

PL02[®] alleviates joint Pain and Inflammation, and subchondral bone deterioration in the Monosodium iodoacetate-induced Osteoarthritis rat model

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Abstract

Osteoarthritis (OA) is the fourth most debilitating multifactorial disease, associated with personal and socioeconomic burdens worldwide. Currently, no safe therapy is available that can effectively prevent the deterioration of cartilage and subchondral bone or reverse existing defects. To address this, we have designed a multitargeted formulation, PL02, consisting of standardized extracts of the *Rosa canina L.* and *Hippophae rhamnoides*, along with collagen peptide, to explore the pharmacological efficacy in the Monosodium iodoacetate-induced (MIA) OA model in the rodents. The results show oral administration of PL02 exhibits antioxidant effects via down-regulating NOS, alleviated pain-related behavior, and reduced inflammation via inhibiting IL-1 β and dependent TNF- α production, downregulating CGRP1 and COX-II. PL02 exerted anti-catabolic and chondroprotective activity by significantly downregulating MMP13 and upregulating BCL2. PL02 exhibited chondrogenic activity by significantly upregulating SOX-9(master regulator of chondrogenesis), Coll-I, and aggrecan, the major collagen and proteoglycan in the articular cartilage and prevented microarchitectural deterioration of subchondral bone. Thus, orally active PL02, a multi-targeted disease-modifying therapy that has not only alleviated pain and inflammation but also effectively arrested cartilage, and subchondral bone deterioration, constitutes a safe novel candidate for OA treatment and management.

Introduction

Osteoarthritis (OA) is a chronic degenerative condition that causes deterioration of the articular cartilage and deformation of the subchondral bones that triggers pain and significantly affects over 300 million people worldwide. This condition is common in older people due to the increasing calcification of bones and loosening of ligaments surrounding joints resulting in chronic pain, joint stiffness, and inflammation. The advancement of the disease results not only in the disruption of cartilage homeostasis and non-cartilaginous components of the joint, like the joint capsule, synovium, subchondral bone, ligaments, and periarticular muscles. The subchondral bone starts to thicken, and osteophytes that emerge and increase friction in joints begin to appear in OA joints, causing elevated inflammation in the synovium, and uneven, enlargement of the joint. (Loeser et al., 2012). Multiple risk factors such as age, obesity, oxidative stress, inflammation, deficiency of essential nutrition, hereditary factors, and trauma may contribute to the development of OA. The first-line therapy used for OA associated pain is nonsteroidal anti-inflammatory drugs; however, with long-term use their efficacy declines, and they can lead to major adverse gastrointestinal and cardiovascular events. Currently, there is no effective therapy for OA; the available options (herbal, NSAID, and steroids) give symptomatic relief for a short period and are poorly tolerated. Therefore, the developing of effective, safe non-opioid treatments for the prevention and resolution of OA pathophysiology is of the utmost urgency. It is well established that oxidative stress and inflammation are involved in the pathogenesis of OA. Both are crucial in inducing cartilage degeneration and subchondral bone deformation, which lead to OA disease progression. Thus, targeting excessive oxidative stress, inflammation, and cartilage regeneration would be a promising approach to alleviate

chronic pain and improve articular cartilage to arrest the progression of diseases.(Murphy et al., 2020; Ren et al., 2022)

Worldwide various research groups have characterized diverse groups of bioactive phytochemicals to discover their pharmaceutical efficiency in treating different kinds of human illnesses (Zhou et al., 2022). Among them, Rosehips are the aggregate fruits of shrubs belonging to the *Rosa* genus of the Rosaceae family that is widely used as necessary dietary antioxidants. The high antioxidant activity is mainly attributed to ascorbic acid; apart from ascorbic acid, various other phytochemicals present, like carotenoids, Tocopherols, polyphenolic compounds, triterpenoic acids are primarily known for their immunomodulatory, hepatoprotective, anti-inflammatory, antitumor, antioxidant and antihyperlipidemic effects in *in-vitro* and *in-vivo* studies. Unsaturated fatty acids found in rosehip seeds, mainly linoleic and α -linolenic acids, have been considered responsible for the inhibitory effects on cyclooxygenase-1 and 2 in *in-vitro* experiments (Bhave et al., 2017; Mármol et al., 2017)

Another critical but less characterized plant is *Hippophae rhamnoides*, which belongs to the family Elaeagnaceae. In India, *Hippophae rhamnoides* is grown in the high-temperate zone and Western Himalayas 2,600–4000 m. primarily in Leh, Laddakh, Himachal Pradesh, Uttarakhand, and Sikkim. The beneficial role of *Hippophae rhamnoides* is established as a natural antioxidant with anti-inflammatory, immunomodulatory, and homocysteine-reducing properties. The phytochemicals present in it have been reported to prevent mitochondrial degeneration, reduce vascular inflammation, and have dilatory and anti-cancer activity. Seabuckthorn (SBT) leaf extract was evaluated in adjuvant-induced arthritis (AIA) rat model and it observed that the SBT leaf extract has a significant anti-inflammatory activity and has the potential for the treatment of arthritis (Bhave et al., 2017; Ganju et al., 2005; Stobdan et al., 2013).

Collagen is the most abundant biomaterial that builds up cartilage, skin, and bone. It has been reported that collagen levels start decreasing by $\sim 1\%$ annually after the age of 25. Compared to whole collagen and gelatin hydrolyzed collagen containing small molecular weight peptides has better bioavailability and has been predicted to have beneficial effects on skin, bone, and joints. (León-López et al., 2019; Upadhyay et al., 2018). Supplementation with hydrolyzed collagen might help restore the collagen levels in the body lost due to aging and a poor diet by stimulating its production in the extracellular matrix of cartilage and other tissues. However, its therapeutic potential has not yet been established.

The MIA-induced OA model is well-established in the literature as the articular injection of the MIA develops the symptoms very much parallel to human OA(Lockwood et al., 2019; Mohan et al., 2011; SR et al., 1994; Udo et al., 2016). Pain becomes the predominant symptom that occurs due to the initial inflammation process. Intra-articular injection of MIA causes chondrocyte cell death, leading to cartilage degeneration and subsequent subchondral bone alterations(Mohan et al., 2011).

Even though the individual therapeutic potential of collagen peptide, Rosehip and *Hippophae rhamnoides* as anti-inflammatory and immunomodulatory agents are known, they have not yet to be considered as first-line therapy for pain and inflammation related to OA and other associated diseases until date. To address this in the present study we have standardized, and characterized the extracts of Rosehip and

Hippophae rhamnoides and developed a formulation PL02 containing extracts and collagen peptides. The MIA-induced animal model of OA was used to evaluate the pharmacological effect of the formulation in the management of OA and mechanism decipher via assessing the level of the anti-inflammatory marker, antioxidant, chemokine, cytokines, gene expression, histological analysis of joint tissue, and microarchitectural analysis of subchondral bone.

Methods

Materials and reagents

Monosodium iodoacetate (MIA), sodium chloride (NaCl), Sephadex G-15 beads were purchased from Sigma-Aldrich (St. Louis, USA). Plant extracts of *Rosa canina* L. and *Hippophae rhamnoides* were a purchased from GMP certified company. Fish Collagen peptide was procured from FSSAI approved company. The XK 16/70 used for collagen desalting and purification was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT) Alkaline phosphatase (ALP), Urea, Creatinine kits for biochemical estimation were purchased from Tulip Diagnostics Private Limited. Lipid Peroxide estimation was done using ELISA kit from Immunotag™ G-Biosciences (Miss The proinflammatory and anti-inflammatory cytokines level y cytokines was checked using Bio-Plex 23 panel Pro™ Cytokine multiplex kit (Bio-Rad, California, USA). Primary and secondary antibodies for TNF- α were procured from Cell Signaling Technology, Danvers, MA. Primers for real-time PCR analysis, designed by NCBI Primer blast and synthesized by Sigma -Aldrich (St. Louis, USA).

Preparation of PL02 Formulation

The formulation PL02 contained hydrolyzed collagen and herbal blend. The herbal blend is composed of the standardization extract of the *Rosa canina* L, *Hippophae rhamnoides* with folic acid, omega 3 fatty acid, omega 7 fatty acid, omega 9 fatty acid, vitamin C and alpha lipoic acid. PL02 formulation was administered orally at 600 mg/kg body weight dissolved in MilliQ water.

Collagen standardisation for quality control

For the enhancement biological activity of the peptide and remove the excess of salts, purification of the peptide was done using Amersham Pharmacia Fast protein liquid chromatography (GE AKTA Explorer) equipped with Pump P-920 controlled by a UPC-900 monitor and IV-908 injection valve for sample. The XK 16/70 column connected to controller was packed with sephadex G-10/15 beads swelled in MilliQ water overnight at 4°C. Collagen peptide was dissolved in MilliQ water, and a stock solution of 500 mg/ml was prepared and passed through the column at a flow rate of 2 ml/min. Peak fractionation was set in the UNICORN method wizard and pump A and B inlet were immersed in sonicated MilliQ. The absorbance (214 nm) of eluted material was continuously measured with the Zn Lamp and fractions were collected using the FRAC-900 fraction collector. The collected fraction lyophilized using Allied Frost Indian fabricated Lyophilizer at 0.02 mbar vacuum pressure.

MALDI-TOF mass spectrometry

Purified collagen peptide powder was further analyzed using Applied Biosystems/MDS SCIEX 4800 MALDI TOF/TOF™ Analyzer (California, USA) equipped with a 355-nm pulsed Nd:YAG laser. All measurements (positive and negative ion detection) were performed in the reflector mode by using delayed extraction conditions. The extraction voltage was 20 kV, and gated matrix suppression was applied to prevent detector saturation by matrix ions with m/z lower than 200. One hundred single laser shots were averaged for each mass spectrum, whereby the laser was randomly moved over the sample in order to average analyte/matrix inhomogeneities: the achievable level of detection (LOD) of MALDI-TOF-MS for a specific compound has been shown to be mainly limited by the level of chemical background noise. Therefore, the use of a larger number of laser shots does not significantly improve spectral quality.

UPLC-MS Analysis

The analysis of formulation was performed using ACQUITY UPLCQTOFMS/MS system (Waters Corp., Milford, MA, USA). The chromatography was carried out with Acquity BEH C18 column (dimension: 100 mm × 2.1 mm, 1.7 μ m; temperature: 25°C). The mobile phase consisted of (A) 0.1% formic acid, (B) acetonitrile, and (C) methanol, and its flow rate was 300 μ l/min. Sample injection volume was 5 μ l. Gradient elution program was optimized as follows: initial 90:10% B: C and increased to 80:20% in 2 min, 50–60% B: C for 1–3 min, 30–70% B: C for 3–6 min, then 10:90% B: C for 1 min, and finally increased quickly to 90–10% in 7–10 min. MS analysis was performed in positive ion modes. Centroid mode data were collected over the m/z range 100–1000 Da with a scan time of 1 s. The Software Respect for Phytochemicals (<http://spectra.psc.riken.jp/>) was used for data analysis

Experimental Design

The experiments are conducted in two rodent models (mouse and rat). Mouse models (Carrageenan induced inflammation and MIA) was used for initial pilot efficacy study are mentioned in the supplementary file. The detailed study performed in rat model is presented in the main manuscript. Wistar rats were randomly allocated into four groups ($n = 10$ each). In the Sham control group (Vehicle; $n = 10$) that received saline instead of MIA, disease control group (MIA; $n = 10$) with OA induction and treating with vehicle, positive control group (MIA + Indomethacin(20mg/kg); $n = 10$) with OA induction and treating with Indomethacin orally, formulation PL02 treated group (MIA + PL02; $n = 10$). Pain and inflammation were assessed by performing behavioral tests on day 3,7,14, 21 and 28. All the animal studies were approved by Institutional animal ethics committee (IAEC#576/21) of National Institute of Immunology and according to ARRIVE (Animal Research: Reporting of *in-vivo* Experiments) guidelines.

MIA induced OA rat model was developed according to the reported procedure. To inject MIA (Sigma, St. Louis, USA) intra-articularly, the right knee of rats was shaved and disinfected with 70% alcohol following anaesthetization using xylazine (10 mg/kg, intraperitoneal) and ketamine hydrochloride (80 mg/kg, intraperitoneal). After local trichotomy, the leg was flexed keeping the knee at a 90° angle; the needle was positioned through the intra-patellar ligament and administered a single intra-articular injection of MIA

0.5 mg/ 25 µl (in 0.9% sterile saline) using a 0.3 ml insulin syringe fitted with a 29G needle. The Sham control group received an injection of 25 µl saline only.

Animals were observed on the mentioned days for pain and inflammation in knee joint and paw. After four weeks of the treatment rats were euthanized, blood, vital organs and the knee joint specimen were collected for the follow-up experiments. Carbon dioxide (CO₂) inhalation method of euthanasia was used, fill rate of about 30–70% of the chamber volume per minute. The blood samples were centrifuged at 12000 rpm for 10 min, and serum was separated from the blood, aliquoted and stored at – 20°C for further analysis. Synovial tissue along with the knee joint was processed for mRNA isolation, lysate preparation, and histological analysis or stored at -80°C for the proceeding study.

Mechanical Allodynia

Von Frey is regarded as the gold standard technique used to evaluate the pain sensitivity of the hind paw of the animals with knee joint arthritis. Paw withdrawal threshold (PWT) is measured in response to increasing pressure stimuli applied by von frey hairs to the plantar surface (Afridi et al., 2019). Electronic von Frey Anesthesiometer (IITC Inc. Life Science, California, USA) consists of rigid Supertips™ mounted on a sensitive probe. On applying pA, connected LCD Readout will display the weight bared by the paw in grams on applying pressure placed in individual cages with a metal grating floor and allowed a 30-minute adaptation before the start of the test. Increasing force was applied to the central plantar region of the hind paw to induce reflecting flexion of the joint. A positive response was defined as a rapid withdrawal of the left hind paw or licking of the paw. The test was repeated three times. The first day of the testing provided a baseline measure of tactile sensitivity. Rats in each group were then tested with von Frey filaments on post-injection 3, 7, 14, 21 and 28.

Measurement Of Knee Edema

The paw diameter was measured after the injection of MIA using electronic Vernier caliper (Insize, India) after MIA injection. The difference between inflamed (left) and right knee joint was calculated indicating the degree of inflammation and was compared with the control group.

Measurement Of Paw Inflammation Using Plethysmometer

Inflammation in the paw was measured using a plethysmometer (Paw Volume) Meter (IITC Inc. Life Science, California, USA). The original device was designed specifically to measure paw volume and its changes (swelling) in rodents aiming to test the efficacy of anti-inflammatory agents (Fereidoni et al., 2000). The left hind paw was dipped in the mercury column attached on a stand and the rise in the mercury level displayed in ml was noted to check for the edema in rat paw.

Biochemical estimation

Blood was withdrawn on day 14 and day 28 through the retro orbital region of rat in the non-anti-coagulant tube. The collected blood samples were centrifuged at 12000 rpm for 15 min at 37°C, and serum was separated from the blood and stored at – 20°C.

Toxicity Assessment:

The measurement of biochemical parameters (Tulip diagnostic Pvt Ltd auto analyzer) Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT), Alkaline phosphatase (ALP), Urea, and Creatinine analysis was performed as per mentioned in the manual of Coral clinical system.

Quantitative analysis of Antioxidant and Anti-inflammatory activity in serum

Lipid peroxide, a biomarker for oxidative damage in cells, was measured in the rat serum using ELISA kit (Immunotag™ G-Biosciences, Missouri, USA). Lipid Peroxidation (LPO) Assay was performed according to the instruction manual provided by the manufacturer. Serum levels of proinflammatory and anti-inflammatory cytokines were analyzed using the Bio-Plex Pro™ Cytokine Standard kit (Bio-Rad, California, USA). The plate was prepared following the instruction manual provided by the manufacturer and read using Bio-Plex 200 Systems (Bio-Rad, California, USA). The data was analyzed on Bio-Plex Manager (acquisition and Analysis) software at low PMT, RP1 instrument settings.

Real time PCR

Total RNA was extracted from bone joint (bone, cartilage and synovium) tissue of all the treatment groups using TRIzol (Invitrogen). 1 µg of total RNA was reverse transcribed using the Verso cDNA synthesis kit (Thermo Fisher Scientific). For real time PCR, data acquisition and analyses were performed using the Light Cycler Real Time PCR system (Roche Diagnostics) and SYBR Green I (Roche Diagnostics, Mannheim, Germany). The relative levels of gene expression were normalized against Beta Actin or GAPDH and plotted as fold change to sham control. Primers were designed using the IDT (Integrated DNA technologies) RT primer designing tool. The sense and antisense primers used are given in supplementary file (Table S2).

In Situ Assay For Biological Activity: Tnf-α Production By SW-982 Cells

SW-982 cells (a human synovial cell line SW-982, HTB-93) procured from ATCC was maintained as per the provider's instructions. The anti-inflammatory activity was evaluated as described by *pasi et al* (Pasi et al., 2015). In brief, for in vitro assays of the biological activity of PL02, SW-982 cells were plated at a density of 10⁵ cells per well in a 24-well tissue culture plate. After 48 h cells were rinsed thoroughly with

PBS, treated with IL-1RA (15ng/ml) or PL02 (1, 2, 5, 10 mg/ml in PBS, centrifuged at 15000 rpm for 15 min at 4C, clear supernatant passed through 0.2µm syringe filter, 200µl/well used for treatment) for 1 h and after that stimulated with 5 ng/ml of human IL-1b (Sigma). After 24 h culture supernatants were analysed for human TNF-α levels using Ultrasensitive ELISA kits from eBioscience as per the manufacturer's instructions.

Histology

On day 28 after dissections organs such as heart, liver, kidney, brain, and left knee joints are fixed with 10% neutral-buffered formalin for 48 h at room temperature. Decalcification of knee joints specimens was done with 20% formic acid for 3 days and then embedded in paraffin. Sections of the tissue specimens were acquired from the paraffin blocks at 5 µm thickness, deparaffinized, and rehydrated in the order of xylene, absolute alcohol, and 50% alcohol. The rehydrated sections were stained with hematoxylin and eosin (H&E) for observation of morphological changes. Besides H& E stain, the sections of knee joints used toluidine blue to evaluate proteoglycan and glycosaminoglycans. The severity of articular cartilage injury at the proximal tibiae was scored using the Mankin score(Ukai et al., 2021). The images were visualized and captured using USB 2.0 Camera Viewer (Leadzoptics Microscope, England, UK).

Ex vivo Microcomputed Tomography Imaging and Analysis

Before imaging, fixed knee joints were further dissected under a dissection microscope to disarticulate them, carefully remove the menisci, and expose the articular cartilage of the tibia and femur. Tibia and femur were subjected to Micro-CT imaging and analysis using µCT (Quantum GXII, Perkin Elmer, USA). Scans were performed using the Aluminium 0.5 + copper 05 filter with the following parameters: time 14 minutes, Voxel Size 36 µm, tube voltage 90 kV, and tube current 180 mA. Sub reconstruction of the acquired image was also performed to generate Sub-volume vox files with a size of 18µm. Analyze 14.0 was used to create serial 2D coronal and axial pictures from 3D reconstructed CT scans (Perkin Elmer, USA). We estimated BMD, BV/TV, BV, Tb.Th, and Tb.Sp using analyse14 software by manually selecting the correct region of interest i.e. lateral and medial subchondral bone in the tibia and femur epiphysis.

Statistical Analysis.

All experiments were repeated at least three times. Data were presented as mean ± standard error for the indicated numbers of independently performed experiments. Statistical significance was assessed by one-way and two-way analysis of variance coupled with a Bonferroni t-test. All statistical analyses were performed using Graph Pad Prism 9.0 for Windows (GraphPad Software, La Jolla, CA, USA).

Results

PI02 Contains Collagen Peptide And Important Secondary Metabolites

Analysis and validation of Collagen peptides have been done using MALDI-TOF. As shown in Figure S1 most of the peak mass observed were < 2 kDa, and the highest peak at m/z 1879.0494 was observed. The ethanolic extract of *Hippophae rhamnoides* and Rosehip in chosen fixed ratio showed several secondary metabolites in UPLCQ-TOFMS/MS analysis (Fig. 1). The Riken Tandem Mass Spectral Database library confirmed the identification of compounds. The identified compounds belong to phenylpropanoid (4coumaric acid and chlorogenic acid hemihydrate), flavonoid (chalcone), an alkaloid (melatonin) group. Mass spectra and nature of identified compounds and their structures and biological activities are summarized in Table 1. The identified compounds have previously been reported for their role in managing pain and inflammation in OA conditions.

PL02 Exhibited No Toxicity In Mice And Rats After 28 Days Of Treatment

The toxicity study was carried out in both mouse and rat models. To evaluate acute toxicity, mice/rats were treated orally with 2g/kg and observed for 24 h. No mortality was observed. Clinical signs (temperature, change in skin, eye color change, general physique, diarrhea, and sedation) were recorded and shown in Table S1. In mice study we observed changes in the body weight on days 1,3,7,10,14 & 28 and biochemical assay on days 14 & day 28. The mean body weight was found significantly ($p < 0.001$) reduced in comparison to sham control and MIA (Figure S2A), however, no physical and behavioral changes were observed. After treatment with PL02 and Indomethacin, the animals followed a similar body weight increased pattern as normal control. In the biochemical assays on days 14 & days 28, the level of SGOT, SGPT, ALP, Urea, and Creatinine showed some significant ($p < 0.001$) changes in comparison to sham control, and MIA treated groups, however, values were within the physiological range (Figure S2B).

Next, we extended our toxicity studies in the rat model of OA, including the histopathological changes in vital organs. The body weights were recorded on day 1,7,14 & 28. We observed significant body weight reduction indicated days in MIA treated group compared to sham control. The PL02 treatment improved body weight at day 14 & day 28 ($p < 0.05$, $p < 0.01$ & $p < 0.001$) (Fig. 3A) in comparison to MIA groups. The mean body weight change in MIA and MIA + Indo group had not shown any behavior or physical changes. The changes in average food intake were significant ($p < 0.01$, $p < 0.001$ & $p < 0.001$) at each time interval for MIA and MIA + PL02 treated group compared to sham control. However, MIA + Indo group showed significant changes ($p < 0.01$, & $p < 0.001$) on day 14 & day 28 in comparison to sham control, MIA, and MIA + PL02 groups (Fig. 3B).

The average water intake volume was also measured during the study. We observed significant reduction in water intake upon MIA injected group ($p < 0.05$ & $p < 0.01$) on day 7, day 14 & day 28, and decrease in intake volume from (10 ± 2.6) to (9 ± 2.7) ml in MIA treated group compared to sham control, MIA + Indo & MIA + PL02 groups (Fig. 3C).

Impaired kidney and liver functions are considered an indicator of toxicity, which might results from consumption of any product ⁽¹⁷⁾. To assess liver and kidney function under different treatment conditions, we performed the biochemical analysis of serum sample. The biochemical estimation of SGOT, SGPT, ALP, Urea & Creatinine on day 14 & day 28 in the serum of rats (Figure S4A) showed significant changes in ALP level [Figure S4A (iii)], Urea level [Figure S4A (iv)] on day 14 and creatinine level [Figure S4A (v)] on day 14 & 28 in MIA group compared to Sham control. However, values were within the normal range. The overall observations collectively depict no toxicity in rats treated with PL02 formulation.

To further confirm the toxicity of PL02 we performed histological analysis of the vital organs harvested from rats on the 28th day, 3–4 hours after behavioral assessment (Figure S3B). In the H& E staining of heart tissue, we did not observe any structural loss of myocytes. The endothelial cell nucleus was observed normal, at 10x [Figure S4B (i)]. The branching cardiac muscle cells and muscle fibers were normal in shape like control in the PL02 group after 28 days of treatment. The brain tissue, especially the hippocampus region, have shown healthy neurons, glial cells in the normal shape, and the dentate gyrus region showed the enrichment of neurons in the MIA + PL02 treated group after 28 days of treatment as in comparison to MIA or control groups [Figure S4B (ii)]. In the liver tissue of all groups, the MIA-treated group showed the sheet of hepatocytes in flaccid tissue compared to the control group. The MIA + PL02 group has not shown any apoptosis after the 28 days of treatment [Figure S4B(iii)]. The MIA group has shown an increased size of the glomerulus in the kidney tissue section at 10X compared to the control group. However, the MIA + PL02 group has shown the same pattern of renal corpuscles, tubules, and collecting ducts compared to the control [Figure S4B (iv)].

Standardization of MIA-Induced animal model of OA

We closely observed animals after MIA injection for the visible sign of inflammation for the first 24h. We have used a plethysmometer to measure edema, Von Frey hair for evaluating mechanical allodynia, vernier caliper to measure knee joint thickness, hot plate to measure hyperalgesia and Rotarod (for mice) for motor function at 1, 3, 5, 7, 14, 21 & 28 days. We observed visible inflammation after 2 hr of MIA injection, and it kept increasing till day seven, and no significant change was observed till day 14. From day 14 to till day 28, we observed a decrease in inflammation, however it was not significant. We checked TNF- α and IL-1 β level in serum at 1, 3, 7, 14, 21, and 28 days. Their levels were significantly high till 28 days (Fig. 4D) compared to sham control. We further characterized model by checking the NF κ B-p65 and IL-1 β mRNA level in the joint tissue at day 7, 14, 21 & 28. No significant change was observed in CTX-II level. We observed significant increase in mRNA level till 28th day (NF κ B-p65, 2.7 ± 0.82 & IL-1 β , 10 ± 1.79 folds) compared to sham control. All the studied parameters indicated successful development of OA model. For detailed studies assessed pain and inflammation symptoms and behavior at 3, 7, 14, 21 & 28 days collected blood and euthanized animals at day 28 for molecular and histological analysis. The MIA-induced animal's joint sections showed OA-like features such as surface discontinuity, loss of proteoglycans, disorientation, and apoptosis of chondrocytes and subchondral bone sclerosis.

PL02 Ameliorated Pain And Inflammation In MIA-induced OA

For analyzing pain sensation in mice, we studied hyperalgesia using the hot plate method, the changes in latency time were found non-significant on day 1 and day 3 in MIA + PL02 treated groups compared to MIA. After ten days post PL02 treatment, a significant change from $p < 0.05$ to $p < 0.0001$ on day 28 (Figure S3A) observed with increase in the latency time from (22.56 ± 6.76) to (50.11 ± 16.54) in comparison to sham control (11.25 ± 4.79) to (25.14 ± 12.56) and MIA (10.25 ± 8.23) to (10.59 ± 16.58) . However, changes in latency time in MIA + PL02 was less significant from $p < 0.05$ to $p < 0.01$ compared to the MIA + Indo group. For motor neuron function assessment, we used the Rotarod test; the latency to fall time was found significantly increase after 14 days of treatment in the MIA + PL02 group from (20.75 ± 12.34) to (298.50 ± 73.44) in comparison to the sham control group (10.75 ± 6.24) to (123 ± 70.03) and MIA (8.80 ± 7.26) to (87.11 ± 30.12) and showed more significant changes after 28 days treatment ($p < 0.0001$) (Figure S3B). The inflammation in the knee area was measured by the vernier caliper of both knees and noted the difference. On day 1 and day 3 post MIA injection, all groups showed a significant difference in knee width and confirmed the development of a model for osteoarthritis. A significant decrease in difference between two knee joint width was observed on day 10 and onwards in PL02 treated group compared to MIA and Indomethacin treated group (Figure S3C). The Mechanical hypersensitivity was measured using the Von Frey method. The MIA + PL02 group has shown more significant ($p < 0.0001$) improvement after 10 days of treatment from (3.65 ± 0.44) to (5.09 ± 1.46) on day 28 in comparison to the MIA treated group (Figure S3D). Thus, initial pilot study on mice model of OA confirmed superior efficacy of PL02 over indomethacin. Next, we extended the study in detail on rat model of OA.

The Plethysmometer was used for the rat model to assess the anti-inflammatory property of the PL02 formulation. By immersing in the mercury column, the change in volume (Δ) of the left paw depicted elevated inflammation in rats administered with MIA. The group administered with PL02 formulation depicted anti-inflammatory activity by significantly reducing the inflammation on day 28 from (9.19 ± 0.39) to (8.07 ± 0.56) in rat's paws in comparison to the MIA group (Fig. 4A).

As illustrated in Fig. 4B, the diameter of the knee joint drastically increases post-MIA injection as compared to the sham control group. The left knee joint administered with MIA showed elevated inflammation. The right knee was used as control. The difference in the right and left knee joint was estimated and used as criteria for evaluating the anti-inflammatory property of the PL02 formulation. We observed slight increase in knee width in sham group after intra-articular injection of saline that revert to normal within a day. The gradual decrease in inflammation in the knee joint was observed in the MIA + PL02 group from (1.6 ± 0.20) to (0.06 ± 0.07) and MIA + Indo group from (1.36 ± 0.20) to (0.13 ± 0.03) as compared to the MIA group from (1.9 ± 0.24) to (0.94 ± 0.15) after 28 days of treatment of PL02 formulation.

Pain being the hallmark of the MIA-induced OA model, the mechanical allodynia was assessed using the von Frey hairs test. The paw withdrawal threshold or the weight bared by rat paw in grams displayed on a

digital meter was noted. Treatment was initiated one day after the MIA injection, and the pain was assessed on days 3, 7, 14, and 28. The analgesic effect of the PL02 formulation was observed from day three onwards, where the paw withdrawal threshold in MIA + PL02 group was higher than in the MIA + Indo treated group. A significant increase ($p < 0.0001$) in paw withdrawal threshold from the 14th day onwards was observed in the MIA + PL02 treated group, and the MIA + Indo treated groups compared to the MIA group. The pain significantly decreased further on the daily administration of formulation for 28 days ($p < 0.0001$) (Fig. 4C).

After 28 days of treatment, blood was withdrawn from the rats and used for cytokines assay using a multiplex analyzer (Fig. 4D). The level of TNF- α was significantly ($p < 0.0001$) decreased in MIA + PL02 group compared to MIA. The level of IL-1 β also significantly reduced upon PL02 treatment compared to the MIA group. The similar pattern was found in IL-10, and IL-17 levels, the group treated with PL02 showed a lower level compared to the MIA group, however, changes were non-significant after analysis. Whereas IFN- γ levels were found significantly ($p < 0.0001$) reduced in MIA + PL02 the group compared to the MIA group.

To further validate the serum cytokine levels we checked the mRNA expression in joint tissue using real-time PCR analysis of pro-inflammatory cytokines, IL-1 β , TNF- α and NF κ B-p65. We used knee joint having a complete synovial capsule to evaluate the effect of PL02 on joint. The mRNA expression of pro-inflammatory mediators IL-1 β (Fig. 4F), NF κ B-p65 (Fig. 4K) and TNF- α (Fig. 4E) decreased significantly in the PL02 treated rats compared with the MIA group ($p < 0.05$) and ($p < 0.0001$), respectively. Chemokines like CCL-2 (Fig. 4G) and CXCL-12 (Fig. 4H) also show significantly declined levels ($p < 0.0001$ & $p < 0.001$) after administration of formulation for 28 days daily in the PL02 treated group in comparison to the MIA group. To further validate the above observation and understand the mechanism we used synovial cell line to study the IL-1 β dependent TNF- α production. The Sw cells when treated with IL-1 β showed significant increase in TNF- α production, which plumed upon pre-treatment with IL-1 receptor antagonist (IL-1RA). Treatment with PL02 showed dose dependent decrease in TNF- α production and at 10mg/ml completely inhibited the IL-1 β induced TNF- α production (Fig. 4L). This indicates that PL02 inhibited the inflammatory cascade at initial stage and inhibited the proinflammatory cytokine production and associated pathology.

It is well established that these pro-inflammatory cytokines enhance the expression of COX-2 and CGRP-1, genes directly involved in pain perception. Therefore, we checked the mRNA expression of these genes in joint tissue. A significant increase ($p < 0.001$) in COX-II & CGRP-1 levels was observed in the MIA group compared to the sham control group. PL02 treatment for 28days significantly reduced their expression ($P < 0.001$) compared to MIA and MIA + Indo group (Fig. 4I-J). The COX-2 and CGRP-1 expression pattern correlated well with pain behavior data (Fig. 4A-C).

PL02 Reduced The Oxidative Stress And Inhibited The Cartilage Destruction

The effect of PL02 on the oxidative stress assessed using biomarker, LPO. The LPO levels were determined in the serum samples collected from the different treatment groups of animals on the 28th - day post-treatment (Fig. 5A). The serum level of LPO in the MIA group was significantly increased to 8.302 nmol/mL compared to the sham control (5.494 nmol/mL). However, the PL02 treatment markedly reduced serum levels of LPO to 6.091 nmol/mL. In real-time PCR analysis of whole joint tissue, a significantly decreased level ($p < 0.01$) of NOS-2 (Fig. 5C) was found in the PL02 group in comparison to the MIA group. The significant high level ($p < 0.0001$) of BCL-2 confirmed the anti-apoptotic property of the PL02 (Fig. 5B) in comparison to MIA. This validates the antioxidant and anti-apoptotic properties of PL02.

Next, we checked the level of protease, mainly Matrix metalloproteinase 13 (MMP13), that attacks the cartilage matrix leading to its destruction. The MMP-13 expression increased post-MIA injection compared to control. A significant downregulation in the level of MMP-13 was observed in PL02-treated animals compared to MIA (Fig. 5D). The reverse pattern was observed for TIMP-2 (Fig. 5E), an inhibitor of the MMPs family involved in the disease process, such as arthritis and metastasis. A significant decrease ($p < 0.001$) in TIMP-2 expression was observed post-MIA injection, which increased significantly ($p < 0.01$) upon PL02 treatment. We have also checked the expression level of collagen-II, the primary collagen, and aggrecan, a major proteoglycan in articular cartilage, indispensable for maintaining hydration which is needed for proper functioning (load bearing properties) of articular cartilage. The loss of aggrecan shows the characteristic of OA; we observed a significant decrease in aggrecan level post-MIA injection. No significant improvement was seen in animals treated with MIA + Indo. Interestingly, 28 days of treatment of PL02 showed (Fig. 5G) a significant high-level expression ($p < 0.0001$) of aggrecan in comparison to MIA. Similarly, a considerable decrease in the fold of the Col-II gene in the MIA group was observed compared to the control group. A significantly increased expression of Col-II was observed in the PL02 treatment group compared to the MIA group. However, no significant change was observed in the Indomethacin-treated group compared to MIA. The expression of COL-II and Aggrecan in the PL02 treated group was comparable to the control.

The increased Aggrecan and Col-II gene expression indicates healthy chondrocytes and cartilage. Subsequently, we determined the mRNA expression level of SOX-9, a master regulator of chondrogenesis. The high level of SOX-9 ensures proliferating but not hypertrophic chondrocytes and is essential for differentiating precursor cells into chondrocytes. The SOX-9 level decreased significantly post MIA injection compared to control. Treatment with indomethacin did not show any improvement in SOX-9 expression. Whereas the 28 days treatment with PL02 in rats showed a significantly high level of SOX-9 (Fig. 5F) compared to MIA. The increased SOX-9 expression is corroborated with increased aggrecan and COL-II expression, which clearly indicate the chondroprotective and regenerative properties of PL02.

PL02 Protected The Articular Cartilage Degeneration

Histological analysis of toluidine blue (Fig. 6A) and hematoxylin& eosin (Fig. 6B) staining of knee joint sections after intra-articular injection of saline or MIA was carried out after euthanizing the rats on Day 28. In the sham control group, where saline was administered into the intraarticular region of the left knee joint, the articular cartilage surface was smooth, and the chondrocytes were arranged orderly in the superficial, mid and deep zone of cartilage. No proliferative changes of chondrocytes are observed, and the chondrons do not appear distorted. After the injection of MIA in the MIA group, the cartilage surface's superficial fibrillation and loss are seen. Chondrosenescence occurred due to MIA injection leading to fragmentation of chondrocytes within the chondron in the deep zone. In contrast, distortion of the complete chondron is seen in the superficial and mid-zone of the cartilage. Loss of superficial matrix parallel to the surface and formation of fissures is also evident in H&E-stained distal epiphyseal cartilage of the tibia of the left-knee joint in rats. Damage to the bone due to the shape of a subchondral bone cyst is observed. In the Indomethacin treated group, the cartilage destruction was restricted, and only a few deformations of chondrons were observed. On treating the rats with the PL02 formulation daily, for a period of 28 days, shows improved macroscopic changes in the articular cartilage as compared to MIA. The surface was smooth, no cartilage surface irregularities were seen, and the chondrocytes were arranged in parallel zones in well-structured lacunae, confirming cartilage protective property of PL02. Next, we studied the level of proteoglycan and glycosaminoglycan in the joint tissue section using toluidine blue staining. The toluidine blue staining showed that the MIA group's proteoglycan and glycosaminoglycan content were significantly decreased. MIA + PL02 treatment protected the cartilage from damage, and a significantly increased level of proteoglycan compared to the MIA group (Figure. 6A) was observed. The improvement in articular cartilage along with proteoglycan upon PL02 treatment was even better than the sham control, indicate that PL02 not only preventing chondrocytes apoptosis but also induce regeneration. Our histological observations were in line with our gene expression profile and substantiated the chondroprotective and regenerative nature of PL02.

Subchondral Bone Analysis

After histopathological analysis, we analyzed the subchondral bone microarchitecture using Micro-CT. All the rats treated with MIA showed pathological changes in subchondral bone in the injected knee. In contrast, the contralateral control knee showed no OA-like changes in the tibial subchondral bone microarchitecture. However, we did not observe significant changes in microarchitecture of subchondral region of distal/proximal femur bone of joint post MIA injection. Therefore, we analysed tibial subchondral bone in detail.

Figure 7a shows the data comparison of the tibial subchondral trabecular bone microarchitectural parameters of the control knee, the MIA-injected, MIA + PL02, and MIA + Indo treated knee after completion of 28 days of treatment. We observed more significant changes in the medial compartment of the Tibia than in the lateral; therefore, considered medial and total changes for interpretation. There was a substantial increase in BV and BV/TV in the MIA-injected tibia compared to the control tibia, in both the medial and the total compartments (15% and 12%, respectively, $P < 0.05$ for both parameters). However,

no such increase in bone volume fraction was observed in animals treated with PL02 and was comparable to the sham control. Tb-Th's trabecular parameter slightly increased post-MIA treatment; however, the difference was insignificant. A significant decrease in Tb. Sp was observed in MIA-treated group compared to sham control. This decrease in Tb. Sp was prevented in PL02 treated group.

The MIA injected knee showed a significant increase in the tibial subchondral plate (both medial and total compartments) compared to the control knee. The calculated value for subchondral plate thickness was 140 ± 25 & 120 ± 9 μm for MIA injected and sham control, respectively. The subchondral plate thickness of 115 ± 15 μm was observed in the PL02 treated tibia, similar to the sham control. Moreover, we have also observed increased porosity of the tibial subchondral plate in the MIA-injected knee compared to the sham control and PL02-treated knee. The %d MIA-Control was 48, and MIA + PL02-Control was 20, $p < 0.001$.

Figure 7B shows the lateral and coronal view of the tibia. As can be seen in the Micro-CT image, increased sclerosis was observed in MIA treated tibia compared to the control. Sclerosis is reduced upon indomethacin treatment. Interestingly no sclerosis was observed in PL02 treated animal's knees. Thus, the overall analysis of tibial subchondral bone indicated that MIA injection induced subchondral pathology reported in humans, which was prevented by PL02 treatment.

Discussion

For a decade, the progress of scientific research in drug discovery has become more precise and steadier, preferring and considering a multitarget approach over a single target for chronic degenerative and metabolic diseases. Therapies that act on a single target give only symptomatic relief with moderate to severe side effects on long-term use. One such chronic medical problem is Osteoarthritis (OA). Without effective treatment, the burden of Osteoarthritis (OA) will continue to rise unsustainably. The prevalent covid-19 pandemic positively impacts OA and increases the disease burden manifold. Despite cost-intensive research for many years all over the world, no effective therapy has been developed to halt, delay or reverse the OA progression or give symptomatic relief that is long lasting. Hence, surgical intervention like joint replacement is preferred by patients for improved pain sensation and quality of life. The development of novel therapy that is multitargeted, safe, and can address symptoms long-lasting as well as prevent or reverse the loss of joint tissue is the need of the hour. To address this, we have leveraged our knowledge/expertise in traditional medicine and bone joint physiology to develop a multitargeted formulation PL02 (patented trademark) for effective & long-lasting treatment of OA. The botanical ingredients used for formulation are already utilized individually as nutraceuticals to support human health, and are sold almost everywhere in the world (Kuatsienu et al., 2017; Upadhyay et al., 2019; Zhou et al., 2022), however, never considered as front-line therapy. The combination present in designed PL02 is unique with defined composition, and extraction process at standardized temperature, containing the maximum amounts of flavonoid-rich secondary metabolites. These metabolites are identified through UPLCMS, like alpha-lipoic acid, kaempferol, Resveratrol, Carnosine, Orientin, and so on (Table S1). The standardized combination has shown a superior synergistic effect in the animal model of OA.

The primary purpose of our study was to use a non-trauma animal model that mimics the tissue-level pathophysiology of human OA, which could be used to study the effect of disease-modifying therapy on cartilage and subchondral bone. To prove the concept, a low-dose MIA-induced rat and mouse model of OA mimics articular cartilage, and subchondral bone pathology of human OA was used (Aüillo-Rasser et al., 2020). The MIA-induced OA model is less invasive, robust and preferred over surgical-OA model where variation in symptom and infection is major problem. After dose optimization, we studied acute and subacute (28 days) toxicity of the PL02 and did not observe any toxic effect in the serological and histopathological study. Next, we went ahead to study the efficacy of PL02 on different symptoms associated with OA, like Inflammation & pain. Treatment with PL02 has significantly alleviated the pain and inflammation in a week compared to indomethacin. The superior anti-inflammatory and analgesic activity of PL02 could be due to the presence of polyphenols and other secondary metabolites. Alpha-lipoic acid in the formulation might have played a significant role in controlling inflammation by lowering serum levels of IL-1 β , IL-6, TNF- α , IL-17, and IL-23 (Lv et al., 2019). Lipoic acid supplementation prevents inflammatory bone loss by inhibiting COX-2 activity and PGE₂ production (Gómez et al., 2021). Kaempferol alleviates inflammatory iNOS and Cox-2 movements and attenuates NF- κ B activation (Zhuang et al., 2017).

Edema is one of the manifestations of inflammation, so we measured knee joint width to compare the anti-inflammatory property of PL02. In mice and rat data, the knee joint difference (edema) significantly reduced after 14 days of oral PL02 treatment (Fig. 2). This indicates that PL02 prevented inflammation-associated cell infiltration and fluid accumulation in the knee joint. Another symptom of inflammation is thermal and mechanical hypersensitivity. The threshold of mechanical hypersensitivity assessed using the Von Frey system was higher in the PL02-treated group than in MIA and MIA + Indo groups. The reduced edema and increase in mechanical threshold confirm the superior anti-inflammatory and analgesic properties of PL02 over Indomethacin. We extended our study to the molecular level, to substantiate the behavior data. The level of pro-inflammatory cytokines both in serum (TNF α , IL-1 β , IL-10, IL-17, and IFN- γ) and mRNA level in joint tissue (TNF α , IL-1 β , CCL2, CGRP1 and CXCL-12). The data clearly corroborated the symptoms profile and indicated that PL02 significantly ameliorated inflammation and pain by modulating the cytokines level in serum and joint tissue compared with MIA or MIA + Indo group. The greater anti-inflammatory effect might be due to the synergistic effect of several secondary metabolites present in PL02 as described above. After observing the higher beneficial effect of PL02 on MIA-induced symptoms of OA, we ventured into understanding the molecular mechanism. Evidently, IL-1 is a potent inducer of cartilage and bone damage than other cytokines particularly TNF- α . Both IL-1 and TNF- α are key inducers of matrix degrading enzymes that cause rapid and extensive cartilage damage. Hence, inhibition of IL-1 induced inflammatory cascade by PL02 has a more pronounced effect on cartilage and bone damage which ultimately is the goal of therapy in OA.

The oxidative stress theory explains that the degradation of ECM and the apoptosis of chondrocytes are the primary pathogenesis events in OA. Inflammation leads to reactive oxygen species (ROS) generation by activating several signaling pathways. The ROS acts as a signalling molecule as well as a mediator of

inflammation(Gómez et al., 2021). ROS activates signaling pathways by acting as second messengers, activating MMPs, inducing cell death, and degrading the matrix, which ends in cartilage degradation by inhibiting cell migration, growth factors bioactivity, and matrix synthesis(Grässel & Muschter, 2020). Molecules with antioxidant properties have been shown to neutralize ROS and break the chain of ROS-mediated damage to the cartilage and synovial capsule. Quantitation of LPO, a product of ROS-mediated oxidation of membrane lipid and mRNA level of an enzyme like NOS-2, clearly indicated the strong antioxidant property of PL02 over indomethacin. This could be attributed to the synergistic effects of the various polyphenols/catechins present in PL02.

The degradation of articular cartilage and bone matrix components is the hallmark of OA. Among the earliest are type II collagen and the proteoglycan aggrecan, leading to cartilage structure and function loss. Destruction of cartilage is the main feature of MIA-induced OA, and it has been shown that a single injection is sufficient to cause > 33% loss of cartilage tissue. The major challenge for modern medicine is prevention or regeneration of cartilage to improve the mechanical property of joint. However, various secondary metabolites of plant origin have shown potential of stalling the cartilage tissue damage. Type 2 collagen, the main component of the cartilage extracellular matrix, has been upregulated by flavonoid Diosmin, present in citrus fruits, Chondrocyte specific differentiation marker SOX-9 depicts increased activity in the presence of Diosmin. It is also under clinical trials for its analgesic properties(Chen et al., 2019). Matrix metalloproteinase involved in cartilage degradation includes MMP-1, MMP-3, and MMP-13. Clinical data reported that OA patients with articular cartilage destruction have high MMP13 expression (Q. Hu & Ecker, 2021), suggesting that elevated MMP13 might be associated with cartilage degradation. This was confirmed with studies on MMP13 transgenic mice, which developed a spontaneous OA-like articular cartilage destruction phenotype (Park et al., 2020). Similarly, in our study the increased MMP13, decreased expression of aggrecan and collagen II, the main components of ECM, exhibited ongoing cartilage damage in MIA-induced osteoarthritis. After the treatment with PL02, the expression of MMP13 was significantly decreased, and significantly increased levels of aggrecan and collagen II were observed. As these secondary metabolites are present in good quantity in herbal extract used to formulate PL02, we observed significantly increased expression of SOX-9 ($p < 0.0001$), aggrecan ($p < 0.001$), and a significant decrease in MMP-13 ($p < 0.01$) in PL02 treated group compared to disease control, indicating a protective effect on PL02 on cartilage degradation after MIA injection in the rat. This constitutes vital evidence for the disease-modifying potential of PL02 (Xia et al., 2019). The histopathological analysis has further substantiated the present claim.

The histological analysis of the joint section stained with H & E and toluidine blue showed normal healthy cartilage in control with the normal distribution of chondrocytes in ECM. MIA injection leads to proteoglycan loss, viable chondrocytes, and proliferation zone(Wang et al., 2022). Whereas healthy cartilage with normally distributed chondrocytes in proteoglycan and glycosaminoglycans rich ECM with visible proliferation zone confirmed the protective effect of PL02 at the tissue level. ECM plays a central role in providing structural and biochemical support to the neighboring cells in cartilage and meets the different roles in chondrogenesis. It is well established that the survival of chondrocytes is anchorage-dependent. Thus, cartilage degradation and chondrocyte apoptosis are closely connected. The apoptosis

of chondrocytes and the destruction of ECM will undoubtedly impair cartilage integrity(Ren et al., 2022). The increased expression of anti-apoptotic BCL-2 upon PL02 treatment suggests that PL02 could suppress apoptosis of chondrocytes by inhibiting inflammation-induced ECM degradation. Thus, macroscopic and histological examination confirmed that orally administered PL02 protected cartilage from destruction.

The subchondral bone not only supply nutrient and metabolism to cartilage, but the primary function is also to provide physiological and non-physiological shock absorption and support the overlying cartilage; hence, any factors that disturb bone cell metabolism, architecture, and structural integrity, might make the bone more susceptible to abnormal loading or even induce aberrant reactions to average physiological load. The structural changes in subchondral bone observed in OA have been long regarded as an adaptation of bone to the biomechanical changes observed in articular cartilage (Fang et al., 2022; Y. Hu et al., 2021). Recently, various pre-clinical and clinical studies demonstrated that alterations in bone structure might even precede and mediate cartilage pathology, and OA progression is associated with temporal changes in bone structure(Lockwood et al., 2019; Mohan et al., 2011; SR et al., 1994; Udo et al., 2016). It has been reported that in early OA, accelerated bone turnover leads to bone plate thinning and increases porosity, whereas the trabecular compartment shows increased trabecular spacing and decreased bone volume fraction. Progression of OA is accompanied by subchondral bone plate thickening, increased trabecular thickness, and increased bone volume fraction(Y. Hu et al., 2021; Mohan et al., 2011). Therefore, it was imperative to check the effect of PL02 on the subchondral bone micro-architecture of the femur and tibia head at the knee joint. We observed more significant changes in the tibia than the femur. A significant difference was observed in the medial compartment than in the lateral compartment in the tibia. The detailed analysis showed a significant increase in bone sclerosis, plate thickness, porosity, bone volume fraction, Tb-Th, and a decrease Tb-Sp in the MIA group. This confirms that MIA induced model in our study mimics the human disease condition and demonstrates disease progression in the tibial subchondral bone over the study duration, and it was similar to as reported by Mohan et al. (Mohan et al., 2011). Interestingly, PL02 treatment has prevented disease progression to subchondral bone. No thickening /sclerosis of the subchondral plate was observed, whereas sclerosis was present in the indomethacin-treated group.

Conclusion

A novel concept of considering OA as a multifaceted disease involving the whole joint and not only cartilage or synovium has been presented. This makes OA a highly heterogeneous disease, any therapeutic targeting a single target or joint tissue may not be effective. PL02 reduces pain and inflammation and protects cartilage damage by preventing apoptosis of chondrocytes and abnormal turnover of subchondral bone (Fig. 9). Thus, in the present study, PL02 significantly alters disease progression and may successfully prevent joint replacement in the advanced disease stages.

List Of Abbreviations

OA

Osteoarthritis

MIA

Monosodium iodoacetate-induced

i.a.

Intrarticular

SBT

Seabuckthorn

AIA

adjuvant-induced arthritis

SGOT

Serum glutamic oxaloacetic transaminase

SGPT

Serum glutamic pyruvic transaminase

ALP

Alkaline phosphatase

LOD

level of detection

LPO

Lipid Peroxidation

H&E

hematoxylin and eosin

BMD

Bone mineral density

BV/TV

Bone volume fraction/ total volume

BV

bone volume

Tb.Th

trabecular thickness

Tb.Sp

trabecular spacing

Micro-CT (μ CT)

micro-computed tomography

ECM

extracellular matrix

Declarations

Contributions

PU & SG, Identified the ingredients for the formulation, designed the research, analyzed the final data & wrote the manuscript. PU, ASN & DK conducted most of the experiments, including articular injection, collecting the animal samples, molecular study, and analysis data. ASN and DK performed bone scanning in μ CT and analyzed the data under the supervision of SG. VA purchased the formulation for commercialization, helped with the label claim, and protected it with a trademark. All authors agreed on the final version of the manuscript.

Competing interest

PU was employed by the National Institute of Immunology on the fellowship of Young Scientists funded by the Department of Health Research, Ministry of Health, and Family Welfare Government of India. DK was a student at the National Institute of Immunology. ASN is a Ph.D. student at the National Institute of Immunology. SG is employed by the National Institute of Immunology, Department of Biotechnology Govt of India New Delhi, India. This study received funding from a Core grant from the National Institute of Immunology, Department of Biotechnology, New Delhi, India. VA is the director of Purobien Life sciences Pvt Ltd. The company is going to commercialize the product as a dietary supplement. The authors declare that the research was conducted without commercial or financial relationships that could not be construed as a potential conflict of interest.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

All data generated or analyzed during this study have been mentioned in the result and its supplementary information files.

Role of the funding source

This work was supported by grants from the National Institute of Immunology (core grant), Department of Biotechnology, New Delhi, India; later, the formulation details as an IPR were transferred to a company

M/s Purobien Lifesciences for commercialization in the nutraceutical category.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

References

1. Afridi, R., Khan, A. U., Khalid, S., Shal, B., Rasheed, H., Ullah, M. Z., Shehzad, O., Kim, Y. S., & Khan, S. (2019). Anti-hyperalgesic properties of a flavanone derivative Poncirin in acute and chronic inflammatory pain models in mice. *BMC Pharmacology and Toxicology*, 20(1), 1–16. <https://doi.org/10.1186/s40360-019-0335-5>
2. Aüllo-Rasser, G., Dousset, E., Roffino, S., Zahouani, H., Lecurieux-Clerville, R., Argenson, J. N., & Chabrand, P. (2020). Early-stage knee OA induced by MIA and MMT compared in the murine model via histological and topographical approaches. *Scientific Reports* 2020 10:1, 10(1), 1–10. <https://doi.org/10.1038/s41598-020-72350-7>
3. Bhawe, A., Schulzova, V., Chmelarova, H., Mrnka, L., & Hajslova, J. (2017). Assessment of rosehips based on the content of their biologically active compounds. *Journal of Food and Drug Analysis*, 25(3), 681–690. <https://doi.org/10.1016/J.JFDA.2016.12.019>
4. Chen, Y., Yang, K., Lu, D., Wu, W., Wang, C., & Tsai, M. (2019). The chondroprotective effect of diosmin on human articular chondrocytes under oxidative stress. *Phytotherapy Research*, 33(9), 2378–2386. <https://doi.org/10.1002/ptr.6425>
5. Fang, C., Guo, J. wei, Wang, Y. jun, Li, X. qun, Zhang, H., Cui, J., Hu, Y., Jing, Y. ying, Chen, X., & Su, J. can. (2022). Diterbutyl phthalate attenuates osteoarthritis in ACLT mice via suppressing ERK/c-fos/NFATc1 pathway, and subsequently inhibiting subchondral osteoclast fusion. *Acta Pharmacologica Sinica*, 43(5), 1299–1310. <https://doi.org/10.1038/S41401-021-00747-9>
6. Fereidoni, M., Ahmadiani, A., Semnanian, S., & Javan, M. (2000). An accurate and simple method for measurement of paw edema. *Journal of Pharmacological and Toxicological Methods*, 43(1), 11–14. [https://doi.org/10.1016/S1056-8719\(00\)00089-7](https://doi.org/10.1016/S1056-8719(00)00089-7)
7. Ganju, L., Padwad, Y., Singh, R., Karan, D., Chanda, S., Chopra, M. K., Bhatnagar, P., Kashyap, R., & Sawhney, R. C. (2005). Anti-inflammatory activity of Seabuckthorn (*Hippophae rhamnoides*) leaves. *International Immunopharmacology*, 5(12), 1675–1684. <https://doi.org/10.1016/J.INTIMP.2005.03.017>
8. Gómez, X., Sanon, S., Zambrano, K., Asquel, S., Bassantes, M., Morales, J. E., Otáñez, G., Pomaquero, C., Villarroel, S., Zurita, A., Calvache, C., Celi, K., Contreras, T., Corrales, D., Naciph, M. B., Peña, J., & Caicedo, A. (2021). Key points for the development of antioxidant cocktails to prevent cellular stress

- and damage caused by reactive oxygen species (ROS) during manned space missions. *Npj Microgravity* 2021 7:1, 7(1), 1–19. <https://doi.org/10.1038/s41526-021-00162-8>
9. Grässel, S., & Muschter, D. (2020). Recent advances in the treatment of osteoarthritis. *F1000Research*, 9. <https://doi.org/10.12688/F1000RESEARCH.22115.1>
 10. Hu, Q., & Ecker, M. (2021). Overview of MMP-13 as a Promising Target for the Treatment of Osteoarthritis. *International Journal of Molecular Sciences*, 22(4), 1–22. <https://doi.org/10.3390/IJMS22041742>
 11. Hu, Y., Chen, X., Wang, S., Jing, Y., & Su, J. (2021). Subchondral bone microenvironment in osteoarthritis and pain. *Bone Research* 2021 9:1, 9(1), 1–13. <https://doi.org/10.1038/s41413-021-00147-z>
 12. Kuatsienu, L. E., Ansah, C., & Adinortey, M. B. (2017). Toxicological evaluation and protective effect of ethanolic leaf extract of *Launaea taraxacifolia* on gentamicin induced rat kidney injury. *Asian Pacific Journal of Tropical Biomedicine*, 7(7), 640–646.
 13. León-López, A., Morales-Peñaloza, A., Martínez-Juárez, V. M., Vargas-Torres, A., Zeugolis, D. I., & Aguirre-Álvarez, G. (2019). Hydrolyzed Collagen—Sources and Applications. *Molecules*, 24(22). <https://doi.org/10.3390/MOLECULES24224031>
 14. Lockwood, S. M., Lopes, D. M., McMahon, S. B., & Dickenson, A. H. (2019). Characterisation of peripheral and central components of the rat monoiodoacetate model of Osteoarthritis. *Osteoarthritis and Cartilage*, 27(4), 712–722. <https://doi.org/10.1016/J.JOCA.2018.12.017>
 15. Loeser, R. F., Goldring, S. R., Scanzello, C. R., & Goldring, M. B. (2012). Osteoarthritis: A disease of the joint as an organ. In *Arthritis and Rheumatism* (Vol. 64, Issue 6, pp. 1697–1707). Arthritis Rheum. <https://doi.org/10.1002/art.34453>
 16. Lv, M., Zhang, W., & Qian, J. (2019). Therapeutic effect of α -lipoic acid on osteoarthritis patients and its influence on TLR4/NF- κ B and IL-23/IL-17 signaling pathway. In *Int J Clin Exp Med* (Vol. 12, Issue 1).
 17. Mármol, I., Sánchez-De-Diego, C., Jiménez-Moreno, N., Ancín-Azpilicueta, C., & Rodríguez-Yoldi, M. (2017). Therapeutic Applications of Rose Hips from Different Rosa Species. *International Journal of Molecular Sciences*, 18(6). <https://doi.org/10.3390/IJMS18061137>
 18. Mohan, G., Perilli, E., Kuliwaba, J. S., Humphries, J. M., Parkinson, I. H., & Fazzalari, N. L. (2011). Application of in vivo micro-computed tomography in the temporal characterisation of subchondral bone architecture in a rat model of low-dose monosodium iodoacetate-induced osteoarthritis. 13(6), R210. <https://doi.org/10.1186/AR3543>
 19. Murphy, M. P., Koepke, L. S., Lopez, M. T., Tong, X., Ambrosi, T. H., Gulati, G. S., Marecic, O., Wang, Y., Ransom, R. C., Hoover, M. Y., Steininger, H., Zhao, L., Walkiewicz, M. P., Quarto, N., Levi, B., Wan, D. C., Weissman, I. L., Goodman, S. B., Yang, F., ... Chan, C. K. F. (2020). Articular cartilage regeneration by activated skeletal stem cells. *Nature Medicine* 2020 26:10, 26(10), 1583–1592. <https://doi.org/10.1038/s41591-020-1013-2>

20. Park, D. R., Kim, J., Kim, G. M., Lee, H., Kim, M., Hwang, D., Lee, H., Kim, H. S., Kim, W., Park, M. C., Shim, H., & Lee, S. Y. (2020). Osteoclast-associated receptor blockade prevents articular cartilage destruction via chondrocyte apoptosis regulation. *Nature Communications* 2020 11:1, 11(1), 1–11. <https://doi.org/10.1038/s41467-020-18208-y>
21. Pasi, S., Kant, R., Gupta, S., & Surolia, A. (2015). Novel multimeric IL-1 receptor antagonist for the treatment of rheumatoid arthritis. *Biomaterials*, 42, 121–133. <https://doi.org/10.1016/J.BIOMATERIALS.2014.11.041>
22. Ren, X., Liu, H., Wu, X., Weng, W., Wang, X., & Su, J. (2022). Reactive Oxygen Species (ROS)-Responsive Biomaterials for the Treatment of Bone-Related Diseases. *Frontiers in Bioengineering and Biotechnology*, 9, 1381. <https://doi.org/10.3389/FBIOE.2021.820468/BIBTEX>
23. SR, C., FW, B., JW, P., JM, C., & TL, Y. (1994). Quantitative assessment of tactile allodynia in the rat paw. *Journal of Neuroscience Methods*, 53(1), 55–63. [https://doi.org/10.1016/0165-0270\(94\)90144-9](https://doi.org/10.1016/0165-0270(94)90144-9)
24. Stobdan, T., Targais, K., Lamo, D., & Srivastava, R. B. (2013). Judicious use of natural resources: A case study of traditional uses of Seabuckthorn (*Hippophae rhamnoides* L.) in trans-Himalayan Ladakh, India. *National Academy Science Letters*, 36(6), 609–613. <https://doi.org/10.1007/S40009-013-0177-4>
25. Udo, M., Muneta, T., Tsuji, K., Ozeki, N., Nakagawa, Y., Ohara, T., Saito, R., Yanagisawa, K., Koga, H., & Sekiya, I. (2016). Monoiodoacetic acid induces arthritis and synovitis in rats in a dose- and time-dependent manner: proposed model-specific scoring systems. *Osteoarthritis and Cartilage*, 24(7), 1284–1291. <https://doi.org/10.1016/J.JOCA.2016.02.005>
26. Ukai, T., Sato, M., Wasai, S., Takahashi, T., Omura, H., & Watanabe, M. (2021). Comparison of properties determined using electromechanical assessment (Arthro-BST™) with macroscopic and histological properties in symptomatic human articular cartilage of the hip. *Arthritis Research and Therapy*, 23(1), 1–10. <https://doi.org/10.1186/S13075-021-02611-X/FIGURES/6>
27. Upadhyay, P., Sadhu, A., Singh, P. K., Agrawal, A., Ilango, K., Purohit, S., & Dubey, G. P. (2018). Revalidation of the neuroprotective effects of a United States patented polyherbal formulation on scopolamine induced learning and memory impairment in rats. *Biomedicine and Pharmacotherapy*, 97.
28. Upadhyay, P., Shukla, R., & Mishra, S. K. (2019). Acute and sub-acute toxicity study of hydro-alcoholic leaves extract of *Reinwardtia indica* in rats. *Biomedicine and Pharmacotherapy*, 111, 36–41. <https://doi.org/10.1016/j.biopha.2018.12.056>
29. Wang, Z., Le, H., Wang, Y., Liu, H., Li, Z., Yang, X., Wang, C., Ding, J., & Chen, X. (2022). Instructive cartilage regeneration modalities with advanced therapeutic implantations under abnormal conditions. *Bioactive Materials*, 11, 317–338. <https://doi.org/10.1016/J.BIOACTMAT.2021.10.002>
30. Xia, T., Gao, R., Zhou, G., Liu, J., Li, J., & Shen, J. (2019). Trans-Cinnamaldehyde Inhibits IL-1 β - Stimulated Inflammation in Chondrocytes by Suppressing NF- κ B and p38-JNK Pathways and Exerts

31. Zhou, D., Zhang, H., Xue, X., Tao, Y., Wang, S., Ren, X., & Su, J. (2022). Safety Evaluation of Natural Drugs in Chronic Skeletal Disorders: A Literature Review of Clinical Trials in the Past 20 years. *Frontiers in Pharmacology*, 12, 3884. <https://doi.org/10.3389/FPHAR.2021.801287/BIBTEX>

32. Zhuang, Z., Ye, G., & Huang, B. (2017). Kaempferol alleviates the interleukin-1 β -induced inflammation in rat osteoarthritis chondrocytes via suppression of NF- κ B. *Medical Science Monitor*, 23, 3925–3931. <https://doi.org/10.12659/MSM.902491>

Tables

Table 1 is not available with this version

Figures

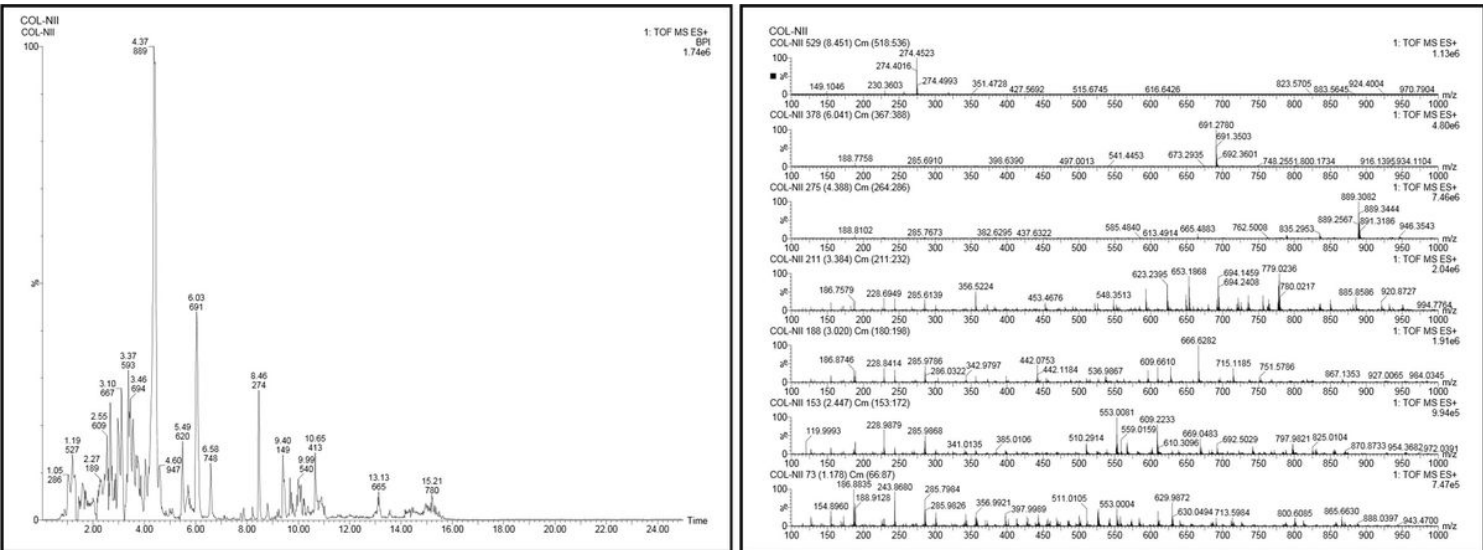


Figure 1

Image illustrated the UPLCMS chromatogram of the PL02 Formulation

Treatment Plan

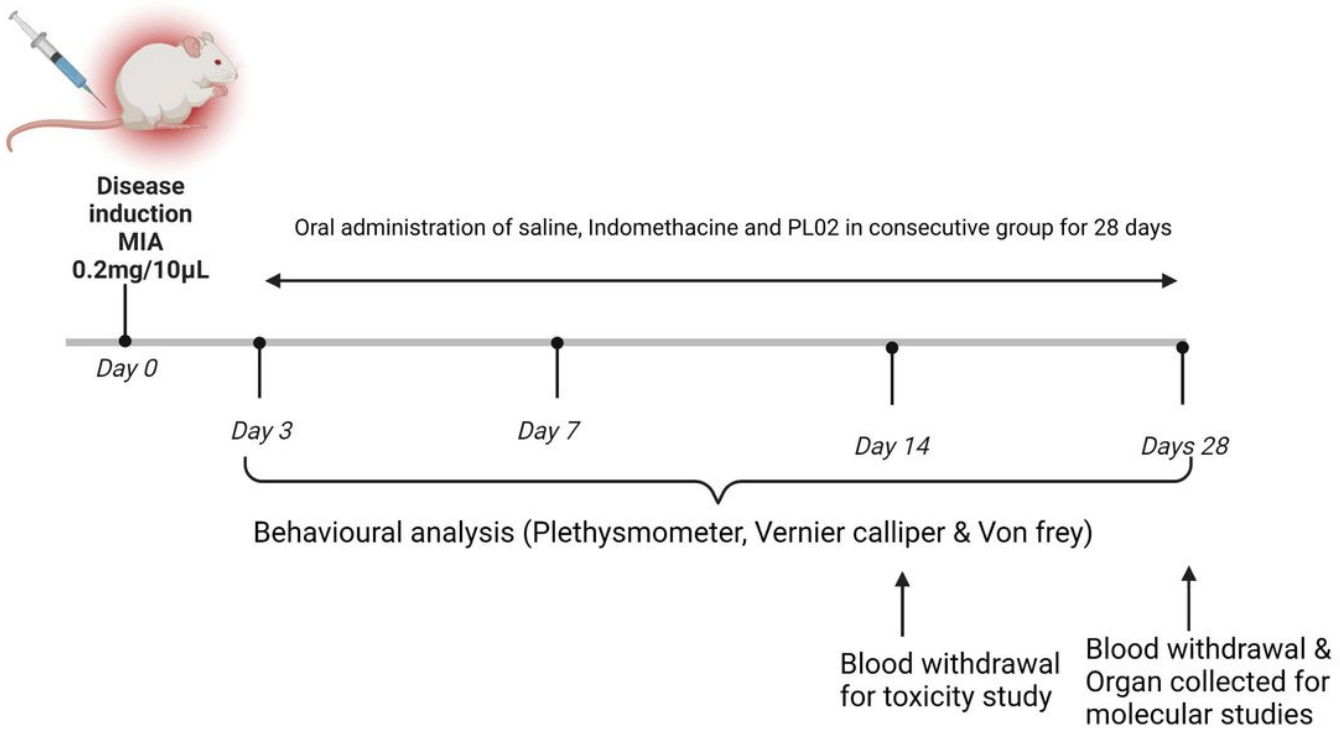


Figure 2

Image illustrated the treatment strategy and analysis plan chart of the study.

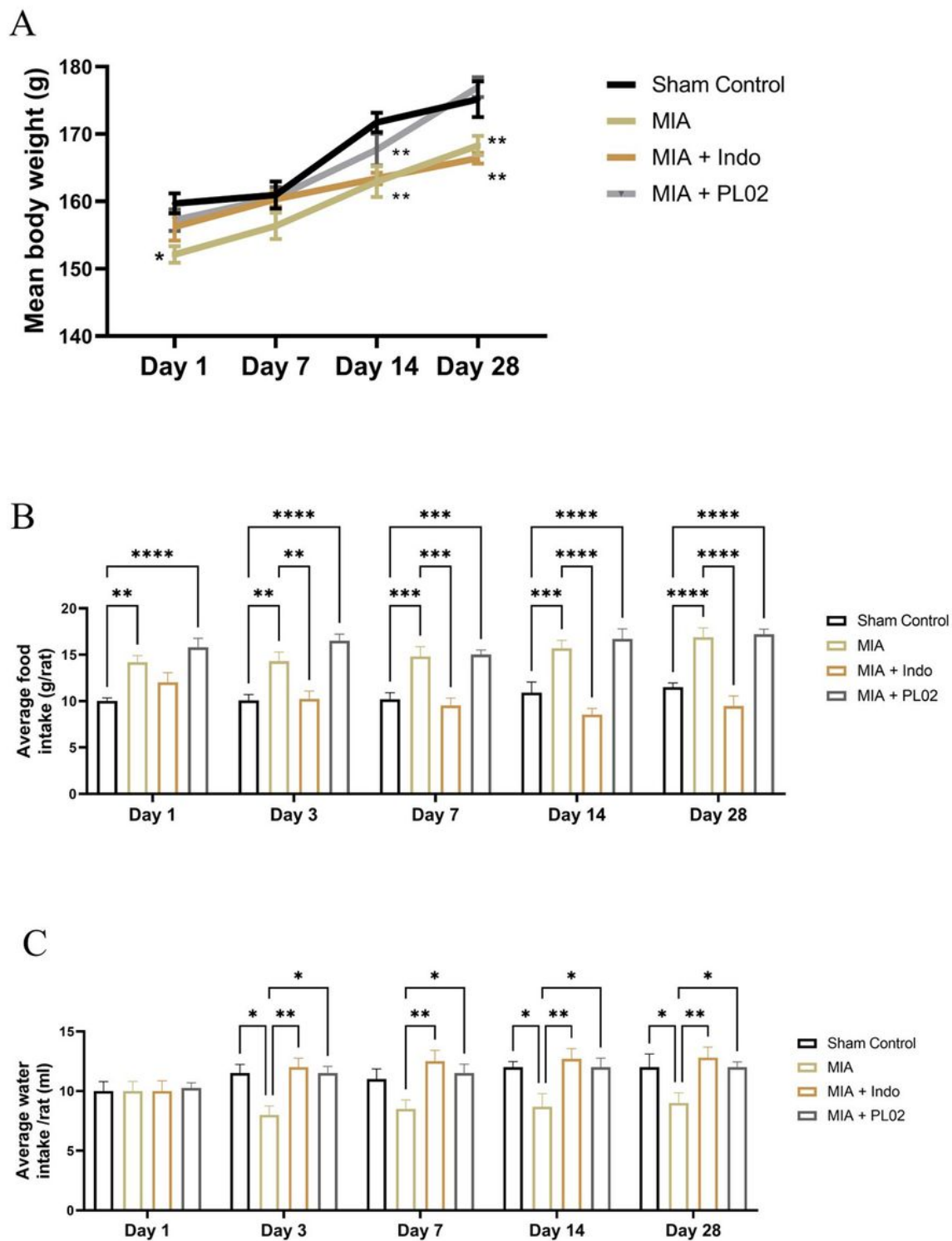


Figure 3

PL02 showed no toxicity after 28 days treatment

(A) Average body weight recorded at days1, 3, 7, 14 and 28 (n=10)

(B) Average food intake was recorded at days 1, 3, 7, 14 and 28 (n=10)

(C) Average water intake volume was recorded at days 1,3,7,14 and 28 (n=10)

Statistical analysis was performed using Two-way ANOVA with Bonferroni

post hoc test. Data presented as means $\pm$ SEM. *P < 0.05, **P<0.01, ***P<0.001

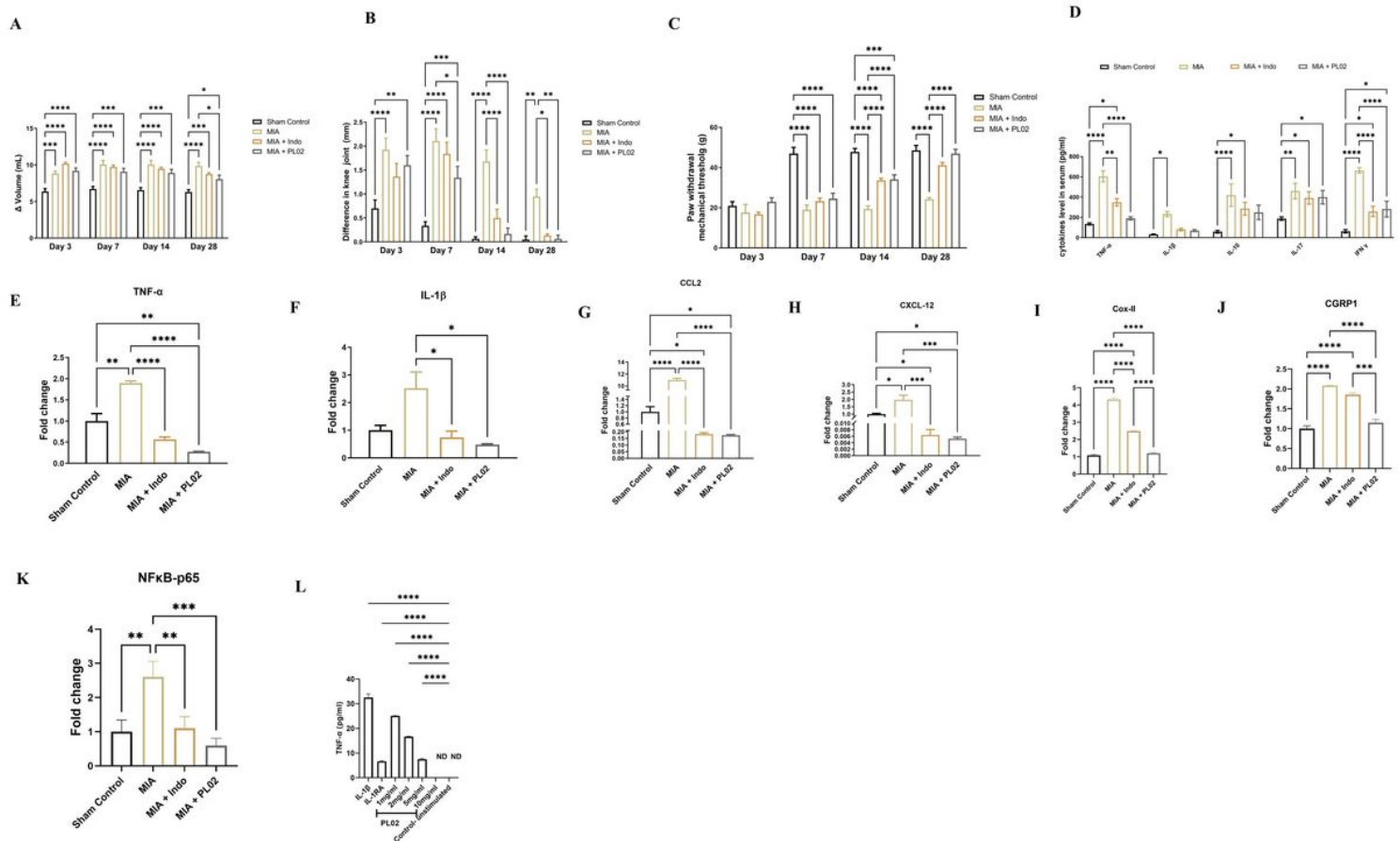


Figure 4

PL02 helps in ameliorated the pain and inflammation (A) Plethysmometer used to measure the inflammation of paw edema at day 3, 7, 14 and 28 (n=10) (B) Vernier caliper used to the measure the inflammation on knee joint at day 3, 7, 14 and 28 (n=10) (C) Von frey was performed for mechanical allodynia to measure the pain at day 3, 7, 14 and 28 (n=10) (D) Serum cytokines levels measured through ELISA method after 28 days treatment (n=6) (E) (F) (G) (H) (I) (J) (K) Quantitative real-time PCR analysis of TNF- α, IL-1β, CCL2 CXCL2, Cox II, CGRP1 and NF κB-p65 mRNA levels (n=6). (L) In situ assay for biological activity: TNF-α production by SW-982 cells (n=3)

Statistical analysis was performed using Two-way ANOVA and one way ANOVA with Bonferroni post hoc test. Data presented as means $\pm$ SEM. *P < 0.05, **P<0.01 and ***P<0.001

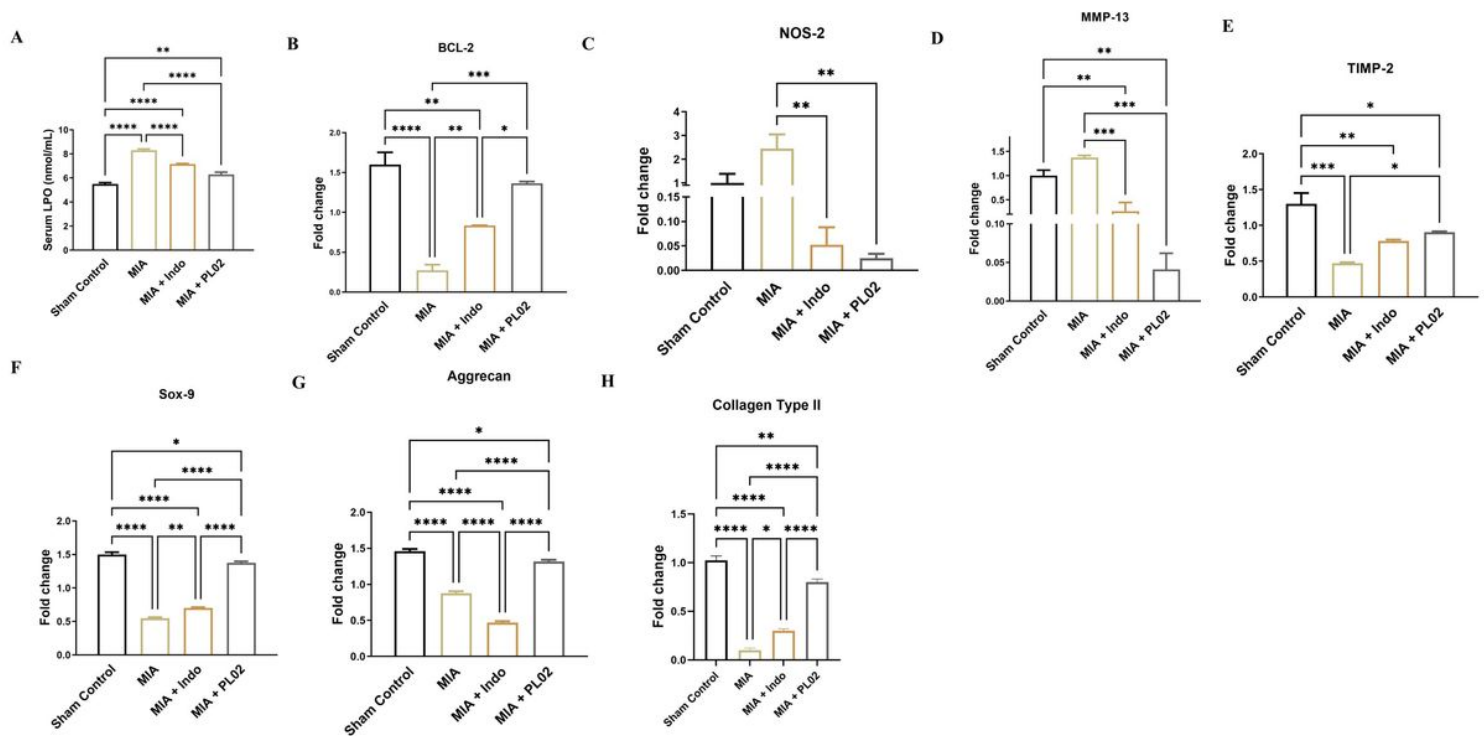


Figure 5

PL02 inhibit cartilage damage by reducing oxidative stress chondrocyte apoptosis and induced chondrogenesis **(A)** Serum level of LPO measured after 28 days treatment (n=6) **(B, C, D, E, F, G & H)** Quantitative real-time PCR analysis of BCL-2, NOS-2, SOX-9, MMP-13, TIMP-2, Aggrecan and Collagen Type II mRNA levels (n=6).

Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc test. Data presented as means $\pm$ SEM. *P < 0.05, **P < 0.01 and ***P < 0.001

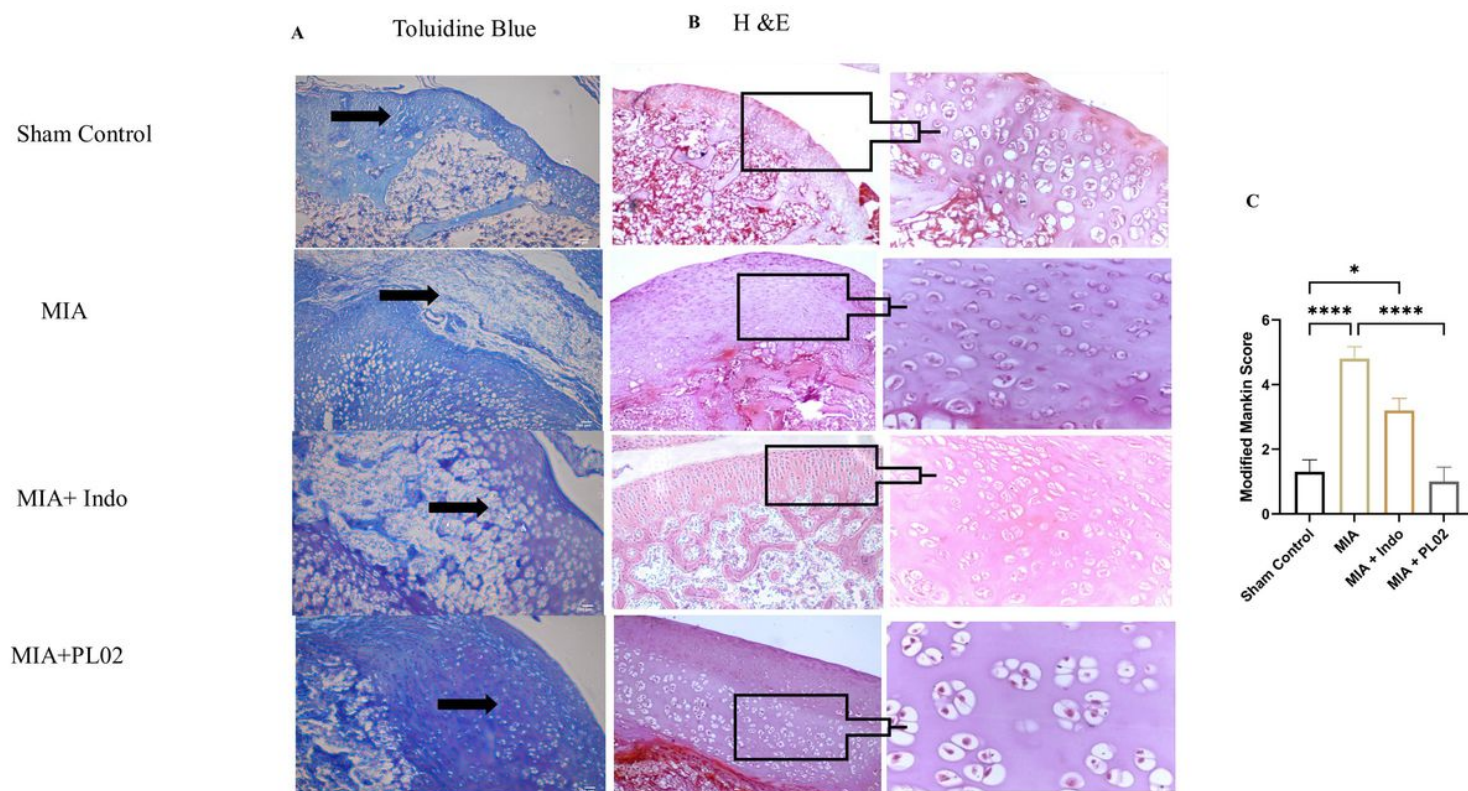


Figure 6

Histological analysis of joint tissue (A) Toluidine blue **(B)** H & E staining of cartilage section was performed to assess the proteoglycan and glycosaminoglycans. **(C)** Modified Mankin score. Scale bar 100 μ m

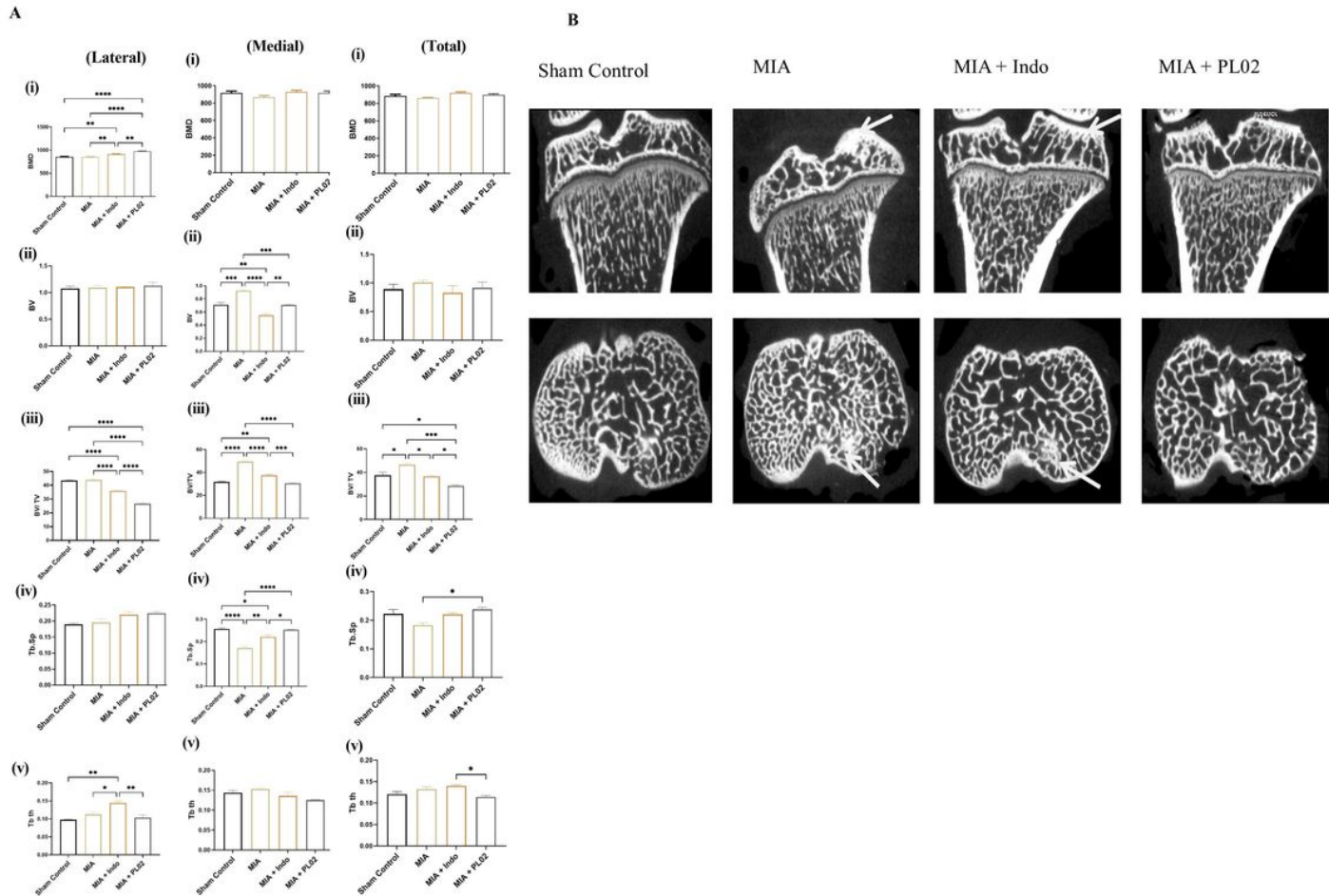


Figure 7

Microarchitectural analysis of subchondral bone (A) : Bar plot showing morphometric parameters of subchondral trabecular bone determined by micro-CT in the control tibiae and in the MIA-injected, MIA injection with treatment, tibiae. There was a statistically significant increase over time in BV, BV/TV, Tb.Th, and Tb.N, and a decrease in Tb.Sp, in both the control and MIA-injected tibiae. Error bars = SD.

BV, bone volume; BV/TV, bone volume fraction; CT, computed tomography; MIA, monosodium iodoacetate; TB. N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness.

$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, between control tibiae and MIA-injected tibiae.

B: Coronal and axial micro-CT images of control and different treated animal tibia at 4 weeks post treatment, The MIA-injected tibia showed altered subchondral bone architecture, with sclerosis on the medial tibial compartment (indicated by arrow), whereas the control tibia and MIA+PL02 treatment showed no sclerosis at 4weeks. Micro-CT, micro-computed tomography; MIA, monosodium iodoacetate.

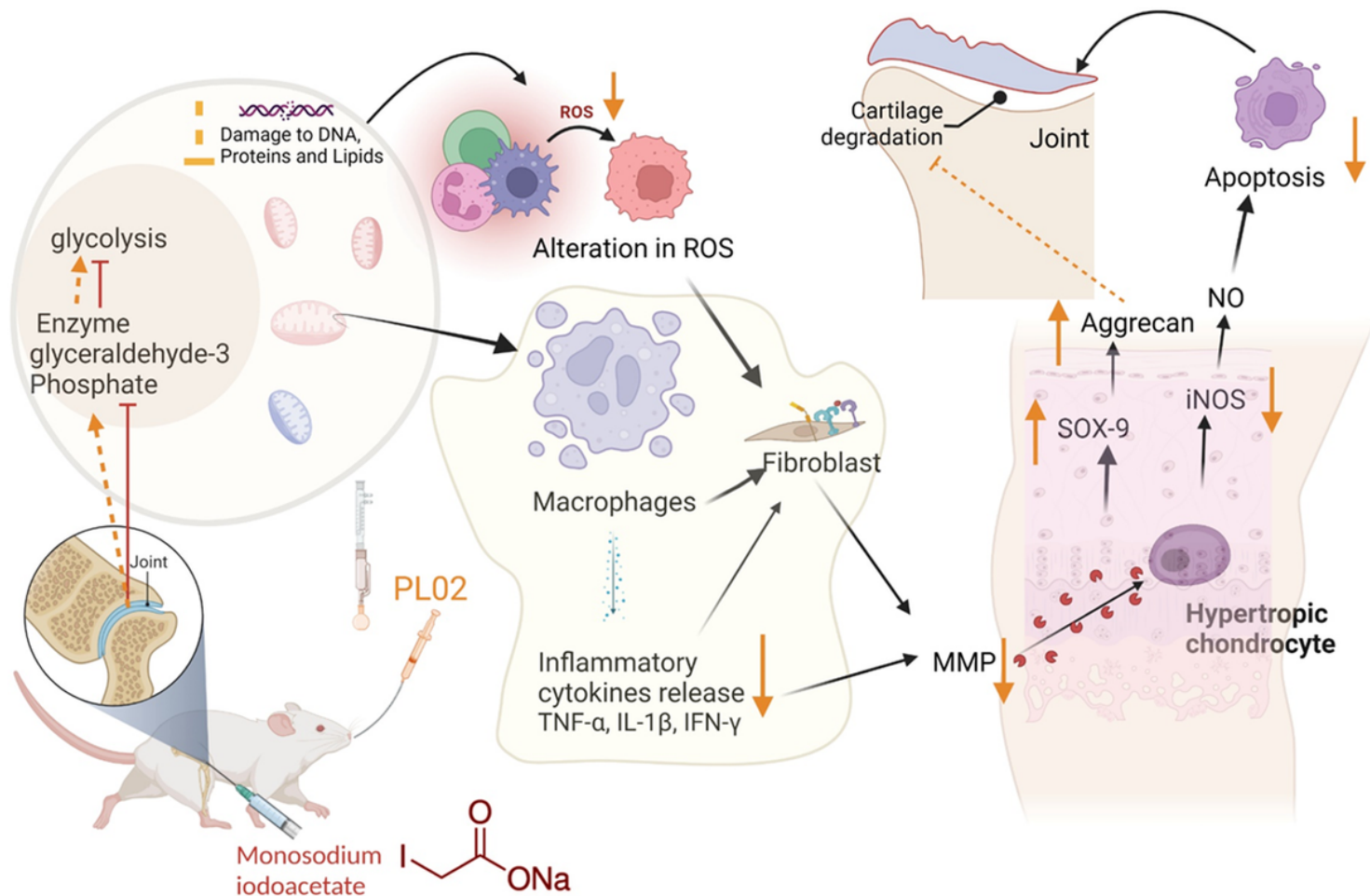


Figure 8

Mechanistic Pathway of PL02® in treatment and management of OA

Supplementary Files

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