 µM decreased TNF-α, respectively. PM at concentrations 30, 20, 10 µM decreased IL-6, respectively.

## DISCUSSION

The investigation highlights the potential of naturalcompunds produced from plants and their partially synthesized derivatives, such as PM 208-209, as promising primary molecules in the process of discovering new drugs.[13,14] Anti-inflammatory agents face challenges in meeting the demand for improved medications to treat various inflammatory diseases. When there is inflammation, COX-2 is overexpressed, which encourages the synthesis of prostaglandins and other key bioactive inflammatory mediators.[15,16] LPS is a potent immune cell activator, initiating inflammatory cascades involving macrophages, cytokines, prostaglandins, iNOS, and COX-2.[17,18] The creation of anti-inflammatory compounds requires limiting the uncontrollably released cytokines and inflammatory mediators.[19-21] An important factor in inflammation and inflammatory disorders is oxidative damage and ROS. It is well known that LPS causes free radicals and oxidative stress in activated macrophages.[20] Our research revealed the presence of similar substances to *P. mullesua*, indicating

that they possess the ability to suppress pro-inflammatory mediators in RAW 264.7. This evidence-based study aims to evaluate the ability of PM 208-209 to effectively and efficiently reduce inflammation. PM 208–209 demonstrated a significant decrease in responses to inflammation in macrophages activated with LPS, while preserving their morphology and lifespan. Within our experimental configuration, PM 208-209 administration resulted in a substantial decrease in the generation of NO induced by LPS. This effect was shown to be similar to that of its original compound and much superior to that of the conventional dexamethasone. The production of NO by iNOS is frequently associated with the causation of several inflammatory diseases. PM 208-209's antioxidant capacity was evaluated using H2DCFDA dye to prevent internalized oxidative stress and the formation of ROS in murine macrophages caused by LPS. The findings showed that treatment with various PM 208–209 concentrations reduced the production of ROS. In order to assess the therapeutic efficacy of PM 208-209, we further examined its anti-inflammatory properties in animal model. In light of this, PM 208-209 is a promising anti-inflammatory drug with a good safety profile, and more research on this drug in models of chronic inflammatory diseases is required.

## CONCLUSION

Ultimately, we discovered that PM 208-209 effectively suppressed LPS-induced macrophage inflammatory reactions, including NO synthesis. There are a lot of synthetic anti-inflammatory drugs available, but there is a chance they will be toxic or have undesirable side effects. Thus, examining the anti-inflammatory properties of natural substances or their derivatives could result in the creation of safer medications for the management of inflammatory conditions. The research utilized RAW-264.7 cells that had been activated with LPS to comprehensively investigate the effect that PM 208-209 had on important inflammatory mediators. These mediators included pro-inflammatory cytokines, NO, and ROS. The study's findings suggest a promising future for PM 208-209 as a novel anti-inflammatory drug, providing impetus for continued research and development in the pursuit of safer and more effective medications for inflammatory disorders.

## AUTHORSHIP CONTRIBUTION

AK- Conceptualization, Investigation; VKS - Writing – review & editing, Supervision. DS - Investigation, Validation. ZA - Supervision, Funding acquisition, Project administration.

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