Chapter 7

Effects of Berbamine hydrochloride on the expression of pro-inflammatory markers and its scratch healing potential.

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Studying the effects of Berbamine hydrochloride on the expression of pro-inflammatory markers and its scratch healing potential.

7.1 Background

We have investigated the potential therapeutic effects of Berbamine hydrochloride (BER) on inflammation and wound healing. Inflammation, while vital for the body's defense mechanism, can lead to chronic conditions when prolonged [239]. Traditional remedies, particularly plant-derived formulations, offer promising avenues for managing inflammation with fewer side effects than conventional modern drugs [240]. BER, a compound derived from the Traditional Indian herb *Berberis aristata*, has shown anti-inflammatory properties by inhibiting the NF-κB signaling pathway, crucial in inflammation regulation. [241], [242] Furthermore, BER has demonstrated anti-tumor activities, suggesting its potential in treating inflammatory diseases. [243]Here we have elucidated BER's effects on pro-inflammatory markers' expression, such as interleukins and tumor necrosis factor-alpha (TNF-α).

The study utilizes in vitro cell-based scratch assays to investigate the potential of Berbamine hydrochloride (BER) in promoting scratch healing, a critical aspect of wound healing. To understand its impact on inflammation and wound healing processes, monoclonal antibodies specific to Cyclooxygenase-2 (COX-2) and tumor necrosis factor-alpha (TNF-α) are employed. Upon treatment of berbamine hydrochloride on the THP-1 Human monocyte cell line, there is a notable decrease in the expression of pro-inflammatory markers COX-2 and TNF-α, as observed through flow cytometry analysis. These results are compared against lipopolysaccharide (LPS) as a positive standard for validation.

The findings of the study shed light on the therapeutic potential of BER in managing inflammation and promoting wound healing. Importantly, BER demonstrates effectiveness in reducing the expression of key pro-inflammatory markers, suggesting its potential as an

alternative treatment with potentially fewer adverse effects compared to conventional modern drugs.

7.2 Introduction

Herbal treatments and remedies have been integral to the management of various ailments since ancient times. Despite an abundance of literature highlighting their medicinal benefits, there is a lack of established methods for evaluating the effectiveness of plant materials concerning their phytochemical, pharmacological, and therapeutic properties. Plant-derived medicines have been employed in human healthcare for millennia, with widespread use in China and India. Inflammation serves as a crucial protective mechanism in the initial defense line of the body against microbial infections and injuries. This process involves the recruitment of various white blood cells, including monocytes, neutrophils, macrophages, dendritic cells, and lymphocytes, to the site of damage. These cells produce cytokines like interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, promoting immune cell activation and infiltration to combat infections. However, prolonged inflammation is linked to numerous non-communicable diseases (NCDs) such as rheumatoid arthritis, diabetes, cardiovascular diseases, inflammatory bowel disease, and cancers. The World Health Organization (WHO) identifies NCDs as a leading cause of global mortality.[244] The nuclear factor (NF)-κB plays a vital role in inflammation regulation by synthesizing inflammatory mediator proteins. Downregulating the NF-κB pathway is a major target for mitigating chronic inflammation and associated diseases.[245] Conventional drugs like COX inhibitors, including nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids, are commonly used for pain and inflammation but may cause adverse effects. Consequently, research is focusing on exploring new drugs and compounds with fewer side effects for the prevention and treatment of inflammatory diseases. Medicinal plants are being investigated for their potential to modulate

pro-inflammatory mediators and increase anti-inflammatory cytokines like IL-10, offering promising alternatives to conventional treatments.

Berbamine (BER) is a unique bisbenzlisoquinolines alkaloid derived from the traditional Indian plant *Berberis aristata*. Approximately two decades ago, investigations found that BER and its derivatives have anti-inflammatory capabilities by inhibiting the production and release of inflammatory mediators in vitro via macrophages or neutrophils. Furthermore, BER had an inhibitory impact on beneath the skin air pouch inflammation in vivo experiments. Considering these discoveries, the specific pharmacological processes behind its anti-inflammatory effect were not completely explored at the time, limiting BER's possible therapeutic uses. [246], [247]. Over the past few years, BER and its derivatives have been shown to have anti-tumor activity against a variety of malignancies, especially lymphoma, myeloma, lung, and breast cancer. Anti-tumor studies show that BER strongly suppresses the NF-κB signaling pathway, which is critical in the inflammatory response.[248], [249], [250] Therefore, BER and its derivatives may exert anti-inflammatory effects by inhibiting the NF-κB signaling pathway in macrophages and neutrophils, suggesting their potential as agents for treating inflammatory diseases. [251]

Various in vitro and animal models exist for testing the potential wound-healing properties of novel therapeutic compounds derived from these medicinal plants. Among these models is the in vitro cell-based scratch assay, a well-established and cost-effective method that aids in the early understanding of how newly developed therapeutic agents contribute to wound healing. Through this test, researchers can assess cell migration and intercellular communication by creating a scratch on a cell monolayer and capturing time-lapse photos at regular intervals, commonly referred to as a scratch assay

The wound healing process consists of several phases, involving inflammation, cell proliferation, and contraction of the collagen lattice. Furthermore, a buildup of free radicals

that lack oxygen or microbial infections might slow the healing process. Over the last 15 years, researchers have developed in vitro assays to target all of these processes. The many stages of wound healing converge, and a plant-based cure should preferably impact at least two separate processes before being experimentally validated for traditional usage.[252], [253] In vitro tests can be used to assess the impact of pharmacological drugs on various processes, such as fibroblast growth or oxidative stress reduction. Several in vitro approaches have been developed to assess the effects of substances on the synthetic cascade that leads to the generation of prostaglandins and leukotrienes. Inhibiting NF-κB production has been frequently used to investigate anti-inflammatory efficacy.[254] However, its application specifically in the study of wound-healing plants has not been extensively explored.

Cyclooxygenase-2 (COX-2/COX2), sometimes referred to as prostaglandin G/H synthase 2 (PGHS-2), is the target of the AS67 monoclonal antibody. The COX-2 enzyme is encoded by the PTGS2 gene, which codes for prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase). The enzyme is found as a homodimer on the inner and outer membranes of the nuclear envelope as well as the lumenal surface of the endoplasmic reticulum. COX-2 converts arachidonic acid into prostaglandin H2. Prostaglandin synthases then convert the PGH2 intermediate into a number of physiologically active prostanoids, including Prostaglandin E2 (PGE2) and Thromboxane A2. Under normal settings, most tissues do not express COX-2.[255], [256] Inflammation, cellular stress, growth factors, tumor promoters, hormones, bacterial endotoxins, and inflammatory cytokines like interleukin-1α all cause an increase in COX-2 expression. Numerous cell types, such as fibroblasts, endothelial cells, monocytes, ovarian follicles, and mesenchymal stromal cells, can activate COX-2. Immunoassay shows that while COX-2 and Cyclooxygenase-1 (COX-1) have structural

similarities, the AS67 antibody does not cross-react with recombinant human COX-1 protein.[257]

Tumor Necrosis Factor (TNF) serves as a highly effective mediator in the realms of juxtacrine, paracrine, and endocrine signaling, playing crucial roles in both inflammatory and immune processes. Its regulatory influence extends to the growth and differentiation of diverse cell types within the biological milieu. Particularly noteworthy is its cytotoxic capability when acting synergistically with Interferon-gamma (IFN-γ), exhibiting a targeted impact on transformed cells.

The cellular orchestration of TNF involves its secretion by activated monocytes, macrophages, and other cell types, including B cells, T cells, and fibroblasts. This underscores the versatile nature of TNF as it operates within the intricate network of cellular communication, contributing to the modulation of inflammatory responses and immune functions across various tissues and physiological contexts.[258], [259] The multifaceted roles of TNF underscore its significance in orchestrating cellular behaviour and immune dynamics, highlighting its potential as a pivotal target for therapeutic interventions in conditions characterized by dysregulated inflammatory and immune processes.

The monoclonal antibody MAb11 exhibits a high degree of specificity as it selectively binds to the human tumor necrosis factor (TNF), also referred to as TNF-α. This targeted binding capability makes MAb11 a valuable tool in research and diagnostic applications, allowing for precise identification and detection of TNF within biological samples.[260]

The MAb11 hybridoma, the cell line used to produce this monoclonal antibody, was generated using a recombinant form of human TNF as the immunogen. The use of recombinant TNF as the immunizing agent ensures that the resulting monoclonal antibody is tailored to recognize and interact specifically with the human TNF protein. This specificity is critical for avoiding

cross-reactivity with other proteins and enhancing the accuracy and reliability of experimental results.

By employing MAb11, researchers can harness its ability to bind specifically to TNF, facilitating the isolation, quantification, and analysis of this important cytokine in various experimental settings. This targeted monoclonal antibody thus contributes to the advancement of our understanding of TNF's role in physiological and pathological processes, offering a precise tool for investigating and manipulating TNF-related pathways in scientific and medical research.

7.3 Methodology

7.3.1 Anti-inflammatory assay on THP1 cells-

In our experimental setup, we utilized the THP-1 Human monocyte cell line obtained from the National Centre for Cell Science (NCCS) in Pune, India. The cells were cultured in 1x phosphate-buffered saline (DPBS) from HiMedia, India. To stimulate cellular responses, we employed Lipopolysaccharide (LPS) also sourced from HiMedia. For cell detachment and maintenance, trypsin-EDTA solution from HiMedia was utilized. Following experimental treatments, cells were fixed using a 2% Paraformaldehyde solution, also obtained from HiMedia.

To facilitate intracellular staining, we prepared a 0.5% BSA solution in 1x DPBS and a subsequent 0.1% Triton-X 100 solution in the BSA mixture. The experimental setup involved the use of 15mL and 1.5 mL centrifuge tubes from Abdos, India, as well as 5mL tubes from Tarsons, India, for sample processing. The immunostaining process included PE Mouse Anti-Human COX-2 (Catalog No. 565125) and FITC Mouse Anti-Human TNF (Catalog No. 562082) antibodies sourced from BD Biosciences. Additionally, BD GolgiStop™, a Protein

Transport Inhibitor containing Monensin (Catalog No. 554724) from BD Biosciences, was used to arrest protein transport within the cells.

Flow cytometric analysis was conducted using the Cytomics FC500 Flow cytometer from Beckman Coulter, USA. The acquired data were subsequently analyzed using FlowJo X 10.0.7, a powerful analysis software. This comprehensive approach allowed us to investigate and understand the cellular responses and expressions of COX-2 and TNF in the context of our experimental conditions.

7.3.2 Analyzing COX-2 and TNF-α expression by Flow cytometry-

Cells should be cultivated for 24 hours at 37°C in a CO_2 incubator with a density of 3 x 10^5 cells/2 mL in a 6-well plate. For an extra 24 hours at 37°C in a CO_2 incubator, incubate the cells containing the required concentration of experimental compounds in 2 mL of culture medium. Add 2 µl of BD GolgiStopTM, a Protein Transport Inhibitor with Monensin, to cells after they have been stimulated with LPS (2 µg/mL) to aid in the accumulation of expressed cytokines within the cell. In a CO_2 incubator, cells should be cultured for 6–8 hours at 37°C. After the treatment, transfer the cells to 5 mL tubes and centrifuge at 300 x g for five minutes at 25°C. Decant the supernatant carefully, then wash the cells again with PBS. Drain the PBS altogether. Incubate for 20 minutes with 0.5 mL of 2% Paraformaldehyde solution, then wash with 0.5% BSA (bovine serum albumin) in 1X phosphate-buffered saline. Next, add 0.1% Triton-X 100 to a 0.5% BSA solution and incubate for 10 minutes. Rinse again with 0.5% BSA in 1X PBS. Add the indicated antibody dilution to 100µl of cell solution, pipette well, and incubate for 30 minutes in the dark at room temperature (25°C). Finally, wash with 0.5% BSA, add 0.5 mL of PBS, carefully mix, and analyze. If samples will not be examined immediately, ensure complete mixing right before analysis.

7.3.3 Scratch healing in L929 cells

We employed the L929 mouse fibroblast cell line, which we obtained from the National Centre for Cell Science (NCCS) located in Pune, India, for our experimental study. In order to supply vital nutrients and promote cell development, the cells were cultivated in Dulbecco's Modified Eagle Medium with High Glucose (DMEM-HG), which was supplemented with 10% Fetal Bovine Serum (FBS). The T-25 cell culture flasks from Biolite, Thermo Scientific, and 12-well cell culture plates from Nunc, Thermo Scientific, were utilized for cell proliferation and experimental treatments.

Cell maintenance and subculture involved the use of phosphate-buffered saline (DPBS) from HiMedia and trypsin from HiMedia for efficient detachment. Sterile 5ml/15 ml centrifuge tubes from Tarsons were employed for sample preparation and centrifugation steps. Microscopic observations and imaging were conducted using the XDFL series microscope from Sunny Instruments, China, to monitor cell morphology and experimental outcomes.

For quantitative analysis of wound healing assays, ImageJ software with the MRI Wound Healing Tool plugin was employed. This software facilitated accurate and efficient measurement of wound closure, allowing us to assess the migratory behavior of the L929 cells in response to specific experimental conditions. The combination of advanced cell culture techniques, reliable laboratory consumables, microscopy, and sophisticated analysis software contributed to a comprehensive and insightful exploration of cellular responses and behavior in our experimental model.

In 12-well plates, cultivate cells to create a monolayer. When the cells are 70–80% confluent, they begin to scrape to generate a wound. Use 1 milliliter of DPBS to wash the monolayer twice. Depending on the pace of cell migration, add 1 ml of medium—with or without test drugs—to each well and incubate for 24 or 48 hours. Using an inverted phase contrast microscope, take pictures at regular intervals of 0 hours, 12 hours, 24 hours, and 48 hours, as

needed. Comparing the initial gap area (0h) to the end gap area (12h or 24h) yields the percentage of cell migration.

7.4 Results and Discussion-

7.4.1 COX-2 Expression –

A decrease in COX-2 expression was observed upon pre-treatment of the cells with Berbamine hydrochloride at non-toxic concentration for 24h prior to LPS stimulation, as compared to only LPS induced cells, significant decrease was seen in the Mean fluorescence intensity of COX-2 PE and also a decrease in number of COX-2 high cells. A reduction in the expression of Cyclooxygenase-2 (COX-2) corresponds to a concurrent decrease in the inflammatory response following treatment with the Berbamine hydrochloride. COX-2 is an enzyme involved in the synthesis of prostaglandins, which are mediators of inflammation. [261]When the expression of COX-2 is downregulated, it implies a diminished capacity to produce prostaglandins, leading to a modulation of the inflammatory cascade.[262]

Table 7.1 Observed fold change in COX-2 expression before and after pre-treatment of berbamine hydrochloride.

S.No.	Sample Name	Geometric mean fluorescence intensity (MFI) of PE COX-2 (FL2-A parameter)	Fold change in COX-2 expression	% of Cells	
				COX-2 low	COX-2 high
1	**Untreated (Control)**	280	1	97.4	2.6
2	**LPS induced - 2 µg/mL**	1370	4.9	38.7	61.3
3	**Berbamine hydrochloride 25µg/mL + LPS**	799	2.9	80.7	19.3

The observed correlation suggests that Berbamine exerts an inhibitory effect on COX-2 expression, potentially through mechanisms such as transcriptional regulation or post-translational modifications. This downregulation of COX-2 is likely to result in a dampened inflammatory response, as the production of pro-inflammatory prostaglandins is curtailed.[263], [264]

This finding has important implications, especially in the context of inflammatory disorders or conditions where excessive inflammation is a contributing factor. The ability of the berbamine hydrochloride to modulate COX-2 expression and, subsequently, the inflammatory response, may signify its therapeutic potential as an anti-inflammatory agent. Further investigations into the precise mechanisms by which the berbamine influences COX-2 expression could provide valuable insights into its mode of action and contribute to the development of targeted interventions for inflammatory conditions.

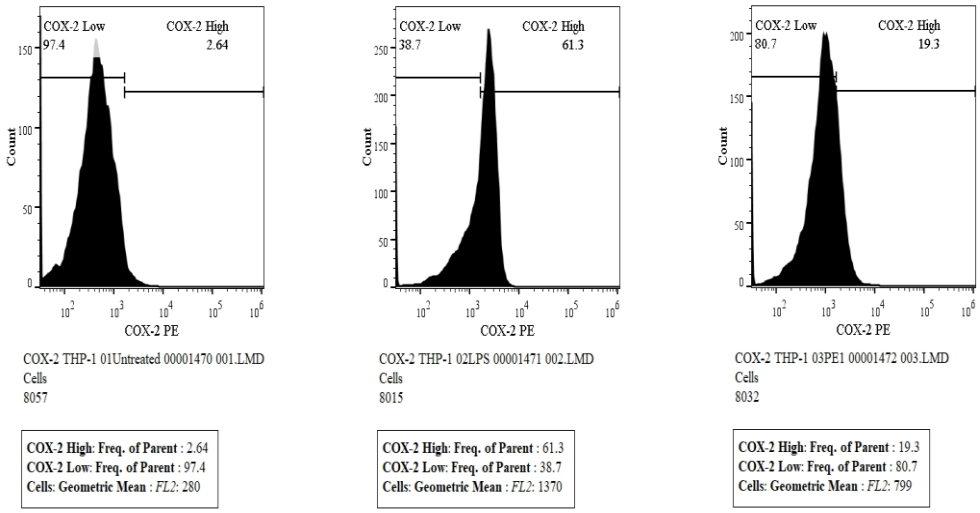

Figure 7.1 -The quantification of COX-2 expression levels within the cell population. The anti-human COX-2 antibody is conjugated to PE fluorochrome (FL2 detector) which binds to the COX-2 protein which is a marker of inflammation. PE (yellow) fluorescence was collected in the FL2 detector using a 575nm band pass filter.

7.4.2 TNFα expression-

The observed decrease in TNFα expression following pre-treatment with Berbamine hydrochloride at a non-toxic concentration 24 hours prior to LPS stimulation suggests a potential anti-inflammatory effect of the sample. TNFα, a pro-inflammatory cytokine, plays a crucial role in mediating inflammatory responses. The decrease in TNFα production was evidenced by a reduction in both the mean fluorescence intensity of FITC fluorochrome and the percentage of TNFα high cells. FITC fluorochrome, conjugated to the anti-human TNFα antibody, facilitates the detection of TNFα protein via fluorescence microscopy or flow cytometry. Upon binding to TNFα, the fluorescence signal is emitted and detected in the FL1 detector using a 525nm band pass filter. This detection method enables the quantification of TNFα expression levels within the cell population.

The observed reduction in TNFα expression indicates that berbamine may possess anti-inflammatory properties, potentially mitigating the inflammatory response induced by LPS stimulation. This finding highlights the therapeutic potential of berbamine in modulating inflammatory pathways and suggests its possible utility in the treatment of inflammatory conditions. Further elucidation of the underlying mechanisms involved in the observed decrease in TNFα expression, as well as additional studies to evaluate the broader anti-inflammatory effects of berbamine, are warranted. Overall, these findings contribute to our understanding of the potential therapeutic applications of berbamine in managing inflammatory disorders and underscore the importance of further investigation into its anti-inflammatory mechanisms of action.

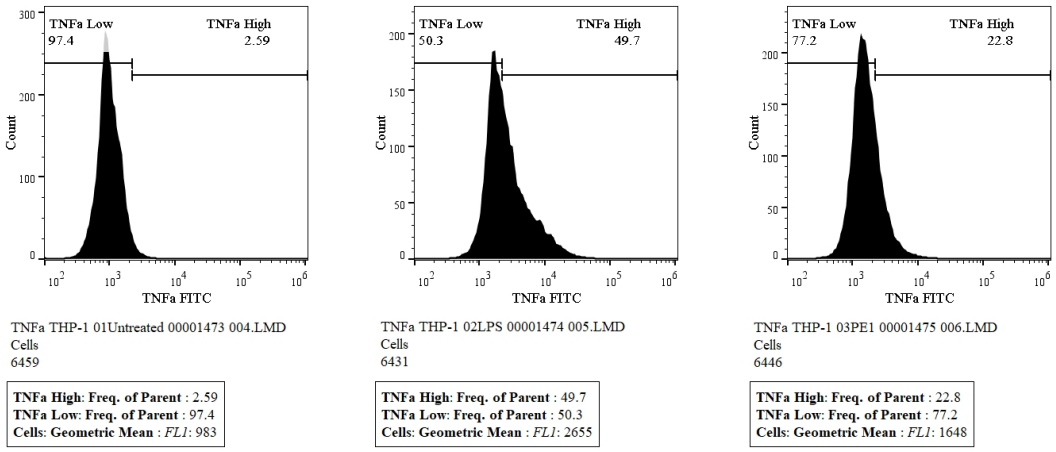

Figure 7.2- The quantification of TNFα expression levels within the cell population. Cell population showed decrease in TNFα production was evidenced by a reduction in both the mean fluorescence intensity of FITC fluorochrome and the percentage of TNFα high cells. FITC fluorochrome, conjugated to the anti-human TNFα antibody.

Table 7.2 Observed fold change in COX-2 expression before and after pre-treatment of berbamine hydrochloride.

S.No.	Sample Name	Geometric mean fluorescence intensity (MFI) of FITC TNFα (FL1-A parameter)	Fold change in TNFα expression	% of Cells	
				TNFα low	TNFα high
1	Untreated (Control)	983	1	97.4	2.6
2	LPS induced - 2 µg/mL	2655	2.7	50.3	49.7
3	Berbamine hydrochloride 25µg/mL + LPS	1648	1.7	77.2	22.8

7.4.3 Cell migration and scratch healing-

In our investigation into the wound healing properties of Berbamine hydrochloride, we conducted a scratch-wound assay utilizing L929 cells. Initially, the control group displayed a

moderate 83.49% wound closure, indicating a baseline level of cellular migration. Upon the introduction of Berbamine hydrochloride at concentrations of 25ug/ml and 50ug/ml, we observed a significant enhancement in wound closure. Specifically, after 12 hours, the healing percentages were noted to be 97.13% and 98.34% respectively.

Table 7.3: Cells treated with berbamine hydrochloride at 25 & 50µg/mL showed significant increase in wound healing as compared to the control untreated cells.

Sample			Wound area (in sq. µm)		% wound healing	Average (% wound healing)
			0h	12h		
Control	**Well 1**	Reading 1	1234772	162656	86.83	83.49± 2.51
		Reading 2	1263238	243182	80.75	
	Well 2	Reading 1	1163531	193006	83.41	
		Reading 2	1170254	199175	82.98	
Berbamine 25µg/ml	**Well 1**	Reading 1	1075566	52953	95.08	97.13±2.69
		Reading 2	1105407	13104	98.81	
	Well 2	Reading 1	1264140	68112	94.61	
		Reading 2	1120871	0	100.00	
Berbamine 50µg/ml	**Well 1**	Reading 1	1149221	24494	97.87	98.34±1.15
		Reading 2	1213188	22958	98.11	
	Well 2	Reading 1	1058111	27795	97.37	
		Reading 2	1136917	0	100.00	

These findings strongly suggest that Berbamine hydrochloride facilitates cell migration, as evidenced by the accelerated closure of the scratch wound. The dose-dependent effect further reinforces this conclusion, with higher concentrations of Berbamine hydrochloride yielding greater improvements in wound healing efficacy. This observed augmentation in wound closure may be attributed to the enhanced cell motility induced by Berbamine hydrochloride, potentially mediated through alterations in cytoskeletal dynamics or signaling pathways.

7.4.4 Analysis of cell migration in L929 cells-

A straightforward, reliable technique for determining the polarity, tenacity, and speed of fundamental cell migration characteristics is the scratch-wound test. After cells have grown to confluence, a thin "wound" is made by scraping with the tip of a pipette. After polarizing, cells on the wound edge migrate into the wound region. The experiment examines the two ways in which a sheet of cells migrates in reaction to the formation of a tiny wound. Cells may separate from the sheet or keep their consistent front. When a scratch is inserted, the affected area moves in a prescribed manner perpendicular to the wound boundary, starting from an upright posture. The deviations from this path are easily quantifiable, providing normalized data for speed and direction consistency.

7.5. Conclusion

The observed decrease in COX-2 expression upon pre-treatment with Berbamine hydrochloride signifies a potential anti-inflammatory effect of the sample. COX-2, an enzyme crucial in prostaglandin synthesis, is intricately involved in mediating inflammatory responses. When COX-2 expression is downregulated, it indicates a reduced capacity for prostaglandin production, thereby modulating the inflammatory cascade. The correlation observed suggests that Berbamine exerts an inhibitory effect on COX-2 expression, possibly through

transcriptional regulation or post-translational modifications. This downregulation of COX-2 is likely to dampen the inflammatory response, as the production of pro-inflammatory prostaglandins is curtailed. These findings hold significant implications, particularly in the context of inflammatory disorders where excessive inflammation is a contributing factor. The ability of Berbamine hydrochloride to modulate COX-2 expression and subsequently the inflammatory response underscores its therapeutic potential as an anti-inflammatory agent. Further investigations into the precise mechanisms underlying the influence of Berbamine on COX-2 expression could provide valuable insights into its mode of action and contribute to the development of targeted interventions for inflammatory conditions.

Similarly, the observed decrease in TNFα expression following pre-treatment with Berbamine hydrochloride suggests its potential as an anti-inflammatory agent. TNFα, a pro-inflammatory cytokine, plays a pivotal role in mediating inflammatory responses. The reduction in TNFα production, evidenced by decreased mean fluorescence intensity of FITC fluorochrome and percentage of TNFα high cells, indicates that Berbamine may possess anti-inflammatory properties, mitigating the inflammatory response induced by LPS stimulation. These findings highlight the therapeutic potential of Berbamine in modulating inflammatory pathways and suggest its possible utility in the treatment of inflammatory conditions. Further elucidation of the underlying mechanisms involved in the observed decrease in TNFα expression, along with additional studies to evaluate the broader anti-inflammatory effects of Berbamine, are warranted.

Furthermore, in our investigation into the wound healing properties of Berbamine hydrochloride, we conducted a scratch-wound assay utilizing L929 cells. The significant enhancement in wound closure observed upon treatment with Berbamine hydrochloride suggests its potential in facilitating cell migration. The dose-dependent effect further reinforces this conclusion, with higher concentrations of Berbamine hydrochloride yielding greater

improvements in wound healing efficacy. This observed augmentation in wound closure may be attributed to the enhanced cell motility induced by Berbamine hydrochloride, potentially mediated through alterations in cytoskeletal dynamics or signaling pathways.

The scratch-wound assay, a simple yet reproducible assay, provides valuable insights into basic cell migration parameters such as speed, persistence, and polarity. By introducing a scratch to initiate migration, the assay allows for the study of cell migration in response to wound formation. Berbamine hydrochloride's ability to accelerate wound closure in this assay underscores its potential in promoting tissue repair and wound healing. Further investigations into the underlying mechanisms involved in Berbamine-mediated enhancement of wound closure could provide valuable insights for the development of novel therapeutics targeting wound healing processes.

In conclusion, the findings from our study highlight the multifaceted therapeutic potential of Berbamine hydrochloride, ranging from its anti-inflammatory properties to its ability to promote wound healing. These observations pave the way for further research into the mechanisms of action underlying Berbamine's effects and its potential clinical applications in the management of inflammatory disorders and wound healing.