

Phytochemical analysis of Citrus japonica enrich extract and investigation of Anti-inflammatory activity in Rat Model

zunera Chauhdary

zunerach@yahoo.com

Government College University Faisalabad <https://orcid.org/0000-0003-4191-8697>

Naheed Akhtar

Government College University Faisalabad

Maryam Afzaal

Government College University Faisalabad

Mamoona Tariq

Government College University Faisalabad

Rabia Munir

Government College University Faisalabad

Uzma Saleem

Punjab University: Panjab University

Research Article

Keywords: Inflammation, Tangeretin rich extract, Tangeretin loaded nanoparticles, Solvent emulsification method, Antioxidants, Zeta sizer, Formaldehyde, Carrageenan, HPLC

Posted Date: June 17th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-6772850/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Phytochemical analysis of *Citrus japonica* enrich extract and investigation of Anti-inflammatory activity in Rat Model

Naheed Akhtar¹, Maryam Afzaal¹, Zunera Chauhdary², Mamoon Tariq¹, Rabia Munir³, Uzma Saleem⁴

1Department of Biochemistry, Government College University, Faisalabad

2Department of Pharmacology, Faculty of Pharmaceutical Sciences, Government College University Faisalabad

3Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Government College University Faisalabad

4 Department of Pharmacology, The University of Punjab

Corresponding author: Zunera Chaudhary

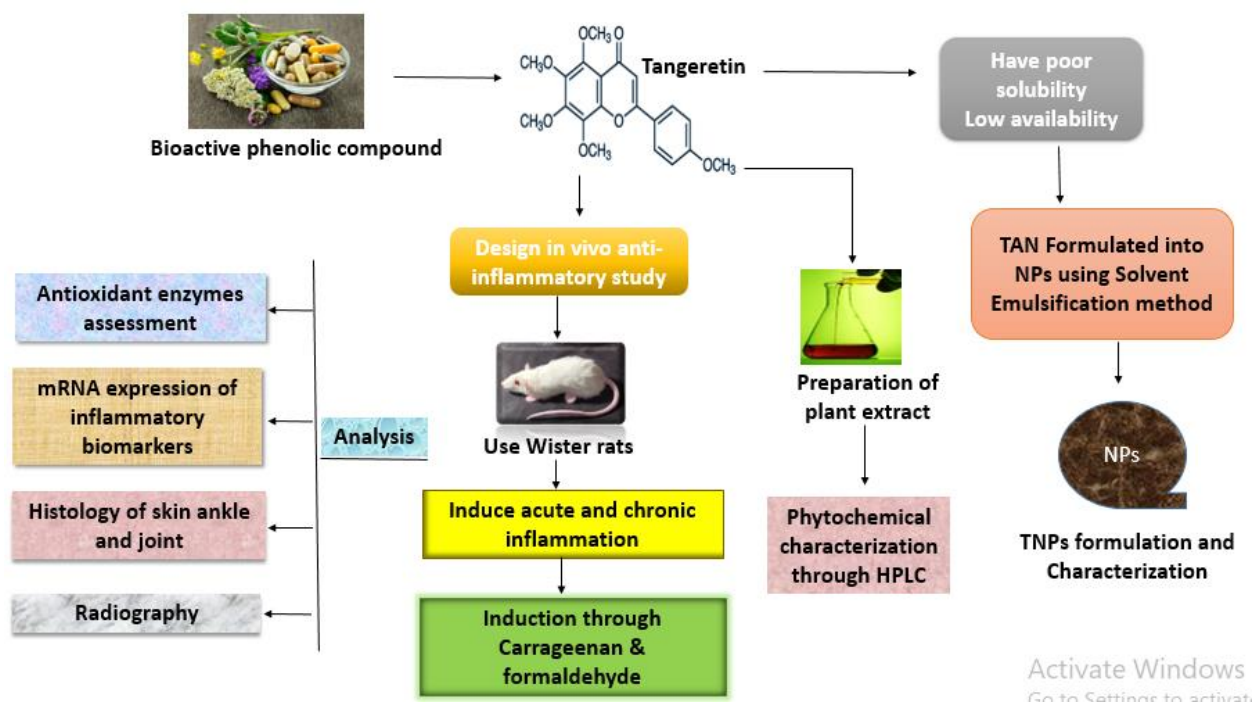
Email address: zunerach@yahoo.com

Abstract

Tangeretin (TAN), a naturally occurring polymethoxylated flavone derived from *Citrus japonica* peel, exhibited wide biomedical applications. Tangeretin is a hydrophobic compound, it has low solubility and availability, which limits its therapeutic potential. The *in vivo* anti-inflammatory effect of Tangeretin rich extract (TRE) & Tangeretin loaded nanoparticles (TNPs) (200µg/kg, 400µg/kg, 800µg/kg) were assessed against diclofenac and ibuprofen (10mg/kg) in adult male and female Wister rats using carrageenan and formaldehyde induced inflammation model. The effectiveness of both Tangeretin rich extract and Tangeretin loaded nanoparticles on formaldehyde evoked hallmarks were assessed through biochemical, hematological, radio-graphical and histopathological examination. The ELSA was carried out to assess the expression of interleukin-6, myeloperoxidase, prostaglandins E2S, nitric oxide and TNF- α . Level of antioxidant enzymes in liver tissue homogenate were also assessed, the concentration of CRP and RA factor also examined. Furthermore, qPCR was used to access the regulatory effect on inflammatory biomarkers. Tangeretin rich extract and Tangeretin loaded nanoparticles significantly elevate ($p < 0.001$) the level of antioxidant enzyme in contrast to pathological inflammation group. Tangeretin rich extract and Tangeretin-loaded nanoparticles significantly decreased paw swelling and paw

diameter ($p < .001$) in formaldehyde and carrageenan induced inflammation model. Tangeretin rich extract and Tangeretin loaded nanoparticles significantly suppressed the Messenger Ribonucleic Acid expression of pro-inflammatory biomarker also reduced the concentration of antioxidant enzymes in liver tissue homogenate. Therefore, work proved Tangeretin rich extract and Tangeretin loaded nanoparticles possess great potential to prevent and treat inflammatory ailments.

Graphical abstract



Highlights

Citrus japonica extract and Tangeretin were assessed to check its potent anti-inflammatory activity in experimental rat model.

Tangeretin rich extract was prepared and HPLC used to detect phytochemical present in extract.

Tangeretin formulated into NPs through solvent emulsification method which enhanced its absorption rate and solubility.

Tangeretin rich extract and Tangeretin loaded nanoparticles decrease paw diameter and paw swelling also elevate the level of antioxidant enzymes by attenuating the pro-inflammatory biomarkers.

Therefore, study imply that Tangeretin rich extract and Tangeretin loaded nanoparticles exhibit promise to cure and manage the inflammatory ailments meriting future investigations.

Keywords: Inflammation, Tangeretin rich extract, Tangeretin loaded nanoparticles, Solvent emulsification method, Antioxidants, Zeta sizer, Formaldehyde, Carrageenan, HPLC.

Introduction

Word inflammation itself was originated from Latin word *inflammare* (Basra et al., 2019) which mean “burned”. The primary component of body’s defense mechanism is inflammation (Taniguchi and Karin, 2018). Body use the inflammation as its non-specific response against the infection (viruses, bacteria, parasites), autoimmune reactions, local injury (radiation, burn), and for hypersensitive response, identify the external attackers and begins the defensive response healing. The inflammation can be recognized at the tissue level by the existences of key signs such as pain (dolor), tumor (swelling), heat (calor) loss of function (function laesa) along with redness (swelling) (Cavaillon, 2021). Important microcirculatory processes such as cellular infiltration, deviation in vascular permeability and liberation of certain signals or inflammatory mediators occurs during the process of inflammation. Wound become worsen and severe and infection could turn deadly in the absence of inflammatory response. The “aim” of inflammatory response is first and foremost, the destruction of inciting agents and consequently start healing as transitory replacement (Menzel et al., 2021).

Acute and chronic are two methods of inflammation (Abudukelimu et al., 2018). As acute inflammation is progressed it ultimately turned into chronic form of inflammation, which leads towards the progression of chronic diseases. In acute inflammation, blood vessels dilate, blood flow increase in vessels along with infiltration of inflammatory cells particularly neutrophils and macrophages. Acute phase lasts for a limited duration (Ullah et al., 2025). In acute phase, the damaged tissues released the cytokines which activate the immune cells and PGs function as a healing response. Chronic inflammation is considered as an adaptive response that possess widespread and extensive nature affecting normal functioning and cellular hemostasis (Muzamil et al., 2021). Chronic inflammation stayed for extended time period (months-years) showing long-lasting outcomes. When external bodies stayed in the body for long time or when immune system become over reactive then it caused chronic conditions. Chronic inflammation is associated with different diseases (Lazou et al., 2020) such as diabetes (Lee et al., 2018), cardiovascular disorders

(Soehnlein and Libby, 2021), neurodegenerative ailments (Kronsten et al., 2022), and their prevalence with the passage of time being a significant factor contributing to death.

When a foreign molecule enters into the body it stimulates the receptors present on the surface of cell ultimately various signaling cascades begins. They produce various inflammatory mediators eventually recruit the inflammatory cells (Chen et al., 2017). Many kind of pattern recognition receptor are used to recognize the pathogens and external invaders in which Toll like receptor (TLRs) often used (Fitzgerald and Kagan, 2020). Myeloid differentiation factor-88 is used for the passage of PAMPs and DAMPs alongside TLRs which activate signaling cascades and leads to beginning the activator protein-1 and nuclear factor κ B. Following receptor recognition, different process of inflammation began such as MAPK, $\text{NF-}\kappa\text{B}$ and JAK- signal transduction, and activates of transcription pathway (Kim et al., 2023).

Mediators of inflammation such as interleukin-6, interleukin- 1β and tumor necrosis factor alpha combines with co-stimulatory molecules along with chemokines and regulate the influx of effectors cells (e.g. monocytes, neutrophils, macrophages) towards tissue damage. Neutrophils release various toxic products from cytoplasmic granules like proteinase, reactive nitrogen species along with reactive oxygen species and exert cytotoxic action. These toxic elements are detrimental for both pathogen and immune cells to avoid persistent microbial infection. Lastly, it produces cardinal signs of inflammation. At termination, resolution of inflammation which is began through activated macrophages. During acute phase of inflammation, LTs (proinflammatory in character), PGs are released later on transform into lipoxins, increase the monocyte infiltration and inhibit further recruitment of neutrophils (Laurindo et al., 2023). Dysregulation among the reactive oxygen species production and removal within body produce oxidative stress which produce pro-inflammatory biomarker and link with the development of various inflammatory disorders. Also control the gene expression of inflammatory markers via the modulation of different pathways like $\text{NF-}\kappa\text{B}$ pathway, MAPKs, pathways (Morris et al., 2022).

Synthetic drugs like steroidal and non-steroidal medication used to cure the inflammation and inflammation associated conditions across the world (Ali et al., 2023). However, the use of these drugs as anti-inflammatory agents is somewhat restricted because they provoke undesirable side effects on kidney, bronchus, mucosa and cardiovascular system (Bindu et al., 2020). These widely

used drugs have been associated to serious deadly consequences. Thus, there is an ongoing demand to develop innovative, cheap therapy to treat the inflammatory disorders with least side effect.

For millennia, natural substances from conventionally used pharmacological plants have been served as a potent remedy for a wide range of ailments, offering effective treatment with negligible adverse effects (Ahmed et al., 2021). Tangeretin (TAN) (4',5,6,7,8-penta methoxyflavone) (Nonglang et al., 2023) is a major poly-methoxyflavone occurs chiefly in the citrus genus, especially in the peels of mandarin oranges (*Citrus reticulata*) and sweet oranges (*Citrus sinensis*) belonging to the family *Rutaceae* (Mizuno et al., 2019). Ethno-pharmacological examination on Tangeretin have demonstrated that it possesses various biological and functional properties such as anti-inflammatory, anti-atherogenic, anti-carcinogenic, hepatoprotective, antioxidant activities (Agrawal and Murti, 2022) also have action on neurological and proliferative fronts. Several mechanistic studies suggest that Tangeretin regulate various cell signaling cascade like downregulate the ROS pro-inflammatory mediators, MAPKs, NF-KB and JNK, and AKT activation (Shi et al., 2022a). Tangeretin rich extract (TRE) possess various phenolic compound which has been recognized through high performance liquid chromatography (HPLC) and elaborated in (Table 3) along with their biological activities.

As Tangeretin is a valuable bioactive with various biochemical and therapeutic applications however, as it is a hydrophobic compound so its therapeutic application become limited due to its low solubility and bioavailability (Hu et al., 2020). To overcome these constraints, a nanoparticle approach was used as it has the ability to deliver the drugs very effectively to the targeted site (Afzal et al., 2022). In this study, Tangeretin loaded nanoparticles (TNPs) has been prepared in order to increase their solubility and bioavailability (Chen et al., 2014). The purpose of this research was to access the therapeutic potential of Tangeretin rich extract (TRE) as well as Tangeretin loaded nanoparticles (TNPs) in carrageenan and formaldehyde induced paw inflammation model.

Material and method

Chemicals

Tangeretin rich extract, carrageenan, formaldehyde, ethanol, stearic acid, Poloxamer-188 (Sigma Aldrich), Tween-80, distilled water, methanol, Trizole, diclofenac, ibuprofen, chloroform,

isoflurane, cDNA kit (thermo scientific) cyber green (SYBER Green master mix of Bio-Rad), Primers (Thermo Fisher Scientific-US).

Tangeretin loaded Nanoparticles (TNPs) preparation

Solvent emulsification method

Nanoparticles have been used to deliver the hydrophobic compounds also it increased the solubility and stability of these compounds very effectively. This Polymer emulsification method was commenced by Vanderhoff in 1979 to produce the polymeric nanoparticles (Mendoza-Muñoz et al., 2016). A successive step in the preparation includes to dissolving the lipid into an organic solvent before combining in aqueous phase that contain surfactant and following the evaporation of solvent. The prepared pre-emulsion treated with probe sonicator. At room temperature, aqueous solid lipid nanoparticles formed. The organic solvent which we used in this process produce concentrated suspension which leads towards the additional evaporation and ultra-filtration process (Duong et al., 2020). A complete method of preparing the nanoparticles of hydrophobic compound Tangeretin SLNPs described in this study.

Organic phase and aqueous phase preparation

Very first, organic phase was prepared. For this, 0.4g of stearic acid was measured using weight balance and pored it into a clean vial then 5ml of ethanol was added into the vial. After this, 0.33g of Tween-80 was measured and was added into the vial that already have (stearic acid + ethanol) as emulsifying agent. Then 0.1g of Tangeretin (TAN) was measured and introduced into the solution. At last, to confirm the proper mixing, shifted this phase on hot plate for 15 minutes at 50°C. Aqueous phase was attained by measuring 0.536g of Polaxamer-188 and distilled water (20ML) and pure into a beaker. A magnet was placed into the beaker and shifted this phase on a magnetic stirrer at 300rpm for 30 minutes to form a clear solution (De Gaetano et al., 2021).

Process of Emulsification

2mL of organic phase was taken and added dropwise into 20mL of aqueous phase using micropipette. Heated this for 30 minutes then again placed on magnetic stirrer at 700rpm to reduce the size of particles. To get more homogeneity in particle size, prepared formulation was treated with probe sonicator. The probe of sonicator was washed with methanol to get rid from any contamination. Then emulsion was treated with probe sonicator, its amplitude was set at 60 watts,

pulse mode was ON for 1-sec and OFF for 2-sec for 15 minutes with the break interval of 5 minutes. After this process, Tangeretin loaded nanoparticles (TNPs) was formed (Figure 1).

Characterization of prepared nanoparticles

A liquid sample of prepared nanoparticles (TNPs) was send to National Textile University (NTU) Faisalabad Pakistan, for zeta sizer to check either our drug (Tangeretin) is loaded or not, and to determine the size of nanoparticles and to check its polydispersity index (dos Santos Souza et al., 2022). And this was evaluated through (Malvern instruments Ltd. ver 7.11).

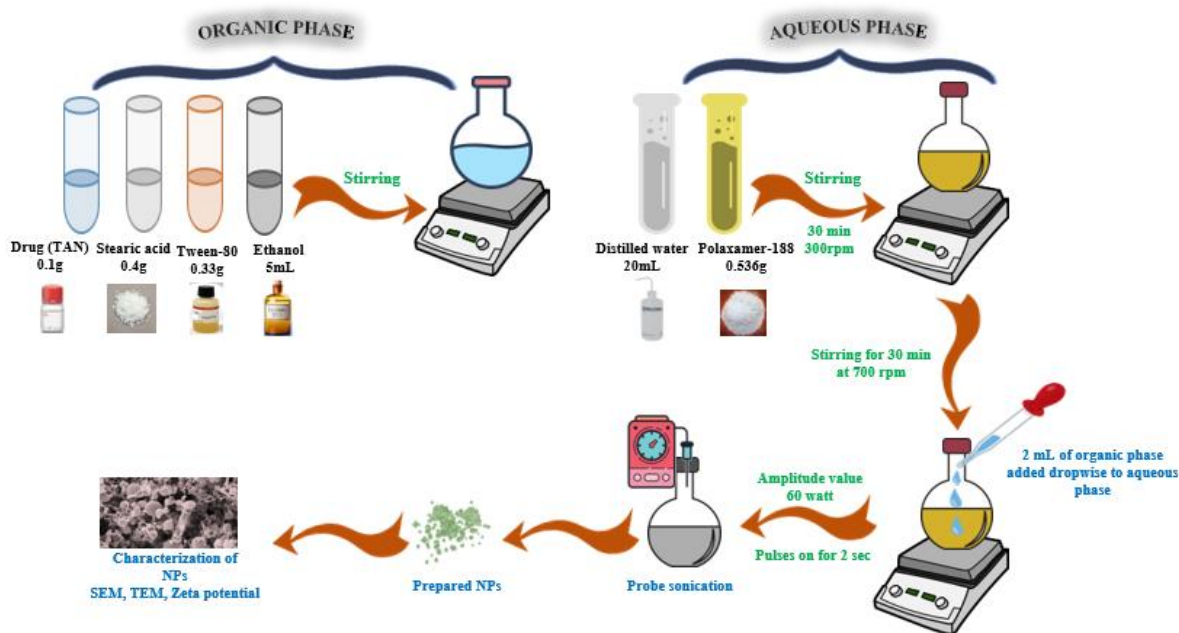


Figure 1: Diagrammatic illustration of Tangeretin Loaded Nanoparticles (TNPs) through Solvent Emulsification method.

Collection of *Citrus Japonica* peels

Citrus japonica peels were collected from local fruit market of Faisalabad following this, they were cleaned and thoroughly washed and then certified to air dry. For the next 24 hours, the peels were indorsed to rest in an oven set at 45°C after being stored at room temperature for 12 hours. Then peels were placed in a mortar pastel and creased and later were treated with a grinder to get a fine powder.

Treatment with Microwave-assisted extraction (MAE) method

The fine powder of peels was subjected to microwave-assisted extraction method. For this 300g of the peels powder was mixed in 500 mL of ethanol in a cleaned beaker and retained in microwave at 70°C (900W) for three rounds lasting for 6 minutes with a one-minute break. Following filtration, rotatory evaporator was used to abolish the extra solvent (ethanol) at 45°C from extract and to achieve a semi-solid extract (Farrukh et al., 2022a).

Characterization of phytochemicals through High-performance liquid chromatography (HPLC)

Using a 0.2 m Miller-HV membrane filter, the Tangeretin rich extract (TRE) were concentrated with methanol before being analyzed using HPLC. It comprised the Shimadzu LC-10A HPLC, a diode array detector called SPD-10AV, a column oven raised as CTO/20A and an auto-sampler identified as SIL-20A. Mutually, guard column (KJO 4282, Phenomenex) as well as the analytical column (5 n 100 (250 + 4.60 mm, Merck, Germany) were put to use. Mobile phase comprises two solvent methanol: 10% of acetonitrile (30:70) and double distilled water along 0.5% of glacial acetate (solution B). They distinguish the analytes by correlating the elution times and samples with standards. The concentration was quantified through external standard approach. Compounds are adequately separated through HPLC since their partition value and resolution are analyzed (Saleem et al., 2021). The phenolic compound in the extract was recognized by matching the UV-visible as well as retention time of the chromatographic peaks with authentic standards at 280 nm (Mushtaq et al., 2023). The analysis by HPLC took place in the Central Hi-Tech Laboratory at the University of Agriculture, Faisalabad, Pakistan.

In vivo evaluation of anti-inflammatory potential

Animal husbandry

Wister rats of both sexes (male and female) were selected whose body weight was 100-150g. And were maintained in animal house at Government College University Faisalabad (GCUF) Pakistan. Rats was treated with parameters such as relatively 50% humidity and 22±2°C temperature. Prior to experimentation, rats under-went a one-week acclimatization period and food and water was provided to rats. Allocated the rat into 08 groups (n=10) and the research was continued with the permission of Ethical Review Committee at Government College University Faisalabad, Pakistan under the Reference No. (GCUF/ERC/495).

Animal model of acute inflammation model

Animal model of acute inflammation was produced through carrageenan (0.1ml of 1% w/v) to determine the anti-inflammatory potential of Tangeretin rich extract (TRE) and Tangeretin loaded nanoparticles (TNPs) (Figure 2). In central area of right hind paw carrageenan was injected. Paw volume was measured at 0 hour then at 1,2,3,4,5,6 hours using digital Vernier caliper (Arham Shabbir et al., 2018).

Healthy: Animals received distilled water (1ml/kg) orally.

Pathological inflammation model (PIM): Animals in this group were injected with carrageenan (0.1mL of 1% w/v).

NSAID-I: Animals received 10mg/kg of standard drug diclofenac orally.

NSAID-II: Animals treated with ibuprofen at a dose of 10mg/kg via oral route.

TRE-1mg/kg: Tangeretin rich extract at a dose of 1mg/kg via oral route given to animals.

TNPs-200µg/kg: Tangeretin loaded nanoparticles TNPs- 200µg/kg was administered to animals via oral route.

TNPs-400µg/kg: Tangeretin loaded nanoparticles TNPs- 400µg/kg was given to animals orally.

TNPs-800µg/kg: Animals received Tangeretin loaded nanoparticles TNPs- 800µg/kg using oral gavage needle.

$$\% \text{ inhibition} = \frac{(K_t - K_0)_{\text{disease control}} - (K_t - K_0)_{\text{treated}}}{(K_t - K_0)_{\text{Treated}}} \times 100$$

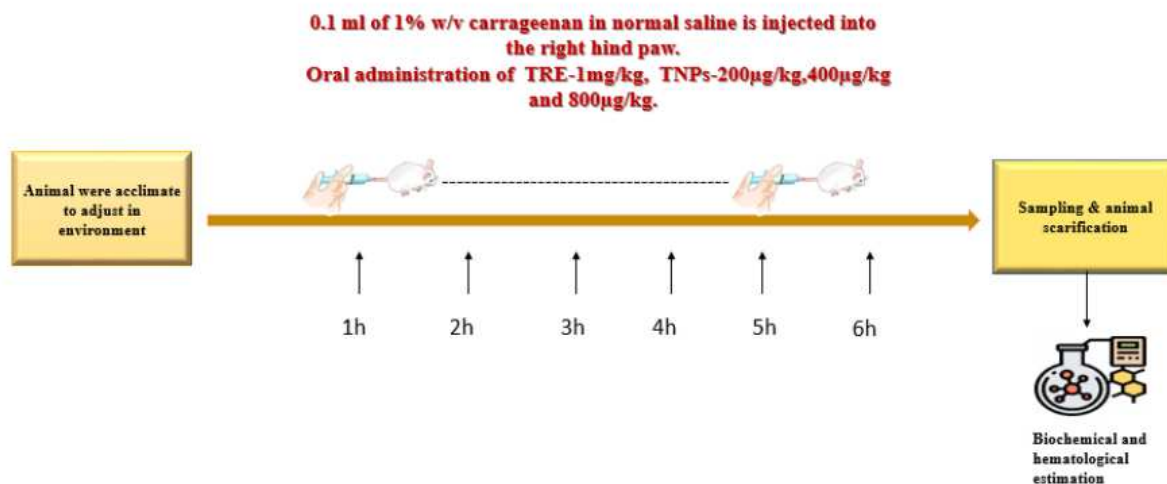


Figure 2: Timeline diagram of Anti-inflammatory effect of Tangeretin rich extract (TRE) & Tangeretin loaded nanoparticles (TNPs) induced by Carrageenan.

Chronic Inflammation model

0.2mL of formaldehyde (2% v/v in normal saline) was injected in sub planar region of right hind paw to produce chronic inflammation in rats. A digital Vernier caliper and electrical weighing scale used to assess the paw diameter and body weight of animals (Chinnasamy et al., 2019).

Healthy: 1mg/kg of distilled water was given to animals orally.

Pathological inflammation model (PIM): A 0.2mL of formaldehyde injection was given to animals by using insulin syringe.

NSAID-I: A dose of 10mg/kg of diclofenac was given to animals orally.

NSAID-II: Ibuprofen at a dose of 10mg/kg was given to animals

TRE-1mg/kg: Tangeretin rich extract (TRE-1mg/kg) was administered to animals orally.

TNPs-200 µg/kg: Tangeretin loaded nanoparticles TNPs-200 µg/kg was given to animals.

TNPs-400µg/kg: Animals were administered with TNPs-400µg/kg orally.

TNPs-800µg/kg: Tangeretin loaded nanoparticles TNPs-800 µg/kg was given to animals via oral gavage needle.

Animals went asleep using chloroform after 12 days of treatment. And were sacrificed on final day. Blood was drawn for the biochemical and hematological analysis (Figure 3).

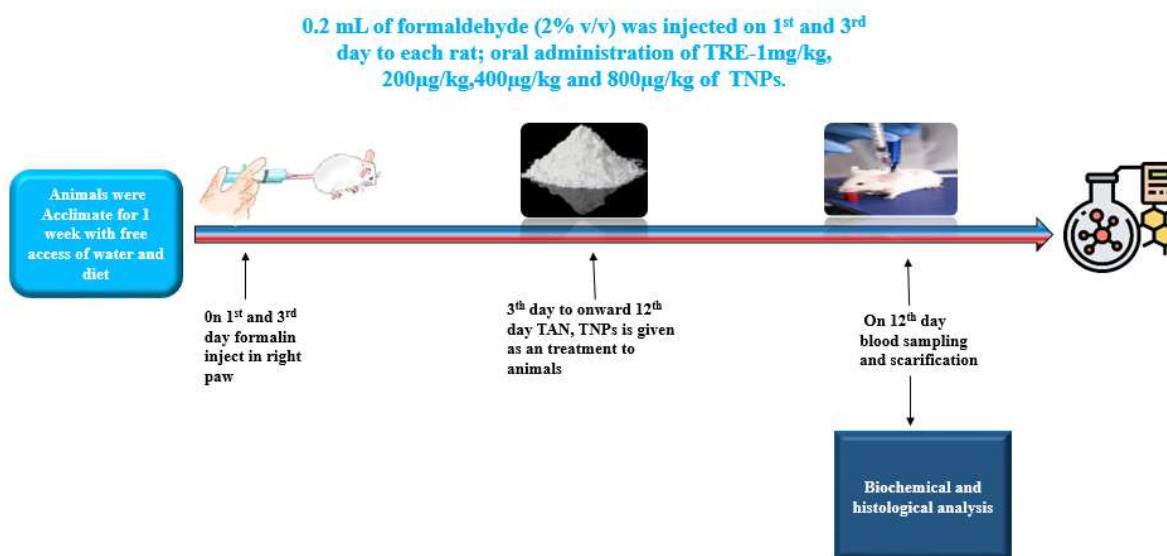


Figure 3: Timeline diagram of Anti-inflammatory potential of Tangeretin rich extract (TRE) & Tangeretin loaded nanoparticles (TNPs) induced by formaldehyde.

Anesthesia protocol

According to the animal's body weight, euthanasia was carried out. Rats were anesthetized with inhalant isoflurane (3.5-4%) by using a precision vaporizer after getting 100% oxygen. Using cervical dislocation method, animals whose body weight was less than 200g were euthanized. Commercially available guillotines were followed by trained professionals and the anesthetic method followed the American Veterinary Medical Association's (AVMA) rule (Underwood and Anthony, 2020).

Hematological and biochemical analysis

On the 12th day after anesthesia, blood samples were collected via heart puncture just before the animals were euthanized. Blood was collected in EDTA vials for hematological and for biochemical analysis (C-reactive protein, aspartate aminotransferase, alkaline aminotransferase, alkaline phosphate, gamma glutamate, rheumatoid factor) also for TNF- α analysis, serum was isolated from blood (taken in Eppendorf tubes) by centrifugation at 15000 rpm for 15 minute at 4°C using the kit manufacturer's protocol (Roche Products®, Switzerland).

Histopathology

Amputated rat paws were placed in sterilized container having 10% formalin solution for histopathological analysis and liver tissue were embedded in paraffin and microtome was used to cut the tissue into 5µm thickness. Histopathological changes were observed in the skin, knee and in ankle using Accu-scope 3000-LED microscope followed the hematoxylin and eosin (H&E) staining. Slides were observed under 4x and 10x (Pandey et al., 2018).

Radiographic analysis

For radiographic examination, and to check the bone degradation, joint constriction, and soft tissues swelling, hind paws from all groups of animals were taken and analyzed (Alamgeer et al., 2017).

Assessment of IL-6 and TNF-Alpha

To determine the level of Interleukin-6 and Tumor necrosis factor alpha serum was used isolated from blood. Vender procedure was used through commercially available kits (Elabsciences Biotechnology® China) for interleukin-6 (Catalog No: E-EL-R0015) and for tumor necrosis factor alpha (Catalog No: E-EL-R0019) (Saleem et al., 2023).

Liver homogenate preparation

Liver tissues were isolated and washed with normal saline (0.9%). Then mixed in phosphate buffer whose PH was 7.4 at a ratio of (1:10 w/v) then centrifuged at 4000 rpm for 30 min at 4°C to get the upper layer (Akhtar et al., 2016).

Superoxide dismutase level (SOD) assessment

100µL of tissue homogenate was mixed with 1.2mL of 0.05M sodium phosphate solution. Then mix with 0.1mL of phenazine methosulphate and 0.3mL of nitro-blue tetrazolium, followed by incubation around 30 minutes at 30°C. Successively, 0.1mL of glacial acid and 0.4mL of n-butanol were added in mixture. This mixture was centrifuged at 1000 rpm for 15 minutes. Then supernatant was collected and absorbance were taken at 560nm using spectrophotometer and expressed as unit/mg (Mir et al., 2019).

Estimation of Malondialdehyde (MDA)

The level of lipid peroxidation was determined using Malondialdehyde. 200µL of tissue homogenate was mixed with (200µl, 8.1%) of sodium dodecyl sulfate then 1.5mL of thio-barbituric acid and 1.5mL of acetic acid was added. Then diluted with 4mL of purified water. Heated this mixture at 90°C for 20 minutes using water bath. After cooling, 1mL of distilled water and 5mL of n-butanol added till a yellow hue appeared. Centrifuged this mixture at 4000rpm for 10minutes. Upper layer was separated and absorbance were recorded at 532nm. And were expressed as mg/ml (Saleem et al., 2014).

$$\text{Concentration of MDA} = \frac{\text{Absorbance} \times \text{volume mixture}(V_t) \times 100}{\text{Aliquot volume} \times \text{tissue weight} \times 1.56 \times 10^5}$$

Here, 1.56×10^5 is molar extinction coefficient, V_t is the total volume of assay mixture and V_u is aliquot volume.

Measurement of Catalases activity

CAT level was determined by taking 50µL of tissue homogenate mixed with 50ml of phosphate buffer (1.95ml). then combined with 30ml of hydrogen peroxide. The absorbance were recorded at 240nm (Bhangale and Acharya, 2016). Level of CAT was expressed as IU/µL

$$\text{CAT level} = \text{OD/E} \times \text{sample volume (mL)} \times \text{protein weight (mg)}$$

Glutathione level (GSH) assessment

To assess GSH level, 1mL of tissue homogenate and 1mL of potassium chloride were taken and mixed with 4mL cold distilled water. 1mL of Trichloroacetic acid (TCA) was added into the mixture following centrifugation for 10 minutes at 3000rpm. The upper layer was separated and incorporated into 0.001M DTNB and (0.4M, 4mL) of tris-buffer. Absorbance were recorded at 412nm and GSH concentration was expressed as µ/mg protein (Saleem et al., 2019).

$$\text{GSH Concentration} = \frac{\text{Dilution factor} \times \text{absorbance}}{\text{Extinction coefficient}}$$

Here, D is the dilution factor, A is the absorbance and E is the extinction coefficient ($C = 13000 \text{ M}^{-1} \text{ CM}^{-1}$).

Assessment of Glutathione peroxidase level (GPx)

Glutathione peroxidase level were measured by taking 0.1mL of liver tissue homogenate and combined with 0.2mL of sodium azide, 0.2mL of EDTA and distilled hydrogen peroxide (H₂O₂). 1ml of 10% of Trichloroacetic acid (TCA) was incorporated to stop the reaction following centrifugation at 2000 rpm for 10 minutes. Then the obtained top layer was treated with 4mL of disodium hydrogen phosphate and 0.5mL of DTNB was added. Spectrophotometer was used to note the absorbance at 420nm (Saleem et al., 2020b) and GPx level were expressed as μ /mg protein.

Real time quantitative polymerase chain reaction (qPCR) assessment

To find out the expression of inflammatory biomarkers real time quantitative polymerase chain reaction was used. The RBCs lysis buffer treated blood followed by RNA extraction via Trizole method. Extracted RNA sample was evaluated using nanodrop spectrophotometer (Saleem et al., 2020a). Isolated RNA was altered into cDNA with Revert Aid cDNA Synthesis Kit, followed the manufacturer protocol. Glyceraldehyde 3 phosphate (GADPH) served as housekeeping gene, and PCR system was used to produce quantitative real time PCR. The procedure entailed by adding 5 μ L of cDNA, 0.5 μ l of forward and reverse primers, and 5 μ l of syber green master mix to a PCR plate, which was then placed into a qRT-PCR machine for amplification. The apparatus was programmed for 40 cycles, including denaturation at (95 °C), and extension at (72 °C) and annealing (60 °C) (Coura et al., 2015) to facilitate amplification and to measure gene expression level (Table 1).

Table 1: List of primers with their base sequence.

Biomarkers	Base Sequence	Primer	Accession no.
TNF- α	ATG GGC TCC CTC TCA TCA GT	Forward	NM-012675.3
	GCT TGG TGG TTT GCT ACG AC	Reverse	
IL-6	CCC ACC AGG AAC GAA AGT CA	Forward	NM-012589.2
	ACT GGC TGG AAG TCT CTT GC	Reverse	
IL-4	CAACAAGGAACACCGAG	Forward	NM-201270.1

	TTTCAGTGTTGTGAGCGTGG	Reverse	
IL-1 β	GTC CTC TGC CAA GTC AGG TC CAG GGA GGG AAA CAC ACG TT	Forward Reverse	NM-031512.2
NF-K β	AGGCCATTGAAGTGATCCAG CAGTGAGGGACTCCGAGAAG	Forward Reverse	NM-001276711.1
COX-2	AAAGGCCTCCATTGACCAGA TCGATGTCATGGTAGAGGGC	Forward Reverse	NM-017232.3
IL-10	ATAACTGCACCCACTTCCCA TTTCTGGGCCATGGTTCTCT	Forward Reverse	XM-006249712.4

Estimation of leukotrienes B4 (LTB₄), prostaglandins E2 (PGE₂), Nitric oxide and Myeloperoxidase (MPO)

Animals were sacrificed and serum samples were collected, according to kits' manufacturer protocol serum level of leukotrienes B4 (LTB₄), prostaglandins E2 (PGE₂), nitric oxide and myeloperoxidase (MPO) were estimated. ELISA kits of LTB₄, PGE₂, Nitric oxide and myeloperoxidase were used Zokeyo, Wuhan Zokeyo Biotechnology Co., Ltd., China.

Statistical analysis

The data was analyzed using GraphPad Prism version 8.4.3, and the results were expressed as mean $\pm$ standard error of the mean (M $\pm$ SEM). One way and two-way ANOVA was employed with Bonferroni post hoc test.

Results

TNP-SLNs characterization

Zeta sizer

The prepared Solid lipid nanoparticles of Tangeretin was evaluated based upon their intensity to find out their particle size, polydispersity index through Zeta sizer (Figure 4). The result showed

that size of nanoparticles was (239.1d. nm) and its polydispersity index was (0.409) showed in (Table 2). TNPs found to be stable, uniform and demonstrated a low PDI value and particle size.

Table 2: Tangeretin-loaded nanoparticles (TNPs) properties.

Properties	Values
Z-Average (d.nm)	239.1
PDI	0.409
Intercept	0.907

Results

	Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 239.1	Peak 1: 203.7	98.1	68.58
Pdl: 0.409	Peak 2: 5560	1.9	6.104e-5
Intercept: 0.907	Peak 3: 0.000	0.0	0.000
Result quality : Good			

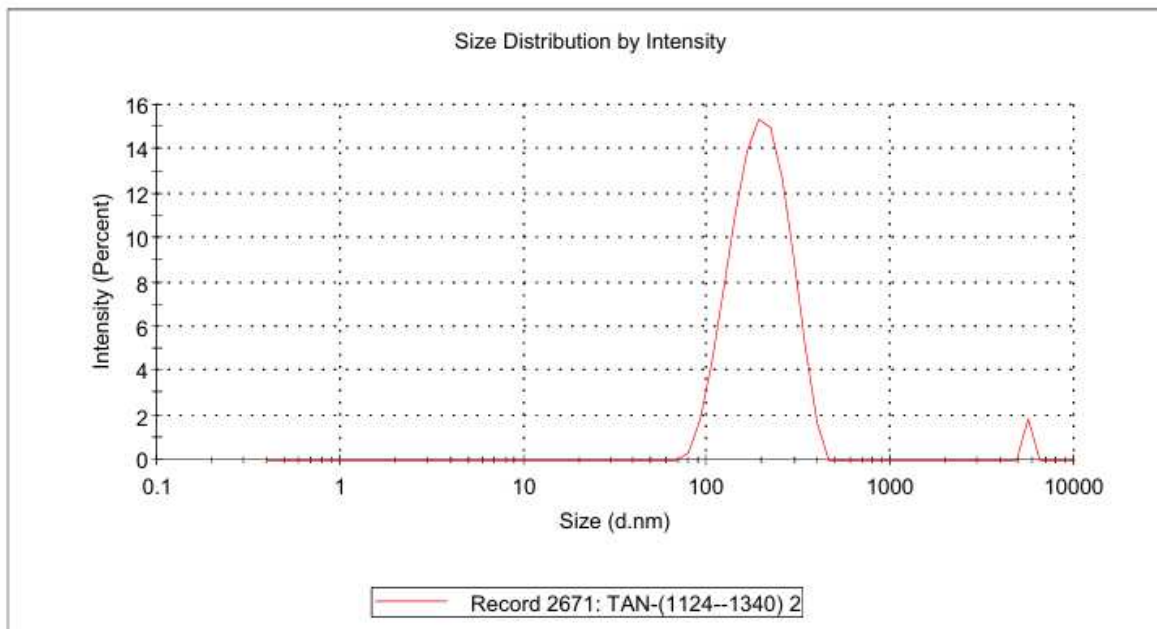


Figure 4: Size distribution diagram of TNPs based upon intensity.

Characterization of phytochemicals through HPLC

A high performance liquid chromatography (HPLC) device was employed to identify the phytochemical compounds in Tangeretin rich extract. It was distinguished that Tangeretin, Gallic acid, Vanillic acid, benzoic acid, Chlorogenic acid, p-coumeric acid, m-coumeric acid, Sinapic acid was found in the examined Phytoconstituents and their peaks shown in (Figure 5). These phenolic compounds hold anti-inflammatory and anti-oxidant activities, can prevent inflammatory intermediaries as well as neutralize free radicals, consequently exhibited a potential in treating inflammation in carrageenan and formaldehyde induced rats after being given in Tangeretin loaded nanoparticles (TNPs) as well as Tangeretin rich extract (TRE) (Table 3).

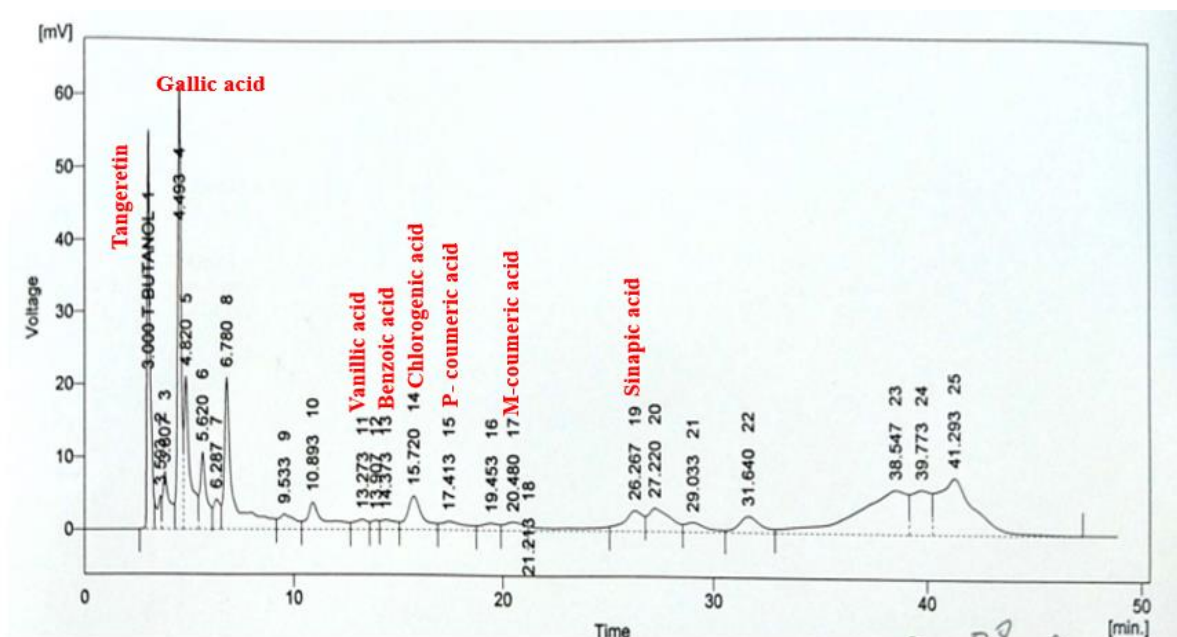


Figure 5: HPLC scan indicated Phytoconstituents of Tangeretin rich extract.

Table 3: Detected phytochemicals with their biological effects.

Sr. No	Identified compounds	Retention time	Area (m V.s)	Area %	Amount (ppm)	Biological effect
--------	----------------------	----------------	--------------	--------	--------------	-------------------

01	Tangeretin	3.000	566.314	9.0	30.08	Tangeretin lessen inflammatory response and oxidative damage by triggering the Sesn2/Keap1/Nrf2 activation (Peng et al., 2024).
02	Gallic acid	4.493	710.157	11.3	25.56	Gallic acid regulates NF-KB and MAPK cascades alleviate inflammatory response by hindering the expression of inflammatory mediators (Bai et al., 2021).
03	Vanillic acid	13.273	60.422	1.0	3.72	Vanillic acid considerably lowered oxidative stress induction and reactive oxygen species production initiated by LPS and fMLP , terminating the cytokine-mediated inflammation and elastase activation (Magiera et al., 2025).
04	Benzoic acid	14.373	65.237	1.0	6.89	Benzoic acid elicits anti-inflammatory capacity by targeting COX-2 binding, hindering NF-KB cascade and by suppressing the inflammatory cytokines production like interleukin-

						1 β , and tumor necrosis factor alpha (Tjahjono et al., 2021).
05	Chlorogenic acid	15.720	226.323	3.6	17.63	Chlorogenic acid exhibits anti-inflammatory potential by blocking the NF-KB, IKK and I κ B complex from being triggered, preventing NF-KB from going into the cell nucleus and binding to its genes (Huang et al., 2023).
06	p-coumeric acid	17.413	91.535	1.5	1.19	p-coumeric acid moderated oxidative stress by nullifying free radicals and reactive oxygen species (Iftikhar et al., 2022).
07	m-coumeric acid	20.480	71.513	1.1	8.58	Using the ARF/Nrf2 signaling cascade, m-coumeric acid recruits the expression of genes for antioxidant reaction and was noted to exist as free carboxylic acids, amides and esters (Roychoudhury et al., 2021).
08	Sinapic acid	26.267	178.369	2.8	2.32	Sinapic acid lessened interleukin-1 β excretion by suppressing NLRP3

						inflammasomes activation and subsequent caspases-1 activation (Lee et al., 2021).
--	--	--	--	--	--	---

Effect of Tangeretin rich extract and Tangeretin loaded nanoparticles on the weight of animals

As inflammation progressed, animals body weight decreased significantly. As animals treated with formaldehyde they showed a considerable decrease in their body weight ($p < 0.001$) when they were compared with healthy animals. However, standards drugs and treatment with TRE-1mg/kg, TNPs-200 μ g/kg, 400 μ g/kg and 800 μ g/kg revamped the body weight substantially ($p < 0.001$) when compared to pathological inflammation group (PIM). And showed a dose dependent recovery (Table 4).

Table 4: Body weight response

Weight in gram				
Groups	1 st day	4 th day	8 th day	12 th day
Healthy	120 $\pm$ 0.02	132 $\pm$ 0.01	143 $\pm$ 0.01	150 $\pm$ 0.03
PIM	124 $\pm$ 0.04 ^{ns}	119 $\pm$ 0.03 [#]	114 $\pm$ 0.02 ^{##}	102 $\pm$ 0.02 ^{###}
NSAID-I	127 $\pm$ 0.03 ^{ns}	130 $\pm$ 0.04 [*]	135 $\pm$ 0.01 ^{**}	143 $\pm$ 0.12 ^{***}
NSAID-II	129 $\pm$ 0.02 ^{ns}	131 $\pm$ 0.01 [*]	136 $\pm$ 0.011 ^{**}	142 $\pm$ 0.02 ^{***}
TRE-1mg/kg	130 $\pm$ 0.01 ^{ns}	132 $\pm$ 0.02 [*]	138 $\pm$ 0.04 ^{**}	145 $\pm$ 0.03 ^{***}
TNPs-200	130 $\pm$ 0.01 ^{ns}	134 $\pm$ 0.01 [*]	139 $\pm$ 0.05 ^{**}	146 $\pm$ 0.04 ^{***}
TNPs-400	132 $\pm$ 0.02 ^{ns}	135 $\pm$ 0.11 [*]	140 $\pm$ 0.03 ^{**}	148 $\pm$ 0.13 ^{***}
TNPs-800	133 $\pm$ 0.04 ^{ns}	134 $\pm$ 0.03 [*]	140 $\pm$ 0.04 ^{**}	149 $\pm$ 0.04 ^{***}

Effect of TRE & TNPs on body weight of animals produced due to the induction of formaldehyde was (ns) non-significantly and ***($p < 0.001$) in contrast to PIM and ### in contrast to healthy.

Effect of Tangeretin rich extract and Tangeretin loaded nanoparticles on acute inflammation animal model

Acute inflammation in rat model was induced through carrageenan and results showed that after administration of carrageenan paw volume was increased (Table 5). Treatment with TRE-1mg/kg and TNP-200µg/kg, 400µg/kg, and 800µg/kg showed a remarkable reduction ($p<0.001$) in paw inflammation due to its anti-inflammatory potential when compared with pathological inflammation group (PIM) (Figure 6). Standards drugs (NSAID-I & NSAID-II) also reduce paw inflammation but higher doses of TNPs showed more remarkable reduction than Tangeretin rich extract and standard drugs. TNPs at the dose level of 200µg/kg, 400µg/kg and 800µg/kg exhibit percentage of inhibition 62%, 65%, 75% sequentially.

Table 5: Effect of Tangeretin rich extract & Tangeretin loaded nanoparticle on paw inflammation (mm) in rats.

Groups	1h	2h	3h	4h	5h	6h
Healthy	4.23±0.01***	4.20±0.01***	4.21±0.12***	4.25±0.2***	4.27±0.11***	4.29±0.02***
PIM	3.68±0.01***	3.40±0.02	3.19±0.1	2.89±0.04	2.17±0.19	2.99±0.10
NSAID-I	5.15±0.18***	5.08±0.19***	5.00±0.2***	5.14±0.011***	4.68±0.10***	4.66±0.18***
NSAID-II	5.15±.15***	5.07±.17***	5.01±0.1***	5.13±0.110***	4.69±.11***	4.68±0.17***
TRE-1mg/kg	5.28±0.10***	5.27±0.14***	5.30±0.16***	5.27±0.12***	5.31±0.01***	5.22±1.11***
TNPs- 200µg/kg	5.30±0.11***	5.30±0.10***	5.31±0.18***	5.32±0.10***	5.34±0.14***	5.26±0.01***
TNPs- 400µg/kg	5.39±0.01***	5.35±0.01***	5.38±0.03***	5.39±0.06***	5.38±0.15***	5.40±0.17***
TNPs- 800µg/kg	6.36±0.02***	6.37±0.02***	6.26±0.02***	6.14±0.07***	6.15±0.18***	6.19±0.19***

P <0.001 and ns (non-significant) in relation to carrageenan induced inflammation in rats.

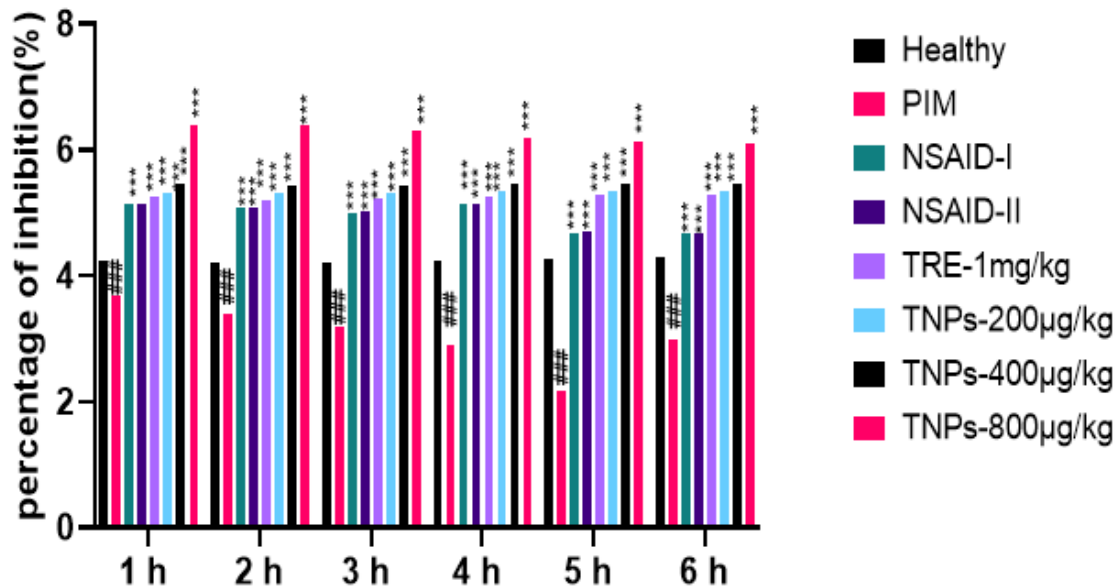


Figure 6: Outcomes of TRE & TNPs on % inhibition on paw inflammation produced by carrageenan was significantly *($p < 0.001$) in contrast to PIM, ### in contrast to healthy.**

Anti-inflammatory effect of Tangeretin rich extract and Tangeretin loaded nanoparticles in formalin induced paw inflammation

Animal model of chronic inflammation was induced through formaldehyde and treatment was given to animals using standards drugs, Tangeretin rich extract and Tangeretin loaded nanoparticles. When formaldehyde was injected it significantly raised the level of inflammation in hind paw ultimately paw volume was also increased (Figure 7). The treatment with TRE-1mg/kg exhibits a reduction in paw inflammation. Following this, TNPs-200µg/kg and 400µg/kg significantly reduced the inflammation than Tangeretin rich extract showed a dose dependent response. However, treatment with TNPs-800µg/kg exhibited a remarkable reduction in paw inflammation than TRE-1mg/kg, TNPs-200, TNPs-400µg/kg, and standard drugs (NSAID-I & NSAID-II) ibuprofen and diclofenac. Therefore, TRE, TNPs and standard drugs showed a potent reduction ($p < .001$) in paw inflammation and in paw volume in contrast to pathological inflammation group (PIM) (Table 6).

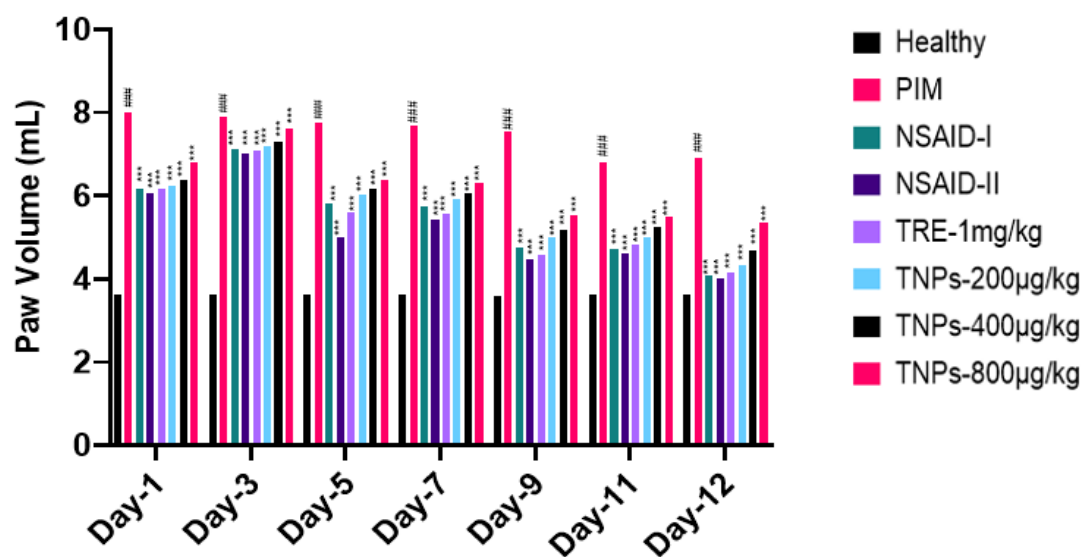


Figure 7: The outcomes of TRE & TNPs on paw edema induced by formaldehyde was significantly *** ($p < 0.001$) in contrast to PIM and ### in contrast to healthy.

Table 6: Anti-inflammatory effect of Tangeretin rich extract (TRE) & Tangeretin loaded nanoparticles (TNPs) on paw edema induced by formaldehyde (mm).

Group	Day 01	Day 03	Day 05	Day 07	Day 09	Day 11	Day 12
Healthy	3.63±0.01***	3.62±0.02***	3.61±0.12***	3.63±0.11***	3.60±0.1***	3.62±0.2***	3.61±0.01***
PIM	3.63±0.01	7.99±0.03	7.89±0.04	7.76±0.02	7.68±0.01	6.80±0.04	6.92±0.02
NSAID-I	3.95±0.03***	6.17±0.05***	7.09±0.03***	5.80±0.04***	5.76±0.03***	4.72±0.05***	4.03±0.01***
NSAID-II	3.93±0.02***	6.08±0.03***	7.01±0.05***	5.00±0.02***	5.44±0.05***	4.46±0.04***	4.00±0.01***
TRE-1mg/kg	3.82±0.02***	6.15±0.01***	7.09±0.05***	5.59±0.03***	5.58±0.04***	4.59±0.01***	4.17±0.02***
TNPs-200µg/kg	3.88±0.01***	6.25±0.01***	7.19±0.01***	6.01±0.05***	5.93±0.02***	5.00±0.01***	4.35±0.04***
TNPs-400µg/kg	3.70±0.05***	6.39±0.04***	7.30±0.02***	6.17±0.03***	6.08±0.05***	5.19±0.03***	4.69±0.05***
TNPs-800µg/kg	3.72±0.05***	6.79±0.03***	7.62±0.04***	6.38±0.02***	6.31±0.04***	5.53±0.01***	5.36±0.02***

P <0.001 and ns (non-significant) in contrast to formaldehyde-induced inflammation model.

Effect of Tangeretin rich extract and Tangeretin loaded nanoparticles on liver function parameters

The concentration of liver enzymes alanine aminotransferase, aspartate aminotransferase, alkaline aminotransferase and gamma glutamate transferase was assessed in formaldehyde induced inflammation model. Pathological inflammation group (PIM) exhibited a significant increase (p<0.001) in the levels of aminotransferase, aspartate aminotransferase, alkaline phosphate and gamma glutamate transferase when compared with healthy animals. The level of these enzymes was raised due to elevated oxidative stress which ultimately leads towards hepatic injury. Treatment with standard drugs (NSAID-I & NSAID-II), TRE-1mg/kg, TNPs-200µg/kg, 400µg/kg, 800µg/kg decreased (p<0.001) the level of these enzymes towards their normal range in a dose dependent manner than pathological inflammation group (PIM). However, higher doses of TNPs-400µg/kg and 800µg/kg showed more remarkable reduction and lower doses and standard drugs (Figure 8).

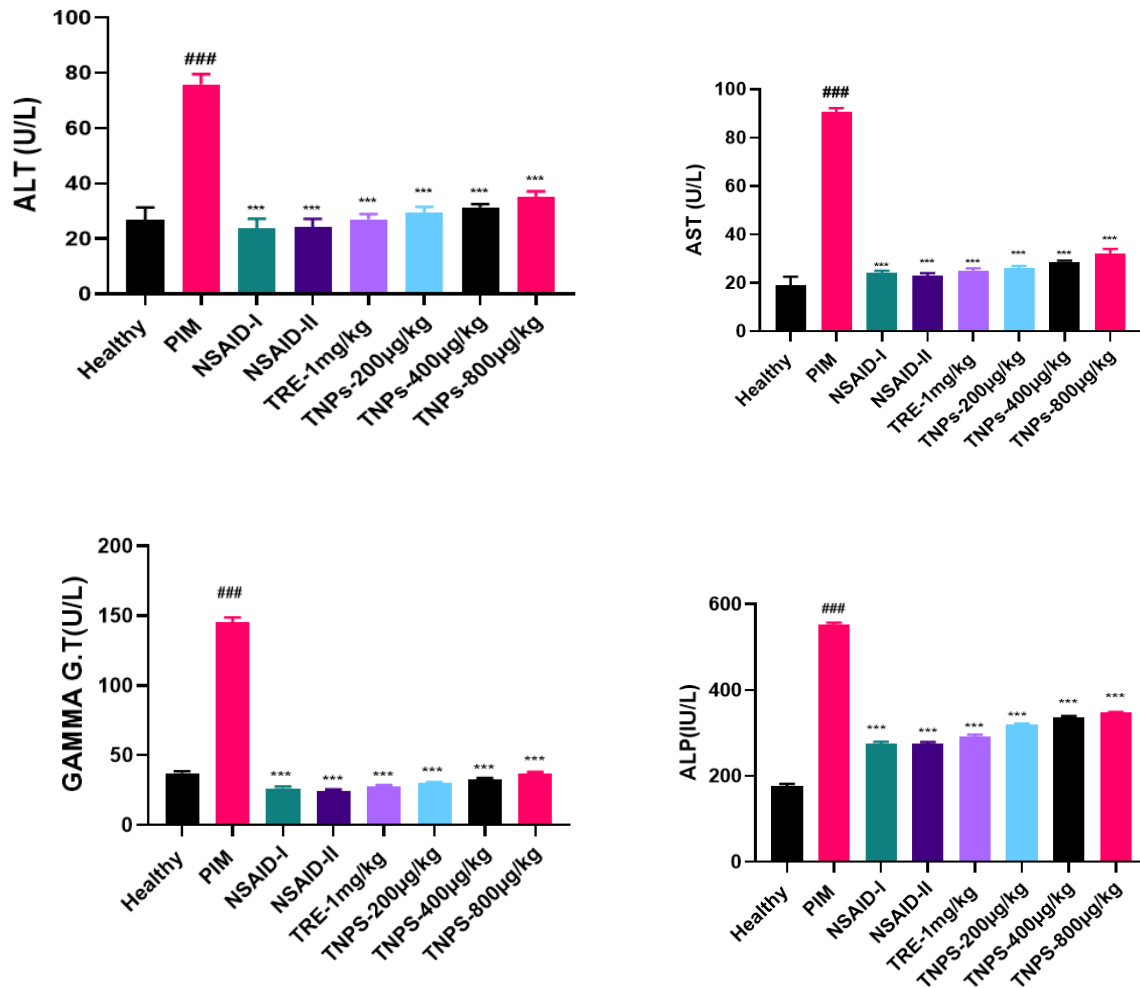


Figure 8: Effect of TRE & TNPs liver parameters. A: Alanine aminotransferase (ALT), B: Aspartate transaminase (AST), C: Gamma-glutamyl transferase, D: Alkaline phosphatase (ALP) ns (non-significant) *($p < 0.001$) in contrast to PIM, and ### in contrast to healthy.**

Effect of Tangeretin rich extract and Tangeretin loaded nanoparticles on hematological analysis

The level of rheumatoid factor (RA) and C-reactive protein (CRP) raised ($p < 0.001$) due to the formaldehyde exposure in pathological inflammation group (PIM) when compared with healthy animals. When treatment was given to animals through standard drugs (NSAID-I & NSAID-II), TRE-1mg/kg, TNPs-200µg/kg, 400µg/kg, and 800µg/kg they significantly reduced ($p < 0.001$) the level of CRP and RA factor (Figure 9).

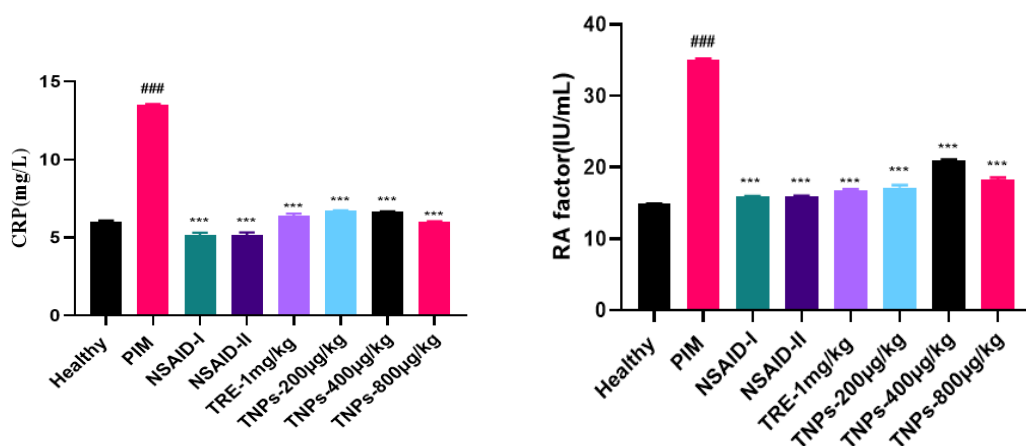
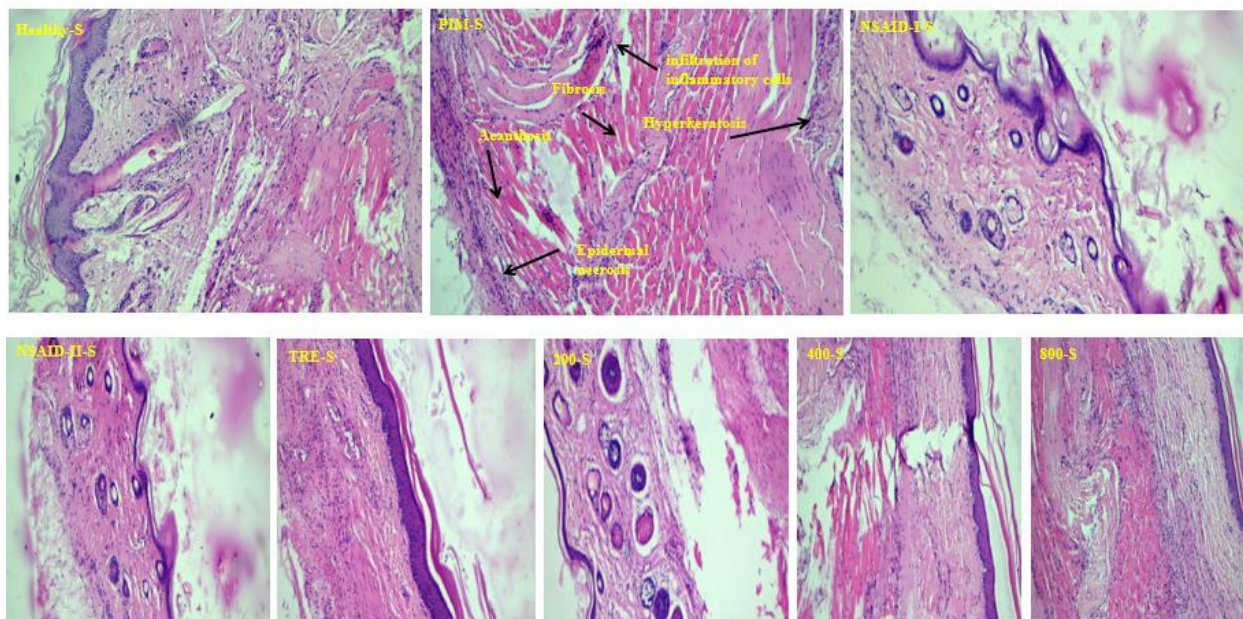


Figure 9: The effect of TRE & TNPs on C-reactive protein (CRP) and RA factor showed a significant difference ($p < 0.001$) in contrast to pathological inflammation group and ### in contrast to healthy.**

Histopathological examination

The histopathological analysis was performed under Accu-scope 3000-LED microscope. In pathological inflammation group (PIM), fibrosis, epidermal necrosis, hyperplasia of synovial lining, acanthosis, hyperkeratosis, synovium and strong intra-articular inflammation was observed due to administration of formaldehyde and carrageenan when compared with animals in healthy group. Massive inflammation cell infiltration was also observed in pathological inflammation group of skin, knee and ankle. When treatment was given to animals via standard drugs (NSAID-I & NSAID-II), TRE-1mg/kg, TNPs-200µg/kg, 400µg/kg and 800µg/kg they significantly alleviate the pathological features in treated groups. Tangeretin rich extract and standard drugs showed good results but when treatment was given via TNPs doses it showed more effective result in alleviating inflammation (Figure 10,11,12).

(A)



(B)

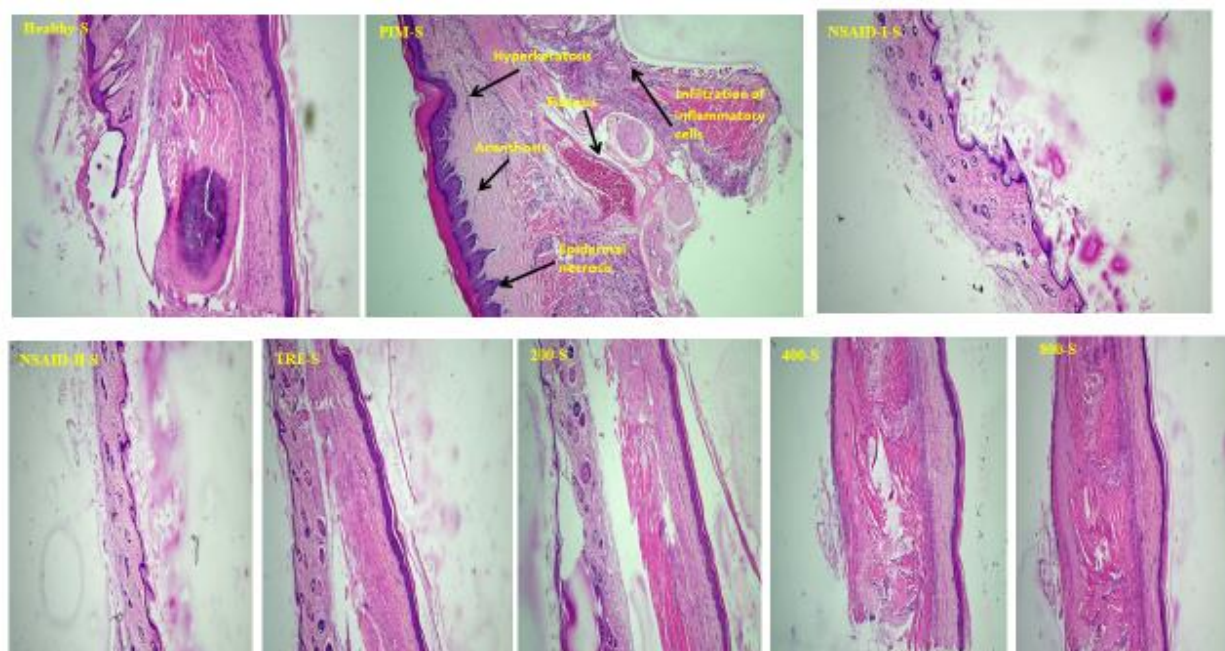
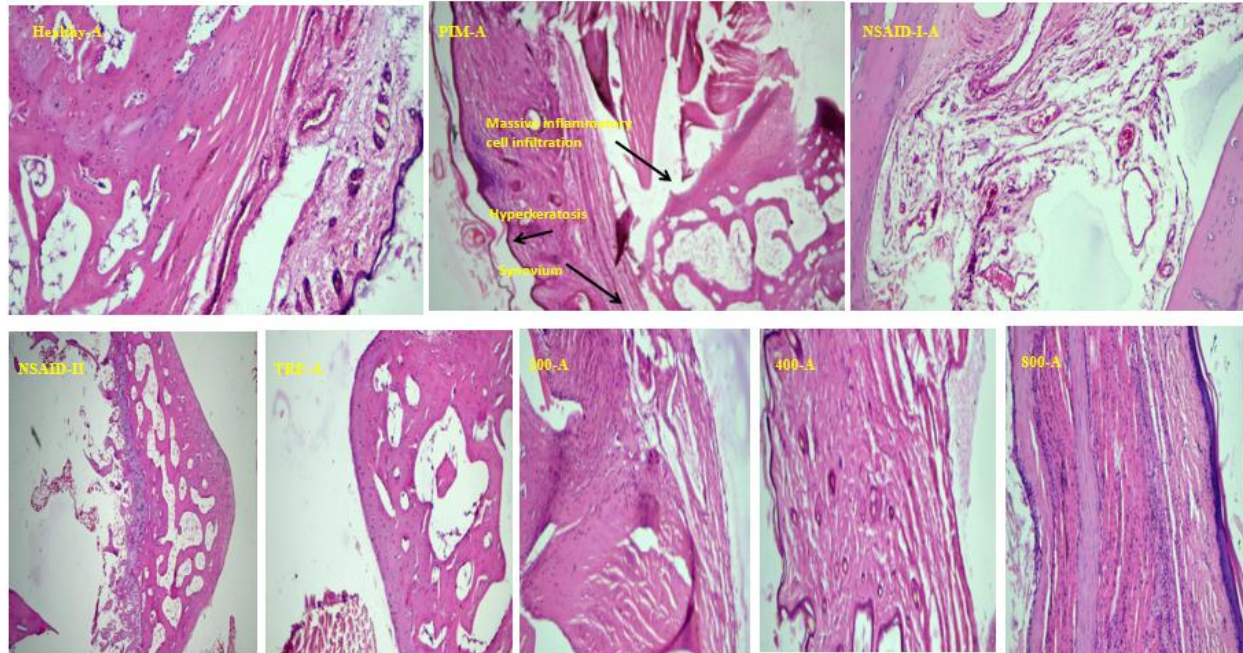


FIGURE 10: Effect of Tangeretin rich extract (TRE) & Tangeretin loaded nanoparticles (TNPs) on histological changes in rat's paw skin after formaldehyde exposure. Here, the (A) represent the figures capture at 10X, while (B) shows figures capture at 4X.

(A)



(B)

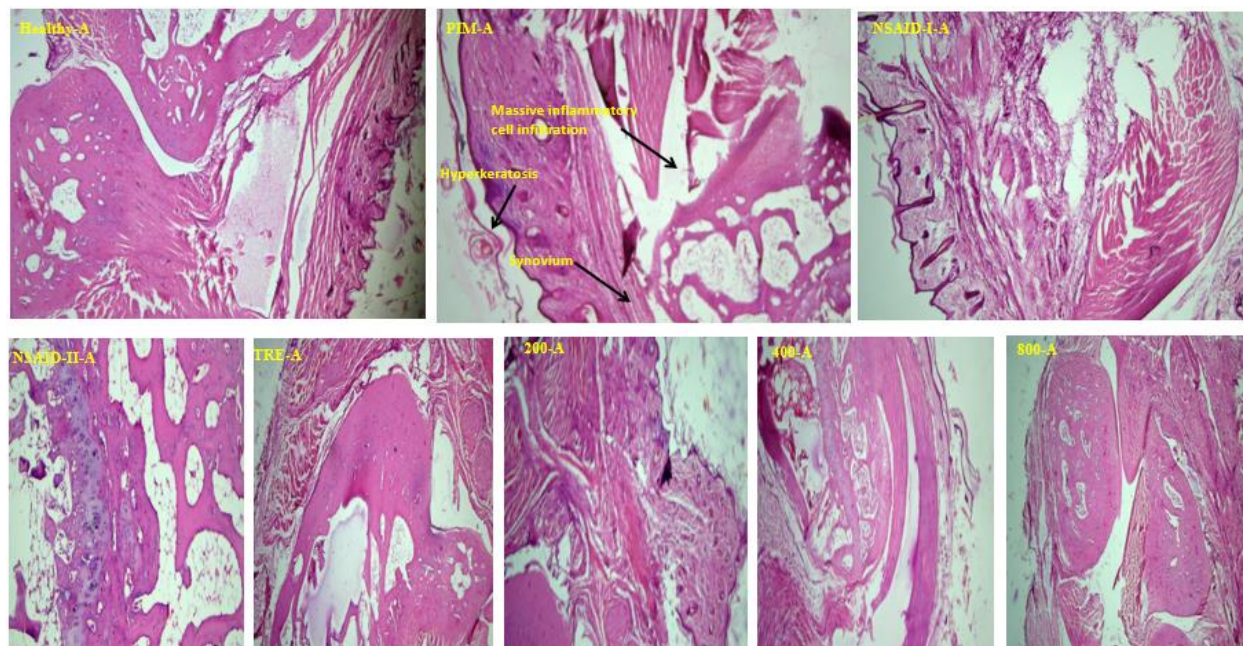
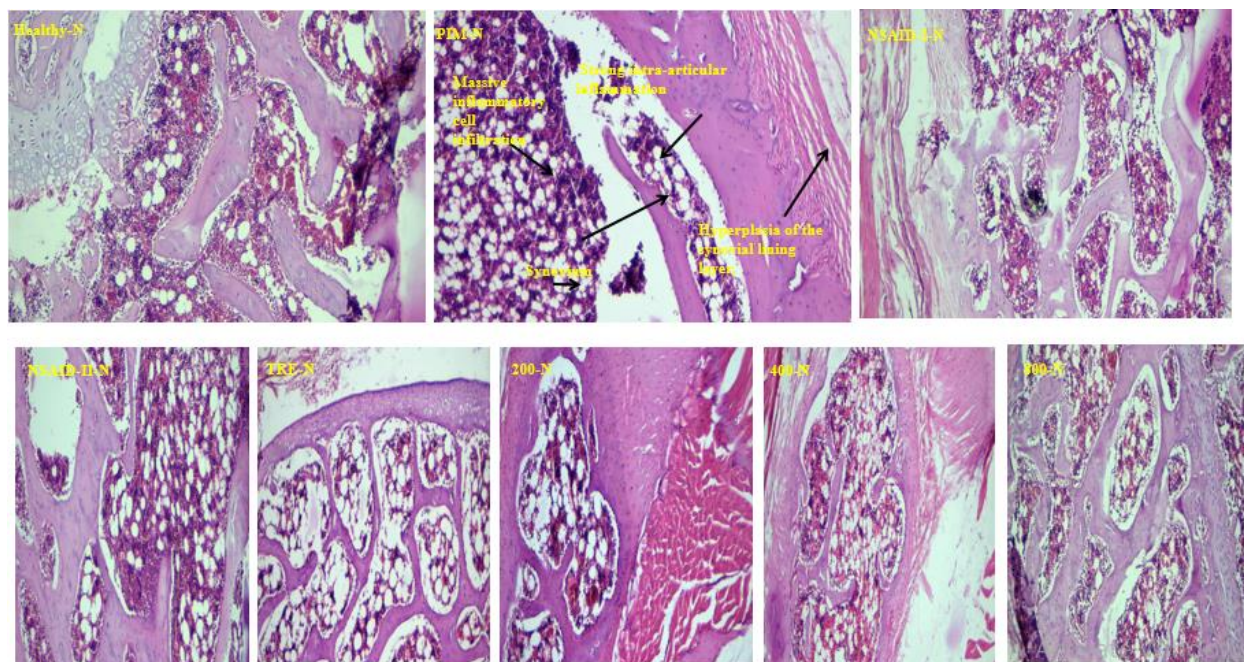


FIGURE 11: Effect of Tangeretin rich extract (TRE) & Tangeretin loaded nanoparticles (TNPs) on histological changes in rat's ankle after formaldehyde exposure. Here, the (A) represent the figures capture at 10X, while (B) shows figures capture at 4X.

(A)



(B)

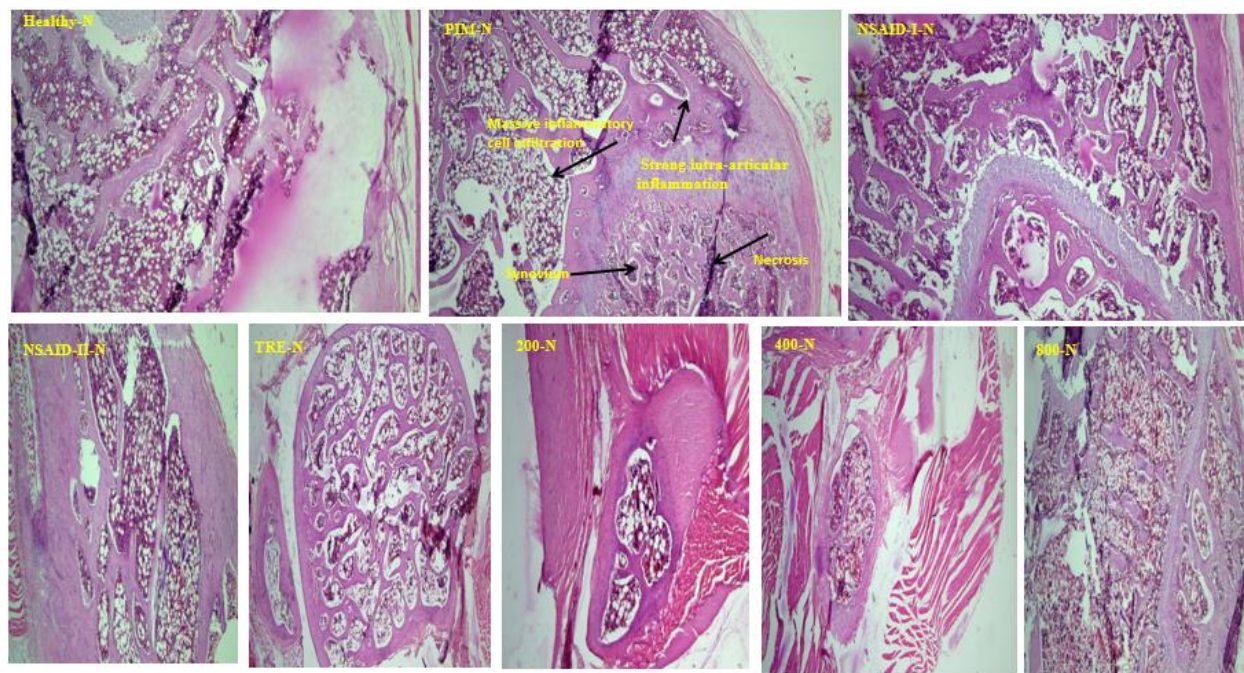


FIGURE 12: Effect of Tangeretin rich extract (TRE) & Tangeretin loaded nanoparticles (TNPs) on histological alterations in rat's knee following formaldehyde exposure. Here, the (A) represent the figures capture at 10X, while (B) shows figures capture at 4X.

Effect of Tangeretin rich extract and Tangeretin loaded nanoparticles on antioxidant enzymes

The level of antioxidant enzymes was assessed after formaldehyde administration. The result showed that the level of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH) was significantly decreased ($p < 0.05$) in pathological inflammation group (PIM) than healthy group. While the level of malondialdehyde (MDA) was raised in PIM in contrast to healthy animals. standard drugs (NSAID-I & NSAID-II), TRE-1mg/kg, TNPs-200 μ g/kg, 400 μ g/kg and 800 μ g/kg significantly raised ($p < 0.05$) the level of first line of antioxidant enzymes while lowered the level of MDA in contrast to pathological inflammation group (PIM). And they showed their effect in dose depend manner. Higher doses of TNPs showed more remarkable result than standard drugs and Tangeretin rich extract (Table 7).

Table 7: Effect of TRE and TNPs on antioxidant enzymes

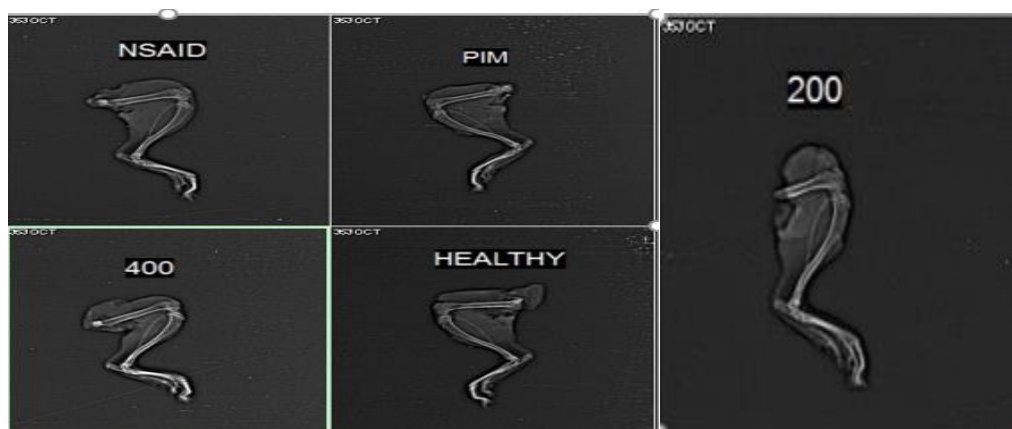
Groups	SOD(IU/ μ L)	CAT(IU/ μ L)	GSH(μ /mg protein)	MDA(TBA mg/ml)	GPx (μ /mg protein)
Healthy	0.27 $\pm$ 0.002	1.04 $\pm$ 0.01	0.57 $\pm$ 0.003	2.90 $\pm$ 0.4	7.30 $\pm$ 0.004
PIM	0.08 $\pm$ 0.001 ^{###}	0.67 $\pm$ 0.03 ^{###}	0.22 $\pm$ 0.002 ^{###}	4.78 $\pm$ 0.06 ^{###}	5.32 $\pm$ 0.001 ^{###}
NSAID-I	0.19 $\pm$ 0.004 ^{***}	0.81 $\pm$ 0.02 ^{***}	0.44 $\pm$ 0.004 ^{***}	3.0 $\pm$ 0.08 ^{***}	6.163 $\pm$ 0.003 ^{***}
NSAID-II	0.17 $\pm$ 0.03 ^{***}	0.80 $\pm$ 0.01 ^{***}	0.45 $\pm$ 0.04 ^{***}	3.01 $\pm$ 0.05 ^{***}	6.161 $\pm$ 0.03 ^{***}
TRE-1mg/kg	0.18 $\pm$ 0.002 ^{***}	0.85 $\pm$ 0.04 ^{***}	0.47 $\pm$ 0.003 ^{***}	2.97 $\pm$ 0.006 ^{***}	6.71 $\pm$ 0.05 ^{***}
TNPs-200 μ g/kg	0.19 $\pm$ 0.003 ^{***}	0.92 $\pm$ 0.04 ^{***}	0.50 $\pm$ 0.001 ^{***}	2.98 $\pm$ 0.06 ^{***}	6.77 $\pm$ 0.006 ^{***}
TNPs-400 μ g/kg	0.21 $\pm$ 0.002 ^{***}	1.01 $\pm$ 0.06 ^{***}	0.56 $\pm$ 0.001 ^{***}	2.84 $\pm$ 0.01 ^{***}	7.21 $\pm$ 0.001 ^{***}

TNPs- 800µg/kg	0.23±0.003***	1.02±0.05***	0.58±0.002***	2.89±0.02***	7.28±0.003***
---------------------------	---------------	--------------	---------------	--------------	---------------

Effect of TRE and TNPs on anti-oxidant enzymes *($p < 0.05$) in contrast to PIM, and ### in contrast to healthy.**

Radio graphical findings

The radio graphical features were observed in inflammation model group due to formaldehyde administration. Intensified radiopaque areas with expanded shadow around ankle and knee joint was observed in PIM group. Also a mouth-eaten appearance of bony structure which was characterized by bone mineralization, joint space narrowing, bone erosion and osteophyte formation was seemed in pathological inflammation group (PIM). When treatment was given to animals with standard drugs (NSAID-I & NSAID-II), TRE-1mg/kg, TNPs-200µg/kg, TNPs-400µg/kg, and 800µg/kg showed a dose dependent recovery in alleviating the pathological features (Figure 13).



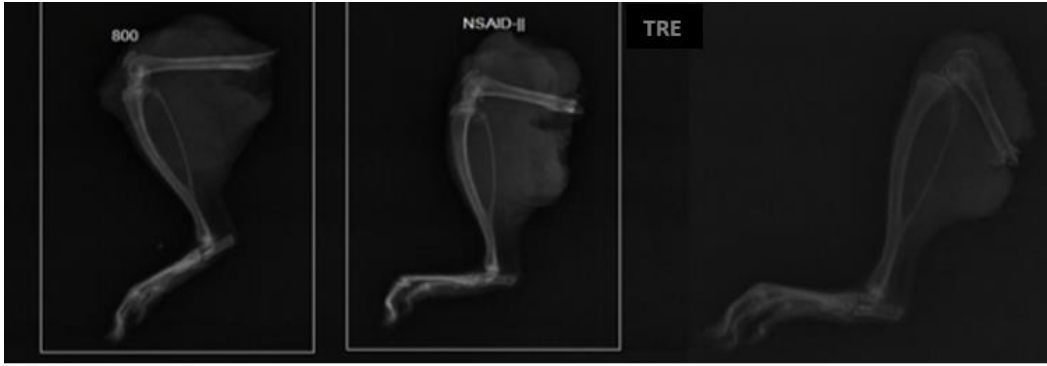


Figure 13: Radiographic images of rat limbs subjected to formaldehyde-induced inflammation and treated with TRE & TNPs.

Consequences of Tangeretin rich extract and Tangeretin loaded nanoparticles on serum level of inflammatory biomarkers

Formaldehyde administration resulted in increased level of Tumor necrosis factor alpha as well as interleukin-6 in pathological inflammation group (PIM) than healthy animals. When treatment was given to animals with TRE-1mg/kg, TNPs-200µg/kg, 400µg/kg, 800µg/kg it decreased the level of tumor necrosis factor alpha and interleukin-6 by mitigating the inflammation in contrast to pathological inflammation group. Standard drugs ibuprofen and diclofenac also decreased the level of these inflammatory markers but current findings showed that more significant results were shown by TNPs higher doses than all other treatments (Figure 14).

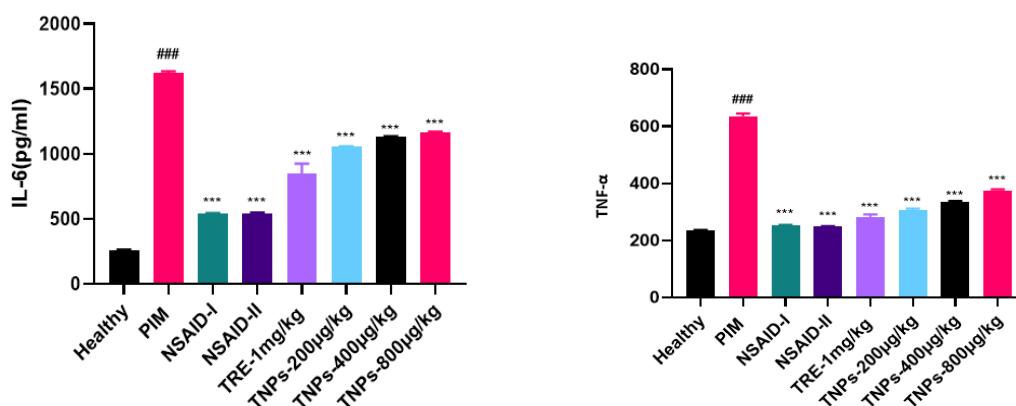


Figure 14: The effect of TRE & TNPs on serum level of IL-6 and TNF-alpha showed a significant difference (*) $p < .001$ in contrast to pathological inflammation group and ### in contrast to healthy.**

Gene expression analysis

The mRNA expression of inflammatory biomarkers was elevated in response to formaldehyde administration. The level of interleukin-6, interleukin-1 β , tumor necrosis factor alpha, cyclooxygenase-2, and NF-Kb was considerably increased ($p < 0.001$) in pathological inflammation group (PIM) when compared with animals in healthy group. While the expression of interleukin-4 and interleukin-10 was remarkably decreased in PIM group in contrast to healthy group. Treatment with standard drugs, TRE-1mg/kg, TNPs-200 μ g/kg, 400 μ g/kg, 800 μ g/kg decreased the level of pro-inflammatory markers while increased the level of anti-inflammatory cytokines than pathological inflammation group (PIM) (Figure 15).

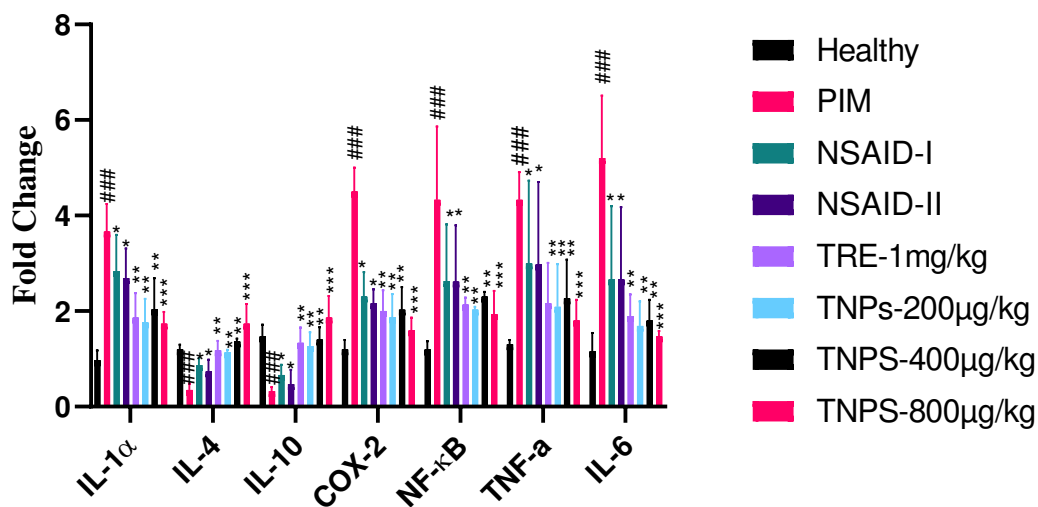


Figure 15: Effect of TRE and TNPs on mRNA expression of inflammatory biomarkers * ($p < 0.001$) in contrast to PIM and ### in contrast to healthy.**

Effect of TRE and TNPs on serum level of pro-inflammatory mediators prostaglandins E2, nitric oxide, myeloperoxidase and leukotrienes B4

In inflammation model group PIM, serum level of pro-inflammatory cytokines significantly increased compared to healthy animals. TRE and TNPs in dose dependent manner significantly ($p < 0.001$) decreased level of these pro-inflammatory mediators in treatment groups compared to PIM group manifesting anti-inflammatory effect of TRE and TPNs (Figure 16).

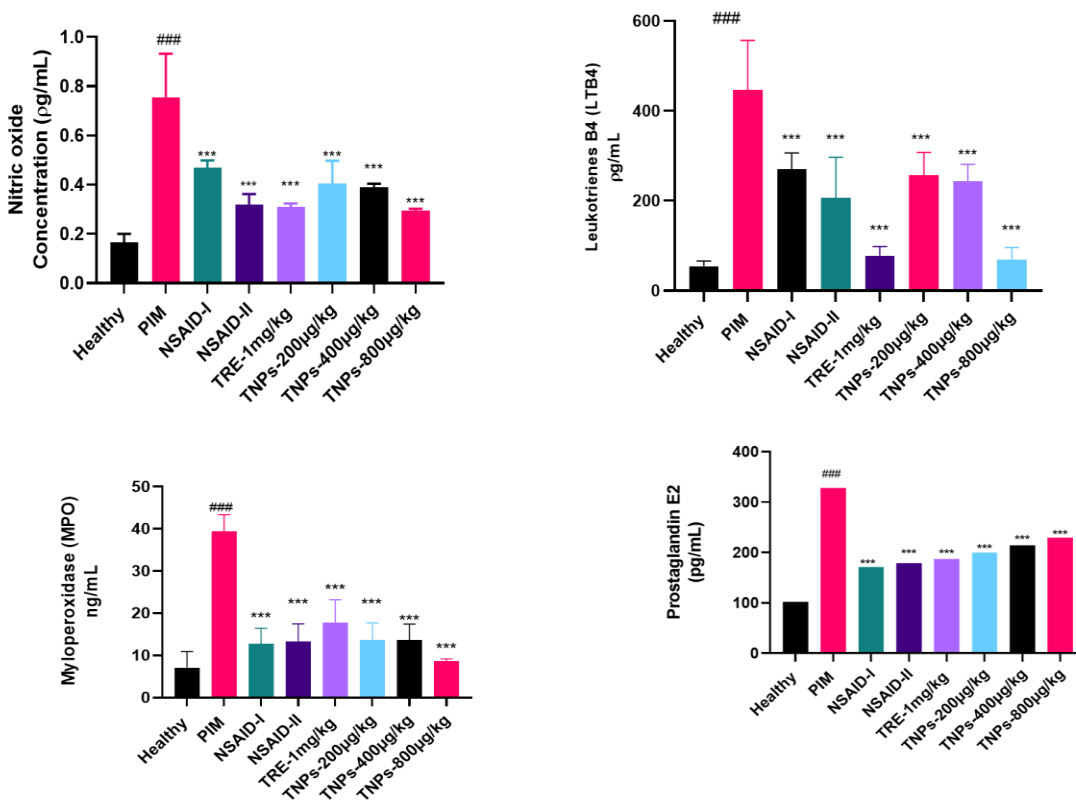


Figure 16: Effect of TRE and TNPs on expression of pro- inflammatory mediators. * ($p < 0.001$) in contrast to PIM and ### in contrast to healthy.**

Discussion

The human body use inflammatory response as its central defense system to fight against the external attackers and pathogens within vertebrates and invertebrate organism. Abandoned and extreme inflammation disturb the homeostasis thus triggers the cell and organ ailments and tissue dysfunction also increases the risk of chronic diseases (Feehan and Gilroy, 2019). Nearly all human disorders including autoimmune conditions and hepatitis, asthma, inflammatory bowel conditions and other illnesses exist due to the inflammation. Proper and early diagnosis become crucial for effective control of persistent inflammatory diseases (He et al., 2023).

Synthetic drugs such as steroidal and non-steroidal medications have been used to cure the inflammatory ailments. The wide range of these NSAIDs comprises ibuprofen and diclofenac with aspirin and naproxen (Stiller and Hjemdahl, 2022). These drugs inactivate those enzymes which generate the inflammatory prostaglandins and triggers the pain as well the inflammation in body.

The administration of corticosteroids and its strains remains one of the methods used for the reduction of tissue inflammation. Metformin serve as the treatment for dyslipidemia and low grade inflammation. These drugs decrease levels of fibrinogen, interleukin-1 β , tumor necrosis factor alpha along with high sensitive C-reactive protein in circulating blood to reduce complications (Muzamil et al., 2021). Unnecessary intake and prolonged use of these drugs resulted in severe complications which surpass their therapeutic value. The consumption of these drugs eventually produce various side effects like stomach ulcer, gastrointestinal issues with kidney or liver dysfunction and diabetes, hypertension and various other illnesses (Tu et al., 2022). A transition from synthetic drugs to natural anti-inflammatory agents become essential because these drugs have shown harmful outcomes. The global scientific community has been explored various natural plants with anti-inflammatory activities with insignificant adverse effect. A number of plants species along strong anti-inflammatory power have been studied such as Berberine-rich extract (BRE) (Naz et al., 2022), *Launaea spinose* (Asif et al., 2020), as well as *Gerbera jamesonii* (Ullah et al., 2025).

The natural bioactive compound which we used in this study is Tangeretin rich extract (TRE) and we have selected this because it has ability to decrease the oxidative stress as well as inflammation (Lee et al., 2016) through the modification of NF-KB and Nrf2 signaling pathways (Shi et al., 2022b). This natural bioactive is chiefly present in citrus fruits principally in the peels of tangerines and in various citrus specimens. This bioactive possess a broad range of biomedical and pharmaceutical actions such as anti-inflammatory effect, antioxidant activity, antibacterial effect, hepatoprotective, anticancer and antidiabetic effect, anti-mutagenic properties (Agrawal and Murti, 2022). Among the chemical substance of Tangeretin rich extract (TRE) the phenolic bioactive compounds discovered through High performance liquid chromatography was Tangeretin, Gallic acid, Vanillic acid, Benzoic acid, Chlorogenic acid, p-coumeric acid, m-coumeric acid, Sinapic acid and literature proved that they all have various biological activities presented in (Table 3).

As Tangeretin have anti-inflammatory activity which have been reported in various studies such as in allergic rhinitis by regulating T cells (Xu et al., 2019) and asthma through regulation of phosphoinositide-3-kinase pathway (Liu et al., 2017a) and Respiratory Syncytial Virus inflammation (Xu et al., 2015) by inhibiting NF-Kb pathway and interleukin-1 β . Study reported

that Tangeretin alleviated cisplatin-induced renal inflammation through the suppression of NF- κ B activation and lower the secretion of IL-1 β and TNF- α (Arab et al., 2016). It was also reported that Tangeretin limits cancer cell proliferation and stimulate autophagy by suppressing the PI3K/AKT/mTOR and MAPK pathways (Arafa et al., 2021).

(Li et al., 2023b) exposed that Gallic acid lowers the level of reactive oxygen species and regulate Nrf2/HO-1/NQO-1 signaling cascade in RAW264.7 and HCEC cell lines, exerts its anti-inflammatory and antioxidant activity. (Magiera et al., 2025) found that Vanillic acid robustly counters oxidative as well as inflammatory responses by lessening oxidative damage and inflammatory markers while at the same time elevating the antioxidants present in plasma and preventing essential biomolecules from impairment. (Kalaycı et al., 2021) elaborated that benzoic acid derivatives (6e,6c,6f) impede Acetylcholinesterase along with homocysteine with great effectiveness and confirmed affinity, and both of these are key constituent of Alzheimer disease. (Li et al., 2023a) reported that Chlorogenic acid (CGA) showed defensive benefits beside hepatic I/R liver injury by regulating pro-oxidant state, swelling and death of cells caused by mitochondrial impairment. CGA administration diminished AST, ALT, ROS and pro-inflammatory cytokines level in liver while sustained the antioxidant system and retained liver histology regular. (Moradi et al., 2023) p-coumeric acid helps resolve liver harm from ischemia reperfusion injuries by relieving oxidative burden, swelling, and autophagosomal degradation. It boosted the antioxidant defense and returns alanine transaminase, aspartate transaminase to their normal values. These findings propose that p-CA can help to guard the liver against sudden environmental harm. (Rostami et al., 2022) reported that Sinapic acid inhibits acetaminophen from causing acute hepatic injury by nullifying reactive oxygen species as well as by reducing liver enzyme activity while boosting antioxidant defense and mitochondrial function. Sinapic acid also has impact on NF- κ B/Nrf2/HO-1 cascade, raises sirtuin-1 and sustain the lysosomal enzymes. This designate that Sinapic acid supports to protect liver.

Inflammatory effect can be produced using various chemical compounds. As study reported that Serotonin and Histamine was used for the induction of inflammation in hind paw (Asif et al., 2020). In current research, carrageenan and formaldehyde was used to induce the acute and chronic inflammation. Here, in present research, Tangeretin rich extract and Tangeretin loaded nanoparticles was used to access its therapeutic potential in inflammation rat model.

Nanotechnology development has established decisive methods to combat inflammatory associated diseases via diagnosis, treatment, and prevention mechanism. Nanoparticles work for multiple purposes across pharmaceutical applications such as they function as vaccine delivery vehicles and for delivery system at targeted site which enhance its drug delivery effectiveness (Huang et al., 2024). As Tangeretin is a hydrophobic compound due to this it has low solubility rate. To overcome this problem, we have formulated the Tangeretin into nanoparticles in order to enhance its solubility and bioavailability (Wang et al., 2025).

Earlier study reported that Tangeretin nanoparticles have been successfully prepared through Solvent emulsification method to increase their therapeutic value in food products (Chen et al., 2014). Existing literature reported that Tangeretin is a useful bioactive compound which have been used earlier to treat various ailments (Agrawal and Murti, 2022). But, here the utilization of Tangeretin loaded nanoparticles (TNPs) and Tangeretin rich extract (TRE) in paw inflammation model is totally a novel technique. In present study, SLNPs of Tangeretin has been prepared using solvent emulsification method to access its anti-inflammatory potential in rat model. The prepared TNPs was evaluated using Zeta sizer to determine the nanoparticle size and polydispersity index. The analysis revealed that TNPs exhibited a small size range (239.1 d. nm) along low PDI value (0.409). TNPs consistency is effective for targeting the inflamed tissues, mandatory for alleviating inflammation in rat model.

Existing literature reported that Tangeretin showed anti-inflammatory potential by inhibiting the various inflammatory mechanisms such as (NF- κ B) pathway also create a balance between the inflammatory cytokines. NF- κ B an important mediator of inflammation is activated via TLRs and through TNF-alpha and IL-6 cytokines and activates the IKK complex. Which leads to the phosphorylation of IK β protein (Liu et al., 2017b). It allows the NFK β p65/p50 to enter into nucleus and triggers the transcription of pro-inflammatory cytokines. While Tangeretin disrupt this cascade by inhibiting IKK complex and stop the phosphorylation of IK β protein. Ultimately, NF- κ B sustain in the cytoplasm and reduce the transcription of inflammatory cytokines (Shu et al., 2014).

Studies reported that Cupressuflavone (CUF) extracted from *Cupressus macrocarpa* have showed its anti-inflammatory activity against carrageenan induced inflammation model (Al-Sayed et al.,

2018). Here, in current study, animals handled acute inflammation by the administration of carrageenan while treated with TRE and TNPs along with standard drugs. When inflammatory response start, administration of carrageenan initiates the production of serotonin and histamine then further triggers the production of bradykinin and prostaglandins as reported in existing literature (Naz et al., 2022). As a result, vascular permeability enhanced which start the synovial cells to proliferate. When treatments were given to animals, it started inactivation of pro-inflammatory biomarkers (IL-6, IL- β , TNF- α , COX-2) by hindering the signaling NF-KB pathway (Shu et al., 2014) and eventually regulates the synovial cell growth. Current results showed that Treatment with Tangeretin rich extract and Tangeretin loaded nanoparticles significantly lessen the paw diameter of rats by reducing the inflammation. Standards drugs also showed remarkable reduction while TNPs higher doses 400 μ g/kg and 800 μ g/kg show more significant and efficient result by reducing paw diameter.

Formaldehyde induced anti-arthritis activity of *Sarcococca saligna* extract has been reported in literature (Farrukh et al., 2022b). Paw inflammation was produced in rats by using formaldehyde for the induction of chronic inflammation. When the formaldehyde was injected in right hind paw it begins the inflammatory response and synovial tissues begins to expand as inflammatory cells neutrophils as well as macrophages invade the affected area. Results of present study indicated an increase in paw swelling because of improvement in vascular permeability resulted in enlarged paw diameter. As the inflammation improved through formaldehyde it resulted in fibrosis, bone erosion, pannus development along with hyperkeratosis in pathological inflammation group (PIM) have been analyzed by histological examination. Histological examination of pathological inflamed group (PIM) of knee and ankle revealed that the prolonged inflammation triggers joint deterioration and articular damage along with strong intra-articular inflammation. Treatments with TRE and TNPs showed a remarkable reduction in pannus development intra-articular inflammation in dose dependent manner.

Leukotrienes have marked role in chronic inflammation as participate in recruitment of leukocytes and other inflammatory mediators as potent lipid mediator. Treatment with TRE and TNPs significantly decreased the level of LTB₄ and inhibition of 5-lipoxygenase mediated and neutrophils mediated inflammation signaling cascades. Inducible nitric oxide synthase and cyclooxygenase -2 induce up-regulation in nitric oxide and prostaglandins E₂ in PIM group

resulting in vasodilation and increased vascular permeability. However, tangeretin loaded nanoparticles and TRE extract decreased serum level of these pro-inflammatory mediators through inhibitory effect on cyclooxygenase-2 and inducible nitric oxide. Tangeretin loaded nanoparticle showed greater inhibitory effect against chronic inflammation markers due to higher cellular uptake and sustained pattern of release imparting stability of formulation. In PIM chronic inflammation model group, the level of myeloperoxidase significantly increased due to oxidative stress and neutrophils infiltration resulting in persistent neutrophils activation in inflamed group. Current study revealed that treatment with TRE and nanoparticles significantly decreased neutrophils derived oxidative insult. Nanoparticles showed greater efficacy due to improved pharmacokinetic parameters and targeted delivery resulting in more bioavailability and inhibition of inflammatory signaling cascades (Manthey and Bendele, 2008). Current findings are consistent to previous reports of polymethoxylated flavonoids inhibitory effect on inflammation and increase the significance of Nano-technology to increase efficacy of bioactive compounds (Chavda et al., 2022, Manthey and Bendele, 2008, Yue et al., 2024).

Existing literature revealed that (Hwang et al., 2004) formaldehyde injection elevated the level of pro-inflammatory cytokines such as IL-6, TNF- α , IL-1 β resulted in T and B cells activation. Results of present study revealed that repeated formaldehyde injection in pathological inflammation group (PIM), the level of pro-inflammatory biomarkers raised while anti-inflammatory cytokines level decreased. This resulted in cytokines imbalance. Treatments with TRE and TNPs lowers the expression of tumor necrosis factor alpha, interleukin-6 and interleukin-1 β (Yang et al., 2020) while inhibiting the nuclear factor kappa B pathway, Cox-2 and increases level of anti-inflammatory cytokines ultimately balance pro and anti-inflammatory cytokines resulted in lessen the inflammation. The TNPs showed more significant result than pure compound and standard drugs.

The body weight decline usually correlates with inflammation, swelling and existing literature reported that decline in body weight interrupts the nutrients absorption from gastrointestinal tract while, the absorption these dietary nutrients reduce pain and enables the rats to relax (Jalalpure et al., 2011). In current research, the formaldehyde exposure in pathological inflamed group (PIM), showed a remarkable weight reduction in animals due to inflammation. Results of present study showed that the treatments with TRE and TNPs elevate the inflammation by enhancing nutrients

absorption and showed a remarkable increase in animal's body weight. Standard drugs also restored the body weight however, higher dose of TNPs showed more significant results than lower doses.

Elevated amount of oxidative stress produced by the body in response to inflammation. The inflation of oxidative stress in inflammatory process was because of formaldehyde treatment resulted in diminished activity of antioxidant enzymes. In literature, it is demonstrated that formaldehyde increase the protein and lipid peroxidation (Gerin et al., 2016). In current study, when formaldehyde was injected into hind paws, the levels of catalases, superoxide dismutase, glutathione was significantly decreased in pathological inflammation group (PIM) while the level of malondialdehyde increased noticeably because of oxidative stress. When treatment was given to animals, it recovered the level of these enzymes by upregulating the Nrf2 pathway and raised the level of catalases, superoxide dismutase, glutathione and glutathione peroxidase and the level of malondialdehyde was remarkably reduced by reducing the lipid peroxidation and oxidative stress (Li et al., 2019). Standard drugs and Tangeretin rich extract showed good effect but Tangeretin loaded nanoparticles with higher doses show more effective result.

The amount of pro-inflammatory cytokines raised substantially during the inflammation process. During inflammation the inflammatory cells produce Tumor necrosis factor-alpha as well as interleukin-6 (Akhter et al., 2022). This Interleukin-6 forms acute phase protein as it functions as inflammation indicator. This protein exerts its biological effects through the T and B immune cells (Gabay, 2006). Tumor necrosis factor alpha simultaneously triggers the production of oxidative stress at the effected site while attract the leukocyte towards epithelium surface.

In current study, when formaldehyde was injected in hind paws it increased the paw inflammation by increasing the reactive oxygen species ultimately it activates the signaling pathways NF-KB, JAK/STAT3. Due to this, the level of pro-inflammatory cytokines IL-6 and TNF-alpha in pathological inflamed group (PIM) was increased. When it was treated with Tangeretin rich extract and Tangeretin loaded nanoparticles it reduced the oxidative stress and inflammation ultimately lower the level of cytokines IL-6 and TNF-alpha by inhibiting the NF-KB pathway (Li et al., 2015). The standards drugs also lower the level of cytokines but most significant result was obtained from higher dose of TNPs.

Earlier studies proved that when cytokine level raised it direct the liver to produce C-reactive protein especially IL-6 stimulates the CRP production in liver (Farrukh et al., 2022b). When formaldehyde was injected it elevated the level of inflammatory cytokines and ultimately enhanced the production of CRP in liver. As a result, high production of CRP through liver tissues that bound to phosphocholine structure then inflicting to harm the tissue. Formaldehyde stimulates the immune system to form immune complex which trigger the B lymphocytes stimulation which then develop into plasma cell ultimately produced rheumatoid factor (Coulie and Van Snick, 1985). When treatment was given to animals it significantly reduced the CRP as well as RA factor by reducing the inflammation and by inhibiting the inflammatory markers.

Existing literature proved that Curcuminoids showed a significant reduction on liver function parameters (ALT, AST, ALP) (Saleem et al., 2023). In current research, when formaldehyde was injected in hind paw of pathological inflamed group (PIM), it raised the level of liver enzymes alanine aminotransferase, aspartate aminotransferase, alkaline phosphate as well as gamma glutamate transferase. Because, when formaldehyde injected, it produces the reactive oxygen species and raised the oxidative stress in the liver ultimately it destroyed the liver tissues. When treatment was given to animals, TRE & TNPs neutralize reactive oxygen species and lessens the oxidative stress and restore the AST, ALT, ALP, and gamma glutamate transferase level because of its anti-oxidant and anti-inflammatory properties. Also TRE & TNPs demonstrated its hepatoprotective activity by restoring the level of these markers (Ijaz et al., 2022). Tangeretin loaded nanoparticles showed a significant reduction but its highest dose more effectively improves the level of these liver enzymes.

Therefore, findings proved that Tangeretin rich extract and Tangeretin loaded nanoparticles have shown successful inflammation fighting properties due to its therapeutic applications. Therefore, it should be used as future scientific research as new anti-inflammatory therapeutic option.

Conclusion

In current study, it was observed that formaldehyde and carrageenan remarkably lowered the body weight, elevate paw inflammation and paw diameter and raised the level of pro-inflammatory cytokines. Tangeretin possess potent anti-inflammatory, antioxidant and hepatoprotective activity which was examined through hematological, biochemical, histopathological, radio-graphical analysis. Tangeretin rich extract and Tangeretin loaded nanoparticles showed its therapeutic effect

by the inhibition of NF-kb pathway and lessen the level of pro-inflammatory cytokines (IL-6, TNF- α , IL-1 β). By owing its anti-inflammatory activity, Tangeretin rich extract and Tangeretin loaded nanoparticles decreased the level of CRP and RA factor by decreasing the inflammation. It lessens the oxidative stress and recover the antioxidant enzymes level SOD, CAT, GPx by lowering the level of MDA. Also due to its hepatoprotective activity, Tangeretin rich extract and Tangeretin loaded nanoparticles decreased the liver enzymes ALP, AST, ALT and gamma glutamate transferase. Further, TRE and TNPs treatment recover the body weight of animals which was decreased due to inflammation. In contrast to standard anti-inflammatory drugs ibuprofen and diclofenac, Tangeretin rich extract and Tangeretin loaded nanoparticles particularly in its loaded nanoparticle form showed more significant and remarkable effects on paw inflammation model due its enhanced solubility and bioavailability. Therefore, current study concluded that Tangeretin loaded nanoparticles present a promising and a safer site for treating inflammatory conditions.

Conflict of Interest

No conflict of interest is found.

Authors contribution

All authors were helped to plan the study. Preparation of initial material was carried out by ZC, MA, RM and US. Experimental work was carried out by MA, NA. Data analysis was performed by MA, MT, & ZC. The final manuscript was reviewed by all authors.

Correspondence Author

Correspondence to Zunera Chaudhary.

Funding

No funding provided by anyone.

References

CNS Neurosci Ther.

ABUDUKELIMU, A., BARBERIS, M., REDEGELD, F. A., SAHIN, N. & WESTERHOFF, H. V. 2018. Predictable irreversible switching between acute and chronic inflammation. *Frontiers in immunology*, 9, 1596.

- AFZAL, O., ALTAMIMI, A. S., NADEEM, M. S., ALZAREA, S. I., ALMALKI, W. H., TARIQ, A., MUBEEN, B., MURTAZA, B. N., IFTIKHAR, S. & RIAZ, N. 2022. Nanoparticles in drug delivery: From history to therapeutic applications. *Nanomaterials*, 12, 4494.
- AGRAWAL, K. K. & MURTI, Y. 2022. Tangeretin: A biologically potential citrus flavone. *Current Traditional Medicine*, 8, 31-41.
- AHMED, S. R., RABBEE, M. F., ROY, A., CHOWDHURY, R., BANIK, A., KUBRA, K., HASSAN CHOWDHURY, M. M. & BAEK, K.-H. 2021. Therapeutic promises of medicinal plants in Bangladesh and their bioactive compounds against ulcers and inflammatory diseases. *Plants*, 10, 1348.
- AKHTAR, M. F., ASHRAF, M., JAVEED, A., ANJUM, A. A., SHARIF, A., SALEEM, A., AKHTAR, B., KHAN, A. M. & ALTAF, I. 2016. Toxicity appraisal of untreated dyeing industry wastewater based on chemical characterization and short term bioassays. *Bulletin of Environmental Contamination and Toxicology*, 96, 502-507.
- AKHTER, S., IRFAN, H. M., ALAMGEER, JAHAN, S., SHAHZAD, M. & LATIF, M. B. 2022. Nerolidol: a potential approach in rheumatoid arthritis through reduction of TNF- α , IL-1 β , IL-6, NF-kB, COX-2 and antioxidant effect in CFA-induced arthritic model. *Inflammopharmacology*, 30, 537-548.
- AL-SAYED, E., GAD, H. A., EL-SHAZLY, M., ABDEL-DAIM, M. M. & NASSER SINGAB, A. 2018. Anti-inflammatory and analgesic activities of cupressuflavone from Cupressus macrocarpa: Impact on pro-inflammatory mediators. *Drug Development Research*, 79, 22-28.
- ALAMGEER, UTTRA, A. M. & HASAN, U. H. 2017. Anti-arthritis activity of aqueous-methanolic extract and various fractions of Berberis orthobotrys Bien ex Aitch. *BMC complementary and alternative medicine*, 17, 1-16.
- ALI, K. A., MAITY, A., ROY, S. D., PRAMANIK, S. D., DAS, P. P. & SHAHARYAR, M. A. 2023. Insight into the mechanism of steroidal and non-steroidal anti-inflammatory drugs. *How Synthetic Drugs Work*. Elsevier.
- ARAB, H. H., MOHAMED, W. R., BARAKAT, B. M. & ARAFA, E.-S. A. 2016. Tangeretin attenuates cisplatin-induced renal injury in rats: impact on the inflammatory cascade and oxidative perturbations. *Chemico-Biological Interactions*, 258, 205-213.
- ARAFA, E.-S. A., SHURRAB, N. T. & BUABEID, M. A. 2021. Therapeutic implications of a polymethoxylated flavone, tangeretin, in the management of cancer via modulation of different molecular pathways. *Advances in Pharmacological and Pharmaceutical Sciences*, 2021, 4709818.
- ARHAM SHABBIR, A. S., BATOOL, S., BASHEER, M., MUHAMMAD SHAHZAD, M. S., KISHWAR SULTANA, K. S., TAREEN, R., JAVED IQBAL, J. I. & SAEED-UL-HASSAN, S.-U.-H. 2018. Ziziphora clinopodioides ameliorated rheumatoid arthritis and inflammatory paw edema in different models of acute and chronic inflammation.
- ASIF, M., MAHRUKH, SAADULLAH, M., YASEEN, H. S., SALEEM, M., YOUSAF, H. M., KHAN, I. U., YASEEN, M. & SHAMS, M. U. 2020. Evaluation of in vivo anti-inflammatory and anti-angiogenic attributes of methanolic extract of Launaea spinosa. *Inflammopharmacology*, 28, 993-1008.
- BAI, J., ZHANG, Y., TANG, C., HOU, Y., AI, X., CHEN, X., ZHANG, Y., WANG, X. & MENG, X. 2021. Gallic acid: Pharmacological activities and molecular mechanisms involved in inflammation-related diseases. *Biomedicine & pharmacotherapy*, 133, 110985.
- BASRA, M., BATOOL, M., FARHAT, F., TAJAMMAL, A. & KHAN, H. 2019. Anti-inflammatory, antithrombotic and molecular docking studies of 1, 3, 4 Oxadiazole derivatives in rats. *J Biomed Sci*, 8, 4.
- BHANGALE, J. O. & ACHARYA, S. R. 2016. Anti-Parkinson activity of petroleum ether extract of Ficus religiosa (L.) leaves. *Advances in Pharmacological and Pharmaceutical Sciences*, 2016, 9436106.
- BINDU, S., MAZUMDER, S. & BANDYOPADHYAY, U. 2020. Non-steroidal anti-inflammatory drugs (NSAIDs) and organ damage: A current perspective. *Biochemical pharmacology*, 180, 114147.

- CAVAILLON, J.-M. 2021. Once upon a time, inflammation. *Journal of Venomous Animals and Toxins including Tropical Diseases*, 27, e20200147.
- CHAVDA, V. P., PATEL, A. B., MISTRY, K. J., SUTHAR, S. F., WU, Z.-X., CHEN, Z.-S. & HOU, K. 2022. Nano-drug delivery systems entrapping natural bioactive compounds for cancer: recent progress and future challenges. *Frontiers in oncology*, 12, 867655.
- CHEN, J., ZHENG, J., MCCLEMENTS, D. J. & XIAO, H. 2014. Tangeretin-loaded protein nanoparticles fabricated from zein/ β -lactoglobulin: Preparation, characterization, and functional performance. *Food chemistry*, 158, 466-472.
- CHEN, L., DENG, H., CUI, H., FANG, J., ZUO, Z., DENG, J., LI, Y., WANG, X. & ZHAO, L. 2017. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, 9, 7204.
- CHINNASAMY, V., SUBRAMANIYAN, V., CHANDIRAN, S., KAYAROHANAM, S., KANNIYAN, D. C., VELAGA, V. S. S. R. & MUHAMMAD, S. 2019. Antiarthritic activity of *Achyranthes aspera* on formaldehyde-induced arthritis in rats. *Open access Macedonian journal of medical sciences*, 7, 2709.
- COULIE, P. G. & VAN SNICK, J. 1985. Rheumatoid factor (RF) production during anamnestic immune responses in the mouse. III. Activation of RF precursor cells is induced by their interaction with immune complexes and carrier-specific helper T cells. *J Exp Med*, 161, 88-97.
- COURA, C. O., SOUZA, R. B., RODRIGUES, J. A. G., VANDERLEI, E. D. S. O., DE ARAÚJO, I. W. F., RIBEIRO, N. A., FROTA, A. F., RIBEIRO, K. A., CHAVES, H. V. & PEREIRA, K. M. A. 2015. Mechanisms involved in the anti-inflammatory action of a polysulfated fraction from *Gracilaria cornea* in rats. *PloS one*, 10, e0119319.
- DE GAETANO, F., CRISTIANO, M. C., VENUTI, V., CRUPI, V., MAJOLINO, D., PALADINI, G., ACRI, G., TESTAGROSSA, B., IRRERA, A. & PAOLINO, D. 2021. Rutin-loaded solid lipid nanoparticles: characterization and in vitro evaluation. *Molecules*, 26, 1039.
- DOS SANTOS SOUZA, L. M., DIBO, M., SARMIENTO, J. J. P., SEABRA, A. B., MEDEIROS, L. P., LOURENÇO, I. M., KOBAYASHI, R. K. T. & NAKAZATO, G. 2022. Biosynthesis of selenium nanoparticles using combinations of plant extracts and their antibacterial activity. *Current Research in Green and Sustainable Chemistry*, 5, 100303.
- DUONG, V.-A., NGUYEN, T.-T.-L. & MAENG, H.-J. 2020. Preparation of solid lipid nanoparticles and nanostructured lipid carriers for drug delivery and the effects of preparation parameters of solvent injection method. *Molecules*, 25, 4781.
- FARRUKH, M., SALEEM, U., AHMAD, B., CHAUHDARY, Z., ALSHARIF, I., MANAN, M., QASIM, M., ALHASANI, R. H., SHAH, G. M. & SHAH, M. A. 2022a. *Sarcococca saligna* hydroalcoholic extract ameliorates arthritis in complete Freund's adjuvant-induced arthritic rats via modulation of inflammatory biomarkers and suppression of oxidative stress markers. *ACS omega*, 7, 13164-13177.
- FARRUKH, M., SALEEM, U., QASIM, M., MANAN, M. & SHAH, M. A. 2022b. *Sarcococca saligna* extract attenuates formaldehyde-induced arthritis in Wistar rats via modulation of pro-inflammatory and inflammatory biomarkers. *Inflammopharmacology*, 30, 579-597.
- FEEHAN, K. T. & GILROY, D. W. 2019. Is resolution the end of inflammation? *Trends in molecular medicine*, 25, 198-214.
- FITZGERALD, K. A. & KAGAN, J. C. 2020. Toll-like receptors and the control of immunity. *Cell*, 180, 1044-1066.
- GABAY, C. 2006. Interleukin-6 and chronic inflammation. *Arthritis research & therapy*, 8, 1-6.
- GERIN, F., ERMAN, H., ERBOGA, M., SENER, U., YILMAZ, A., SEYHAN, H. & GUREL, A. 2016. The effects of ferulic acid against oxidative stress and inflammation in formaldehyde-induced hepatotoxicity. *Inflammation*, 39, 1377-1386.

- HE, R., LI, L., ZHANG, T., DING, X., XING, Y., ZHU, S., GU, Z. & HU, H. 2023. Recent advances of nanotechnology application in autoimmune diseases—A bibliometric analysis. *Nano Today*, 48, 101694.
- HU, Y., LIU, F., PANG, J., MCCLEMENTS, D. J., ZHOU, Z., LI, B. & LI, Y. 2020. Biopolymer additives enhance tangeretin bioavailability in emulsion-based delivery systems: an in vitro and in vivo study. *Journal of Agricultural and Food Chemistry*, 69, 730-740.
- HUANG, J., XIE, M., HE, L., SONG, X. & CAO, T. 2023. Chlorogenic acid: A review on its mechanisms of anti-inflammation, disease treatment, and related delivery systems. *Frontiers in Pharmacology*, 14, 1218015.
- HUANG, Y., GUO, X., WU, Y., CHEN, X., FENG, L., XIE, N. & SHEN, G. 2024. Nanotechnology's frontier in combatting infectious and inflammatory diseases: prevention and treatment. *Signal Transduction and Targeted Therapy*, 9, 34.
- HWANG, S.-Y., KIM, J.-Y., KIM, K.-W., PARK, M.-K., MOON, Y., KIM, W.-U. & KIM, H.-Y. 2004. IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF- κ B-and PI3-kinase/Akt-dependent pathways. *Arthritis Res Ther*, 6, 1-9.
- IFTIKHAR, A., NAUSHEEN, R., MUZAFFAR, H., NAEEM, M. A., FAROOQ, M., KHURSHID, M., ALMATROUDI, A., ALRUMAIHI, F., ALLEMAILEM, K. S. & ANWAR, H. 2022. Potential therapeutic benefits of honey in neurological disorders: the role of polyphenols. *Molecules*, 27, 3297.
- IJAZ, M. U., SHAHAB, M. S., SAMAD, A., ASHRAF, A., AL-GHANIM, K., MRUTHINTI, S. S. & MAHBOOB, S. 2022. Tangeretin ameliorates bisphenol induced hepatocyte injury by inhibiting inflammation and oxidative stress. *Saudi Journal of Biological Sciences*, 29, 1375-1379.
- JALALPURI, S. S., MANDAVKAR, Y. D., KHALURE, P. R., SHINDE, G. S., SHELAR, P. A. & SHAH, A. S. 2011. Antiarthritic activity of various extracts of *Mesua ferrea* Linn. seed. *Journal of ethnopharmacology*, 138, 700-704.
- KALAYCI, M., TÜRKES, C., ARSLAN, M., DEMIR, Y. & BEYDEMİR, Ş. 2021. Novel benzoic acid derivatives: Synthesis and biological evaluation as multitarget acetylcholinesterase and carbonic anhydrase inhibitors. *Archiv der Pharmazie*, 354, 2000282.
- KIM, H.-J., KIM, H., LEE, J.-H. & HWANGBO, C. 2023. Toll-like receptor 4 (TLR4): new insight immune and aging. *Immunity & Ageing*, 20, 67.
- KRONSTEN, V. T., TRANAH, T. H., PARIANTE, C. & SHAWCROSS, D. L. 2022. Gut-derived systemic inflammation as a driver of depression in chronic liver disease. *Journal of hepatology*, 76, 665-680.
- LAURINDO, L. F., SANTOS, A. R. D. O. D., CARVALHO, A. C. A. D., BECHARA, M. D., GUIGUER, E. L., GOULART, R. D. A., VARGAS SINATORA, R., ARAÚJO, A. C. & BARBALHO, S. M. 2023. Phytochemicals and regulation of NF- κ B in inflammatory bowel diseases: an overview of in vitro and in vivo effects. *Metabolites*, 13, 96.
- LAZOU, A., IKONOMIDIS, I., BARTEKOVA, M., BENEDEK, T., MAKAVOS, G., PALIOURA, D., CABRERA FUENTES, H. & ANDREADOU, I. 2020. Chronic inflammatory diseases, myocardial function and cardioprotection. *British Journal of Pharmacology*, 177, 5357-5374.
- LEE, E. H., SHIN, J. H., KIM, S. S. & SEO, S. R. 2021. Sinapic acid controls inflammation by suppressing NLRP3 inflammasome activation. *Cells*, 10, 2327.
- LEE, Y. S., WOLLAM, J. & OLEFSKY, J. M. 2018. An integrated view of immunometabolism. *Cell*, 172, 22-40.
- LEE, Y. Y., LEE, E. J., PARK, J. S., JANG, S. E., KIM, D. H. & KIM, H. S. 2016. Anti-Inflammatory and Antioxidant Mechanism of Tangeretin in Activated Microglia. *J Neuroimmune Pharmacol*, 11, 294-305.

- LI, K., FENG, Z., WANG, L., MA, X., WANG, L., LIU, K., GENG, X. & PENG, C. 2023a. Chlorogenic acid alleviates hepatic ischemia–reperfusion injury by inhibiting oxidative stress, inflammation, and mitochondria-mediated apoptosis in vivo and in vitro. *Inflammation*, 46, 1061-1076.
- LI, K., GONG, Q., LU, B., HUANG, K., TONG, Y., MUTSVENE, T. E., LIN, M., XU, Z., LU, F. & LI, X. 2023b. Anti-inflammatory and antioxidative effects of gallic acid on experimental dry eye: in vitro and in vivo studies. *Eye and Vision*, 10, 17.
- LI, X., XIE, P., HOU, Y., CHEN, S., HE, P., XIAO, Z., ZHAN, J., LUO, D., GU, M. & LIN, D. 2019. Tangeretin inhibits oxidative stress and inflammation via upregulating Nrf-2 signaling pathway in collagen-induced arthritic rats. *Pharmacology*, 104, 187-195.
- LI, Y.-J., ZHANG, T., TU, J.-X., LI, G. & ZHOU, Y. 2015. Tangeretin inhibits IL-1 β induced proliferation of rheumatoid synovial fibroblasts and the production of COX-2, PGE2 and MMPs via modulation of p38 MAPK/ERK/JNK pathways. *Bangladesh Journal of Pharmacology*, 10, 714-725.
- LIU, L.-L., LI, F.-H., ZHANG, Y., ZHANG, X.-F. & YANG, J. 2017a. Tangeretin has anti-asthmatic effects via regulating PI3K and Notch signaling and modulating Th1/Th2/Th17 cytokine balance in neonatal asthmatic mice. *Brazilian Journal of Medical and Biological Research*, 50, e5991.
- LIU, T., ZHANG, L., JOO, D. & SUN, S.-C. 2017b. NF- κ B signaling in inflammation. *Signal transduction and targeted therapy*, 2, 1-9.
- MAGIERA, A., KOŁODZIEJCZYK-CZEPAS, J. & OLSZEWSKA, M. A. 2025. Antioxidant and Anti-Inflammatory Effects of Vanillic Acid in Human Plasma, Human Neutrophils, and Non-Cellular Models In Vitro. *Molecules*, 30, 467.
- MANTHEY, J. A. & BENDELE, P. 2008. Anti-inflammatory activity of an orange peel polymethoxylated flavone, 3', 4', 3, 5, 6, 7, 8-heptamethoxyflavone, in the rat carrageenan/paw edema and mouse lipopolysaccharide-challenge assays. *Journal of agricultural and food chemistry*, 56, 9399-9403.
- MENDOZA-MUÑOZ, N., ALCALÁ-ALCALÁ, S. & QUINTANAR-GUERRERO, D. 2016. preparation of polymer nanoparticles by the emulsification-solvent evaporation method: from vanderhoff's pioneer approach to recent adaptations. *Polymer Nanoparticles for Nanomedicines: A Guide for their Design, Preparation and Development*, 87-121.
- MENZEL, A., SAMOUDA, H., DOHET, F., LOAP, S., ELLULU, M. S. & BOHN, T. 2021. Common and novel markers for measuring inflammation and oxidative stress ex vivo in research and clinical practice—which to use regarding disease outcomes? *Antioxidants*, 10, 414.
- MIR, N. T., SALEEM, U., ANWAR, F., AHMAD, B., ULLAH, I., HIRA, S., ISMAIL, T., ALI, T. & AYAZ, M. 2019. Lawsonia Inermis markedly improves cognitive functions in animal models and modulate oxidative stress markers in the brain. *Medicina*, 55, 192.
- MIZUNO, H., YOSHIKAWA, H. & USUKI, T. 2019. Extraction of nobiletin and tangeretin from peels of shekwasha and ponkan using [C2mim][[(MeO)(H) PO2] and centrifugation. *Natural Product Communications*, 14, 1934578X19845816.
- MORADI, M., FARBOOD, Y., MARD, S. A., DIANAT, M., GOUDARZI, G., KHORSANDI, L. & SEYEDIAN, S. S. 2023. p-Coumaric acid has pure anti-inflammatory characteristics against hepatopathy caused by ischemia-reperfusion in the liver and dust exposure. *Iranian Journal of Basic Medical Sciences*, 26, 164.
- MORRIS, G., GEVEZOVA, M., SARAFIAN, V. & MAES, M. 2022. Redox regulation of the immune response. *Cellular & Molecular Immunology*, 19, 1079-1101.
- MUSHTAQ, S., HANIF, U., SHAH, M. A., MAZHAR, N., CHAUHDARY, Z., QASIM, M., MUNEEB, M., CHAUDHARY, M. & DAR, M. 2023. Neuroprotective evaluation of Marsilea quadrifolia L. and Salvinia molesta DS Mitchel in aluminium chloride induced Alzheimer disease. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 51, 13228-13228.
- MUZAMIL, A., TAHIR, H. M., ALI, S., LIAQAT, I., ALI, A. & SUMMER, M. 2021. Inflammatory process and role of cytokines in inflammation: An overview. *Punjab Univ. J. Zool*, 36.

- NAZ, I., MASOUD, M. S., CHAUHDARY, Z., SHAH, M. A. & PANICHAYUPAKARANANT, P. 2022. Anti-inflammatory potential of berberine-rich extract via modulation of inflammation biomarkers. *Journal of Food Biochemistry*, 46, e14389.
- NONGLANG, F. P., ROY, D., NEWAR, D. & BHAN, S. 2023. Tangeretin-a special polymethoxyflavones: An insight into its pharmacological and therapeutic effects on diabetes.
- PANDEY, H., SRIVASTAVA, S., KUMAR, R. & TRIPATHI, Y. B. 2018. Preclinical acute and repeated dose toxicity of *Pueraria tuberosa* (PTWE) on charles foster rats. *Int J Pharm Sci Res*, 9, 4572-4581.
- PENG, B., HU, J., SUN, Y., HUANG, Y., PENG, Q., ZHAO, W., XU, W. & ZHU, L. 2024. Tangeretin alleviates inflammation and oxidative response induced by spinal cord injury by activating the *Sesn2/Keap1/Nrf2* pathway. *Phytotherapy Research*, 38, 4555-4569.
- ROSTAMI, A., BALUCHNEJADMOJARAD, T. & ROGHANI, M. 2022. Sinapic acid ameliorates paracetamol-induced acute liver injury through targeting oxidative stress and inflammation. *Molecular biology reports*, 49, 4179-4191.
- ROYCHOUDHURY, S., SINHA, B., CHOUDHURY, B. P., JHA, N. K., PALIT, P., KUNDU, S., MANDAL, S. C., KOLESAROVA, A., YOUSEF, M. I. & RUOKOLAINEN, J. 2021. Scavenging properties of plant-derived natural biomolecule para-coumaric acid in the prevention of oxidative stress-induced diseases. *Antioxidants*, 10, 1205.
- SALEEM, A., SALEEM, M. & AKHTAR, M. F. 2020a. Antioxidant, anti-inflammatory and antiarthritic potential of *Moringa oleifera* Lam: An ethnomedicinal plant of Moringaceae family. *South African Journal of Botany*, 128, 246-256.
- SALEEM, U., AHMAD, B., AHMAD, M., HUSSAIN, K. & BUKHARI, N. I. 2014. Investigation of in vivo antioxidant activity of *Euphorbia helioscopia* latex and leaves methanol extract: a target against oxidative stress induced toxicity. *Asian Pacific Journal of Tropical Medicine*, 7, S369-S375.
- SALEEM, U., CHAUHDARY, Z., BAKHTAWAR, Z., ALQAHTANI, J., FARRUKH, M., ALSHARIF, I., BAKOBAH, T. A., SHAH, M. A., BLUNDELL, R. & PANICHAYUPAKARANANT, P. 2023. Curcuminoids-enriched extract and its cyclodextrin inclusion complexes ameliorates arthritis in complete Freund's adjuvant-induced arthritic mice via modulation of inflammatory biomarkers and suppression of oxidative stress markers. *Inflammopharmacology*, 31, 3047-3062.
- SALEEM, U., CHAUHDARY, Z., RAZA, Z., SHAH, S., RAHMAN, M.-U., ZAIB, P. & AHMAD, B. 2020b. Anti-Parkinson's Activity of *Tribulus terrestris* via Modulation of AChE, α -Synuclein, TNF- α , and IL-1 β . *ACS omega*, 5, 25216-25227.
- SALEEM, U., RAZA, Z., ANWAR, F., AHMAD, B., HIRA, S. & ALI, T. 2019. Experimental and computational studies to characterize and evaluate the therapeutic effect of *Albizia lebbek* (L.) seeds in Alzheimer's disease. *Medicina*, 55, 184.
- SALEEM, U., SHEHZAD, A., SHAH, S., RAZA, Z., SHAH, M. A., BIBI, S., CHAUHDARY, Z. & AHMAD, B. 2021. Antiparkinsonian activity of *Cucurbita pepo* seeds along with possible underlying mechanism. *Metabolic Brain Disease*, 36, 1231-1251.
- SHI, Y., CHEN, J., LI, S., WU, Y., YU, C., NI, L., XIAO, J., SHAO, Z., ZHU, H. & WANG, J. 2022a. Tangeretin suppresses osteoarthritis progression via the *Nrf2/NF- κ B* and *MAPK/NF- κ B* signaling pathways. *Phytomedicine*, 98, 153928.
- SHI, Y., CHEN, J., LI, S., WU, Y., YU, C., NI, L., XIAO, J., SHAO, Z., ZHU, H., WANG, J., WANG, X. & ZHANG, X. 2022b. Tangeretin suppresses osteoarthritis progression via the *Nrf2/NF- κ B* and *MAPK/NF- κ B* signaling pathways. *Phytomedicine*, 98, 153928.
- SHU, Z., YANG, B., ZHAO, H., XU, B., JIAO, W., WANG, Q., WANG, Z. & KUANG, H. 2014. Tangeretin exerts anti-neuroinflammatory effects via *NF- κ B* modulation in lipopolysaccharide-stimulated microglial cells. *Int Immunopharmacol*, 19, 275-82.
- SOEHNLEIN, O. & LIBBY, P. 2021. Targeting inflammation in atherosclerosis—from experimental insights to the clinic. *Nature reviews Drug discovery*, 20, 589-610.

- STILLER, C. O. & HJEMDAHL, P. 2022. Lessons from 20 years with COX-2 inhibitors: Importance of dose–response considerations and fair play in comparative trials. *Journal of internal medicine*, 292, 557-574.
- TANIGUCHI, K. & KARIN, M. 2018. NF- κ B, inflammation, immunity and cancer: coming of age. *Nature Reviews Immunology*, 18, 309-324.
- TJAHJONO, Y., KARNATI, S., FOE, K., ANGGARA, E., GUNAWAN, Y. N., WIJAYA, H., SUYONO, H., ESAR, S. Y., HADINUGROHO, W. & WIHADMADYATAMI, H. 2021. Anti-inflammatory activity of 2-((3-(chloromethyl) benzoyl) oxy) benzoic acid in LPS-induced rat model. *Prostaglandins & Other Lipid Mediators*, 154, 106549.
- TU, Z., ZHONG, Y., HU, H., SHAO, D., HAAG, R., SCHIRNER, M., LEE, J., SULLENGER, B. & LEONG, K. W. 2022. Design of therapeutic biomaterials to control inflammation. *Nature Reviews Materials*, 7, 557-574.
- ULLAH, M. S., AMJAD, A., CHAUHDARY, Z., SALEEM, U. & AKHTAR, N. 2025. Phytochemical studies of *Gerbera jamesonii* and evaluation of anti-inflammatory potential in formaldehyde-induced arthritis in rats. *Inflammopharmacology*, 1-19.
- UNDERWOOD, W. & ANTHONY, R. 2020. AVMA guidelines for the euthanasia of animals: 2020 edition. Retrieved on March, 2013, 2020-1.
- WANG, W., JIA, T., ZHANG, Y. & ZHOU, Y. 2025. Progress of Researches on Pharmacological Effects and Bioavailability of Tangeretin. *Journal of Oleo Science*, 74, 13-23.
- XU, J.-J., LIU, Z., TANG, W., WANG, G.-C., CHUNG, H. Y., LIU, Q.-Y., ZHUANG, L., LI, M.-M. & LI, Y.-L. 2015. Tangeretin from citrus reticulate inhibits respiratory syncytial virus replication and associated inflammation in vivo. *Journal of Agricultural and Food Chemistry*, 63, 9520-9527.
- XU, S., KONG, Y.-G., JIAO, W.-E., YANG, R., QIAO, Y.-L., XU, Y., TAO, Z.-Z. & CHEN, S.-M. 2019. Tangeretin promotes regulatory T cell differentiation by inhibiting Notch1/Jagged1 signaling in allergic rhinitis. *International immunopharmacology*, 72, 402-412.
- YANG, T., FENG, C., WANG, D., QU, Y., YANG, Y., WANG, Y. & SUN, Z. 2020. Neuroprotective and anti-inflammatory effect of tangeretin against cerebral ischemia-reperfusion injury in rats. *Inflammation*, 43, 2332-2343.
- YUE, L., LI, N., YE, X., XIU, Y. & WANG, B. 2024. Polymethoxylated flavones for modulating signaling pathways in inflammation. *International Immunopharmacology*, 143, 113522.