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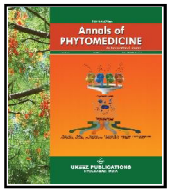

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The *in vivo* antiarthritic activity of guggulosomes prepared using gold nanoparticles generated from stem extract of *Tinospora cardifolia* (Thunb.) Miers

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Abstract

The aim of our study is to evaluate antiarthritic activity of guggulosomes prepared using gold nanoparticles (GNP) generated from stem extract of *Tinospora cordifolia* (Thunb.) Miers and to determine its mechanism of action. In India, *T. cordifolia* and guggul are well-known and regularly used to manage a range of ailments, such as arthritis. Arthritis was induced by complete freunds adjuvant (CFA) in wistar albino rats and various parameters related to arthritis were recorded. Assessment of body weight, paw diameter, alterations in haematologicaland biochemical parameters as well as the histopathological examination were also carried out. Serum levels of cytokines pro-inflammatory marker, rheumatoid factor (RF), C-reactive protein (CRP) and oxidative stress mediators were assessed, in addition to liver function. Oral administration of guggulosome at a dose of 250 mg/kg for a period of 21 days produced significant (p<0.01and p<0.001) anti-inflammatory effect analogous to that of marketed product Rumalaya at the same dose. This was compared to stem extract and GNP both at a dose of 50mg/kg. Haematological and antioxidant anomalies in the CFA rats were reversed to normal, while weight consistently increased. Guggulosomes also significantly attenuated RF (25.2 ± 1.07%, p<0.001), CRP (12 ± 7.45%, p<0.01), TNF-a (35.5 ± 3.14%, p<0.001), IL-1b (25.9 ± 6.13%, p<0.001) and IL-6 (20.56 ± 12.18%, p<0.001) levels in CFA induced arthritis model compared to positive control group. These results were supported by histological evaluations of the joint. The analysis of various arthritic assessment parameters used in this study revealed that guggulosome made using GNP has fewer adverse effects on liver function and is efficacious in the treatment of rheumatoid arthritis. The findings also suggested that guggulosome presents notable antiarthritic activity that is mediated by suppressing proinflammatory cytokines. We conclude that guggulosome prepared using GNP generated from stem extract of *T. cordifolia* was found to give a synergistic effect and hence has therapeutic potential as an antiarthritic agent.

1. Introduction

Rheumatoid arthritis (RA) is a chronic, crippling, progressive, autoimmune disease of unknown cause with a life time prevalence (Wasserman, 2011). It is a systemic disease affecting multiple joints symmetrically. Most of the time, the condition affects women between the ages of 30 and 50 (Guo *et al*., 2018). RA is a widespread health problem throughout the globe and the rate at which the number of people suffering from this disease condition worldwide is increasing, is worrisome. RA affects 1-2% of the population worldwide. Several drugs available are non-steroidal anti-inflammatory drugs (aceclophenac, diclophenac), steroids (glucocorticoid), disease modifying antirheumatoid drugs (methotrexate, cyclosporin A) and biologicals (adalimumab, anakinra, tocilizumab) for treating moderate to severe cases of RA (Pratima *et al*., 2021). The gastrointestinal side effects attributed to these compounds include nausea, stomach discomfort, ulcers, abdominal pain and even gastrointestinal bleeding (Fraenkel *et al*., 2021). Corticosteroid medications causes weight gain, risk of infection, facial puffiness, muscle weakness, drug dependency and destruction of large joints such as hips. Major disadvantages of biologicals is that, on chronic usage, patients become refractive to these drugs and the efficacy of treatment declines, moreover cost of these drugs are prohibitive (Radu and Bungau, 2021).

Nowadays, researchers are focusing on traditional system of medicine for the discovery of drugs. Physicians and patients alike have acknowledged that compared to allopathic medications, herbal medicines have a reduced incidence of adverse effects. In order to develop new drug delivery technologies that would further increase the effectiveness of plant actives/extracts due to increased bioavailability and/or better drug targeting, pharmaceutical scientists have shifted their focus to designing a drug delivery system for herbal medicines using a scientific approach. *T. cordifolia* which belongs to Menispermaceae family is a large, deciduous, climbing shrub, found throughout India and also in Srilanka, Burma, Nepal and China (Devi *et al*., 2018; Khatun *et al*., 2016). It is commonly known as Amruta, a term attributed to its ability to impart youthfulness, exuberance, longevity, and hence included in wide range

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of Ayurvedic products since time immemorial (Sinha *et al*., 2004). It is known to possess antipyretic, antistress, anti-inflammatory, antiallergic, antiarthritic, antidiabetic, antioxidant, hepatoprotective, radioprotective and immunomodulatory activity (Patni, 2015).

An eco-friendly alternative to biological and chemical processes that reduces the upkeep of the septic environment and eliminates the production of harmful byproducts is the green synthesis of GNPs using plant extract (Teimuri-Mofrad *et al*., 2017). Herbal gold nanoparticles synthesized from different plant extracts have been found to enhance various activities like *in vitro* antioxidant (Boruah *et al*., 2021), *in vitro* cytotoxicity (Phukan *et al*., 2021), anti-inflammatory, anticancer (Sun *et al*., 2017) and antimicrobial activities(Bhau *et al*., 2015).

Guggul is an ingredientin several traditional Ayurvedic formulations used to treat inflammation, hypercholesteremia and skin conditions. Guggul is an oleo gum resin derived from the *Commiphora mukul* plant (Ragavi and Surendran, 2021). Guggulosomes are the vesicular drug delivery systems wherein the guggul lipids when triturated with the drug solution, form drug entrapped vesicles (Verma *et al*., 2010). These guggulosomes have been shown to enhance the activity of the encapsulated drug in a synergistic manner (Agarwal and Sundari, 2010). Taking these facts into consideration, the present study has been designed to compare the antiarthritic potential and its changes in haematological and biochemical parameters of *T. cordifolia* extract, GNP synthesized using *T. cordifolia* extract and guggulosome prepared using the synthesized GNP, in Freunds adjuvant induced arthritic rats. This study was aimed to provide a better cure in patients of rheumatoid arthritis.

2. Materials and Methods

2.1 Preparation of *Tinospora cordifolia* (Thunb.) Miers stem extract

Fresh stems of *Tinospora cordifolia* (Thunb.) Miers were collected from Mumbai, India and authenticated by Dr. Rajendra D.Shinde, Director of Blatter Herbarium St. Xaviers College, Mumbai (Specimen number P/D/2868/2018). The washed and dried stems were milled to a coarse powder, 1 kg of the dried powder was extracted with 5 lit of hydroalcoholic solvent 1:1, by maceration at room temperature. Rotary evaporator was used at 60°C to remove the alcohol from the filtered hydroalcoholic extract and the aqueous extract was dried to a semisolid mass (TC 101) which was stored in a refrigerator at 4°C for further studies.

2.2 Drugs and chemicals

Guggul was purchased from M/s. Yucca Enterprises, Mumbai. Guduchi tablet and Rumalaya tablet were purchased from local Ayurvedic Medical store, Mumbai. CFA was obtained from Sigma Chemical Company (St. Louis, MO, USA).

2.3 Synthesis of gold nanoparticles

1 mM gold chloride solution and 1.25% of TC 101 was prepared in distilled water (DW). Filtered TC 101 extract and gold chloride solution was mixed in 1:9 ratio and kept on a magnetic stirrer at 400 rpm for 3 h at room temperature for synthesis of GNP 101.The formation of violet coloured solution confirmed the formation of GNP 101. UV-VIS scan of the GNP 101 solution, was also recorded. The solution was centrifuged at 10,000 rpm for 15 min. Supernatant was discarded and pellets were washed thrice with DW (Abbasi *et al*., 2014). Finally, the GNP 101 pellets were dried at 37°C, powdered and stored for further studies.

2.4 Purification of guggul

Guggul was purchased from M/s. Yucca Enterprises, a reputable herbal drug supplier in Mumbai, India. Guggul was purified by tying in a muslin cloth and immersing in hot DW till the muslin cloth was devoid of hard guggul remains. After throwing the unwanted remains in the muslin cloth, the solution was heated till a soft mass is obtained which solidifies on cooling. Purified guggul thus obtained was powdered and used to make guggulosome (Dave *et al*., 2017).

2.5 Formulation of GNP guggulosome

The powdered GNP 101 was accurately weighed and triturated with DW to make a smooth slurry of GNP 101. To prepare guggulosome, the powdered guggul was triturated with the prepared GNP 101 slurry for 10 min (Verma *et al*., 2010). Adjust the volume with distilled water and sonicate continuously till complete entrapment of GPN 101 is achieved to obtain GNP 102 guggulosome, which is observed by centrifuging the sonicated guggulosome at 10,000 rpm for 20 min and taking the absorbance of the supernatant at 546 nm using JASCO V-630 spectrophotometer. This GNP 102 guggulosome was dried and used for further animal study.

3. Antiarthritic study of GNP guggulosome

3.1 Experimental animals

Clean polypropylene cages were utilised to house the animals. Three animals were put per cage under standard conditions of temperature (24°C ± 2°C), relative humidity (50% ± 5%) and light (12 h light/12 h dark cycle) in Institute's Animal house. Prior to the experiment, animals that were purchased from outside were given 8-10 days to get used to the surroundings in our animal house while having unlimited access to food and water. The rats were housed in stainless steel top grill cages with pelleted meal facilities. Corncob was utilised as bedding, and it was changed twice a week. Standard pelleted food and water were given to the animals as and when needed.

3.2 Experimental design

The antiarthritic activity in albino rats was evaluated using Freund's adjuvant-induced arthritis model. Animals were randomly divided into six groups of six animals each (n = 6). To induce arthritis, a single injection of 0.1 ml of complete Freund's adjuvant (CFA) was injected into the planter area of the left hind paw. Once daily oral dosing of all six groups (except group I and II) was started from day 1, 30 min before adjuvant injection and continued till 21st day post injection (Augustine *et al*., 2013).

3.3 For determination of dose of TC 101

Animal experimental protocol was reviewed and approved by Institutional Animal Ethics Committee (Protocol No. KMKCP/IAEC 14/2017) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), guidelines were followed for use and care of experimental animals in research. Animals for study were purchased from Bharat Serum and Vaccines Pvt. Ltd. Thane, Mumbai. The study was carried out at K.M. Kundnani College of Pharmacy, Colaba, Mumbai, Albino wistar rats having 150-200 g weight of either sex was selected for the study.

Animals were allocated into six groups (6 rats for each group) designed as follows:

Group I

Positive control (PC) which were administered single dose of 0.1 ml CFA into the planter region of the left hind paw.

Group II

Negative control (NC) were administered saline.

Group III

CFA induced arthritic rats were administered marketed product (M) Guduchi Himalaya tablet (100 mg/kg) as reference standard.

Groups IV, V, VI

CFA induced arthritic rats were be administered 25 mg/kg, 50 mg/kg, 100 mg/kg, respectively of the extract TC 101.

Using a vernier calliper, paw thickness was measured on days 1, 7, 14, and 21. The percentage inhibition of the paw thickness was then determined. The dose of TC 101 at which maximun inhibition was observed was selected as dose for further studies. Extract of TC 101 was used for the synthesis of GNP 101. GNP 101 was used to prepare GNP 102 guggulosome. As GNP 101 and GNP 102 are derivatives of TC 101, for all further antiarthritic studies, same dose as of TC 101 was used for both GNP 101 and GNP 102.

3.3.1 Statistical analysis

The values obtained were expressed as mean ± SEM (n = 6). Statistical significance was determined using one-way ANOVA, followed by Dunnett's t-test. *p<0.05; **p<0.01compared with positive control, ***p<0.001 compared with vehicle control. Values of p<0.05 was considered significant. ANOVA: Analysis of variance, SEM: Standard error of the mean.

3.4 For antiarthritic study of TC 101, GNP 101 and GNP 102

Animal experimental protocol was reviewed and approved by Institutional Animal Ethics Committee (Protocol No. BVC/IAEC/08/2019) and CPCSEA guidelines were followed for use and care of experimental animals in research. The animals used for the study were obtained from animal house of Bombay Veterinary College, Parel. Female albino wistar rats having 140-200 gm weight were selected for the study.

Animals were allocated into six groups (6 rats for each group) designed as follows:

Group I

Positive control (PC) group were administered single dose of 0.1 ml CFA into the planter region of the left hind paw.

Group II

Negative control (NC) which were administered saline.

Group III

CFA induced arthritic rats were administered marketed product (M) Rumalaya tablet (250 mg/kg) as reference standard.

Group IV

CFA induced arthritic rats were administered 50 mg/kg of the extract TC 101

Group V

CFA induced arthritic rats were administered 50 mg/kg of GNP 101.

Group VI

CFA induced arthritic rats were administered 250 mg/kg of guggulosome GNP 102 containing 50 mg of GNP 101.

Using a micrometre gauge, paw thickness was measured on days 1, 7, 14, and 21 and body weight of each group was recorded using a digital balance. Blood was withdrawn for assessment of biochemical parameters on 1st, 14th, 21st days by retro-orbital puncture under mild anaesthesia using 1 ml of isoflurane USP in absorbent cotton kept in the anaethesia induction chamber. On 22nd day, the rats were euthanized by cervical decapitation under isoflurane anaesthesia.The blood was then transferred into an EDTA and non-EDTA tubes. For the antioxidant study, the liver was excised and phosphate buffer was used to prepare the homogenate. The left hind paw were amputated above the knee joint and preserved in 10% formalin for histological examination (Nagarkar and Jagtap., 2017). Except for negative control group animals, all the animals were sacrificed. Dehydrated tissues were processed and paraffin wax was used to embed them. Haematoxylin and eosin staining was applied to 4 µm sections, which provided the localisation of inflammatory cells present and destruction of joint when observed under light microscope (Saleem *et al*., 2020). Following biochemical parameters in serum were estimated using diagnostic kits:

1. Biochemical parameters like ESR, WBC, RBC, hemoglobin, ALP, AST, ALT, Rheumatoid factor, C-reactive protein.
2. Biochemical markers like TNF-α, IL-1β and IL-6 were quantitatively determined.
3. Tissue antioxidant level SOD, GSH, GPX were determined

The percentage inhibition of the paw thickness was calculated by the formula:

$$\% \text{ Inhibition} = \{1 - (\text{drug treated/negative control})\} \times 100$$

Hematological parameters were estimated by using a hematology analyzer from Diatron MI PIC, Hungary. Serum was separated from the blood of experimental animals by allowing it to clot at room temperature for 30 min. Using ELISA kits and following the manufacturer's instructions, the protein concentration of serum proinflammatory cytokines such TNF-α, IL-6 and IL-1β was measured. Using assay kits supplied by Sigma Aldrich and following the manufacturer's instructions, the levels of SOD, GPX and GSH were assessed (Aiyalu *et al*., 2016; Kamal *et al*., 2021; Kim *et al*., 2016).

3.4.1 Statistical analysis

The values obtained were expressed as mean ± SEM (n=6). Statistical significance was determined using one-way ANOVA, followed by Duncans multiple test by using statistical software Sigma plot 14.5.*p<0.01; **p<0.001compared with positive control. Values of p<0.01 was considered significant.

4. Results

Formation of violet coloured GNP 101 solution using TC 101 along with the UV-VIS scan of the GNP 101 solution, confirmed the formation of GNP. Guggulosome made using GNP 101 was found to have an

entrapment efficiency and % cumulative drug release of 95.54 ± 0.38 and 80.15 ± 2.26, respectively.

The anti-inflammatory activity of TC 101 at the doses of 25 mg/kg, 50 mg/kg, 100 mg/kg and marketed Guduchi tablet at a dose of 100 mg/kg on CFA-induced arthritic paw edema was examined against PC group of rats and was found to be significant at $p<0.05$, $p<0.01$ and $p<0.001$, respectively as shown in Figure 1. In the 21 days study, it was found that all the treatment groups showed significant $p< 0.001$ decrease in the chronic inflammation induced by adjuvant as demonstrated by reduction in paw thickness. Rats treated with marketed product at the dose of 100 mg/kg (Guduchi) showed significant decrease in joint diameter ($p<0.05$, $p<0.01$ and $p<0.001$) from day 0 to day 21 of the study as compared to PC group rats. Treatment with TC 101 at the doses of 25 mg/kg, 50 mg/kg and 100 mg/kg also showed significant decrease ($p<0.05$, $p<0.01$and $p<0.001$) from day 0 to day 21 of the study as compared to PC group of rats. On comparison, it was found that the percentage of inhibition expressed by both 50 mg/kg and 100 mg/kg of TC 101 were similar at 22 ± 0.051% and 23 ± 0.043%, respectively, and was found to be as active as marketed at 22 ± 0.0035%. Therefore, it was decided to choose 50 mg/kg as the dose of TC 101 for further studies.

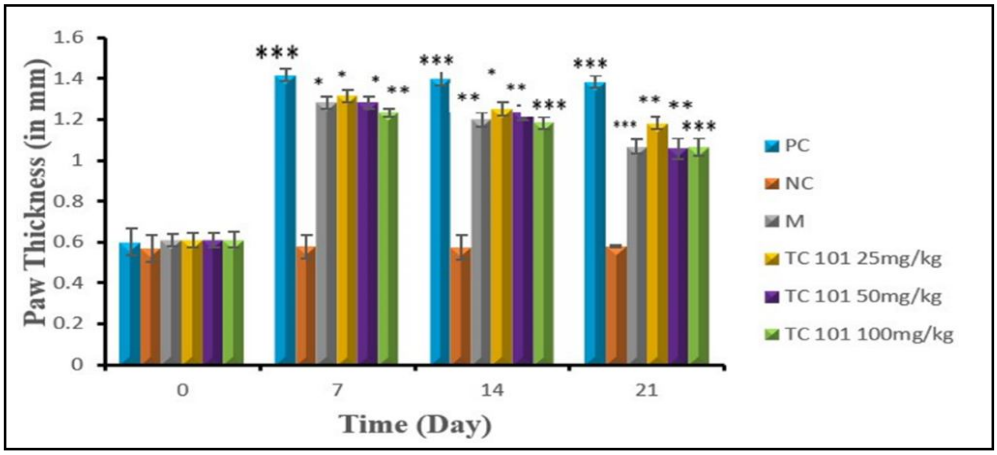


Figure 1: Effect of TC 101 herbal extract on arthritic model. PC: Positive control, NC: Negative control, M: Marketed guduchi tablet 100 mg/kg, TC 101: *Tinospora cordifolia* stem extract at dose 25, 50 and 100 mg/kg. Values are expressed as mean ± SEM (n = 6). *$p<0.05$; **$p<0.01$ compared with PC, ***$p<0.001$ compared with NC.

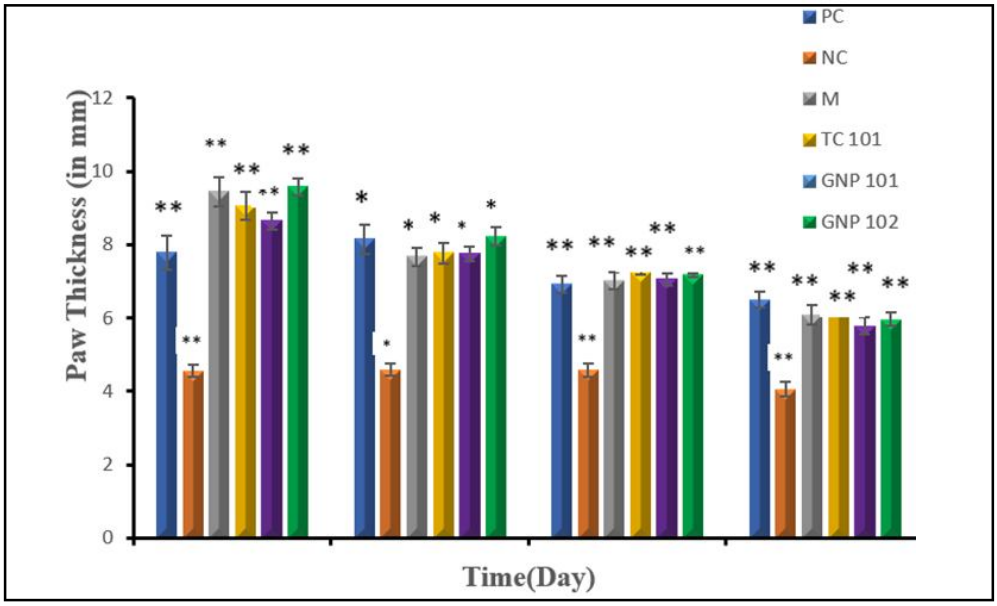


Figure 2: Effect of TC 101, GNP 101 and GNP 102 on arthritic model. PC: Positive control, NC: Negative control, M: the animal group that was given marketed Rumalaya tablet 250 mg/kg, TC 101: The animal group that was given *Tinospora cordifolia* stem extract at dose 50 mg/kg, GNP 101: The animal group that was given gold nanoparticle synthesized using *Tinospora cordifolia* stem extract at dose 50 mg/kg and GNP 102: the animal group given guggulosome made using gold nanoparticle synthesized using *Tinospora cordifolia* stem extract and guggul at 250 mg/kg. Values are expressed as mean ± SEM (n=6). *$p<0.01$; **$p<0.001$ compared with PC.

The activity of TC 101, GNP 101 and GNP 102 at the doses of 50 mg/kg, 50 mg/kg and 250 mg/kg, respectively and marketed Rumalaya at the dose of 250 mg/kg on CFA-induced arthritic paw edema was examined against PC group rats and was found to be significant at $p<0.01$ and $p<0.001$, respectively, as shown in Figure 2. In the 21 days study, it was found that, all the treatment groups significantly $p<0.001$ decrease the chronic inflammation induced by adjuvant as demonstrated by reduction in paw thickness. Rats treated with marketed product 250 mg/kg (Rumalaya) showed significant decrease in joint diameter ($p<0.01$and $p<0.001$) from day 0 to day 21 of the study as compared to PC group rats. Treatment with TC 101 (50 mg/kg), GNP 101 (50 mg/kg) and GNP 102 (250 mg/kg) also showed significant decrease in paw thickness of 24.6 ± 0.23%, 33.1 ± 0.24% and 37.6 ± 0.19% at $p<0.01$and $p<0.001$, respectively, from day 0 to day 21 of the study as compared to PC group of rats. On comparison, it was found that the highest percentage of inhibition was expressed by GNP 102 at a dose of 250 mg/kg at 37.6 ± 0.19% and more inhibitory than marketed product at 35.5 ± 0.27%.

The average gain in body weight on days 7, 14, 21 was compared to day 0 as shown in Figure 3. Marketed group, TC 101 group, GNP101 group and GNP 102 group showed an increase in body weight, whereas PC group showed loss in body weight of 5.4 ± 4.37% compared to NC group. The total increase in the body weight was the least in TC 101 at 14.5 ± 6.93%, while GNP 102 showed the maximum increase in body weight at 21.5 ± 6.35% and in marketed product, the increase in weight was 17.3 ± 3.9%. All the groups showed a steady increase in body weight except for PC group rats.

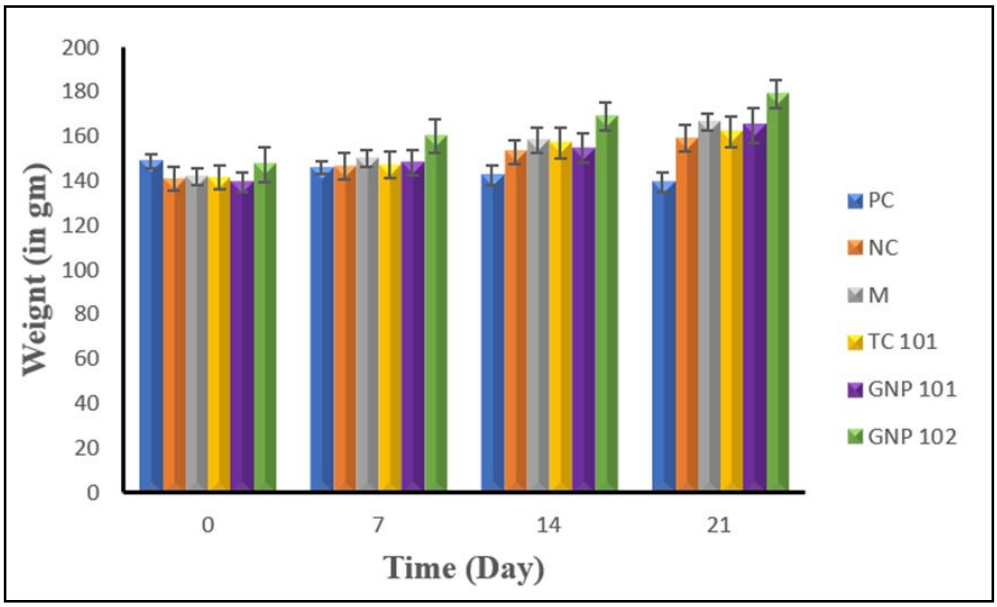


Figure 3: Effect of TC 101, GNP 101 and GNP 102 on body weight of arthritic model. PC: Positive control, NC: Negative control, M: The animal group that was given marketed Rumalaya tablet 250 mg/kg, TC 101: The animal group that was given *Tinospora cordifolia* stem extract at dose 50 mg/kg, GNP 101: The animal group that was given gold nanoparticle synthesized using *Tinospora cordifolia* stem extract at dose 50 mg/kg and GNP 102: The animal group given guggulosome made using gold nanoparticle synthesized using *Tinospora cordifolia* stem extract and guggul at 250 mg/kg. Values are expressed as mean ± SEM (n=6).*$p<0.01$; **$p<0.001$compared with PC.

The results of the change in haematological parameters are shown in Table 1. In the PC group,WBC and ESR levels increased significantly ($p<0.01$and $p<0.001$) in contrast to RBC where there was no significant change. While haemoglobin levels decreased significantly ($p< 0.01$) compared with NC group. Characteristic haematological alterations such as decrease in WBC, ESR and increase in haemoglobin levels were observed when treated with marketed, GNP 101 and GNP 102, when compared to PC group. While the level of RBC was found to be same in GNP 102, it increased in marketed group and decreased in TC 101 and GNP101 group, respectively.

The marketed, GNP 101, and GNP 102 group remarkably counteracted the ESR and WBC count, which was drastically increased in the PC group, restoring it back to normal, thus proving its significant roles in severe arthritic conditions. Thus, the marketed, TC 101, GNP 101 and GNP 102 groups improved the altered haematological parameters.

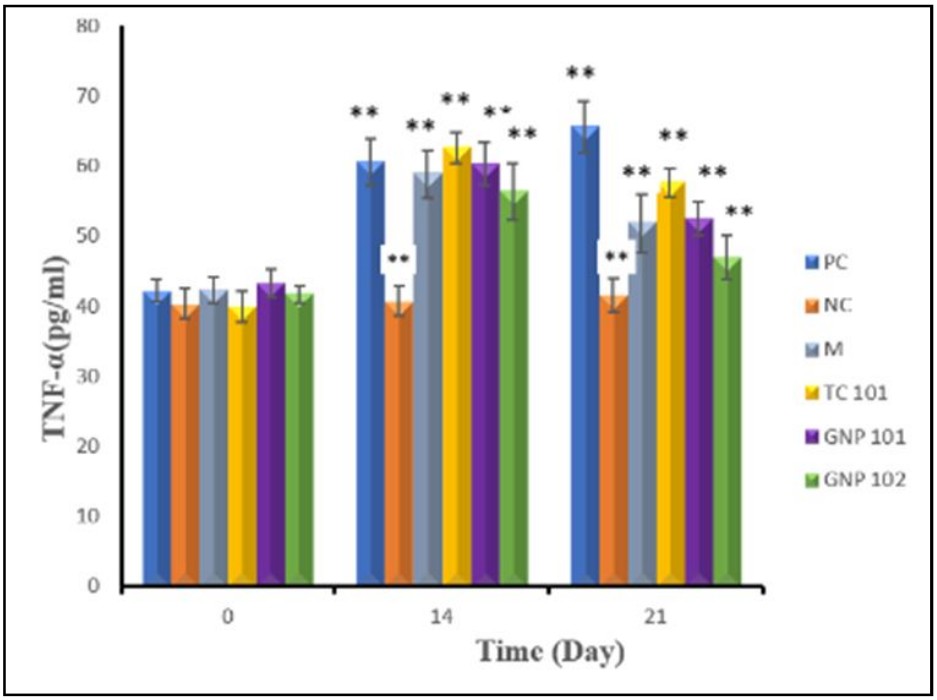
The serum levels of inflammatory cytokines such TNF-α, IL-6 and IL-1β in different experimental groups is shown in Figure 4(a), (b) and (c). Measurement of the serum cytokine levels in various experimental groups demonstrated that there was a significant elevation of 36.5 ± 3.696%, 46.7 ± 5.04%, 48.5 ± 6.08% at $p<0.01$and $p<0.001$ in serum levels of TNF-α, IL-6 and IL-1β, respectively, in PC group rats when compared with NC group rats. Upon treatment with TC 101, GNP 101, GNP 102 and Rumalaya. TNF-α, IL-6 and IL-1β serum levels were noticeably reduced ($p<0.01$and $p<0.001$), when compared to the untreated counterparts in the 21 days study. On comparison, it was found that the highest decrease in the levels of TNF-α was expressed by GNP 102 at a dose of 250 mg/kg of 35.5 ± 3.14% at $p<0.001$ and even more active than marketed at 32.8 ± 4.15%, $p<0.001$ compared to PC group rats. Reduction in the levels of IL-6 and IL-1β by GNP 102 at a dose of 250 mg/kg was found to be 20.56 ± 12.18%, $p<0.001$ and 25.9 ± 6.134%, $p<0.001$ compared

to Rumalaya which gave 14.11 ± 6.78%, $p<0.001$ and 24 ± 3.83%, $p<0.001$decrease in the levels of IL-6 and IL-1β on day 21 post CFA injection. Thus, the inhibitory effect of GNP 102 was found to be greater than Rumalaya.

Table 1: Effect of TC 101, GNP 101 and GNP 102 on RBC, WBC, Hb and ESR

Groups	RBC 10^6/cu.mm			WBC 10^3/cu.mm		
	0DAY	14 DAY	21 DAY	0 DAY	14 DAY	21 DAY
Positive control	7.1 ± 0.25	6.9 ± 0.21	6.8 ± 0.19	9.9 ± 0.51	10.9 ± 0.69	12.2 ± 0.47
Negative control	7.0 ± 0.25	6.8 ± 0.26	6.5 ± 0.34	9.7 ± 0.84	9.7 ± 0.71	9.8 ± 0.67
Marketed	7.0 ± 0.45	6.8 ± 0.39	6.9 ± 0.26	8.6 ± 0.54	10.1 ± 0.64	10.8 ± 0.69
TC 101	6.2 ± 0.14	6.2 ± 0.13	6.2 ± 0.12	9.8 ± 0.58	10.2 ± 0.52	10.7 ± 0.65
GNP 101	6.6 ± 0.28	6.5 ± 0.34	6.7 ± 0.3	8.8 ± 0.33	9.3 ± 0.35	10.3 ± 0.411
GNP 102	6.6 ± 0.31	6.7 ± 0.24	6.8 ± 0.25	8.1 ± 0.55	8.8 ± 0.54	10.2 ± 0.78
Groups	**Hb (g/dl)**			**ESR mm/h**		
	0 DAY	**14 DAY**	**21 DAY**	**0 DAY**	**14 DAY**	**21 DAY**
Positive control	11.6 ± 0.29*	11.9 ± 0.25*	10.7 ± 0.50	8.6 ± 0.55*	13.9 ± 0.52**	14.2 ± 0.61**
Negative control	13.2 ± 0.71*	12.8 ± 0.49*	12.6 ± 0.49	9.8 ± 0.26*	9.7 ± 0.24**	9.7 ± 0.25**
Marketed	12.7 ± 0.54*	12.3 ± 0.38*	12.1 ± 0.41	9.6 ± 0.37*	15.2 ± 0.36**	12.6 ± 0.31**
TC 101	10.9 ± 0.46*	10.7 ± 0.58*	10.7 ± 0.55	9.9 ± 0.41*	14.2 ± 0.66**	13.3 ± 0.64**
GNP 101	11.5 ± 0.23*	10.6 ± 0.18*	10.8 ± 0.51	10.1 ± 0.44*	14.4 ± 0.54**	13.3 ± 0.57**
GNP 102	11.6 ± 0.47*	10.9 ± 0.45*	10.9 ± 0.48	10.5 ± 0.25*	13.6 ± 0.31**	13.1 ± 0.36**

RBC: Red blood cell, WBC: White blood cell, Hb: Haemoglobin, ESR: Erythrocyte sedimentation rate. PC: Positive control, NC: Negative control, M: The animal group that was given marketed Rumalaya tablet 250 mg/kg, TC 101: The animal group that was given *Tinospora cordifolia* stem extract at dose 50 mg/kg, GNP 101: The animal group that was given gold nanoparticle synthesized using *Tinospora cordifolia* stem extract at dose 50 mg/kg and GNP 102: The animal group given guggulosome made using gold nanoparticle synthesized using *Tinospora cordifolia* stem extract and guggul at 250 mg/kg. Values are expressed as mean ± SEM (n=6). *$p<0.01$; **$p<0.001$ compared with PC.



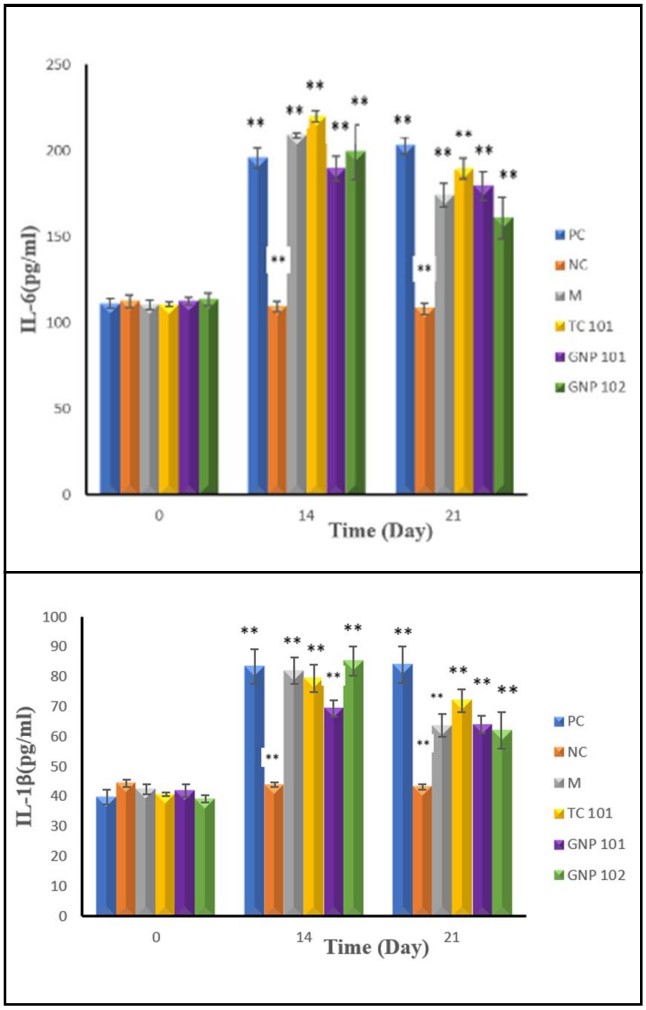


Figure 4: **Effect of TC 101, GNP 101 and GNP 102 on cytokines in complete Freund's adjuvant (CFA) induced rat model. (a) Effect on TNF-α: Tumor necrosis factor, (b) Effect on IL-6: interleukin-6 and (c) Effect on IL-1 β: interleukin-1β. PC: Positive control, NC: Negative control, M: the animal group that was given marketed Rumalaya tablet 250 mg/kg, TC 101: The animal group that was given *Tinospora cordifolia* stem extract at dose 50 mg/kg, GNP 101: The animal group that was given gold nanoparticle synthesized using *Tinospora cordifolia* stem extract at dose 50 mg/kg and GNP 102: The animal group given guggulosome made using gold nanoparticle synthesized using *Tinospora cordifolia* stem extract and guggul at 250 mg/kg. Values are expressed as mean ± SEM (n = 6). *p<0.01; **p<0.001 compared with PC.**

The most distinctive markers for RA are regarded to be rheumatoid factor (RF) and C-reactive proteins (CRP) antibodies. After the experimental period, RF and CRP values were measured in serum as shown in Figure 5 (a) and (b). Increased RF value of 42.5 ± 0.89%, p<0.001 in serum was observed in PC group rats as compared to the NC group rats. After 21 days treatment with TC 101, GNP 101 and GNP 102, the serum RF value was reduced significantly to 17.2 ± 0.56%, 20.5 ± 0.56%, 25.2 ± 1.07%, p<0.001, respectively, compared to PC group rats and was found to be comparable to that of Rumalaya administered rats at 28.5 ± 1.02%, p<0.001. Thus, it is clear that GNP 102 showed maximum reduction in the serum RF value. Similarly, after the experimental period, increased CRP value of 13 ± 6.42%, p<0.01 in serum was observed in PC group rats as compared to the NC group rats. After 21 days, treatment with TC 101, GNP 101 and GNP 102 to CFA induced rats, the serum CRP value was reduced significantly to 9.06 ± 5.78%, 11.17 ± 8.03%, 12 ± 7.45%, p<0.01, respectively, compared to PC group rats and was found to be comparable to that of Rumalaya administered rats at 11 ± 11.02%, p<0.01. Thus, it is clear that GNP 102 showed maximum reduction in the serum CRP value.

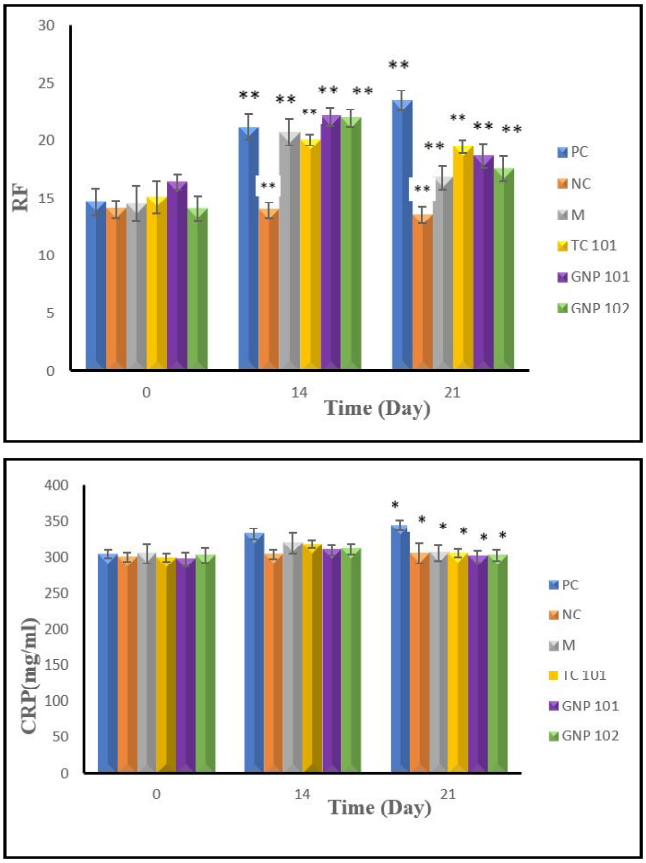


Figure 5: **Effect of TC 101, GNP 101 and GNP 102 on RF and CRP of arthritic model (a) Effect on RF: Rheumatoid factor and (b) Effect on CRP: C-reactive protein. PC: Positive control, NC: Negative control, M: The animal group that was given marketed Rumalaya tablet 250 mg/kg, TC 101: The animal group that was given *Tinospora cordifolia* stem extract at dose 50 mg/kg, GNP 101: The animal group that was given gold nanoparticle synthesized using *Tinospora cordifolia* stem extract at dose 50 mg/kg and GNP 102: The animal group given guggulosome made using gold nanoparticle synthesized using *Tinospora cordifolia* stem extract and guggul at 250 mg/kg. Values are expressed as mean ± SEM (n=6). *p<0.01; **p<0.001compared with PC.**

Markers of liver function include aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP). In PC group rats, serum levels of AST, ALT and ALP resulted in a significant increase to 43 ± 4.06%, 42 ± 1.07% and 41 ± 2.82%, at p<0.001, p<0.001, p<0.01, respectively, against the NC group rats

as shown in Figure 6 (a), (b) and (c). All the treatment groups showed a significant reduction ($p< 0.001$, $p<0.001$, $p<0.01$) in AST, ALP and ALT levels when compared to PC group rats. Amongst all treatment groups, GNP 102 group was found to show 21.5 ± 2.41%,32 ± 8.21%, 34 ± 4.17% at $p< 0.001$, $p<0.001$, $p<0.01$, decrease respectively, in the serum levels when compared to the PC group rats. The marketed product showed similar reduction of 23 ± 4.21%, 33 ± 5.83%, 35 ± 2.33% at $p< 0.001$, $p<0.001$, $p<0.01$, respectively.

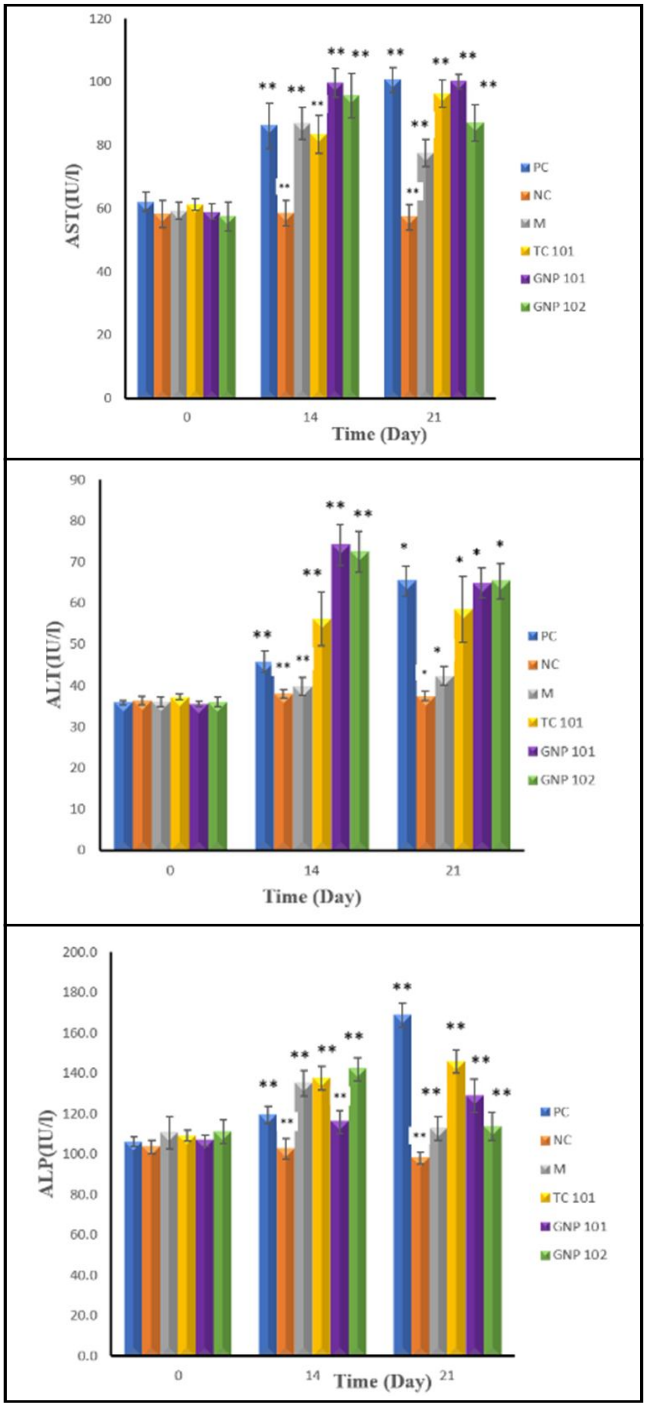
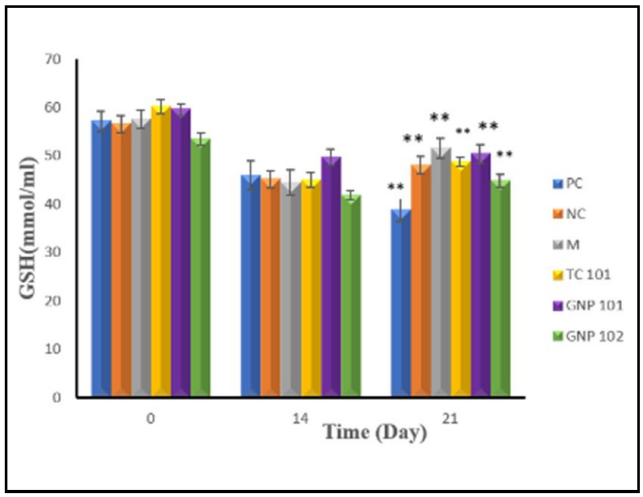


Figure 6: Effect of TC 101, GNP 101 and GNP 102 on transaminase levels in complete freund's adjuvant (CFA) induced rat model. (a) Effect on AST: aspartate aminotransferase; (b) Effect on ALT: Alanine aminotransferase; (c) Effect on ALP: Alkaline phosphatase. PC: Positive control, NC: Negative control, M: The animal goup that was given marketed Rumalaya tablet 250 mg/kg, TC 101: The animal group that was given *Tinospora cordifolia* stem extract at dose 50 mg/kg, GNP 101: The animal group that was given gold nanoparticle synthesized using *Tinospora cordifolia* stem extract at dose 50 mg/kg and GNP 102: The animal group given guggulosome made using gold nanoparticle synthesized using *Tinospora cordifolia* stem extract and guggul at 250 mg/kg. Values are expressed as mean ± SEM (n=6). *$p<0.01$; **$p<0.001$compared with PC.

Results on the alteration of oxidative stress parameters is shown in Figure7 (a), (b) and (c) indicate the levels of GSH (reduced glutathione), GPX (glutathione peroxidase) and SOD (superoxide dismutase) determined in the liver tissue of the rats. In the PC group rats the levels of GSH, GPX and SOD were found to decrease significantly from 51.7 ± 2.16 mmol/ml, 109.7 ± 2.67 U/ml, 10.9 ± 0.39 U/ml to 38.9 ± 2.47 mmol/ml , 92.1 ± 3.02 U/ml, 6.1 ± 1.11 U/ml at $p< 0.001$, $p<0.001$, $p<0.01$, respectively, against the NC group rats from day 0 to day 21, but in all the treatment groups, TC 101, GNP 101 and GNP 102, the values were found to decrease from 60.2 ± 1.52, 59.5 ± 1.19, 53.4 ± 1.34 on day 0 to 45.0 ± 1.49, 49.7 ± 1.68, 41.8 ± 0.92, respectively, on day 14 for GSH (mmol/ml), from 114.5 ± 1.43, 111.2 ± 2.69, 114.8 ± 1.23 on day 0 to 91.5 ± 3.39, 86.1 ± 3.74, 92.1 ± 3.07, respectively, at $p< 0.001$ on day 14 for GPX (U/ml) , from 12.9 ± 0.71, 11.8 ± 1.03, 12.7 ± 0.56 on day 0 to 5.9 ± 0.75, 6.4 ± 0.85, 7.1 ± 0.62, respectively, at $p<0.01$ on day 14 for SOD (U/ml). On day 21 in all the treatment groups, TC 101, GNP 101 and GNP 102 there was a significant increase in all the values 48.7 ± 0.93, 50.3 ± 1.94, 44.8 ± 1.35, respectively, at $p< 0.001$ for GSH (mmol/ml), 95.5 ± 3.57, 96.5 ± 2.41, 103 4 ± 3.38, respectively, at $p< 0.001$ for GPX (U/ml) and 8.6 ± 0.73, 8.8 ± 0.78, 11.1 ± 0.55, respectively, at $p< 0.01$ for SOD (U/ml), indicating that the treatment has the ability to elevate the serum levels of the antioxidant parameters as observed in marketed product group.



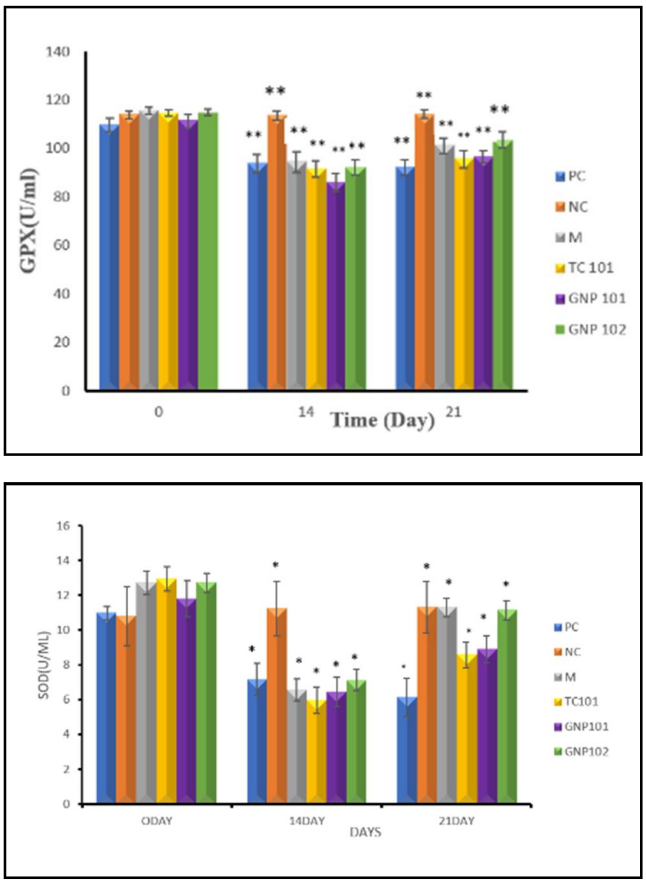


Figure 7: **Effect of TC 101, GNP 101 and GNP 102 on oxidative stress parameters in complete freund's adjuvant (CFA) induced rat model. (a): Effect on GSH: Reduced glutathione; (b): Effect on GPX: Glutathione peroxidase; (c): Effect on SOD: Superoxide dismutase. PC: Positive control, NC: Negative control, M: The animal group that was given marketed Rumalaya tablet 250 mg/kg, TC 101: The animal group that was given *Tinospora cordifolia* stem extract at dose 50 mg/kg, GNP 101: The animal group that was given gold nanoparticle synthesized using *Tinospora cordifolia* stem extract at dose 50 mg/kg and GNP 102: The animal group given guggulosome made using gold nanoparticle synthesized using *Tinospora cordifolia* stem extract and guggul at 250 mg/kg. Values are expressed as mean ± SEM (n=6). *$p<0.01$; **$p<0.001$ compared with PC.**

A histopathological evaluation of the knee joint exhibited multifocal infiltrations of mono and polymorpho nuclear cells at the joint, disturbed architectural pattern, loss of cartilage, osteoblast hyperplasia, accumulation of adipose tissue, congestion of vessels, loss of marrow and disarray in the PC group rats as shown in Figure 8(a). Animals in the NC group showed no evidence of inflammation, bone erosion, cartilage destruction or cellular infiltration as shown in Figure 8(b). M group (250 mg/kg) revealed a substantial protection of the architecture of the joint by reducing focal loss of cartilage, congestion of vessels and loss of marrow as shown in Figure 8(c). The rats treated with TC 101, GNP 101 and GNP 102 showed a remarkable reduction in osteoblast hyperplasia and infiltration of mono and polymorpho nuclear cells at the joint as shown in Figure8 (d,e,f). Amongst the three treated group GNP 102 exhibited action that is comparable to that of marketed group Rumalaya (250 mg/kg).

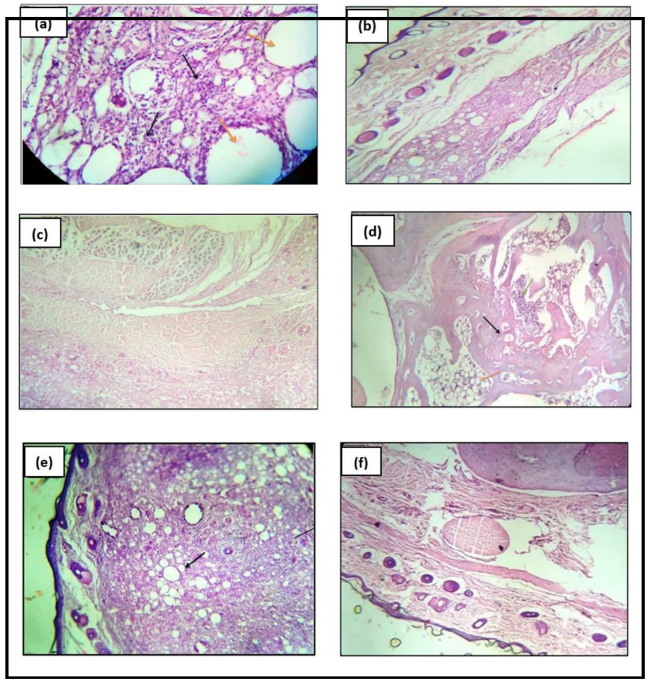


Figure 8: **(a) PC group microphotographs showing accumulation of mono and polymorpho nuclear cells (black arrow) with loss of marrow by deposits of adipose tissue (orange arrow) in rats from study Gr. A [H & E, 100 X] (b) NC group microphotographs showing maintained structural and architectural pattern in paw joint of rats and no significant pathological changes/lesions noticed in animals from this group Gr. B [H & E, 100 X] (c) M group microphotographs showing nearly maintained architectural pattern in rats from study Gr. C [H & E, 100 X] (d) TC 101 group microphotographs showing mild osteoblast hyperplasia (black arrow); accumulation of few mono and polymorpho nuclear cells (green arrow) with mild loss of marrow and deposits of adipose tissue (orange arrow) in rats from study Gr. D [H & E, 100 X] (e) GNP 101 group microphotographs showing disturbed architectural pattern and deposits of adipose tissue (black arrow) in rats from study Gr. E [H & E, 100 X] (f) GNP 102 group microphotographs revealed maintained architectural pattern and mild focal loss of cartilage in rats from study Gr. F [H & E, 100 X].**

5. Discussion

The present study for determination of dose for formulation of GNP was conducted at a daily dose of TC 101 25 mg/kg, 50 mg/kg and 100 mg/kg, respectively, using CFA induced rat paw method. All the treatment groups were found to decrease the paw thickness significantly compared to the PC group. TC 101 at the dose of 50 mg/kg and 100 mg/kg were found to give similar results comparable to marketed guduchi tablet at a dose of 100 mg/kg, indicating that the TC 101 has better activity. Therefore, it was decided to synthesise GNP and conduct antiarthritic activity at a dose of 50 mg/kg. Since

TC 101 is the hydroalcoholic stem extract, guduchi tablet manufactured by Himalaya was used as reference standard for the study as it contains only guduchi stem extract.

T. cordifolia is widely used in folk medicine and Ayurveda as an anti-inflammatory agent. Alkaloids, glycosides, diterpenoid lactones, sesquiterpenoid, steroids, phenolics and polysaccharides are the chemical components that have been reported in the stem (Nair *et al*., 2016; Mohan *et al*., 2016). Previous studies have indicated for the presence of various phytochemicals in *T. cordifolia* extract that have anti-inflammatory activity (Prakash *et al*., 2011). Guduchighana (aqueous concentrated extract) at the dose of 50 mg/kg have shown significant anti-inflammatory activity in comparison with the earlier study of Patgiri *etal*. (2014). As compared to standard, the bioactive fraction of *T. cordifolia* in methanol inhibited the expression of TNF-α and IL-1β in dendritic cell suspensions stimulated with LPS (Jacob *et al*., 2018). From our previous studies, we have proved that formation of GNP enhanced the anti-inflammatory activity of *T. cordifolia* by using the *in vitro* human red blood cell membrane stabilization method and gene expression of cytokine TNF-α by isolated human peripheral blood mononuclear cells using RT-PCR.

The present study demonstrated that TC 101, GNP 101 and GNP 102 at a daily dosage of 50 mg/kg, 50 mg/kg and 250 mg/kg, respectively, significantly attenuated adjuvant-induced polyarthritis as shown by the decrease in paw thickness. In the present investigation, the decreased paw swelling in the drug-treated rats from day 0 onwards may be attributable to the plant extract's ability to provide immunological protection (Shrilakshmi *et al*., 2022; Brijesh *et al*., 2019). When compared to PC-treated rats in the current study, animals treated with GNP 102 showed a significant reduction in inflammation. The study revealed that when compared to rats treated with TC 101 and GNP 101, rats treated with GNP 102 demonstrated a highly significant reduction in paw edoema (paw thickness). The results were comparable to marketed herbal product. The percentage inhibition of inflammation by the GNP 102 and M were 37.6% and 35.5%, respectively. Marketed herbal product Rumalaya tablet is used to treat and control rheumatoid arthritis by reducing pain, inflammation and morning stiffness significantly. Rumalaya tablet was chosen as reference standard because it contains guduchi, guggul and swarnbhasma along with many other herbs (Singh *et al*., 2010). Our formulation GNP102 also contains GNP synthesized from guduchi as well as guggul used to make guggulosome, thus making a good product for study.

The GNP synthesized using stem extract of *T. cordifolia* in our lab gave the following characterization results: UV-VIS spectrum of the aqueous medium containing, gold nanoparticles showed a peak at 546 nm. The hexagonal shaped nanoparticles were well dispersed with particle size ranging from 30-60 nm, that were confirmed by TEM and SEM, respectively, also reported by Abbasi *et al*. (2014). FTIR showed shift in position and intensity of the peaks. HPTLC screening showed that even after forming gold nanoparticles, it retains most of its phytochemical constituents. *In vitro* stability studies have confirmed that gold nanoparticles are stable in biological fluids at physiological pH and also in salt solutions. XRD studies confirmed crystalline nature of the synthesized nanoparticles. Zeta potential value of the synthesized gold nanoparticles is -29 mV at 25° showing good stability of nanoparticles.

Guggul has been used in Ayurvedic medicines for treatment of many ailments such as obesity, atherosclerosis, inflammation, wrinkles, acne *etc* (Jaiswal *et al*., 2016). The purpose of motivation of invention brings into being that guggul being medicinally active ingredient, it could function as a carrier, excipient and synergist (Verma *et al*., 2010). In guggulosomes, GNP 101 was used as drug which has been found to show better anti-inflammatory activity compared to TC 101 in our earlier study. Thus, GNP 101 resembles guggul with anti-inflammatory property due to guggul lipid causing synergistic effect. Guggulosomes are nontoxic and biocompatible. Therefore, the antiarthritic potential of the investigated TC 101, GNP 101 and GNP 102 preparations is a result of their constituents, which show anti-inflammatory activity mediated by inhibition of a variety of molecules involved in inflammation, such as NF-kB, COX-2, 5-LOX, TNF-α, IL-1β, IL-6, matrix metalloproteinases (MMPs), and nitric oxide (Raut *et al*., 2017; Mallavadhani *et al*., 2019; Jain and Gupta., 2006).

Reduced body weight is a key indicator of health in many illness conditions, including rheumatoid arthritis. It is generally recognised that RA causes body mass loss, probably due to inflammatory cytokines, soreness, loss of appetite, inadequate absorption of nutrients through the intestine, enhanced energy expenditure and increased protein catabolism (Shamlan *et al*., 2021). The study's findings demonstrated that, in contrast to the PC group rats, which showed weight loss, treatment with TC 101, GNP 101 and GNP 102 improved the body mass of the treated animals. The restoration of the intestine's ability to absorb nutrients may be the reason for the increased body weight observed during treatment with TC 101, GNP 101 and GNP 102.

In the present study, PC group rats showed reduced Hb levels and increased ESR and WBC counts. Elevation in WBC count plays a vital role in body's defence mechanism (Ekambaram *et al*., 2010). The stimulation of immune system against the invading antigens causes significant increase of WBC count in arthritic rats and the respective decrease in GNP 102 treated groups demonstrated its immunomodulation effect. In relation to the number and size of red blood cells as well as the relative concentration of plasma proteins, particularly fibrinogen and α and β globulins, the ESR is an assessment of the suspension stability of RBCs in plasma (Rajendran and Krishnakumar., 2010). The ESR is commonly utilized as a non-specific marker of inflammatory or threatening conditions in arthritis. ESR and WBC, which markedly increased in the PC group rats, was restored back to normal level after treatment with both GNP 102 and marketed groups thus establishing its crucial role in arthritic conditions.

A T cell-mediated immune response that stimulates the secretion of cytokines and encourages the formation of antibodies, which results in the destruction of the joint, causes RA (Singh *et al*., 2021; Cui. *et al*., 2019). Inhibitors of these cytokines are therefore effective at reducing chronic inflammation, whereas proinflammatory cytokines (particularly IL-1β, IL-6 and TNF-α) play a significant role in the development of arthritis (Foong and Hamid, 2012; Nagarkar and Jagtap, 2017). As shown in the results, the levels of inflammatory cytokine TNF-α, IL 1 β and IL 6 in PC group rats were significantly increased. In our experiments, all the treated groups TC 101, GNP 101 and GNP 102 displayed significant reductions in the levels of inflammatory mediators such as TNF-α, IL-1β, and IL-6 when compared to the PC group rats, especially by GNP 102 at a dose of

250 mg/kg was almost equivalent to that of the marketed product. This result indicates that anti-inflammatory effect of TC 101, GNP 101 and GNP 102 could be associated with its inhibition of TNF α, IL 1 β and IL-6 levels. Amongst all the treatment groups, GNP 102 was found to show maximum reduction. Herbal drugs are safer with lesser side effect than allopathic medications, hence should be considered in the treatment of arthritis.

RF factor is observed positively in 80% of RA patients (Mahajan and Mehta., 2009). Treatment with TC 101, GNP 101 and GNP 102 showed significant decrease in RF value as compared to disease control in CFA-induced arthritic model (Saber *et al*., 2020; Hegde *et al*., 2018). Amongst all, GNP 102 was found to be even better than Rumalaya, which confirms its antiarthritic potential. Similar results were observed with GNP 102 at a dose of 250 mg/kg in the levels of CRP (Petchi *et al*., 2018;Vetal *et al*., 2013).

The hepatoprotective efficacy of GNP 102, as demonstrated by the liver function enzymes AST, ALT, and ALP, was another promising biological benefit of GNP 102 (Gupta and Mohan., 2022; Kamal *et al*., 2021). Additionally, GNP 102 at a dose of 250 mg/kg restored the liver's antioxidant levels, which was disrupted by CFA induced arthritis. Similar results were observed with GNP 102 at a dose of 250 mg/kg in the levels of GSH, GPX and SOD, the oxidative stress markers (Augustine *et al*., 2013; Kshirsagar *et al*., 2014). This might be because GNP 102 has both antioxidant and antiarthritic properties.

Histopathological changes in arthritis are also useful indicators of disease sensitivity (Zhao *et al*., 2016). Histological studies of the paw tissues further support the hypothesis that the suppression of the increase in the thickness of the hind paws is associated with the prevention of the infiltration of mono and polymorphonuclear cells (Subash *et al*., 2012; Djuichou *et al*., 2019). A significant reduction in histological alterations in the joint tissues was accompanied with the inhibition of joint swelling (Patel *et al*., 2021). Severe infiltration of mono and polymorpho neuclear cells is seen in paw tissues of PC group rats and on treatment with TC 101, GNP 101 and GNP 102, there is reduction in the cellular infiltration but GNP 102 formulation at a dose of 250 mg/kg, showed a maximum decrease in neutrophil infiltration. Both these formulations of GNP 101 and GNP 102 have suppressed the macrophage infiltration, edema formation and inflammation in paw tissues. Similarly, the histological analysis of the joints has shown that GNP 102, as well as Rumalaya, revealed maintained architectural pattern and mild focal loss of cartilage in rats. Therefore, by preventing damaged synovial tissue from releasing cytokines, GNP 102's potential to protect against cartilage and synovial membrane deterioration could additionally suppress systemic inflammation.

To conclude TC 101, GNP 101 and GNP 102 at a dose of 50 mg/kg, 50 mg/kg and 250 mg/kg, respectively, revealed a significant decrease in paw thickness, improved blood indices and normalised the haematological and biochemical irregularities. Amongst all, GNP 102 was found to be more effective than marketed Rumalaya tablet. Further, histological studies confirmed anti-inflammatory effect of GNP 102 in CFA induced inflammation. Inflammatory biomarkers reported decreased expression as a result of the treatment. The anti-inflammatory activity of GNP 101 has been enhanced due to synergistic property of guggul. In summary, it is evident from the study that in CFA induced inflammation in rat model, GNP 102 possessed highest anti-inflammatory activity.

6. Conclusion

Guggulosome of GNP prepared by trituration, followed by sonication method was found to enhance oral antiarthritic activity of GNP synthesized using *T. cordiolia*. We evaluated the efficacy of orally administered TC 101, GNP 101 and GNP 102 in CFA-induced arthritis in rat model for a period of 21 days.When used in the treatment of inflammation on paw edema, GNP guggulosome produced the anticipated results. These findings discovered that guggul lipid provides better entrapment and better protection from inflammation, attributed to its promising carrier and due to its synergistic effects with GNP. This study provides strong evidence for promising anti-inflammatrory activity of guggulosome at a dose of 250 mg/kg. Strategy for synergistic action for inflammation could thus be achieved with this delivery system. However, further clinical studies are necessary.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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