

Green-synthesis and characterization of Pueraria tuberosa aqueous extract loaded silver nanoparticles for antiarthritic effect against Freund's complete adjuvant induced in male Wistar rats

Tejas Vijay Nirwane
tejasnirwane1234@gmail.com

Tejas Vijay Nirwane, KLE College of Pharmacy, KLE Academy of Higher Education and Research

Namit Madan Kudatarkar
namitkudatarkar18@gmail.com

KLE College of Pharmacy, KLE Academy of Higher Education and Research

Omkar Annaso Shelar
Omkarshelar579@gmail.com

KLE College of Pharmacy, KLE Academy of Higher Education and Research

Research Article

Keywords: Pueraria tuberosa, Rheumatoid Arthritis, Silver Nanoparticle, Freund's Complete Adjuvant

DOI: <https://doi.org/10.21203/rs.3.rs-6118320/v1>

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Additional Declarations: No competing interests reported.

Green-synthesis and characterization of *Pueraria tuberosa* aqueous extract loaded silver nanoparticles for antiarthritic effect against Freund's complete adjuvant induced in male Wistar rats

Tejas Vijay Nirwane ^{1*}, Namit Madan Kudatarkar, ¹, Omkar Annaso Shelar²

¹ Tejas Vijay Nirwane

Department of Pharmacology, KLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi-590010, Karnataka, India

Correspondence: E-mail: tejasnirwane1234@gmail.com

¹ Namit Madan Kudatarkar

Department of Pharmacology, KLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi-590010, Karnataka, India

Tel: +91 9552880153

namitkudatarkar18@gmail.com

ORCID ID- [0000-0002-1535-5117](https://orcid.org/0000-0002-1535-5117)

²Omkar Annaso Shelar

Department of Pharmaceutics, KLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi-590010, Karnataka, India

Omkarshelar579@gmail.com

Abstract

Rheumatoid arthritis (RA) is an autoimmune chronic illness affecting 0.5% to 1% of the population. Traditional medicinal plants, pivotal in drug discovery, prompted the investigation of *Pueraria tuberosa* (PT) tuber for its anti-rheumatic properties. Aqueous extract of PT and *Pueraria tuberosa* loaded silver nanoparticle (PTAgNP) were investigated for their effects in Freund's Complete Adjuvant induced RA in rats.

In vivo evaluation using Freund's complete adjuvant (FCA) induced arthritis model and *Pueraria tuberosa* tuber extract at a dose (200, 400 mg/kg) and PTAgnP 100mg/kg was used as a treatment for about 21 days. NPs were characterized using UV, XRD, SEM, zetasizer. After 21 days treatment, oxidative stress in paw tissue, biochemical parameter, inflammatory cytokines, x-ray, histopathological analysis of ankle joint were evaluated.

PT and PTAgnP which showed treatment significantly ameliorates the adjuvant induced arthritic scoring, histological alterations, paw volume, elevation of biochemical (AST, ALT, ALP, CRP) and restored the endogenous anti-oxidant (SOD, GSH, MDA) activities. Significant reduction in paw swelling, arthritis score and weight of spleen in treatment groups. Increased in the body weight, indicating improvement of disease condition. Biochemical analyses indicated reduced ESR, WBC and increased level of RBC and Hb suggesting decreased inflammation. There was also a significant decrease in levels of TNF-alpha and IL-6 pro-inflammatory cytokines in treatment groups. Radiological examination showed reduced soft tissue swelling and joint changes in treated groups.

PT and PTAgnPs showed anti-arthritic effects through anti-oxidant activity, reduction of inflammatory markers and improvement in joint parameters. These findings support further exploration of PT and the nanoparticles demonstrated significant antiarthritic

activity against rheumatoid arthritis induced by complete Freund's adjuvant in experimental rats, surpassing the effects of the extract and standard indomethacin, *Pueraria tuberosa* loaded silver nanoparticle showed as a potential source as a novel anti-arthritic drug.

Key Words

Pueraria tuberosa, Rheumatoid Arthritis, Silver Nanoparticle, Freund's Complete Adjuvant

1 Introduction

Inflammation is a common response to tissue injury; it can get out of control and lead to other complications. Certain pro-inflammatory mediators such as leukotrienes, prostaglandins, cytokines or chemokines are produced in response of an inflammatory reaction [1]. Persistent inflammation causes a number of acute and chronic diseases. The prevalence of long-term inflammatory conditions such rheumatoid arthritis (RA), is a major worldwide health issue. The prevalence of rheumatoid arthritis ranges from 0.5 to 1% globally, whereas it ranges from 0.7 to 1% in the Indian population [2].

Numerous autoantibodies, like anti-cyclic citrullinated peptide antibody (ACPA) and rheumatoid factor (RF), are significant diagnosis markers of Rheumatoid arthritis and assessing the progression of bone destruction and the efficacy of treatment [3]. When citrullinated proteins, ACPA and RF combine to produce immunological complexes that activate macrophages and release inflammatory cytokines like interferon (IF)- γ , interleukin (IL) and Tumor necrosis factor (TNF) [4]. These cytokines activate B cells, macrophages and endothelial cells. IgM antibody against IgG (i.e. anti-IgG) is released by activated B-cells; this molecule is known as rheumatoid factor (RF). When macrophages are activated, more cytokines are released, which damage joint tissues and result in the formation of pannus and cartilage vascularization. Ankylosis and joint fibrosis that occur due to the deterioration and degeneration of bone and cartilage [5].

TNF is a major cytokine molecule that regulates immunological function, cell metabolism, apoptosis, inflammation, proliferation and differentiation [6]. This versatile cytokine is primarily produced by natural killer (NK) cells, macrophages and activated lymphocytes.

RA, cancer, psoriasis, ankylosing spondylitis, neurological conditions and inflammatory illnesses are only a some of the conditions that have been linked to TNF- α dysregulation [7].

The production of immunoglobulins was stimulated by IL-6, another important RA regulator. Although fibroblasts, endothelial cells, monocytes and T lymphocytes are the types of cells that can generated IL-6. In the RA synovium, B lymphocytes and synovial fibroblasts secretes IL-6 [8].

RA treatment focuses on reducing joint inflammation and pain due to ineffective medications. Traditional therapies like analgesics, steroids, NSAIDs and anticytokines have low success rates [9]. Herbal medicines which is used by 80% of people according to WHO, Traditional medicine showed their strong anti-inflammatory properties and lower toxicity compared to synthetic drugs. They offer safer, cheaper and effective therapeutic options [10].

Pueraria tuberosa contains diverse phytochemicals effective in treating various diseases. These affordable, accessible plant-based remedies aim to minimize adverse effects [11]. Rich in phytoestrogenic compounds and secondary metabolites like puerarin, genistein and quercetin, it exhibits antihyperglycemic, antioxidant and anti-inflammatory properties [12].

In traditional medicine it is being claimed that the tubers of *Pueraria tuberosa* is utilized to treat rheumatism, whose etiology is thought to be attributable to changes in the immune system's functioning and to reduce joint swelling [13].

Nanotechnology's growing role in medicine encompasses various fields like electronics, healthcare and optics [14]. Silver nanoparticles can be synthesized chemically, biologically, physically, but chemical methods often introduce hazardous chemicals [15]. Green synthesis methods, especially in biomedicine are favoured to avoid toxicity and environmental harm [16]. Despite challenges like low solubility, nano technology offers promising enhancements in drug delivery, protection and bioavailability [17][18].

This technique involves the reduction and stabilization of nanoparticles using plant extracts, enzymes, proteins, antioxidants, flavonoids, glycoproteins, saponins, triglycerides, polysaccharides, terpenes and tannins [19].

The purpose of this investigation is to assess the therapeutic impact of *Pueraria tuberosa* tuber aqueous extract and to characterize and evaluate the effect of *Pueraria tuberosa* loaded silver nanoparticles for oral delivery against arthritic disease by inducing Freund's Complete Adjuvant (FCA) in rats.

2 Materials and Methods

2.1 Collection of Plant specimens

The *Pueraria tuberosa* tuber part (Vidarikand) was collected from KLE Society Ayurveda Pharmacy in Belagavi district, Karnataka, India. It was identified and authenticated by Dr. Divya Khare Authentication expert from Shri B.M.K. Ayurveda Mahavidyalaya Belgaum, Where the specimen deposited under CRF CODE-(BMK/CRF352/2023-24).

2.2 Preparation of Extract

100g *Pueraria tuberosa* tuber powder was mixed with 500ml distilled water in a 2000ml conical flask, macerated for 7-9 days, shaken twice daily. 10ml chloroform was added for preservation. The solvent extract filtered after maceration was heated at 50°C to yield crude extract, stored in the refrigerator for the experimental study [20].

2.3 Characterization of *Pueraria tuberosa* Using Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) was employed using Xevo G2-XS QT system. The solvent composition in channel A composed of Formic acid in water at 0.1%, while channel B contained acetonitrile. Analytical data were processed with MassLynx V4.1 software program. The mass spectrometer handled with a capillary voltage of 3.0 kV, collision energy set at 20 V, source temperature maintained at 150°C and an injection volume of 20 µL.

2.4 Synthesis of silver nanoparticle

Silver nanoparticles were synthesized using 20% v/v PTAE and 2 mM silver nitrate, heated for 80 minutes at 80°C on a magnetic stirrer (REMI 2MLH). The pH was adjusted to 6 for nanoparticle formation. Formation was confirmed by visual color change (fig. 1) and UV-Visible spectroscopy (Shimadzu 1900). Unreacted plant extract was removed by centrifugation at 10,000 rpm for 10 minutes. Further purification involved centrifugation at 14,000 rpm for one hour. The nanoparticles were suspended in distilled water and left to evaporate at room temperature [21, 22].

2.5 Characterization of AgNP

2.5.1 UV- spectral analysis

By employing UV spectrophotometry (Shimadzu 1900) to measure the reaction mixture's color shift in the 300–800 nm range, the synthesis of AgNPs was validated. Additionally, UV-Visible analysis was utilised to optimise several parameters during AgNP production and assess the stability of the produced NPs.

2.5.2 Dynamic light scattering studies

Zetasizer (Malvern., UK) was utilized to examine the zeta. potential and particle size. Characteristics of colloidal dispersion of AgNPs. Prior to analysis, AgNPs were diluted using deionized water.

2.5.3 X-ray diffraction (XRD)

The Ag-NPs X-ray. diffraction. (XRD) was acquired by the use of a BRUKER-binary V3 instrument. The X-ray diffractometer performed at 40 mA current flow and 40 kV voltage with cu K α radiation (1.54060) in a θ -2 θ configuration.

2.5.4 Scanning electron microscope and energy dispersive spectrometer (SEM/w EDS)

SEM was employed to examine the physical traits of the green-synthesised silver nanoparticle. Samples were scanned by applying a SEM Quorum connected to an EDAX system for SEM-EDAX analysis. The EDAX system examined it to confirm that silver was present. Following validation, 500x magnification scanning was performed.

2.6 Animals

Male Wistar rats weighing 150–200g in a healthy state were employed in the research. They were kept in a standard laboratory environment. Water was available at all times, along with a typical pelleted meal for the animals. Following a seven-day period of acclimatization. The Institutional Animal Ethics Committee granted ethical approval (IAEC Reg no. 221/Po/Re/S/2000/CPCSEA) KLEU'S College of Pharmacy, Belagavi, prior to carrying out the experiments.

2.7 Experimental Design

Male Wistar rats were split up to six groups, with six rats in each group.

Group I: Vehicle Control: Normal food and water were given to the animals.

Group II: Disease Control: 0.1. mL of. FCA(Sigma-Aldrich) containing 5mg/ml of Mycobacterium tuberculosis H 37RA S.C. into the sub. plantar region. of the right hind. paw on 0th day.

Group III: Standard group (Indomethacin-10 mg/kg): 0.1 ml of FCA + standard indomethacin (p.o) 10 mg/kg ;dose from 1st day upto 21 days

Group IV: PT-200 mg/kg: 0.1 ml of FCA + 200 mg/kg(p.o.) tuber extract from the 1st day upto 21 days

Group V: PT-400 mg/kg: 0.1 ml of FCA + 400 mg/kg(p.o.) tuber extract from the 1st day upto 21 days.

Group VI: PTAgnP-100 mg/kg: 0.1 ml of FCA + synthesized silver nanoparticle loaded with *Pueraria tuberosa*

Based on a review of the literature, the doses of PT and indomethacin were chosen [23,24].

After the experimental regimen, rats were euthanized with a high dose of anaesthesia. Blood was collected from the retro-orbital plexus, serum separated by centrifugation and used for biochemical assays. Spleen was weighed, immunized paw was preserved in formalin for histological examination.

2.8 Parameter to Evaluate

2.8.1 Physical Parameters

2.8.2 Body weight

On days 0, 7, 14 and 21, following the day of induction, an electronic weighing balance was used to measure the change in body weight [25].

2.8.3 Paw size

From the day of induction, the inflammatory response was measured on days 0, 7, 14 and 21 using a vernier calliper [25].

2.8.4 Spleen weight

Twenty-one days after the treatment, the animals were sacrificed and the spleen was taken out, cleaned in phosphate buffered saline (PBS) and weighed. The ratio (mg/g) of the spleen weight to the body weight was used to express the indices of spleen, respectively [26].

2.8.5 Measurement of Arthritis Score

On days 0, 7, 14 and 21, the same people noted the morphological characteristics of arthritis in every rodent, based on the degree of joint erythema and oedema. The following criteria were applied: normal paw = 0, mild digit erythema and swelling = 1, moderate digit erythema and swelling = 2, severe digit erythema and swelling = 3, and gross deformity and disability to use limbs = 4. The results from each paw were then added to determine the arthritis score [27].

2.8.6 Estimation of haematological parameters

On the 22nd day, blood samples from every experimental animal were taken via the retro-orbital plexus and placed in tubes with anticoagulant (dipotassium EDTA). Measurements were made of the haematological parameters, which include erythrocyte sedimentation rate, haemoglobin, white blood cells and red blood cells [28].

2.8.7 Radiological analysis of ankle joints

To assess cartilage and bone injury, radiographs of experimental and control rat joints were taken. An X-ray machine was used to image of the right hind limb's joints prior to sacrifice. (Acteon Satelec Xmind DC). The X-ray machine performed at 230V with a peak voltage of 70kV, 8mA, 0.050 sec exposure time. The X-ray images were recorded and changes or erosion in the affected ankle joint were analysed.

2.8.8 Estimation of oxidative stress in Paw tissue

The animal's paw tissue was removed and it was cleaned with physiological saline that had been frozen with ice. One gram of paw tissue with a specified weight was homogenized in nine milliliters of 0.1 M Tris HCL buffer solution (pH 7.4). Following a centrifugation of the homogenate, the supernatant was extracted and utilized for the glutathione (GSH) [30], malondialdehyde (MDA) [31] and superoxide dismutase (SOD) [29] assays.

2.8.9 Estimation of biochemical Parameter

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), C-reactive protein (CRP) were measured by utilizing commercial kits through Auto analyzer (AGD CliniPak diagnostic kit, Mumbai).

2.8.10 Estimation of TNF-alpha and IL-6

The evaluation of cytokines was done using the blood serum. The rat kit of TNF- α and IL-6 (KRISHGEN Biosystem, Ashley Ct, Whittier, CA) was used to analyse the amount of TNF- α and IL-6. This made use of the sandwich concept of ELISA and a microliter plate reader set at 450 nm. Using the standard curve, the concentrations of IL-6 and TNF- α (pg/mol) were calculated [32].

2.8.11 Histopathological analysis of ankle joint

Ankle joints were fixed in 10% formalin, dehydrated in alcohol, rinsed for 12 hours and embedded in paraffin. Sections were cut, baked and stained with eosin and haematoxylin. Anti-arthritic effects of treatment doses, *Pueraria tuberosa* extract and PTAgnP were examined under a microscope alongside histological changes from arthritis.

Statistical Analysis

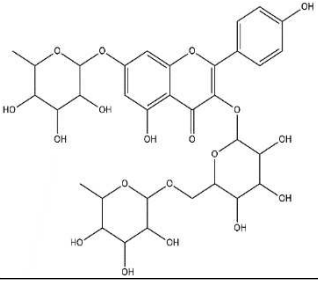
The data were examined by Two-way ANOVA Followed by Tukey's multiple comparison test using Graph Pad Prism Software version 8. Where $^{aa}p < 0.05$, $^{a}p < 0.01$, $^{a}p < 0.001$ when correlated to disease control group; $^{##}p < 0.05$, $^{##}p < 0.01$, $^{#}p < 0.001$ when correlated to normal group; $^{@@}p < 0.05$, $^{@}p < 0.01$, $^{@}p < 0.001$ when correlated to standard Indomethacin considered statistically significant.

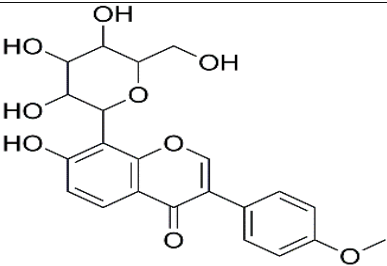
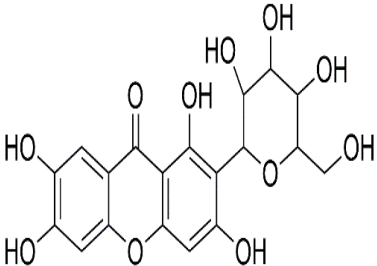
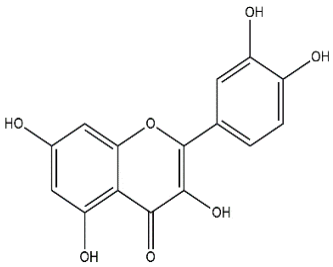
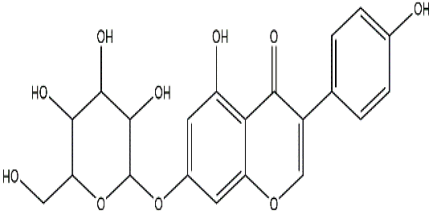
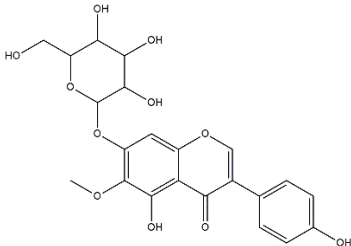
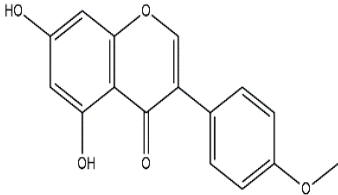
3 Result and Discussion

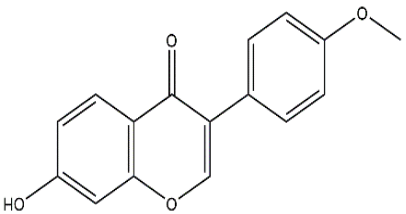
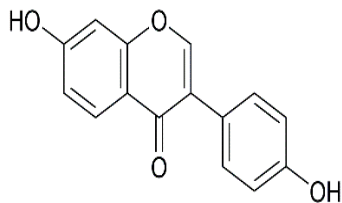
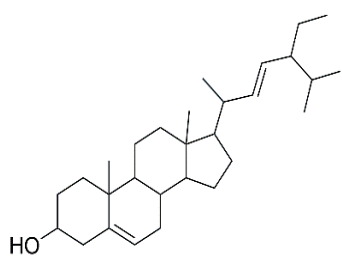
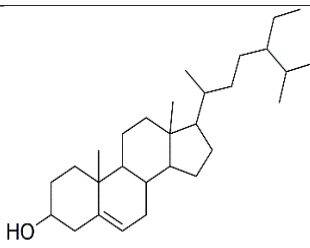
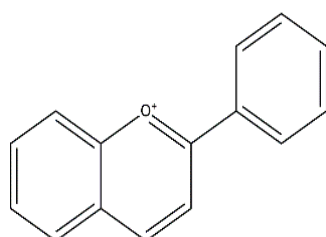
3.1 Characterization of *Pueraria tuberosa* Using Liquid chromatography-mass spectrometry

The LC-MS data was manually sorted to list $[M+H]^+$ m/z values. Molecular weights were used to identify compounds against reference ones in *P. tuberosa* tubers (Table 1). The interpretation revealed the presence of 12 phytoconstituents.

Table 1 The name of compounds identified in *Pueraria tuberosa* by LC-MS

Sr. No.	Compound identified	Molecular formula	Molecular Weight (m/z)	Chemical Structure
1	robinin	C ₃₃ H ₄₀ O ₁₉	740.36	
2	4-methoxypuerarin	C ₂₂ H ₂₂ O ₉	433.18	

				
3	magniferin	C ₁₉ H ₁₈ O ₁₁	445	
4	quercetin	C ₁₅ H ₁₀ O ₇	300.14	
5	genistin	C ₂₁ H ₂₀ O ₁₀	433.1	
6	tectoridin	C ₂₂ H ₂₂ O ₁₁	463.21	
7	biochanin A	C ₁₆ H ₁₂ O ₅	285.1	

8	biochanin B	$C_{16}H_{12}O_4$	269	
9	daidzein	$C_{15}H_{10}O_4$	255	
10	stigmasterol	$C_{29}H_{48}O$	413.23	
11	beta-Sitosterol	$C_{29}H_{50}O$	415.28	
12	anthocyanin	$C_{15}H_{11}O^+$	208.15	

3.2 Synthesis of silver Nanoparticle

The following parameters were used to synthesize AgNPs: temperature of reaction 80°C, 20% (v/v) PTAE, a solution of 2 mM AgNO₃, a reaction incubation period of 80 min and pH 6. By using surface plasmon resonance to cause a colour shift to brown, the production of AgNPs was visually observed.

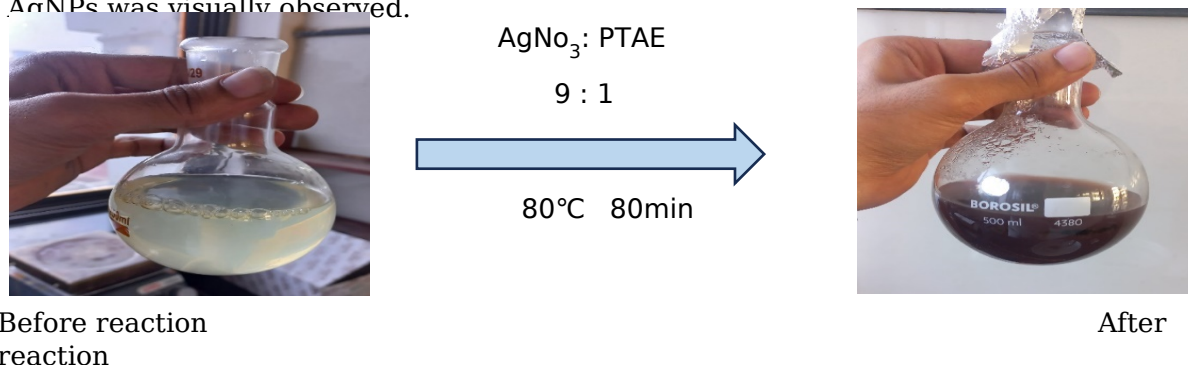


Fig. 1. Color change of reaction mixture of synthesized PT AgNPs

3.3 Characterization of Silver Nanoparticle

3.3.1 UV- spectral analysis

The UV-visible spectrophotometer characterizes silver nanoparticles in the 400–540 nm range. The surface plasmon resonance (SPR) band peaks at 438 nm (fig. 2a), indicating the reduction of Ag⁺ ions to Ag nanoparticles. A single peak suggests spherical shapes [33], while a widened peak indicates polydisperse nanoparticles produced using *Pueraria tuberosa* tuber. A similar outcome for greenly produced silver nanoparticles was reported [34].

3.3.2 DLS Analysis

DLS determined particle size distributions from micron to nanometre by measuring the diffusion coefficient of nanoparticles undergoing Brownian motion. Synthesized silver nanoparticles had a size of 103.2 nm, a polydispersity index of 0.19 (fig. 2c) and a negative zeta potential of -20.47 (fig. 2b), indicating stability and preventing aggregation.

3.3.3 X-ray diffraction (XRD)

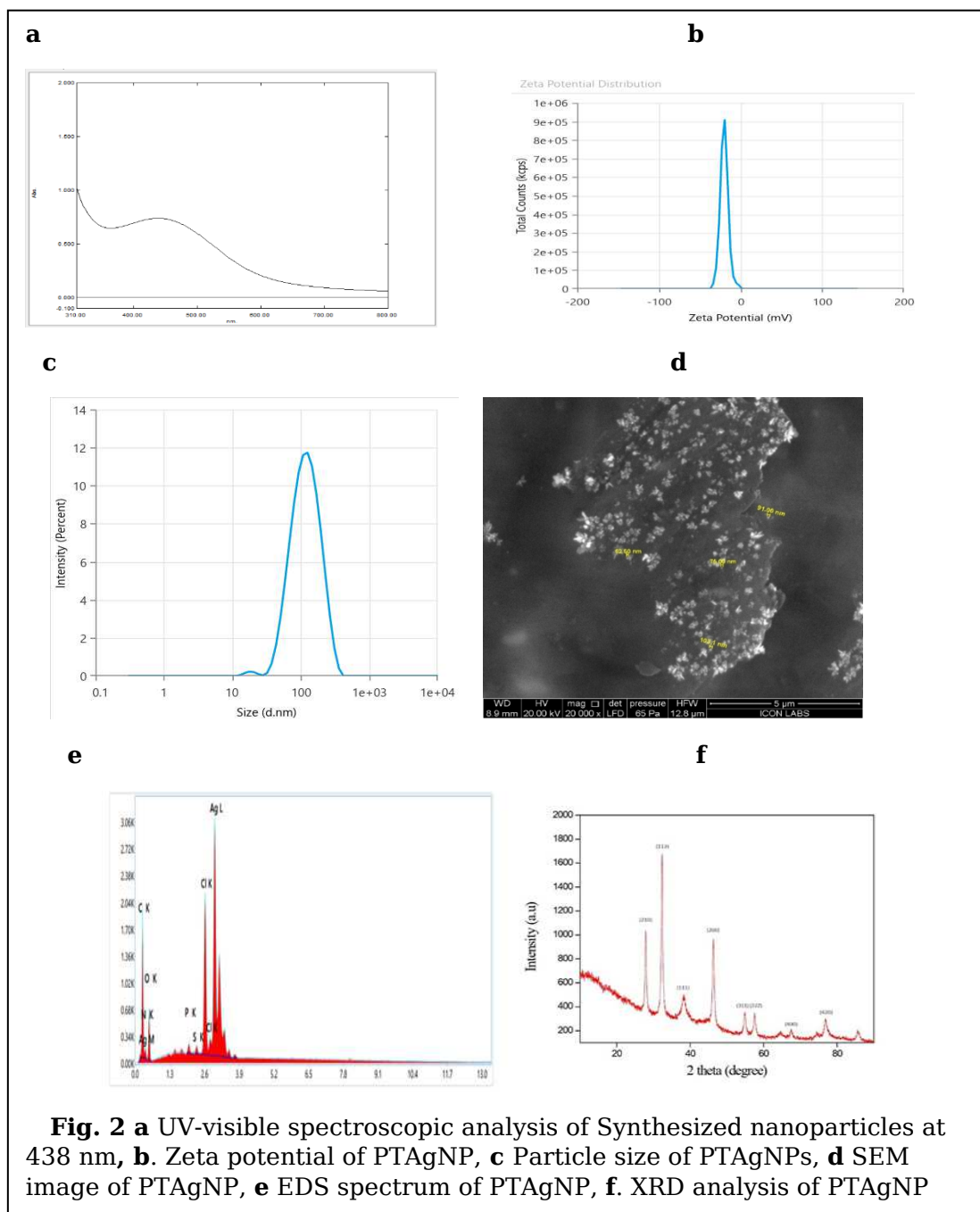
The XRD method assessed crystallinity and size of green synthesized NPs. Analysis of the XRD spectrum (Fig. 2f) revealed peaks at 27.87°, 32.29°, 38.58°, 44.27°, 54.84°, 57.50°, 67.54°, 76.83° which are corresponding to the plane of (210), (113), (111), (200), (311), (222), (400) and (420) respectively corresponding to specific crystal planes, indicating a face-centered cubic structure. Results matched JCPDS File No. 89-3722. Employing Debye-Scherrer's equation estimated AgNPs' average size as 11 to 25 nm.

3.3.4 Scanning electron microscope and energy dispersive spectrometer (SEM/w EDS)

SEM images revealed silver nanoparticles' size (62–104 nm) and spherical in shape. Size variation is due to proteins from *Pueraria tuberosa* tuber extract. Nanoparticles are well-separated and do not clump. Furthermore, the outcomes demonstrated that the produced silver nanoparticle had a spherical in form as seen in fig. 2d.

The EDS technique determines the presence and quantity of elements in a sample. EDS spectra of green synthesized AgNPs showed strong silver signals, with the highest peak

at 3 keV typical of elemental silver (fig. 2e). Small peaks for C, N, O, P, S and Cl were also found.



3.4 Effect of PT and PT loaded NPs on body weight

A gradual reduction in body weight was seen in all the RA induced animals from 1st day to 7th day. Body weight increased from day 7th day to 21st in 200mg/kg, 400mg/kg *Pueraria tuberosa* tuber extract and 100mg/kg *Pueraria tuberosa* loaded silver nanoparticle treatments. (Fig. 3a)

3.5 Effect of PT and PT loaded NPs on paw size measurement

Following the rat hind paw immunization with FCA, the arthritic control group's paw size increased significantly ($P < 0.001$) in comparison to the normal and treatment groups.

When correlated to the disease control group, treatment with PT at 200, 400, PTAgnP 100 mg/kg and standard indomethacin significantly ($P < 0.001$) suppressed paw volume on the 21st day. (Fig. 3b)

3.6 Effect of PT and PT loaded NPs on index of spleen

Weight of spleen per animal body weight was used to establish the relative weight of the spleen.

The relative weight of spleen was significantly ($P < 0.001$) increased in the arthritic control group as correlated to normal group. The relative weight of spleen was significantly ($P < 0.001$) decreased in treatment groups of PT at 200, 400, PTAgnP 100 mg/kg and standard indomethacin (Fig. 3c)

3.7 Effect of PT and PT loaded NPs on arthritis score

When correlated to the disease control group, the PT treatment groups experienced a dose-dependent substantial reduction in arthritis scores. (Fig. 3d)

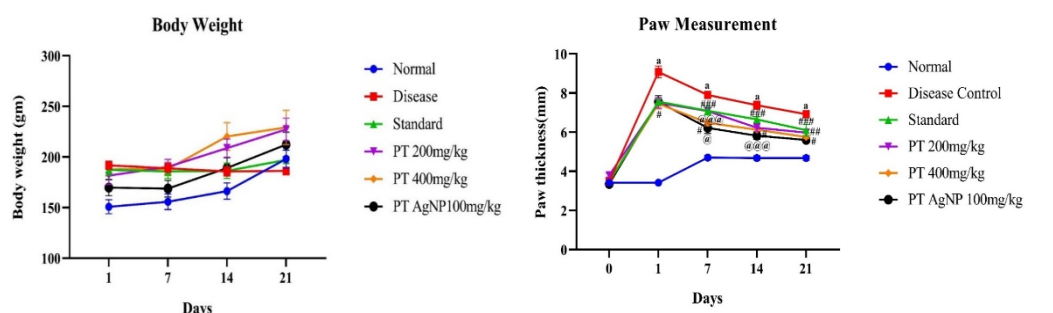


Fig. 3a Body Weight

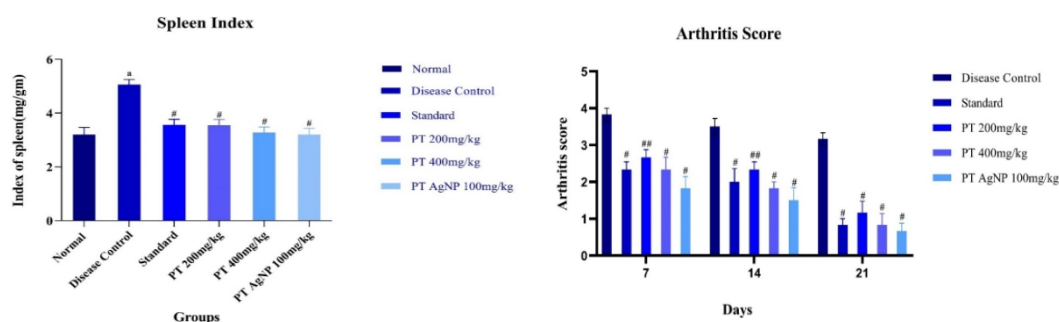


Fig. 3c Indices of Spleen

3.8 Effect of PT and PT loaded NPs on hematological parameters

When comparing the arthritic control group ($13.25 \pm 0.50 \times 10^3/\text{mm}^3$, $9.72 \pm 1.36 \text{ mm/hr}$) to the normal group ($6.44 \pm 0.09 \times 10^3/\text{mm}^3$, $3.63 \pm 0.11 \text{ mm/hr}$), we found a substantial ($p < 0.001$) rise of WBC, ESR and when correlated to the normal group ($6.85 \pm 0.07 \times 10^6/\text{mm}^3$ for red blood cells and $14.05 \pm 0.57 \text{ g/dL}$ for haemoglobin content), a significant ($P < 0.001$) decrease was seen in the level of RBC ($3.91 \pm 0.12 \times 10^6/\text{mm}^3$) and the Hb content ($7.23 \pm 0.21 \text{ g/dL}$) in the arthritic control group. There was a noteworthy decrease in white blood cell counts ($11.09 \pm 0.46 \times 10^3/\text{mm}^3$, $10.96 \pm 0.19 \times 10^3/\text{mm}^3$, $10.86 \pm 0.26 \times 10^3/\text{mm}^3$ and $10.54 \pm 0.17 \times 10^3/\text{mm}^3$ for PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg, respectively) after treatment with PT and indomethacin.

In comparison to the arthritic control group, it also restored the haemoglobin levels in treatment groups ($12.05 \pm 0.53 \text{ g/dL}$, $13.20 \pm 0.55 \text{ g/dL}$, $13.59 \pm 0.60 \text{ g/dL}$ and $13.25 \pm 0.62 \text{ g/dL}$ for PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg,

respectively) and RBC (6.46 ± 0.11 $10^6/\text{mm}^3$, 6.7 ± 0.40 $10^6/\text{mm}^3$, 6.88 ± 0.09 $10^6/\text{mm}^3$ and 6.69 ± 0.08 $10^6/\text{mm}^3$ for PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg, respectively). Comparing the PT and indomethacin treatment groups to the arthritic control group, the ESR significantly decreased (4.96 ± 0.34 mm/hr, 4.46 ± 0.40 mm/hr, 4.20 ± 0.41 mm/hr and 4.43 ± 0.29 mm/hr for PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg, respectively). (Fig. 4) whereas no difference was observed between standard and test.

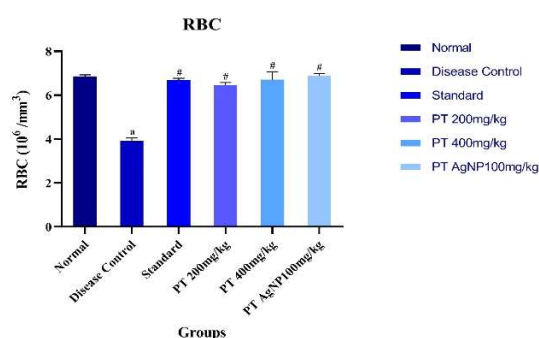


Fig. 4 a
b

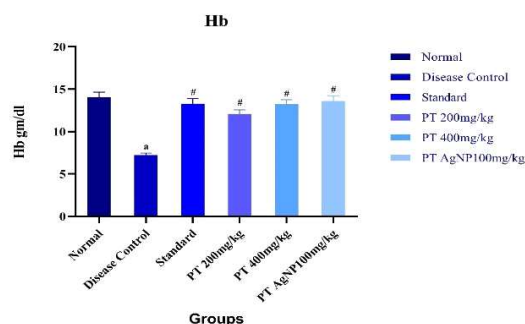


Fig. 4

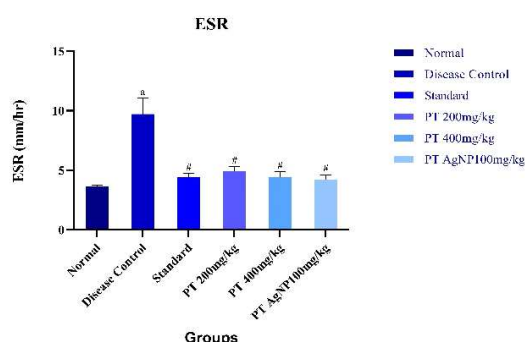


Fig. 4 c

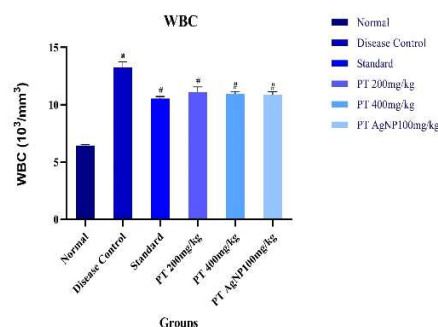


Fig. 4 d

Fig. 4 Hematological parameters: a) Red Blood Cells, b) Haemoglobin, c) Erythrocyte sedimentation rate, d) White Blood Cells

3.9 Effect of PT and PT loaded NPs on the radiograph analysis

The x-ray results showed that there was no soft tissue swelling or bone damage in the animals in the normal group, The radiography pattern of the CFA-induced arthritic rats' hind legs (up to the ankle joint) revealed shortening of the intertarsal joints' articulation

spaces and swelling of soft tissue (phalangeal area), both of which are unequivocal signs of cartilage deterioration. Treatment with indomethacin (10mg/kg), PT AgNP (100mg/kg) and PT (200 and 400 mg/kg) decreased joint/articulation space narrowing and improved the joints' radiographic pattern (Fig. 5).

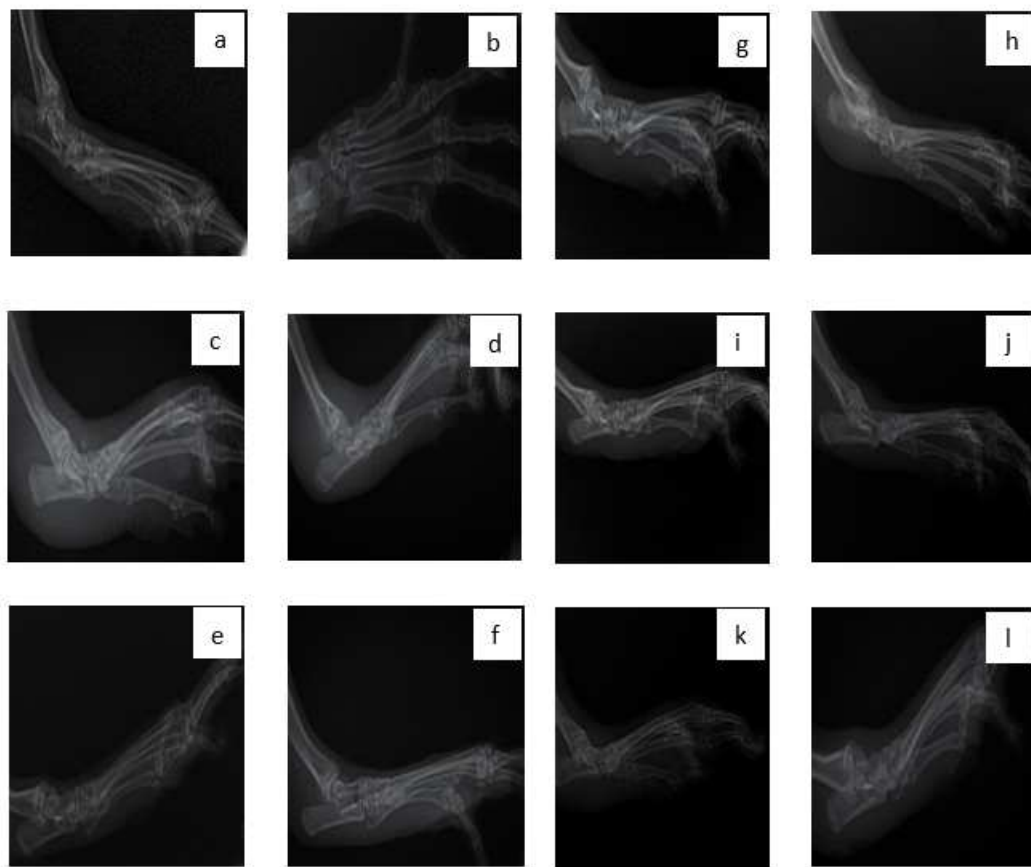


Fig. 5. Radiographical (X-ray) analysis of ankle joint of FCA injected right hand paw on day 21.

(a, b) Normal group

(g, h) PT 200 mg/kg

(c, d) Disease Control group

(I, j) PT 400mg/kg

(e, f) Standard group

(k, l) PT AgNP 100mg/kg

3.10 In-vivo antioxidant assay

3.10.1 Superoxide dismutase (SOD)

Disease control group significantly ($p < 0.001$) decreased SOD level with the mean of 5.98 ± 0.09 as correlated to normal group with mean 12.79 ± 0.09 , whereas PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg displayed significant ($p < 0.001$) increase in SOD level with mean value 7.46 ± 0.15 , 9.58 ± 0.42 , 10.83 ± 0.10 and 8.85 ± 0.39 respectively, when correlated with disease control group. *Pueraria tuberosa* tuber extract with dose PT AgNP 100mg/kg showed statistical significantly ($p < 0.001$) in SOD level when correlated with standard group with the mean value 10.83 ± 0.10 and

PT 200 mg/kg showed statistical significantly ($p < 0.01$) in SOD level when correlated with standard group with the mean value 7.46 ± 0.15 (Fig. 6 a)

3.10.2 Glutathione (GSH)

Disease control group significantly ($p < 0.001$) decreased GSH level with the mean of 27.54 ± 1.63 as correlated to normal group with mean 59.83 ± 2.8 , whereas PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg displayed significant ($p < 0.001$) increase in GSH level with mean value 49.35 ± 1.75 , 54.44 ± 1.73 , 57.08 ± 3.87 and 45.81 ± 1.57 respectively, when correlated with disease control group. PT AgNP 100mg/kg 57.08 ± 3.87 showed statistical significantly ($p < 0.05$) in GSH level when correlated with standard group with the mean value 45.81 ± 1.57 , whereas PT 200 mg/kg, 400mg/kg dose displayed no statistically significant but showed theoretical significance as correlated to the standard group. (Fig. 6 b)

3.10.3 Malondialdehyde (MDA)

Lipid peroxidation marker malondialdehyde (MDA), In disease control group significantly ($p < 0.001$) increased MDA level with the mean of 4.35 ± 0.19 as correlated to normal group with mean 1.45 ± 0.11 , whereas PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg displayed significant ($p < 0.001$) decrease in MDA level with mean value 2.91 ± 0.13 , 2.24 ± 0.22 , 1.49 ± 0.12 and 2.47 ± 0.19 respectively, when correlated with disease control group. PT AgNP 100mg/kg 1.49 ± 0.12 showed statistical significantly ($p < 0.01$) in MDA level when correlated with standard group with the mean value 2.47 ± 0.19 , whereas PT 200 mg/kg, 400mg/kg dose displayed no statistically significant but showed theoretical significance as correlated to the standard group. (Fig.6 c)

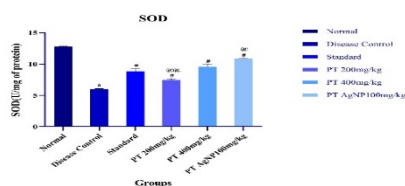


Fig. 6 a

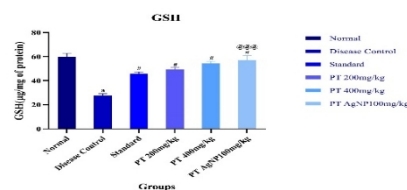


Fig. 6 b

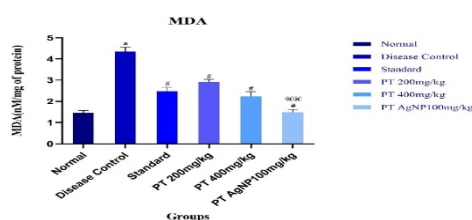


Fig. 6 c

Fig. 6. Antioxidant levels in paw tissue a SOD, b GSH, c MDA

3.11 Biochemical parameters:

3.11.1 Alanine Transaminase (ALT)

ALT level was examined at end of study. Disease control group significantly ($p < 0.001$) elevated serum ALT level with the mean of 97.88 ± 5.71 as correlated to normal group with mean 31.29 ± 1.53 , whereas PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg displayed significant ($p < 0.001$) decrease in ALT level with mean value 68.33 ± 2.14 , 61.39 ± 4.24 , 59.07 ± 2.36 and 66.65 ± 5.32 respectively, when correlated with disease control group. (Fig. 7a)

3.11.2 Alkaline Phosphatase (ALP)

ALP level was examined at end of study. Disease control group significantly ($p < 0.001$) elevated serum ALP level with the mean of 304.33 ± 4.91 as correlated to normal group with mean 161.53 ± 5.60 , whereas PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg displayed significant ($p < 0.001$) decrease in ALP level with mean value 247.34 ± 2.67 , 221.66 ± 5.57 , 191.83 ± 3.70 and 244.06 ± 3.70 respectively, when correlated with disease control group. PT AgNP 100mg/kg 191.83 ± 3.70 displayed statistical significantly ($p < 0.001$) in ALP level when correlated with standard group, whereas PT 400 mg/kg dose displayed statistical significantly ($p < 0.01$) in ALP level when correlated with standard group. (Fig. 7b)

3.11.3 Aspartate Transaminase (AST)

AST level was examined at end of study. Disease control group significantly ($p < 0.001$) elevated serum AST level with the mean of 246.87 ± 3.70 as correlated to normal group with mean 150.74 ± 2.27 , whereas PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg displayed significant ($p < 0.001$) decrease in ALP level with mean value 195 ± 3.60 , 167.93 ± 1.84 , 152.76 ± 1.66 and 189.95 ± 3.57 respectively, when correlated with disease control group. PT 400 mg/kg, PT AgNP 100mg/kg displayed statistical significantly ($p < 0.001$) in AST level when correlated with standard group. (Fig. 7c)

3.11.4 C-reactive protein (CRP)

CRP level was examined at end of study. Disease control group significantly ($p < 0.001$) elevated serum CRP level with the mean of 824.5 ± 4.30 as correlated to normal group with mean 430.83 ± 4.14 , whereas PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg displayed significant ($p < 0.001$) decrease in CRP level with mean value 482.16 ± 3.72 , 461 ± 3.30 , 454.33 ± 7.48 and 451.83 ± 2.79 respectively, when correlated with disease control group. PT 200mg/kg displayed statistical significantly ($p < 0.001$) in CRP level when correlated with standard group. (Fig. 7d)

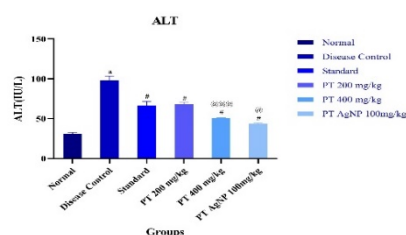


Fig. 7 a

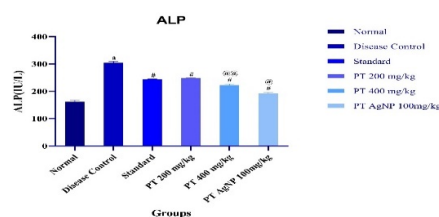


Fig. 7

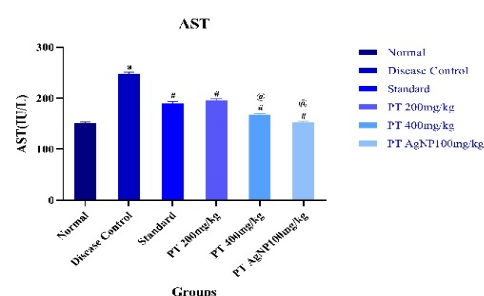


Fig. 7 c

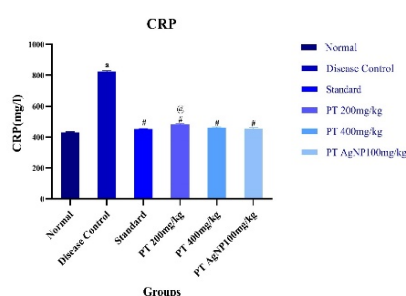


Fig. 7 d

Fig. 7. Biochemical markers **a** Alanine aminotransferases (ALT), **b** Alkaline phosphatase (ALP),

c Aspartate aminotransferases (AST), **d** C- Reactive Protein (CRP)

3.11.5 Effect of PT and PT loaded NPs on serum Pro-inflammatory cytokines TNF-alpha

The significant ($p < 0.001$) raise was observed in the serum TNF- α of FCA injected rats (1623.1 ± 7.80) when correlated to that of the normal group (719.37 ± 5.53). Whereas PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg displayed significant ($p < 0.001$) decrease in TNF-alpha level with mean value 913.24 ± 7.61 , 776.98 ± 14.55 , 741.23 ± 5.22 , 817.91 ± 10.54 respectively. PT 200 mg/kg and PT AgNP 100mg/kg displayed statistical significance ($p < 0.001$) in TNF-alpha level when correlated with standard group. Figure 8a

3.11.6 Effect of PT and PT loaded NPs on serum Pro-inflammatory cytokines IL-6

The significant ($p < 0.001$) raise was observed in the serum IL-6 of FCA injected rats (2914.29 ± 7.82) when correlated to that of the normal group (1212.70 ± 6.60). Whereas PT at 200, 400, PT AgNP 100 mg/kg and indomethacin 10 mg/kg displayed significant ($p < 0.001$) decrease in IL-6 level with mean value 1547.74 ± 5.10 , 1436.35 ± 8.29 , 1393.14 ± 8.09 , 1411.83 ± 8.19 respectively. PT 200 mg/kg displayed statistical significance ($p < 0.001$) in IL-6 level when correlated with standard group. (Fig. 8b)

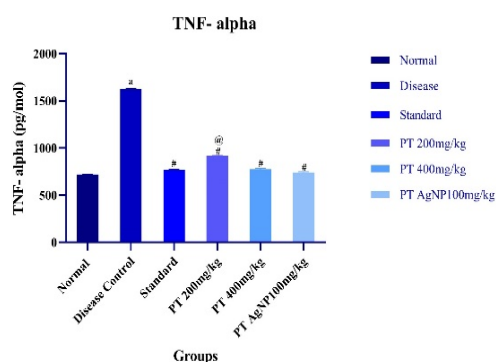


Fig. 8 a

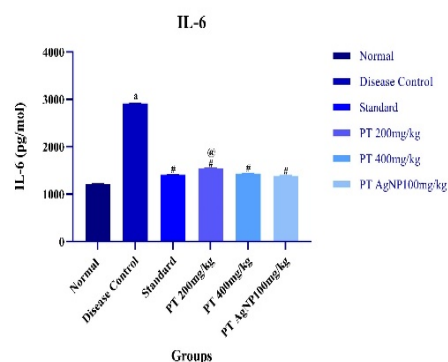


Fig.

Fig. 8 Pro-inflammatory Cytokine **a** TNF-alpha, **b** IL-6

3.11.7 Effect of PT and PT loaded NPs on histopathology of hind paw

The examination of the ankle joint in normal rats revealed normal cartilage, articular joint surface and synovial fluid. There was also no evidence of inflammatory cell infiltration. Also, no inflammation and infiltration of inflammatory cells was observed in Fig. 9 (a1, a2)

Histopathology of hind paw in disease control group Fig. 9 (b1-b4) revealed the presence of typical cartilage and bone damage, infiltration of inflammatory cells, pannus formation, synovial osteoclast formation was observed. We can see layered synovial cell membranes,

increased cell density and improved cellular infiltrates through the joints and synovial hyperplasia. In the above slide we can see osteoclast cells i.e. accountable for rheumatoid arthritis's bone loss.

Histopathology of ankle joint of standard group rats showed moderate inflammatory cells accumulation and observed the osteoclast cells. Furthermore, mild bone reformation in the ankle joint was seen in Fig. 9 (c1, c2) hence it slightly protected against the bone and cartilage destruction and disease is still not fully covered.

Treatment with PT 200mg/kg showed in Fig. 9 (d1, d2) pannus formation and mild inflammatory cell infiltration, fewer cartilage and bone destruction and it prevented against synovial space thickening. Histopathology of ankle joint of PT 400mg/kg group rats showed in Fig. 9 (e1, e2, e3) significantly reduced cartilage and bone destruction, few inflammatory cells, enhance the bone reformation, increased synovial space thinning, no evidence of osteoclast cells. It showed the healing process of disease.

Histopathology of ankle joint of PT AgNP 100mg/kg group rats showed in Fig. 9 (g1, g2) significant protection against cartilage and bone destruction, no evidence of inflammatory cells, enhanced the bone reformation. Rest of the places seems to be normal which indicates the healing potential of the given treatment. Treated with above dose showed reversal of arthritic features.

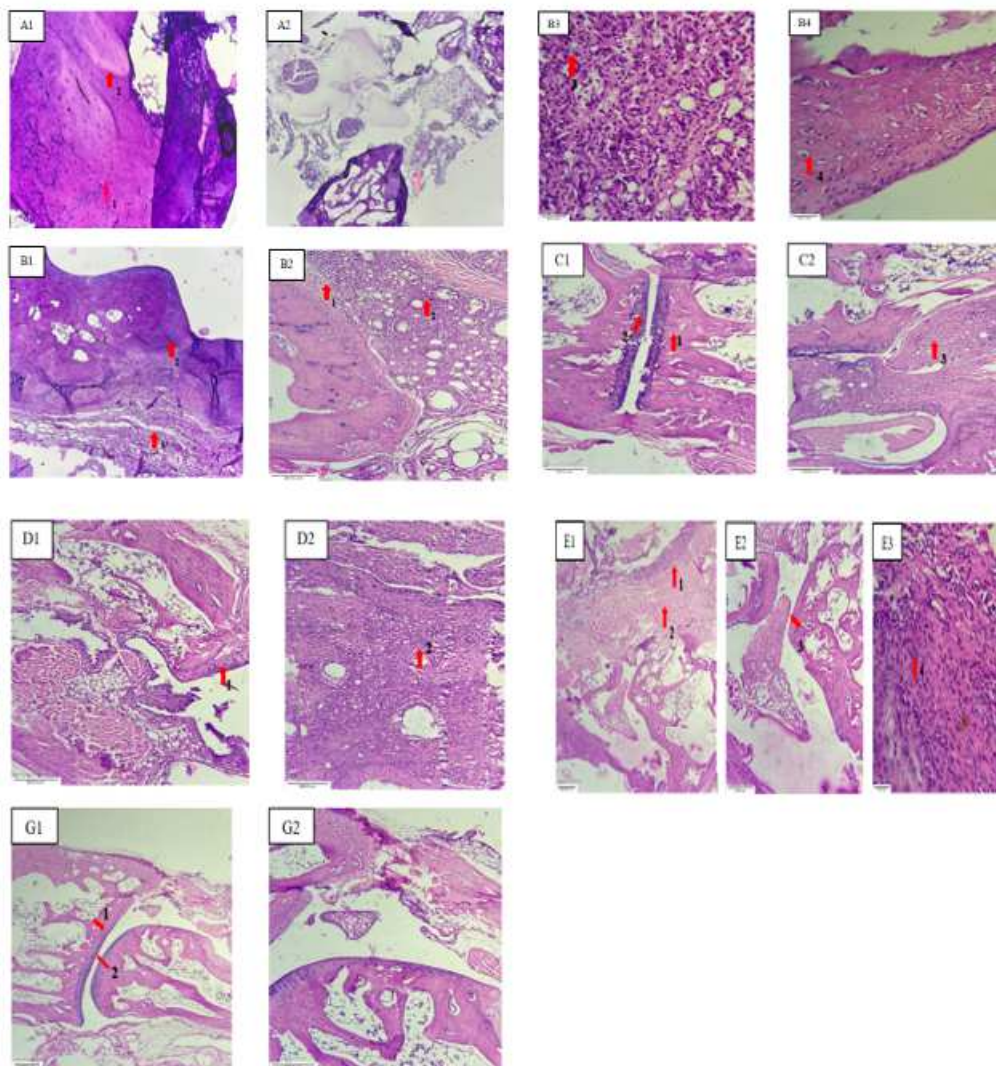


Fig. 9 Histopathological findings of rat ankle joint

The red arrow showing in histopathology image as elaborated in following way.

In normal group 1 normal cartilage, 2 bone, Disease Control group 1 cartilage and bone damage, 2 Pannus formation, 3 Inflammatory cell infiltrate, 4 osteoclast, Standard treatment 1 cartilage and bone, 2 Pannus, 3 Inflammatory cells, PT200mg/kg 1 Pannus formation, 2 Inflammatory cell infiltration, PT400mg/kg 1 Cartilage, 2 Bone, 3 Synovial space, 4 Inflammatory cells, PTAgnP100mg/kg 1 Cartilage and bone, 2 synovial space

3.12 Discussion

Rheumatoid arthritis is a chronic illness marked by inflammatory synovitis in peripheral joints caused by cytokines, which gradually destroys bones and cartilage.

Common symptoms of RA include pain, stiffness, edema, deformity and finally loss of joint function.

Since there is currently no known cure for adjuvant arthritis, patients are typically administered drugs, help to control the disorder's painful symptoms. Currently, non-steroidal anti-inflammatory drugs (NSAIDs), steroids, physical therapy and anti-Tumour Necrosis Factor (Anti-TNF) antibodies are the standard treatments for patients with rheumatoid arthritis.

The severe adverse effects and high cost are the main disadvantages of these drugs. Hence, the need for traditional medicine is raised. These substances not only effectively reduce rheumatoid arthritis symptoms, but they also have adverse effects. One such compound is *Pueraria tuberosa* tuber part, historically and traditionally utilized to alleviate rheumatism and joint swelling, conditions associated with immune system dysregulation [35].

Hence, the goal of this research was to examine if an aqueous extract of *Pueraria tuberosa* tuber (200mg/kg, 400mg/kg) and *Pueraria tuberosa* loaded silver nanoparticle (100mg/kg) might protect rats' arthritic from FCA induced rheumatoid arthritis. The most popular and effective model of arthritis is FCA induced RA model. Inflammatory cytokines are linked to T cells, neutrophils both of which are helper cells are linked to arthritis which caused by FCA [36]. So, replicate human immunity and spontaneous arthritic disease [37,38].

Throughout the synthesis of NPs, phytoconstituents are utilized extensively as effective capping agents, serving a dual function of stability and reduction. Because they include active ingredients that offer the perfect template for NPs green synthesis, they play for a dual purpose. In the present research study, *Pueraria tuberosa* aqueous extract, a highly biocompatible and economical biopolymer was utilized to successfully produce AgNPs green synthesis.

The appropriateness of synthesized PTAgnPs in order to build a nanomedicine delivery system was confirmed by their ideal stability, which allowed them to resist harsh biological environments without losing any of their characteristic spectral features.

To improve the performance of the nanotechnology-based medication delivery system, tiny size of the particles is essential which can ensure increased overall physical stability and improve the loaded contents' in-vivo therapeutic efficacy. The administered medications have longer-lasting therapeutic effects because smaller particles are maintained in the bloodstream longer and do not release their loaded contents as quickly. Additionally, smaller particles have less toxicity than bigger, comparable-sized particles in nature [39, 40]. PTAgnPs were discovered to be in the nano range, indicating both their greater in vivo therapeutic potential and stability.

The possible benefits against arthritis from PT (200,400mg/kg) and PTAgnP (100mg/kg) were easily seen by the deduction of paw size, restoration of body weight and arthritis score as correlated to the disease control group. The leading causes of bone loss in rats having arthritic conditions including, altered bone configuration and resorption of bone due to FCA administration.

It has been reported that a decrease in Hb count in arthritis results due to decreased levels of erythropoietin and premature destruction of red blood cells. In arthritic circumstances, a rise in the corresponding colony stimulating factors mediated by IL-1 β causes a slight increase in WBC count. The FCA-treated experimental rats showed increases in WBC, ESR and rapid losses in Hb and RBC. In addition to preventing the sudden rise in WBC and ESR, the AgNPs demonstrated an apparent rise in RBC, Hb. A little difference was seen in these results, indicating that the AgNPs had superior activity [41].

Oxidative stress in arthritis patients reduces endogenous antioxidants, which worsening the inflammation, especially in the synovium and cartilage. Evaluating enzymatic and non-enzymatic biomolecule concentrations, such as SOD and GSH, showed PT's antioxidant effects. PT significantly restored endogenous antioxidants in test animals, exhibiting antioxidant properties by scavenging free radicals and reducing MDA levels, indicative of lymphocyte infiltration prevention.

In the disease control group, elevated levels of liver marker enzymes ALP, AST and ALT were observed. ALP is a significant marker for liver and bone diseases, released due to localized bone loss during arthritis, leading to elevated serum ALP levels. Treatment with PT and PTAgnPs significantly reduced enzyme levels, comparable to Indomethacin, indicating potential liver function improvement.

An important acute-phase protein generated as a result of inflammation is C-reactive protein (CRP).

It is also a vital non-specific marker protein for inflammatory and infectious diseases [42]. The level of CRP is responsible for the immune system activation in the inflammatory process. In this study, significant CRP elevation was observed in FCA injected group animals. The treatment with PT and PTAgnP notable reduced levels of CRP correlate to the disease control group.

Cytokines including TNF- α , IL-6, IL-1 and IL-17 are the inflammatory mediators associated with RA. These cytokines stimulate chondrocytes and synoviocytes, which causes the production of matrix metalloproteinase into the synovial fluid and the subsequent degradation of the synovial membrane and cartilage [43]. In the FCA induced arthritis model developed in this study, there was an increase in all these indicators supporting the development of RA. Among the cytokines IL-6 and TNF- α were measured in this study, the amount of IL-6 and TNF-alpha, which significantly increase during the development of arthritis were reduced after treatment with *Pueraria tuberosa* extract and its synthesized silver nanoparticles.

4 Conclusion

Our results demonstrated that, *Pueraria tuberosa* and its *Pueraria tuberosa* loaded silver nanoparticle efficiently repaired the damage to the joints and significantly decreased the inflammation of the paws and synovitis in FCA induced arthritis rats. Moreover, the treatment combined to suppress the production of ROS and inhibiting TNF- α and IL-6 pro-inflammatory cytokines. *Pueraria tuberosa* has been shown to have immunomodulatory potential and its newly developed *Pueraria tuberosa* loaded silver nanoparticle invade mechanisms of RA at substantial low doses. The research validates the increased effectiveness of *Pueraria tuberosa* after loading in the formulation of newly synthesized NPs Considering the potential therapeutic benefits of *Pueraria tuberosa*,

these results may lead to the development of novel antiarthritic drugs from the plant *Pueraria tuberosa*, further investigations are warranted to explore its clinical outcomes.

Funding

There was no funding provided by any national or international organisations.

Ethics and consent to participant declarations

Not Applicable

Consent to Publish declaration

Not Applicable

Research Involving Animal

The Institutional Animal Ethics Committee granted ethical approval (IAEC Reg no. 221/Po/Re/S/2000/CPCSEA) KLEU'S College of Pharmacy, Belagavi, prior to carrying out the experiments.

Author Contribution

Tejas Nirwane design, analysed, interpretation of the research work and drafted the manuscript, Namit Kudatarkar helped to design and supervised in research work along with drafted the manuscript, Omkar Shelar participated in synthesize the nanoparticles.

Acknowledgement

The authors thank KLE College of Pharmacy, Belagavi for providing the instruments and software to complete the research work.

References

1. Ye, silada, E.; Üstün, O.; Sezik, E.; Takaishi, Y.; Ono, Y.; Honda, G. Inhibitory Effects of Turkish Folk Remedies on Inflammatory Cytokines: Interleukin-1 , Interleukin-1 and Tumor Necrosis Factor . J. Ethnopharmacol. 1997, 58, 59-73.
2. Busija L, Bridgett L, Williams SR, Osborne RH, Buchbinder R, March L. Osteoarthritis, Best Pract Res Clin Rheumatol. 2010
3. Klareskog L, Malmström V, Lundberg K, Padyukov L, Alfredsson L. Smoking, citrullination and genetic variability in the immunopathogenesis of rheumatoid arthritis. In Seminars in immunology 2011 Apr 1 (Vol. 23, No. 2, pp. 92-98). Academic Press.
4. Clavel C, Nogueira L, Laurent L, Iobagiu C, Vincent C, Sebbag M, Serre G. Induction of macrophage secretion of tumor necrosis factor α through Fc γ receptor IIa engagement by rheumatoid arthritis-specific autoantibodies to citrullinated proteins complexed with fibrinogen. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 2008 Mar;58(3):678-88.
5. Mohan H. Textbook of pathology. Jaypee Brothers Medical Publishers; 2018 Nov 30.
6. Montgomery SL, Bowers WJ. Tumor necrosis factor-alpha and the roles it plays in homeostatic and degenerative processes within the central nervous system. Journal of neuroimmune pharmacology. 2012 Mar;7(1):42-59.
7. Feldmann M, Maini RN. TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. Nature medicine. 2003 Oct 1;9(10):1245-50.

8. Kondo N, Kuroda T, Kobayashi D. Cytokine networks in the pathogenesis of rheumatoid arthritis. *International journal of molecular sciences*. 2021 Oct 10;22(20):10922.
9. Singh VS, Dhawale SC, Shakeel F, Faiyazuddin Md, Alshehri S. Antiarthritic Potential of *Calotropis procera* Leaf Fractions in FCA-Induced Arthritic Rats: Involvement of Cellular Inflammatory Mediators and Other Biomarkers. *Agriculture*. 2021 Jan 15;11(1):68.
10. Perera HDSM, Samarasekera JKRR, Handunnetti SM, Weerasena OVDSJ. In vitro anti-inflammatory and anti-oxidant activities of Sri Lankan medicinal plants. *Industrial Crops and Products*. 2016 Dec;94:610-20.
11. Ali, B.; Mujeeb, M.; Aeri, V.; Mir, S.R.; Faiyazuddin, M.; Shakeel, F. Anti-inflammatory and antioxidant activity of *Ficus carica* Linn. leaves. *Nat. Prod. Res.* 2012, 26, 460-465.
12. Bharti R, Chopra BS, Raut S, Khatri N. *Pueraria tuberosa*: A review on traditional uses, pharmacology, and phytochemistry. *Frontiers in pharmacology*. 2021 Jan 27;11:582506.
13. Kirtikar KR, Basu BD. *Pueraria DC*. *Indian Medicinal Plants*. 2nd ed. Dehradun: Oriental Enterprises, pp. 1104-07, 2001.
14. Kaushik R, Supratim B, Banerjee PC. 'Green' synthesis of silver nanoparticles by using grape (*Vitis vinifera*) fruit extract: characterization of the particles and study of antibacterial activity. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*. 2013;4(1):1271-8.
15. Jain D, Daima HK, Kachhwaha S, Kothari SL. Synthesis of plant-mediated silver nanoparticles using papaya fruit extract and evaluation of their anti microbial activities. *Digest journal of nanomaterials and biostructures*. 2009 Sep 1;4(3):557-63.
16. Gupta PK, Mishra L. Ecofriendly ruthenium-containing nanomaterials: synthesis, characterization, electrochemistry, bioactivity and catalysis. *Nanoscale Advances*. 2020;2(5):1774-91.
17. Bonifacio BV, da Silva PB, Ramos MA, Negri KM, Bauab TM, Chorilli M. Nanotechnology-based drug delivery systems and herbal medicines: a review. *International journal of nanomedicine*. 2014 Dec 9:1-5.
18. Servat-Medina L, Gonzalez-Gomez A, Reyes-Ortega F, Sousa IM, de Cássia Almeida Queiroz N, Zago PM, Jorge MP, Monteiro KM, de Carvalho JE, Román JS, Foglio MA. Chitosan-tripolyphosphate nanoparticles as *Arrabidaea chica* standardized extract carrier: synthesis, characterization, biocompatibility, and antiulcerogenic activity. *International journal of nanomedicine*. 2015 Jun 9:3897-909.
19. Francis G, Thombre R, Parekh F, Leksminarayan P. Bioinspired synthesis of gold nanoparticles using *Ficus benghalensis* (Indian Banyan) leaf extract. *Chem Sci Trans*. 2014;3(1):470-4.
20. Kokate CK. *Practical Pharmacognosy*. Vallabh Prakashan Publication. New Delhi, India. 1999;115.
21. Satpathy S, Patra A, Ahirwar B, Delwar Hussain M. Antioxidant and anticancer activities of green synthesized silver nanoparticles using aqueous extract of tubers of *Pueraria tuberosa*. *Artificial cells, nanomedicine, and biotechnology*. 2018 Nov 12;46(sup3):71-85.
22. Mani A, Vasanthi C, Gopal V, Chellathai D. Role of phyto-stabilised silver nanoparticles in suppressing adjuvant induced arthritis in rats. *International Immunopharmacology*. 2016 Dec 1;41:17

23. Rao NV, Pujar B, Nimbal SK, Shantakumar SM, Satyanarayana S. Nootropic activity of tuber extract of *Pueraria tuberosa* (Roxb).
24. Mali SM, Sinnathambi A, Kapase CU, Bodhankar SL, Mahadik KR. Anti-arthritic activity of standardised extract of *Phyllanthus áamarus* in Freund's complete adjuvant induced arthritis. *Biomedicine & Aging Pathology*. 2011 Jul 1;1(3):185-90.
25. Kaushik S, Jain P, Satapathy T, Purabiya P, Roy A. Evaluation of anti-arthritic and anti-inflammatory activities of *Martynia annua* L. Ethanolic extract. *Clinical Phytoscience*. 2021 Dec;7:1-1.
26. He R, Qian X, Yin J, Zhu Z. Preparation of polychrome silver nanoparticles in different solvents. *Journal of Materials Chemistry*. 2002;12(12):3783-6.
27. Wood FD, Pearson CM, Tanaka A. Capacity of mycobacterial wax D and its subfractions to induce adjuvant arthritis in rats. *International Archives of Allergy and Applied Immunology*. 1969 May 1;35(5):456-67.
28. Delwatta SL, Gunatilake M, Baumans V, Seneviratne MD, Dissanayaka ML, Batagoda SS, Udagedara AH, Walpola PB. Reference values for selected hematological, biochemical and physiological parameters of Sprague-Dawley rats at the Animal House, Faculty of Medicine, University of Colombo, Sri Lanka. *Animal models and experimental medicine*. 2018 Dec;1(4):250-4.
29. Anuradha Nandi & I. B. Chatterjee. Scavenging of superoxide radical by ascorbic acid. *Journal of Biosciences* volume 11, pages 435–441 (1987).
30. ELLMAN GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959 May;82(1):70-7.
31. Nadiger HA, Marcus SR, Chandrakala MV, Kulkarni DD. Malondialdehyde levels in different organs of rats subjected to acute alcohol toxicity. *Indian Journal of Clinical Biochemistry* 1986;133-136.
32. Baia L, Muresan D, Baia M, Popp J, Simon S. Structural properties of silver nanoclusters-phosphate glass composites. *Vibrational spectroscopy*. 2007 Mar 11;43(2):313-8.
33. Singh S, Kumar P. Neuroprotective activity of curcumin in combination with piperine against quinolinic acid induced neurodegeneration in rats. *Pharmacology*. 2016 Jan 30;97(3-4):151-60.
34. Bethu MS, Netala VR, Domdi L, Tartte V, Janapala VR. Potential anticancer activity of biogenic silver nanoparticles using leaf extract of *Rhynchosia suaveolens*: an insight into the mechanism. *Artificial cells, nanomedicine, and biotechnology*. 2018 Oct 31;46(sup1):104-14.
35. Patel J, Doshi N, Bhalerao A, Bonagiri R. Immunomodulatory activity of ethanolic extract of *Pueraria Tuberosa* Immunomodulatory activity of ethanolic extract of *Pueraria Tuberosa* DC. *Int. J. Sci. Eng. Res*. 2016;7(11):708-13.
36. Ahmad N, Fazal H, Abbasi BH, Farooq S, Ali M, Khan MA. Biological role of *Piper nigrum* L. (Black pepper): A review. *Asian Pacific Journal of Tropical Biomedicine*. 2012 Jan 1;2(3):S1945-53.
37. Zhang ZC, Zhang SJ, Jin B, Wu Y, Yang XF, Yu B, Xie QM. Ciclamilast ameliorates adjuvant-induced arthritis in a rat model. *BioMed Research International*. 2015 Apr 27;2015.
38. Berg WB. Lessons from animal models of arthritis over the past decade. *Arthritis research & therapy*. 2009 Oct;11:1-0.
39. K. Rao, S. Aziz, T. Roome, A. Razzak, B. Sikandar, K. S. Jamali, M. Imran, T. Jabri and M. R. Shah, *Artificial cells, nanomedicine, and biotechnology*, 2018, 111.
40. M. Imran, M. R. Shah, F. Ullah, S. Ullah, A. M. Elhissi, W. Nawaz, F. Ahmad, A. Sadiq and I. Ali, *Drug delivery*, 2016, 23, 36533664.

41. Ramaswamy M, Solaimuthu C, Duraikannu S. Antiarthritic activity of synthesized silver nanoparticles from aqueous extract of *Moringa concanensis* Nimmo leaves against FCA induced rheumatic arthritis in rats. *Journal of Drug Delivery and Therapeutics*. 2019 May 15;9(3):66-75.
42. Lapić I, Padoan A, Bozzato D, Plebani M. Erythrocyte sedimentation rate and C-reactive protein in acute inflammation: meta-analysis of diagnostic accuracy studies. *American journal of clinical pathology*. 2020 Jan 1;153(1):14-29.
43. Smolen JS, Aletaha D, Koeller M, Weisman MH, Emery P. New therapies for treatment of rheumatoid arthritis. *Lancet*. 2007; 370 (9602):1861-74.