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Characterization and *in vivo* evaluation of nanoformulations in FCA induced rheumatoid arthritis in rats

Rida Siddique, Faqir Muhammad, Bilal Aslam and M Naeem Faisal
Institute of Physiology and Pharmacology, University of Agriculture, Faisalabad, Pakistan

Abstract: Rheumatoid arthritis is an inflammatory arthropathy, autoimmune in nature, leading to disability of joints involving structural destruction of articular bone and cartilage due to inflammation in synovium resulting in joint stiffness, swelling and pain. Nanomedicine has played a crucial role in improving the efficacy of treatment by controlling the release of pharmacologically active ingredients to increase bioavailability and achieve uniform and targeted delivery of drug. In this study, we prepared celecoxib, gingerol and oleanic acid loaded PLGA nanoparticles by solvent evaporation method and nanoparticles were characterized by particle size, zeta potential, polydispersity index, entrapement efficiency and FTIR. FCA is induced in right hand paw of rats for induction of arthritis. Celecoxib, gingerol and oleanic acid loaded PLGA nanoparticles coated with chitosan were given orally to rats for the evaluation of anti-arthritic effect of this nanoformulation in rats. Animals were divided into six groups for 21 days trial. On 21st day blood samples were collected for evaluation of hematological and lipid profile parameters. The data was subjected to statistical analysis by applying one way ANOVA and tukey test. At the end of study it was concluded that PLGA loaded celecoxib, gingerol and oleanic acid coated with chitosan have excellent effects in minimizing the side effects and increasing the therapeutic efficacy of drugs.

Keywords: PLGA, chitosan, nanoparticles, arthritis.

INTRODUCTION

Rheumatoid arthritis is an autoimmune inflammatory arthropathy; clinically attributed by dysregulation of stromal tissue causing articular dysregulation and chronic inflammation resulting in articular disability (Toussirot *et al*., 2020). Proinflammatory mediators and cytokines like TNF α, IL-6, IL-1*β*, COX-2, OPG and RANK-L play a crucial role in pathogenesis i.e. responsible for chronic inflammation resulting in damage to juxta-articular bone and articular cartilage (de Molon *et al*. 2019). Being an autoimmune disorder, rheumatoid arthritis has no cure but remission of the disease is possible in case of early recognition and a prompt, appropriate treatment (Yang *et al*., 2020). First-line treatment for rheumatoid arthritis includes NSAIDs and DMARDs followed by glucocorticoids; owing to their multiple systemic side effects and less oral tolerance, strict continuous monitoring is essential while undergoing the treatment (Burmester and Pope 2017). In recent years, the use of nanoparticles for controlled drug delivery in the treatment of rheumatoid arthritis has been a huge milestone (Feng and Guo, 2020).

Celecoxib can be orally used for RA; as it is a selective COX-2 inhibitor, have 90% oral bioavailability and causes less adverse effects in GIT and other systemic systems like CVS and renal system in comparison with other NSAIDs (Jabbari *et al.,* 2020). Oleanic acid (OA); isolated from V. angularis, regulates many pharmacological activities like anti-allergic and anti-inflammatory disorders (Choi *et al*., 2016). Gingerol is a compound of ginger from the family of Zingiberaceae. It inhibits the proliferation of synovitis by inhibiting the formation of pro-inflammatory cytokine i.e., TNFα (Bhalekar *et al*., 2019)

Polymers are used for successfully delivering the active particles of the drug. They prevent drugs from degradation, reduce the need for repetitive dosing and help in achieving targeted action (Jeong and Park, 2020). Using PLGA, distinctive polymeric nanoparticles can be developed to target proinflammatory mediators due to its specificity; thus its excellent choice as a polymer in the treatment of an inflammatory sickness (Mir *et al*., 2017).

A complete drug delivery system consists of three components i.e., targeting agent, carrier system and therapeutic moiety. Chitosan is the best carrier of choice in oral drug delivery owing to its biocompatibility, biodegradability, permeation enhancer effect, pH and mucoadhesive conditions (Quinones *et al*., 2018).

Hence, celecoxib, gingerol and oleanic acid are found to have anti-inflammatory activities but are not investigated for treating rheumatoid arthritis. The objective of our present study includes preparation and characterization of celecoxib, gingerol and oleanic acid loaded PLGA nanoparticles and evaluation of the anti-arthritic effect of these nanoformulations in FCA induced arthritic rats.

MATERIALS AND METHODS

PLGA (poly lactic-co-glycolic acid) MW (7000-17,000) and Chitosan MW (140,000-220,000) high-quality

**Corresponding author:* e-mail: faqirmuhammad33@gmail.com

product purchased from Sigma-Aldrich. Celecoxib was gifted by CCL Pvt LTD Pakistan. Oleanic acid and gingerol were purchased from Sigma Aldrich. Polyvinyl Alcohol (PVA) practical grade was purchased from Duksan Pure Chemicals. FCA (Freund complete adjuvant) was purchased from *in vivo* Gen, Germany. All the reagents and chemicals were of analytical grade.

Preparation of different solutions

PVA (2%) solution was prepared by adding distilled water in 2g of PVA to make a total solution of 100ml at room temperature. PLGA in ethyl acetate was prepared by adding 100 mg of PLGA in 2ml of ethyl acetate. A drug in acetone was prepared by adding 5mg of a drug in 5ml of acetone. Chitosan was prepared in 1% acetic acid solution by adding 10ml of 1% of acetic acid in 20mg of chitosan. All the solutions were mixed well during preparation with the help of vortex mixer.

Method of preparation

Double emulsion method was used to prepare drugs (celecoxib, oleanic acid, gingerol) loaded with PLGA nanoparticles. Drug was dissolved in 5ml of distilled water and PLGA 100 mg was dissolved in 2ml of ethyl acetate. Both of these solutions were mixed by ultrasound sonication for 30 seconds. 20mg of cationic TMC (N-Trimethyl Chitosan Chloride) was added to 10ml of 1% acetic acid solution. 10ml of 2% PVA solution was taken in the beaker and prepared double emulsion (w/o/w) was added drop wise into it with continuous magnetic stirring at room temperature for 2 hours. Residual ethyl acetate was removed by vacuum rotatory evaporation followed by centrifugation at 25000 rpm for 30min. As a result, pallets formed and the supernatant was separated (Akhtar *et al*., 2020).

Characterization of nanoparticles

Zeta potential, zeta size and Polydispersity index of celecoxib loaded PLGA nanoparticles, gingerol loaded PLGA nanoparticles and oleanic acid loaded PLGA nanoparticles was performed with zeta sizer (Malvern Instruments, Malvern, UK) (Akhtar *et al*., 2020).

The encapsulation efficiency (EE %) of chitosan coated drug loaded PLGA nanoparticles was measured by UV-visible spectrophotometric analysis. Nanoparticles were centrifuged at 25000 rpm for 20 minutes. The supernatant was removed and measured by UV-visible spectrophotometric analysis. It's calculated by using the following equation.

EF% = Total amount of drug added-free drug /total amount of drug × 100 (Akhtar *et al*., 2020).

To determine the compatibility and presence of active constituents FTIR (Fourier-transform infra-red spectroscopy) (Shimdazu, Japan) was performed for the formulations of celecoxib, gingerol and oleanic acid loaded PLGA nanoparticles (Akhtar *et al*., 2020).

Experimental design

In the experimental research study, 36 healthy rats (n=6) body weight (100 to 200g) were used. Rats were kept in the animal house of the Institute of Physiology and Pharmacology, University of Agriculture Faisalabad. All experiments were approved and conducted following the rules of the institutional Bioethics committee vide letter number 3744/ORIC-UAF.

Rats were divided into 6 groups for 21 days trial. The arthritis was induced by FCA 0.1ml in left hind paw in sub planter region. Signs of severe inflammation were visualized in two days but animals (groups 2-6) didn't receive any treatment except normal diet till seven days to induce arthritis (Mahdi *et al*., 2018). Group 1 (Control group) and Group 2 (arthritic control group) were given normal food and normal saline. Group 3 was treated with celecoxib 5mg/kg. Group 4, 5 and 6 were treated with celecoxib nanoparticles, gingerol nanoparticles and oleanic nanoparticles 5mg/kg continuously for 14 days.

Physical parameters

Body weight of rats was determined on 9th, 12, 15, 18 and 21. Paw diameter was measured with the help of vernier caliper to observe the swelling of paw on 9th, 12th, 15th, 18th and 21st days. The presence of erythema and edema was noticed in contralateral and ipsilateral paw and was scored 0 to 4 (Mahdi *et al*., 2018).

Blood samples collection

On the 21st day of the study, rats were decapitated and blood was collected in gel clot activator tubes for evaluating biochemical and hematological parameters. Blood was centrifuged for 10minutes at 10,000 rpm and serum was separated and stored in a refrigerator at 4°C. EDTA tubes were used to collect blood samples of rats for hematological analysis (Jadhav & Vavia 2020).

Hematology and lipid profile

The blood samples were analyzed by a hematology analyzer (Medonic, Germany) (Mahdi *et al*., 2018). QCA, Spain kit was used to determine the total serum cholesterol, Serum high density lipoprotein-cholesterol (HDL) and serum triglycerides. Low density lipoprotein cholesterol (LDL) and very low density lipoprotein cholesterol (VLDL) were measured by following formulas

LDL- cholesterol = total cholesterol - (triglycerides/5 - HDL- cholesterol)

VLDL - cholesterol= (triglycerides/5)

STATISTICAL ANALYSIS

Data was statistically analyzed at the level of significance ($P<0.05$) by using one way ANOVA and tukey's multiple comparison by using graph pad prism 6.01. Body weight, paw measurement and arthritis score was statistically analyzed by using two way ANOVA.

RESULTS

Characterization of nanoparticles

Size, potential, polydispersity index and encapsulation efficiency

Zeta size of chitosan modified celecoxib loaded PLGA nanoparticles, chitosan modified gingerol loaded PLGA nanoparticles and chitosan modified oleanic acid loaded PLGA nanoparticles average size was found to be 128, 116.5 and 68 (d.nm) (fig. 1 A,B,C) while zeta potential of prepared nanoparticle of celecoxib was + 38.8. Zeta potential of prepared nanoparticle of gingerol was + 8.10 mv. Zeta potential of prepared nanoparticle of oleanic acid was + 48 mv (fig. 1 D, E, F). Nanoparticles of celecoxib, gingerol and oleanic acid showed a good value of poly dispersity index of 0.28, 0.52 and 1.00 that showed homogenecity of nanoformulations (fig. 2 A). Entrapment efficiency of celecoxib loaded PLGA nanoparticles, gingerol loaded PLGA nanoparticles and oleanic acid loaded PLGA nanoparticles were found to be 80.74%, 80.46% and 80% (fig. 2 B). It revealed that drugs were loaded successfully in PLGA nanoparticles.

FTIR

FTIR analysis of celecoxib nanoparticles, gingerol nanoparticles and oleanic acid nanoparticles were shown in fig. 3.

Effect on Body weight

The body weight was constantly decreased as inflammation occurs. As shown in fig: 4-A there was more weight loss in arthritic group as compared to normal group (p<0.05). Contrarily the treatment with celecoxib and other nanoformulations significantly restored the body weight of arthritic group.

Effect on Paw swelling

FCA was administered by subplanter route and inflammation was noticed in primary jaw (injected) with maximum swelling on 8th day. There was a steady increase in paw thickness and volume of arthritic group noticed 8-21 day compared to normal control group (fig: 4-B). Treatments including CEL and other nanoparticles significantly (P<0.05) reduced paw measurement as compared to arthritic group.

Effect on arthritis score

In whole study no swelling was noticed in normal control group. There was constant increase in arthritic index in arthritic group (P<0.05) as illustrated in fig. 4-C as compared to the control group. A substantial decreased (P<0.05) in arthritic score when treated with CEL and other nanoformulations.

Effect on hematological parameters

A substantial reduction (P<0.05) in RBC, Hb and hematocrit was observed and noticeable increase in WBC, ESR and platelets count in arthritic rats as compared to normal control group (fig. 5). Restoration of RBC, Hb and hematocrit level in treated groups was observed as compared to control group whereas WBC, ESR and Platelets count levels was restored in treated groups.

Effect on Serum lipid profile

Mean ± SD values of serum triglycerides (mg/dL), cholesterol (mg/dL), HDL (mg/dL), LDL (mg/dL) and VLDL (mg/dL) has been shown in fig. 6. Induction of rheumatoid arthritis significantly (P<0.05) increased in serum triglycerides, cholesterol and LDL in arthritic group as compared to control group whereas, substantial decrease (P<0.05) in HDL in arthritic group was seen as compared to control group.

DISCUSSION

Size of nanoprticles is an important determinant that affects the rate of release as well as that of absorption of drug. Nanoparticles distribution is important factor in particle size. Previous studies showed that nanoparticles range 1 to 100 nm in size but some studies revealed that size of nanoparticles range even from 1 to 1000 nm due to polymer coating. The sizes of nanoparticles coated with chitosan and loaded with celecoxib, gingerol and oleanic acid were 193.3 (d.nm), 137.5 (d.nm) and 68 (d.nm) respectively which depicts that coating has been done on drug loaded PLGA nanoparticles (Silva *et al*., 2019).

Zeta potential is an important determinant of stability of nanoparticles. Zeta potential of PLGA nanoparticles, loaded with celecoxib, gingerol and oleanic acid was determined as +38.8, +8.10 and +48 respectively. The shift of charge from negative to positive depicts coating of chitosan on drug loaded PLGA nanoparticles (Ibrahim *et al*., 2016).

Poly dispersity index is a value that determines size distribution. Mono dispersity is achieved when PDI value is at the lowest i.e., near zero. The values greater than >0.5 exhibit wide size distribution. Therefore, PDI value of chitosan coated drug loaded PLGA nanoparticles was low as in agreement with Nair *et al*., 2019 and dispersion was homogeneous and stable.

Entrapment efficiency depicts the amount of drug entrapped efficiently in a nanoparticle. The entrapement efficiency of CEL-NP, OLE-NP and GIN-NP was increased. Molecular weight of nanoparticles is another important determinant of entrapment efficiency. The increased molecular weight of PLGA nanoparticles increased the entrapment efficiency of drugs (Elsewedy *et al*., 2020).

To characterize the chemical structure of drug loaded PLGA nanoparticles, FTIR analysis was performed. Almost same values obtained showed that the drugs had a great compatibility with the polymer used. CEL showed

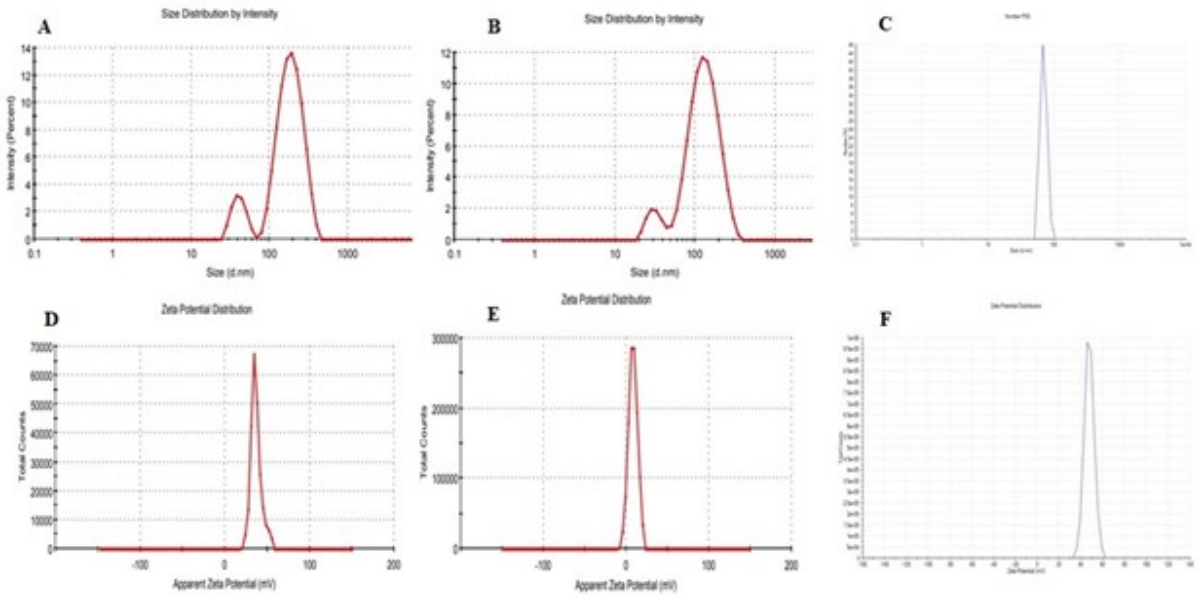

Fig. 1: Zeta size and Potential of PLGA loaded nanoparticles (A): Zeta size of celecoxib loaded PLGA nanoparticles (B): Zeta size of gingerol loaded PLGA nanoparticles (C): Zeta size of oleanic acid loaded PLGA nanoparticles (D): Zeta potential of celecoxib loaded PLGA nanoparticles (E): Zeta potential of gingerol loaded PLGA nanoparticles (F): Zeta potential of oleanic acid loaded PLGA nanoparticles

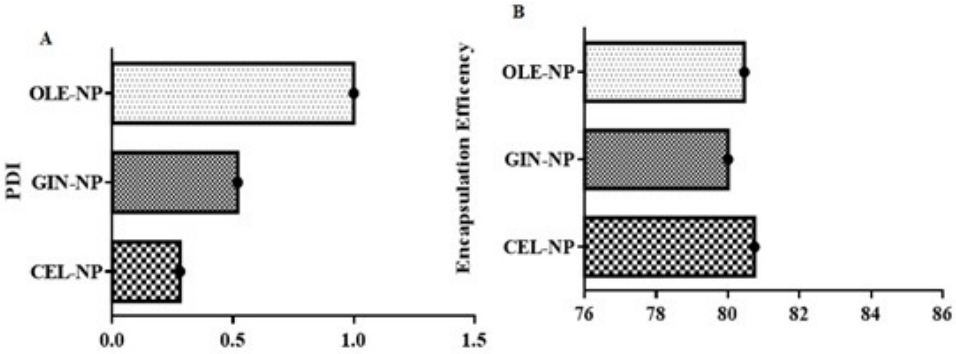

Fig. 2: Polydispersity index and entrapment efficiency of celecoxib, gingerol and oleanic acid loaded PLGA nanoparticles.

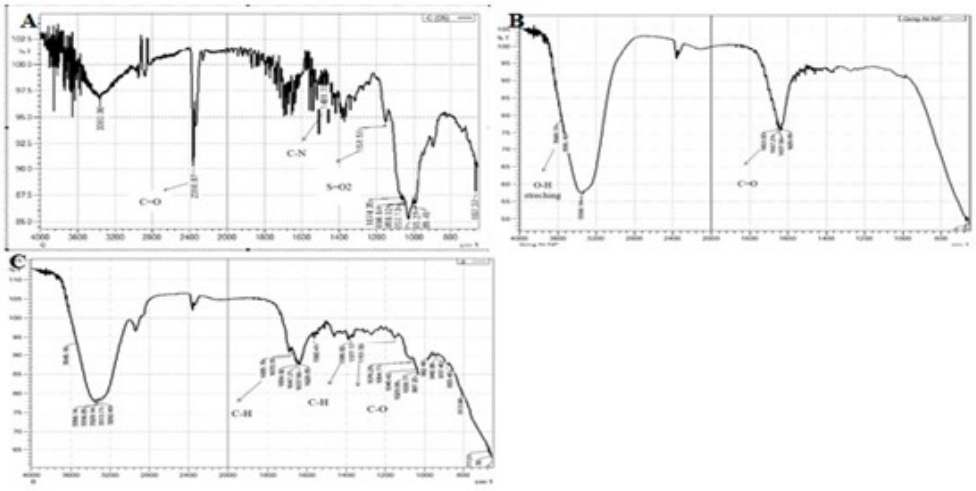

Fig. 3: FTIR analysis of nanoparticles (A): FTIR analysis of celecoxib nanoparticles (B) FTIR analysis of gingerol nanoparticles (C) FTIR analysis of oleanic acid nanoparticles.

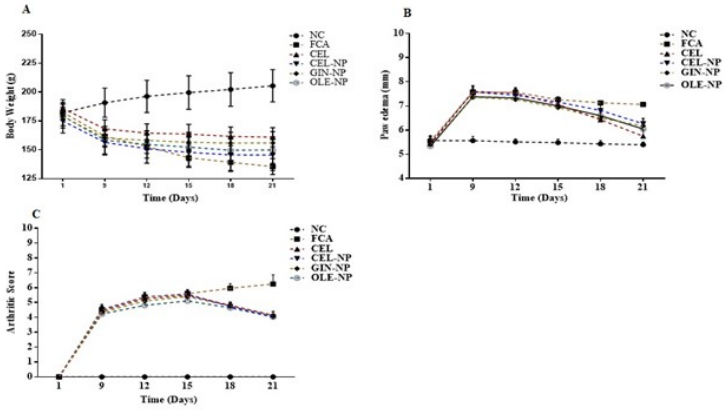

Fig. 4: Trend lines for body weight, paw edema and arthritic score (A): Effect of nanoformulation in FCA induced RA rats on body weight (B): Inhibition of paw edema by nanoformulations in FCA induced RA rats (C): Effect of nanoformulation in FCA induced RA rats on arthritic score. Results were analyzed by two way ANOVA and tukeys multiple comparison test. Results are compared with normal control, arthritic control, and standard drug ($P<0.05$).

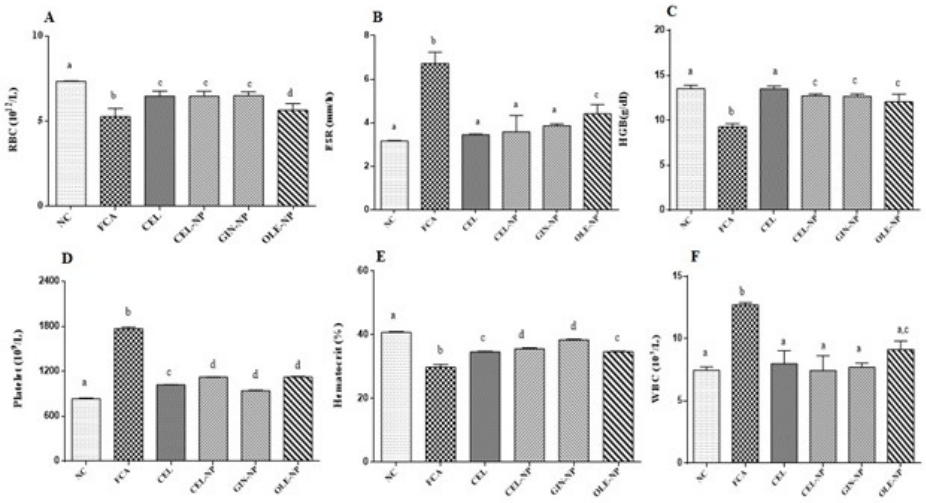

Fig. 5: Effect of nanoparticles on hematological parameters (A): Improved effects of treatments on RBC level in FCA induced arthritic rats (B): Mean serum ESR (mm/h) levels of normal control, FCA induced arthritis group and other treatment groups (C): Effect of nanoformulations on HGB levels of normal control, arthritic control and other treatment groups (D): Mean serum Platelets count levels of normal control, arthritic control and other treatment groups (E): Mean Hematocrit levels of normal control, arthritic control and other treatment groups (F): Reduced effects of treatments on WBC level in FCA induced arthritic rats. Results are compared with normal control, arthritic control, and standard drug. **[a-d]** superscript present that they are significantly different from each other ($P<0.05$).

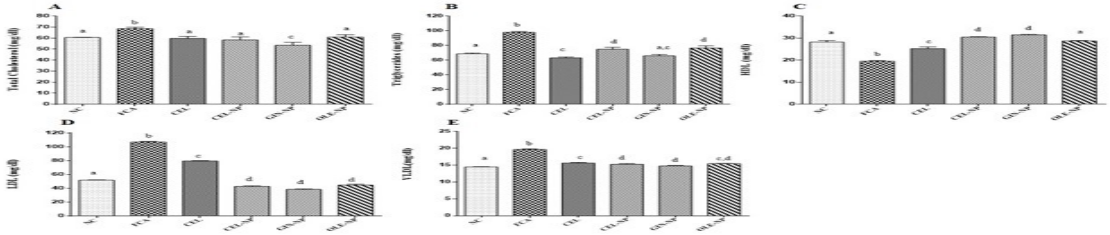

Fig. 6: Effect of nanoformulations on lipid profile in rheumatoid arthritis in rats(A):Reduced effects of treatments on total cholestrol level in FCA induced arthritic rats (B): Reduced effects of treatments on total triglycerides level in FCA induced arthritic rats (C): Improved effects of treatments on HDL level in FCA induced arthritic rats (D): Effect of nanoformulations on LDL levels of normal control, arthritic control and other treatment groups (E): Mean VLDL levels of normal control, arthritic control and other treatment groups. Results are compared with normal control, arthritic control, and standard drug. **[a-d]** superscript present that they are significantly different from each other ($P<0.05$).

symmetric stretching at 1103.28 (S=O2) and asymmetric stretching at 1346.31 (C-N). CEL-NP showed absorption bands at 2360 which represents C-O group. The absorption bands at 1481.33 (C-N) and 1151.50 (S=O2) confirmed the presence of celecoxib. FTIR spectrum of oleanic acid showed asymmetric stretching at 2939.52 (C-H) bonds. The absorption band showed stretching of C-O bond at 1683.86. C-H deformation at 1386.82 and asymmetric deformation peek at 1463. FTIR analysis of gingerol showed stretching at 1716.65 (C-O) bond while absorption bands represent C=C bond at 1384.89. GIN-NP absorption bands represent OH group at 3356.14 and stretching of C=O at 1653 (Chantarodsakun *et al*., 2014). The results found in our study were comparable with those of (Emami *et al*., 2015 and Ghosh *et al*., 2016).

The use of nanoparticles significantly reduced the weight reduction in experimental arthritic rats. Weight reduction is an important feature of arthritis as it is directly proportional to inflammation. Other Contributory factors include decreased food intake, hyperalgesia, allodynia, distress, decreased metabolism of lipids and proteins, muscle proteolysis and decreased intestinal absorption. The use of nanoparticles significantly restored the weight to normal owing to reduced inflammation as revealed in previous studies (Jadhav & Vavia 2020).

The use of nanoparticles significantly reduced the increased paw measurement of arthritic rats that is caused by edematous swelling due to inflammatory mediators such as IL-1, IL-6 and TNFα released from monocytes and macrophages causing increased interleukins infiltration resulting in vasodilatation and edema (Voon *et al*., 2017). The therapeutic use of nanoparticles restored the paw measurement of arthritic rats owing to reduced inflammatory signs i.e. edematous swelling as evidenced from results of previous studies (Jadhav & Vavia 2020).

Arthritic score is an index for determining the severity of inflammation. The arthritic score was significantly raised in experimental arthritic rats. After therapeutic use of nanoparticles, the arthritic score significantly decreased as the inflammatory signs showed a significant reduction due to decreased release of inflammatory mediators like TNFα as evidenced in the results of previous studies (Jadhav & Vavia 2020).

Anemia is another clinical feature of rheumatiod arthritis owing to erosion of joints and other tissues. The arthritic group showed decreased Hb and hematocrit values. The therapeutic use of nanoparticles restored the values of Hb and hematocrit. There was a significant rise in ESR and WBCs values owing to inflammation which results in increased infiltration of leukocytes. The nanoparticles exhibited a significant decrease in inflammatory mediators leading to decrease in leucocytes resulting in decreased inflammation. Therefore, ESR, WBCs values were significantly decreased in arthritic rats after therapeutic use of nanoparticles. Our results were comparable to those of other previous studies as well (Janakiraman *et al*., 2020).

Levels of serum lipid profile showed a significant increase in arthritic group of rats while after the therapeutic use of nanoparticles, the levels of triglycerides and serum cholesterol were decreased as evidenced in a previous study (Aryaeian *et al*., 2020).

CONCLUSION

So the study concluded that celecoxib, gingerol and oleanic acid, when loaded with PLGA and coated with chitosan, showed excellent results in therapeutic efficacy. This therapeutic efficacy might be due to controlled, targeted and prolong release of drug to the inflamed joints. The sustained and targeted release of PLGA nano-particles has excellent results in treating experimental arthritis. So this approach of targeted and sustained release of drug using nanoparticles can be used as a successful strategy in treatment of other inflammatory diseases such as Atherosclerosis, granulomatous diseases and inflammatory bowel disease

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