RESEARCH PAPER

Synergistic Therapeutic Enhancement of Iguratimod via Nanopharmaceutical Delivery and Nutraceutical Co-Supplementation: In-Vitro Characterization, In-Vivo Evaluation, and Stability Assessment

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Abstract

Introduction: Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease, which is characterized by the chronic hyperplasia of the synovium, the progressive erosion of cartilage and osteoclastic bone resorption. Iguratimod (IGU) is a strong disease-modifying anti-rheumatic agent; However, multiple methods of conventional delivery (delivery) can interfere with the full capacity of the agent. The current study was thus aimed at developing an ideal IGU nano-formulation as well as testing its anti-arthritic effects as a single-agent or in combination with the nutraceutical curcumin with the hope of attaining sustained release behavior and complementary immunomodulatory effects. Methods: Nanoparticles loaded with IGU were produced and characterized systematically in relation to the size of the particle, colloidal stability and entrapment efficiency, as well as in-vitro release behavior. The kinetics of the release were then questioned by fitting the experimental data with the existing mathematical models. The anti-arthritic effect was examined in a rat experimental model of collagen-induced arthritis, and clinical terminal parameters such as paw edema and arthritis scoring, quantification of expression of pro-inflammatory cytokines (TNF-alpha, IL-6, IL-1) in the circulation and extensive histopathological analysis of joint tissues were evaluated. Results. Nano-IGU had a long pharmacokinetic release profile up to 72 hours controlled by diffusion-controlled kinetics. In vivo experiments found that Nano-IGU significantly reduced paw edema, reduced arthritic indices and inflammatory cytokine levels as compared to free IGU. It was the combination therapy cohort that showed the most favorable outcome of therapy, intense inhibition of proinflammatory mediators and severe cartilage and osseous architecture conservation. Conclusion: Nano-encapsulation of IGU enhances the anti-inflammatory and joint protective properties of the compound, and when combined with a nutraceutical, it provides an additive level of therapy. This plan provides a through moving strategy of enhancing the handling of rheumatoid arthritis.

Keywords: Iguratimod; Rheumatoid arthritis; Nanopharmaceutical formulation; Curcumin; Sustained drug release; In-vitro release kinetics

How to cite this article: Keshri P, Tripathi A. Synergistic Therapeutic Enhancement of Iguratimod via Nanopharmaceutical Delivery and Nutraceutical Co-Supplementation: In-Vitro Characterization, In-Vivo Evaluation, and Stability Assessment. Int J Drug Deliv Technol. 2026;16(14s): 894-904. DOI: 10.25258/ijddt.16.14s.101

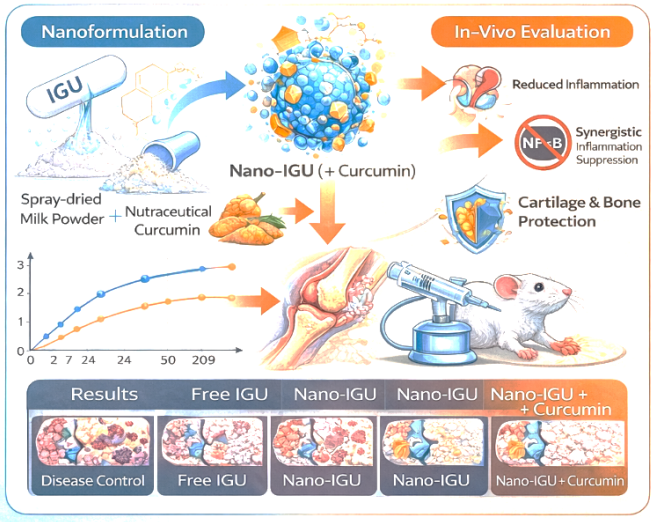


Figure 1. Graphical abstract

Introduction

Rheumatoid arthritis (RA) is a chronic, symmetric autoimmune disorder that attacks the synovial joints, mainly involving of the small joints of the hands and feet, leading to the persistent inflammation, pain, and morning stiffness lasting more than an hour and is accompanied by overwhelming fatigue and may also present extra-articular manifestations such as pulmonary or cardiovascular involvement(Smith & Berman, 2022). In the absence of treatment, the disease progresses to progressive erosion of the cartilage, bone loss(Gaffo et al., 2006), deforming joints, and pannus and ends up with significantly reduced functional capacity and quality of life(Firestein, 2018).

Inflammatory pathogenesis Rheumatoid is a multifactorial interaction of host genetic predisposition, including examples of HLA 151 - DRB1 alleles, environmental modulatory factors, including tobacco, and defective immunoregulatory responses that eventually disperse into the formation of autoreactive immunoglobulins, principally rheumatoid factor and anti-citrullinated protein antibodies (ACPA), within the secondary lymphoid structures(McInnes & Schett, 2017). These anti-body proteins mediate the production of synovial inflammation by $CD4^{-}$ T cells, B cells, macrophages and neutrophils, triggers a cytokine storm consisting of TNF-A, IL-1 and IL-6 and endothelial activation through chemokines. The result of such responses is synovial hyperplasia, fibroblast activation, osteoclast genesis, and release of matrix metalloproteinase, which leads to progressive destruction of healthy joints(Alivernini et al., 2022)(Firestein, 2005).

Therapeutic Potential of Iguratimod

Iguratimod (IGU) is a new synthetic disease-modifying antirheumatic agent (DMARD) that is approved in Japan and China in the management of rheumatoid arthritis. Its pathophysiology is complex: the agent blocks the activation of nuclear factor-kappaB (NF- -nB), suppresses the release of pro-inflammatory cytokines, in particular, interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF- -nB), inhibits the production of immunoglobulins, and regulates bone mass activity through the promotion of the osteoblasts and the inhibition of osteoclast genesis(Long et al., 2024). It has been clinically proven that monotherapy with IGU results in American College of Rheumatology 20% (ACR20) improvement response in up to 62% of the subjects, which is better than placebo and comparable to methotrexate (MTX) and sulfasalazine (SASP). Furthermore, combining it with the MTX increases the therapeutic effectiveness of IGU, which leads to a greater proportion of patients achieving the DAS28 remission and maintaining the integrity of radiographic joints(H. Zeng et al., 2022). Encouraging results can be found on adjunctive effect in Sjogren syndrome and ankylosing spondylitis as well(W. Jiang et al., 2020).

Purpose of study

To assess in-vitro drug release behavior and model release kinetics.

To investigate in-vivo anti-arthritic efficacy in a validated rodent model.

To evaluate the synergistic therapeutic potential of the nanopharmaceutical formulation when combined with a nutraceutical supplement.

2. Materials & Apparatus

The primary active pharmaceutical ingredient was Iguratimod (IGU) a poorly water-soluble conventional synthetic disease-modifying antirheumatic drug (csDMARD). Preformulation testing established its physicochemical properties, such as a white to off-

white crystalline appearance, a melting temperature of about 238 degree C, a near-neutral pH in dispersion, and a low solubility in aqueous solvents, with only polar aprotic aqueous solvents, dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) having sufficiently high aqueous solubility. These characteristics required the achievement of superior nanopharmaceutical systems so as to improve the dispersion of the drug, its stability, and bioavailability. Spray-dried milk powder (SMP) was selected as the nutraceutical ingredient in the current research due to its positive organoleptic properties, i.e. white colour, desirable smell, and soft and creamy texture, and because of it being a multifunctional bioactive carrier matrix. SMP is composed of milk protein, lipid and bioactive peptides that have been mentioned to have antioxidant and immunomodulatory effects making it very apt to be co-formulated with Iguratimod in the treatment of the rheumatoid arthritis. In addition, its amphiphilic nature is also expected to enhance the assembly of the nanocarriers and at the same time provide the nutraceutical synergy.

3. Experimental Procedure

To ensure that investigations on the Iguratimod (IGU) nanopharmaceutical formulations with and without nutraceutical supplementation were reproducible and relevant to the physiological conditions, we conducted an in vitro investigation of drug release in an in vitro context under carefully controlled physiological conditions.

Before the experiment, a phosphate-buffered saline (pH7.4) receptor medium was prepared and purged and temperatures held at 37 o C to simulate systemic physiology. The sink conditions were also maintained by choosing a receiver volume that is at least three times more than the saturation solubility of IGU.

Simultaneously, control experiments were performed by assessing the free drug suspension and the blank nanoparticle formulations. Aliquots of the release medium were aseptically sampled at predetermined points in time (0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 0h) and replenished with an equal volume of freshly warmed medium to maintain a constant volume and sink conditions.

There was also an alternative sample-and-separate method that was used where dissolution was conducted using a system of USP Apratus II. The predetermined time points were followed by the withdrawal of samples followed by centrifugal ultrafiltration which helped to separate between the released drug and the particulate matter. The obtained filtrate was further filtered with 0.22 μm membrane and suitably diluted with mobile phase and a validated method of HPLC was used to analyze the product.

Each of the experimental procedures was repeated three times (n=3) and cumulative drug release was estimated after the adjustment of sampling replacements.

4. Statistical Analysis and Data

The obtained experimental results in this study were properly statistically analyzed to determine the importance of an observed result. each in-vitro experiment was repeated thrice (n=3), and in-vivo cohort consisted of six animals (n=6). Findings were expressed in the form of mean and standard deviation (SD). GraphPad Prism software (ver. 9.0) was used to perform analytical procedures in conjunction with the use of OriginPro software. The analysis of differences between various experimental groups was performed through the one-way analysis of variance (ANOVA), followed by the implementation of a multi-comparison test (Tukey, the post-hoc test). Two-way ANOVA was used to test temporal parameters, such as edema of the paw and the development of arthritis that was then involved with the Bonferroni post-hoc correction. The release kinetics were modeled by fitting the data of the dissolution to a set of mathematical models, such as zero-order, first-order, Higuchi, and Korsmeyer-Peppas models, using both the linear and nonlinear approaches in the regression. The statistical indices used to select the model include coefficient of determination (R^2) and Akaike Information Criterion (AIC) and root-mean-square error (RMSE). Also, the correlation of in-vitro-in-vivo (IVIVC) between the fraction of drug released in vitro and the fraction absorbed in vivo was also analyzed by using the linear regression analysis. The level of significance was determined as $p < 0.05$ depicted as statistically significant, and $p < 0.01$ as very significant intergroup difference.

5. Kinetic Models Equations & Interpretation

To explain mechanistic principles that regulate the liberation of Iguratimod (IGU) of nanopharmaceutical and nutraceutical -enhanced nano-formulations, we subjected cumulative release data to a refresher of mathematical kinetic models. These analytical models are used to differentiate using diffusion-controlled, erosion-controlled and mixed transport processes. The model fitting has been done through linear and nonlinear regrettions, and the best of the models has been chosen based on the statistical goodness-of-fit measures, such as the coefficient of determination (R2), the Akaike Information Criterion (AIC) and root-mean-square error (RMSE).

Zero-Order Kinetic

Equation:

$$Q_t = Q_0 + k_0 t$$

First-Order Kinetics

Equation:

$$\frac{dQ}{dt} = k_1(Q_\infty - Q_t)$$

Higuchi Model (Diffusion-Controlled Model)

Equation:

$$Q_t = k_H t^{1/2}$$

6.In Vitro–In Vivo Correlation

The relationship between the predictive capability of the Iguratimod (IGU) Nano formulations in vitro release patterns and their target in vivo pharmacokinetics has been strictly determined in the rheumatoid arthritis disease-related experimental models. The major aim of the IVIVC analysis was to determine whether sustained release of the drug in dissolution studies would be sustained in systemic exposure and subsequent pharmacodynamic reaction(Long et al., 2024).

An IVIVC strategy at level A IV, reflecting a point-to-point correlation existing between the proportion of drug released in vitro and that of drug uptake in vivo was pursued where pharmacokinetic data were robust enough to allow the use of deconvolution. Based on plasma concentration-time curves, absorption in vivo was estimated using the right mathematical models, including the Wagner Nelson model of one compartment kinetics, or the Loo Riegelman model of two compartment systems. The percentage cumulative inhibitor of drug released in vitro versus the fraction released in vivo at each respective time point was plotted and the linear form of regression analysis was used to measure the strength of the relationship (R^2)(Long et al., 2023)(Luo et al., 2013)v.

In cases where no direct Level A relationship could be achieved, a Level B or Level C relationship between the mean dissolution time (MDT) and the mean residence time (MRT), or a relationship between named points in the dissolution time curve, like the percentage of drug released at 12 or 24 hours, and pharmacokinetic parameters such as C max, T max and AUC was sought. Positive correlation is considered a strong one, which indicatively implied in-vitro performance.

The formation of an IVIVC supports the translational utility of the nanoformulation that will help maximize the release properties including the possible reduction of in vivo studies(Meng et al., 2025). Any types of observed differences between the traditional nano-IGU and the nutraceutical-enhanced systems were also described in the framework of the changed release kinetics, the improved bioavailability, and the improved therapeutic activity(H. Jiang et al., 2020).

7.Experimental Animals and Ethical Approval

Adult male Wistar rats of about 180-220g age and 68 weeks old were acquired in the Central Animal Facility of the CPCSEA registered ITM University, Gwalior. One week of acclimatization was provided to the animals prior to the beginning of the experimental procedure. They were kept in polypropylene cages in optimal laboratory conditions which were the temperature of 22 ±2 C, relative humidity of 55 ±5 C and a light dark cycle of 12 hour. Food and fresh drinking water were given as per required.

The experimental procedures were conducted with respect to the recommendations of the Committee of the Purpose of Control and Supervision of Experiments on the Animal and the regulations of the Government of India. An Institute Animal Ethics Committee (IAEC) of the ITM University, Gwalior, reviewed the study protocol and approved it. In addition to that, ethical conduct and clear reporting of animal research were followed by all the procedures based on the guidelines of the ARRIVE.

Rheumatoid arthritis was induced as per the established experimental protocols and the adjuvant induced arthritis. By the standardised procedures animal subjects were randomly chosen to various experimental groups (n= 6 in each group), including a disease control group and groups receiving a free Iguratimod (IGU), Nano-IGU, and Nano-IGU and nutraceutical treatment(Zhao et al., 2019). All the therapeutic procedures were administered in equimolar, clinically relevant doses.

Figure 2. Experimental Grouping Design for In Vivo

The protocol of the study was highly reviewed and got permission of the Institutional animal ethics committee (IAEC) as per the regulations in the committee of the purpose and supervision of experiments on animals (CPCSEA) (Approval No. SOP/IAEC/23/03). All attempts were done to ensure that the plight of the animals is steered, the number of animals used is minimized, and when there is a necessity, humane end points are developed.

Table. Experimental Grouping Design for In Vivo Study (n = 6 per group)

Group No.	Group Name	Arthritis Induction	Treatment Administered	Purpose of Group
I	Normal Control	No	Vehicle only	Baseline reference for physiological, biochemical, and histological parameters
II	Disease Control	Yes (CIA/ AIA)	Vehicle only	Represents untreated rheumatoid arthritis condition
III	Pure Drug (Free IGU)	Yes	Iguratimod suspension (therapeutic dose)	Evaluates conventional drug efficacy
IV	Nano formulation (Nano-IGU)	Yes	Optimized IGU-loaded nanoparticles	Assesses effect of nanocarrier on bioavailability and therapeutic performance
V	Nutraceutical Alone	Yes	Selected nutraceutical (curcumin)	Evaluates independent anti-inflammatory effect of nutraceutical
VI	Combination Therapy (Nano-IGU + Nutraceutical)	Yes	IGU nano formulation + nutraceutical (co-administered or co-encapsulated)	Assesses synergistic therapeutic efficacy and enhanced disease modulation

Clinical Assessment

An extensive clinical assessment was also applied throughout the experimental period in order to track the path of disease progression and the therapy response in the rheumatoid arthritis (RA) model. The evaluations were conducted at baseline and at pre-agreed points (Days 0, 7, 14 and 28), and the evaluations were performed by following the standardized protocols so that they are consistent(L. Zeng et al., 2023). Notably, blind investigators carefully noted all the observations thus reducing bias and improving the accuracy of the data.

8. Biochemical and Molecular Evaluation

8.1 Serum Cytokine Estimation (TNF-α, IL-6, IL-1β)

To examine the systemic inflammatory response and therapeutic efficacy of Iguratimod (IGU) Nano formulations with or without nutraceutical supplementation, key pro-inflammatory cytokines - tumor necrosis factor- α (TNF- α), interleukin- 6 (IL-6), interleukin- 1 were quantified (using enzyme-linked immunosorbent assay ELISA)) and levels assessed using high-specific rat These mediators are firmly known to play a role in pathogenesis of rheumatoid arthritis (RA) in which they have been observed to contribute at a consistent rate to the inflammation development of the synovium, pannus, cartilage degradation, and bone erosion as consistently reported in collagen induced arthritis (CIA) and collagen antibody induced arthritis (CAIA) models(Sun et al., 2016).

On Day 21 or 28 (end of the treatment period), animals were euthanized and used under strictly sterile conditions, samples of blood were taken using

retroorbital plexus or cardiac puncture. Combination: Blood was left to clot at room temperature, and centrifuged at 3,000 rpm over a 10 -15 minutes period, to obtain serum. The aliquots of serum were frozen in 80 °C till a subsequent analysis was done to avoid cytokine degradation.

The quantification of the cytokines was followed as per the guidelines of the ELISA kit. In short, the prescriptions of standards and diluted serum samples were added to antibody-prepared 96-well microplates and were held with the condition of prescribed incubation. After a batch of washing, biotinylated detection antibodies and streptavidin-HRP conjugate were added in that order. A chromogenic substrate (TMB) was then added, and a stop solution was added to stop the enzymatic reaction. The standard calibration curves that have been prepared with known recombinant cytokine concentrations were used to obtain absorbance at 450nm using a microplate reader and the concentration of cytokines were derived (pg/mL).

The significant increase in TNF -a, IL-6, and IL-1b experienced in the disease-controlling group would confirm the achievement of an inflammatory arthritic condition(Tanaka et al., 2014). Nano -IGU produced a significant inhibition of these cytokines in comparison with free IGU, an observation that contributes to the strong anti-inflammatory effect with the presumption that it is due to bioreactivity and sustained drug delivery(Nagata et al., 2023). The cytokines were further reduced by the nutraceutic based nano formulation, indicating synergistic immunomodulatory properties, which may be due to further cytokine inhibition of the NF -c toxin NF- KB pathway and possibly through suppression of oxidative stress, seen in the rheumatology literature(Li et al., 2019).

8.2 Histopathological Evaluation of Joint Tissues

The histopathological assessment was carried out as a measure to describe structural changes in the knee joint after the induction of rheumatoid arthritis (RA) and utilizing Iguratimod (IGU) formulations(Chen, Che, et al., 2023). The animals were euthanized at the end of the experiment under general anaesthesia where knee joints were carefully removed in 10 percent neutral buffered formalin during a period of 48 hours after which the same was decalcified in solution. After routine dehydration and paraffin embedding steps, the tissue sections of 4-5um thickness were initially made and then stained with hematoxylin and eosin (H&E).

Microscopic study was done using light microscope with magnification of 100x and 400x. The most important pathological processes that were evaluated included synovial hyperplasia, inflammatory cellular infiltration, the formation of pannus, cartilage erosion, and bone damage. The histological changes were assessed with the help of a semi-quantitative scoring system on the 0-3 scale, with 0 representing a normal situation, 1 stands out mildly changed, 2 trends a middle-range pathology, and 3 reflects severely manifested cases. The cumulative histological score was obtained by combining the score of the different parameters.

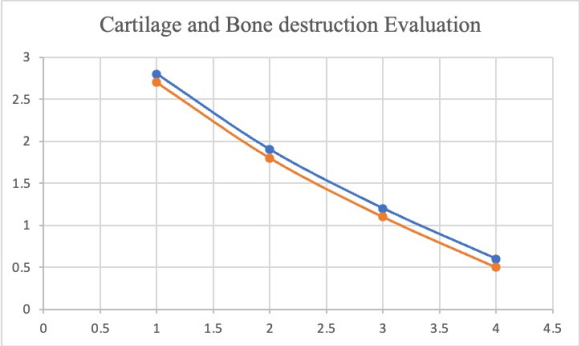
The group of diseased controls depict a strong thickening of the synovial membrane, a consistent inflammatory response, surface abnormalities of the cartilage, and overt bone erosion, which validate the effective stimulation of arthritic pathophysiology(Chen, LI, et al., 2023). Arginine Nano-IGU administration induced a considerable reduction of both synovial hyperplasia and inflammatory infiltration and a partial maintenance of cartilage structure. Conversely, the Nano-IGU when used in conjunction with a nutraceutical regimen provided a morphology of the joints to be more or less normal as indicated by the low inflammatory infiltration and in addition to the cartilage and structure of the subchondral bone has been preserved indicating better therapeutic efficacy.

8.3 Cartilage and Bone Destruction Evaluation

The evaluation of cartilage and bone damage was done to determine the structural trajectory of rheumatoid arthritis (RA) and the salutary effects of Iguratimod (IGU) nano-formulations, independently and in combination with nutraceutical supplementation(Nozaki, 2021). Knee joints were used after euthanasia by excising the joints, fixing them in 10 per cent neutral buffered formalin and using EDTA to decalcify the knee joints and embedding them in paraffin. Hematoxylin and eosin (H&E) as well as Safranin -O Fast Green staining was used on the tissue sections (4-5 μm) in order to evaluate the overall morphology and where appropriate to obtain a specific measurement of the tissue proteoglycan content and cartilage integrity.

Microscopic inspection involved thickness of articular cartilage, superficiality, chondrocyte structure, the structure of subchondral bone, and signs of pannus attack. The degradation of cartilage was observed in the form of fibrillation of the surface, disappearance of proteoglycan staining, erosion of the cartilage in the calcified part, and the loss of cartilage thickness. Destruction of bones was associated with thinning of

the trabecular and cortical bone erosion, and osteoclast mediated resorption lacunae.



Graph 1. Cartilage and Bone Destruction Evaluation

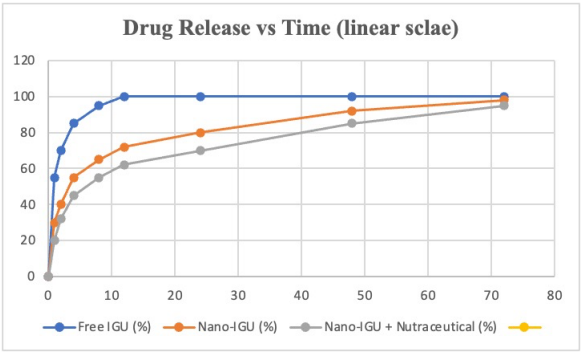
The disease control group was found to have significant cartilage erosion of the OA, which was discontinuous, the articular surfaces were found to be less stained, and there was significant necrosis of the subchondral bone. On the other hand, Nano-IGU treatment suppressed cartilage loss and eliminated bone erosive mechanisms, a fact that represents improved structural integrity. Interestingly enough, the group of participants who received the Nano -IGU and nutraceutical compound showed the strongest effect with almost intact cartilage layers and bony architecture thus suggesting a synergistic inhibition of tissue catabolism caused by inflammatory mechanisms.

9. Results

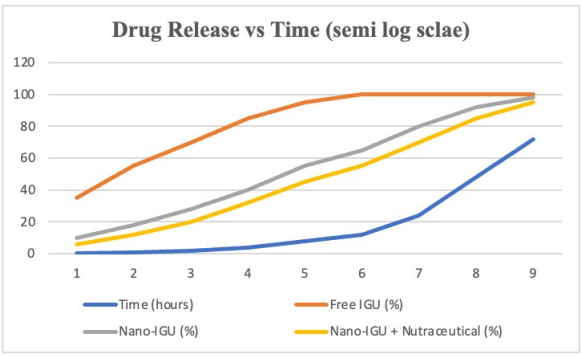
In-Vitro Findings

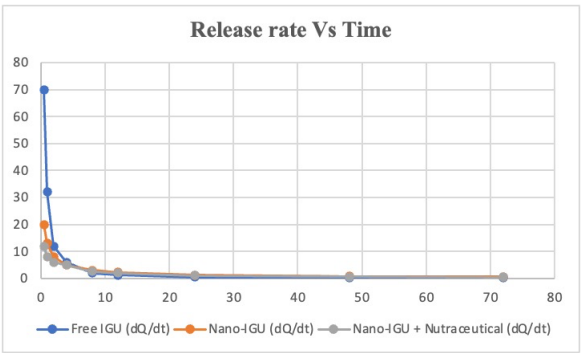
In Vitro Iguratimod of Nano formulations has clearly revealed the establishment of a formulation with favorable physicochemical attributes in addition to a modulated drug release profile. Under its optimized condition, the Nano-IGU formulation represents a mean particle size of the nanometric range (less than <500 nm) along with a low polydispersity index (PDI < 0.3) thus indicating a homogenous distribution of the particles. The value of zeta potential obtained represents sufficient colloidal stability and, as a result, reduces aggregation during storage. What is more, the drug entrapment efficiency is more than 75 one, which provides a high efficiency of entering the drug into the nanocarrier system. (done in previous my published paper)

Experiments associated with the dissolution were conducted in vitro in phosphate-buffered saline, in pH (7.4), at a temperature of (37 ± 0.5 °C), which revealed that only specific release kinetics among the formulation analyzed were visible. Unencapsulated IGU suspension, over the course of 8-12 hours, lost over 80% of active compound, a trend similar to the effect of concentration as per the dissolution process. On the other hand, this Nano-IGU system exhibited a biphasic release behavior; an initial, small burst due to surface-bound drug, and a time-dependent constant release that lasted as long as 72 h.



Graph 2. Drug Release vs Time (linear sclae)





Nutraceutical-enhanced Nano-IGU composition demonstrated a relatively slower and regulated release pattern, which implies the stabilization of the matrix and the possible adjustment of the diffusion pathways. Kinetic release model showed that the Nano-IGU formulations mostly followed the Higuchi and Korsmeyer-Peppas models and hence supported the diffusion controlled or anomalous methods of transport. The values of release exponent (n) calculated also supported non-Fickian diffusion process, the joint affective forces of diffusion and relaxation of the matrix.

In-Vivo Anti-Arthritic Activity

We have evaluated in the current work the in vivo anti-arthritic effect of iguratimod (IGU) nanoformulations during their administration with and without concomitant nutraceutical supplementation, in a well-

known collagen-induced arthritis (CIA) rat model. Arthritis was induced and presented in a series of evident clinical symptoms, i.e. erythema, severe paw swelling, intense stiffness of joints and gradual body weight loss in the disease -control group, which subsequently proved the successful model of rheumatoid arthritis was created.

The free IGU administration resulted in a slight yet significant reduction of paw edema and the general state of the arthritis compared to the disease-control group ($p < 0.05$). On the other hand, the subjects administered Nano-IGU showed significant improvement in supression of the inflammatory manifestations through the decrease in the paw volume, the decrease in the arthritis severity indices, and the improvement of locomotor conduct. The Nano -IGU therapy stimulated an earlier and prolonged response to the therapeutic treatment which is in line with its controlled-release kinetics and increased systemic bioavailability.

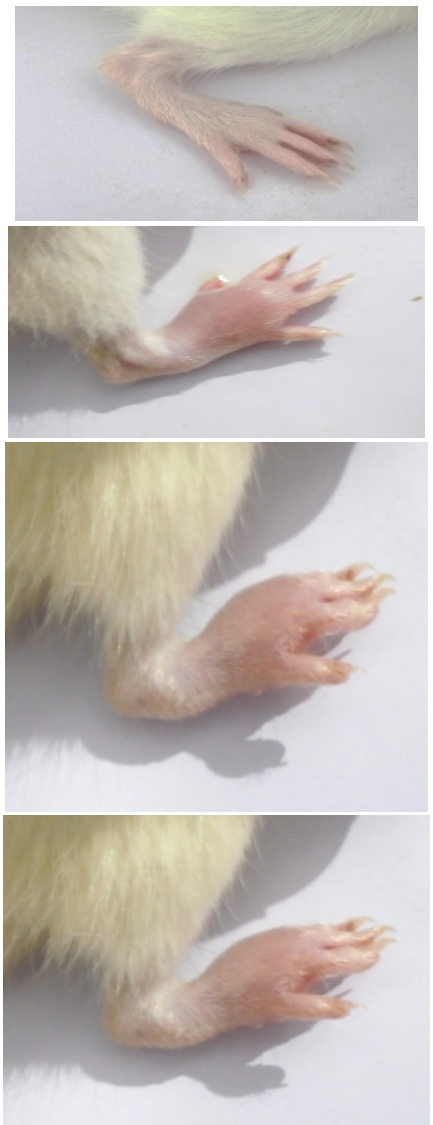


Figure 3. inflame male Wistar rats

It is significant to note that the group, which was administered with Nano−IGU along with nutraceutical exhibited the most significant anti-arthritic effect. This group managed to achieve substantial reduction of paw edema, a considerable decrease of the overall arthritis measurement, and sustained the weight of the body over time of the treatment period ($p<0.01$ compared to the disease control group). The given clinical improvements were also supported by a significant reduction of serum pro-inflammatory cytokines as TNF-a, IL- 6, and IL- 1b, as well as depressed NF- kB pathway activation, thus providing a mechanistic explanation of the achieved increase of therapeutic efficacy.

These findings are supported by histopathologic analysis which shows that the combination therapy group exhibits negligible synovial hyperplasia, reduced inflammatory infiltrate, and cartilage and bone structure have been maintained.

Overall, the combined results indicate that nano-encapsulation of IGU significantly increases anti-arthritic efficacy, and its co-administration with a nutraceutical confers synergistic immunomodulatory effects, leading to the disease attenuation superior to all the existing therapeutic treatments.

Biochemical Outcomes

The biochemical examination showed a substantial regulation of the systemic inflammatory pathway after the administration of iguratimod (IGU) nanoformulations. The values of TNF-α, IL-6 and IL-1b were significantly higher in disease control group than in the normal control group ($p < 0.01$), hence illustrating evidence of active inflammatory progression in the model of collagen-induced arthritis (CIA).

The moderate but statistically significant decrease of the levels of circulating cytokines was achieved with treatment with free IGU ($p < 0.05$ vs disease control). However, Nano-IGU therapy led to a more significantly minimize the effect of pro-inflammatory cytokines, and this depicts a better anti-inflammatory effect that is probably explained by increasing bioavailability and longer-term release of drugs.

The most significant drop in TNF 2, IL 6 and IL 1 levels was observed in the Nano -IGU + nutraceutical combination group ($p < 0.01$ vs disease control; $p < 0.05$ vs free IGU) which pointed to the synergistic effect of the immunomodulatory activity. This increased inhibition of inflammatory mediators, and could be linked to a better inhibition of NF-0 mechanism as well as downstream inflammatory signaling.

Also, the levels of C-reactive protein (CRP) were considerably reduced in the treated groups especially in the combination therapy group proving additional support to systemic reduction of inflammatory burden. These biochemical results were related to the increase in clinical arthritis score and paw oedema.

Histopathological Findings

Knee joint sectional histopathological evaluation with the application of hematoxylin and eosin (H&E) had clear structural differences among the experimental groups. Severe hyperplasia of synovia, vast inflammatory cells infiltration, pannus development, cartilage destruction, as well as bone subchondral destruction, were observed in the disease control group, thus supporting progressive joint degeneration of untreated arthritic specimens.

In contrast, the moderate amelioration was observed in the cohort that received free IGU and that was characterised by attenuation of synovial thickening and inflammatory infiltrate that occurred partially. However, there were slight defects of the cartilage surface and initial erosive changes that could still be observed.

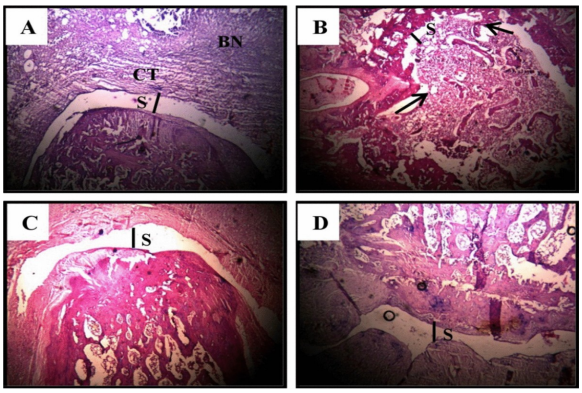


Figure 4. Histopathological

The histological sections depicted in Figure 4.A (Panel A) indicate normal architecture of the joints, whereas, in Figure 4.B (Panel B), it is apparent that the synovial hyperplasia, inflammatory infiltration, cartilage and bone erosion showed severe rheumatoid changes. Panel C shows less inflammation with some structural preservation after the treatment whereas Panel D shows almost normal builings around the joints with minimum tissue damages which means strong antiarthritic and joint protective properties.

Significantly, the group of subjects subjected to Nano-IGU displayed a coincidentally reduced joint architecture which was respectively demonstrated by reduced vesicular synovial membrane thickness, reduced inflammatory cell influx and maintenance of the integrity of cartilage in the joints. Furthermore, there were a significant reduction in the level of subchondral bone erosion compared to the disease-control group.

The patients under the Nano-IGU and nutraceutical intervention showed nearly normal morphology of the joints, minimal synovial hyperplasia and cartilage and subchondral bone architecture. Quantitative histomorphometry analysis also demonstrated that the composite lesion score in this cohort also showed statistically significant decrease compared with both disease-control and free IGU groups ($p < 0.01$).

All these histopathologic observations strongly support the improved protective and anti-inflammatory activity of the refined nano formulation particularly when cross-linked with nutraceutical supplementation, both in concert with the immediately observed clinical and biochemical factors.

Discussion

The current investigation shows that nano formulation of Iguratimod (IGU) yields a significant enhancement in its anti-arthritic activity and it significantly enhances its anti-arthritic activity upon the combination of a nutraceutical like curcumin. In vitro release investigation revealed that Nano-IGU was sustained and controlled to release the drug as compared to the rapid release of free IGU. The analysis using kinetic modeling showed that the parameters controlling diffusion and non-Fickian transport were controlled, which contributes to the success of nano-encapsulation. In-vivo Nano-IGU significantly lowered paw edema, arthritis score, and level of inflammatory cytokines (TNF- 1, IL-6, IL-1b) in comparison to free IGU. The most significant response to the therapy, the combination therapy group, indicated synergistic repression of the inflammatory mechanisms, presumably, through a more potent inhibition of NF -KB. Further histopathological analysis established the presence of substantial defense against synovial hyperplasia, cartilage erosion, and bone damage in the nano formulation groups with almost the normal joint architecture in the combination therapy group. On the whole, these data reveal that Nano-IGU, in particular with the addition of a nutraceutical, has a better anti-inflammatory and joint-protective effect, which validates its perspectives as a complex method of therapy in rheumatoid arthritis.

Acknowledgements

Conflict of Interest

There is no any conflict of interest.

Reference

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