Article

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Development and Evaluation of Apocynin Loaded pH-Sensitive Nanoparticle Based Hydrogel for the Management of Rheumatoid Arthritis

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Abstract: The aim of the current study was to develop and evaluate the therapeutic potential of apocynin (APO) loaded pH-sensitive nanoparticles (NPs) based transdermal hydrogel for management of rheumatoid arthritis (RA). Slightly modified nanoprecipitation technique was used for preparation of polymeric nanoparticles. Optimization was done through design expert software. Optimized APO-NPs were loaded into carbopol-934 based hydrogel as final dosage form and further studied for physicochemical properties. Optimized APO-NPs formulation had a minimum particle size 63.44 nm, polydisperibility index 0.161, and zeta potential -15mV with a maximum encapsulation efficiency of 89%. In-vitro and ex-vivo studies of APO-NPs based hydrogel was performed at pH 5.5 (pH of normal skin) and 6.8 (pH of inflammed joint) showed a pH-responsive sustained drug release and increased penetration in comparison to free APO based hydrogel. The stability studies of APO-NPs based hydrogel were done to strengthen the potential use of the prepared formulation through transdermal route. Assessment and therapeutic efficacy of the prepared pH-sensitive nanocarriers system was evaluated in chronic inflammatory RA mice model. Parameters associated with chronic inflammation were investigated including behavioral changes and histopathological, and radiological x-rays images of joints of mice paws. In-vivo study depicts improvement in behavioral parameter, decline in synovial hyperplasia and bone structure restoration. In conclusion, APO loaded pH-sensitive NPs based transdermal is a promising carrier system that can effectively manage RA.

Keywords: pH sensitive polymer; methacrylate polymers; eudragit L100; polymeric nanoparticles; apocynin; rheumatoid arthritis; carbopol-934 based hydrogel; transdermal drug delivery system

1. Introduction

RA is a chronic autoimmune disease. Its primary characteristics include severe inflammation and swelling of multiple joints and cartilage that relate with symptoms like synovitis, periarticular osteopenia and osteitis [1]. RA disease progression can gradually damage cartilage and bones and the severe inflammation is manifested by imbalance between fabrication and destruction of osteoid due to various other inflammatory cytokine mediators such as IL-6, TNF alpha [2]. The primary sites for RA include joints of knees, wrists, fingers, and legs and that may cause persistent joint pain, swelling and stiffness, and cartilage abrasion leading to permanent physical disability [3–5]. Generally, two approaches are followed for management of RA that include pharmacological and non-pharmacological treatment, depending upon the severity of condition. Initially, the basic goal

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and objectives of non-pharmacological treatment approaches is to improve the quality of life of rheumatoid arthritis patient. It should be considered the first-line treatment therapy and at this stage investigate the Signs and symptoms of the for RA patients. These may include healthy physical activity and routine exercise, and physiotherapy, and other lifestyles.

A variety of conventional pharmacological therapies like NSAIDs include flurbiprofen, ibuprofen, diclofenac, naproxen and piroxicam are the most commonly recommended first-line drugs for the symptomatic pain relief. NSAIDs act by inhibiting cyclooxygenase enzyme and non-selective NSAIDs inhibit the synthesis of prostaglandins. However, the prolong use may lead to moderate-to-severe adverse effects like gastrointestinal stomach ulceration. Similarly, conventional therapies need dose repetition which is not cost effective and has issues of dose adherence.

APO, (4-hydroxy-3-methoxyacetophenone), a phytochemical is an acetovanillone. It is naturally extracted from plants that are native to western Himalaya. It has molecular weight of 166.17g/mol [6]. APO exerts its anti-inflammatory and antioxidant effect by inhibiting NADPH, that produces super oxide O2. Apocynin protects chondrocytes from proteoglycan synthesis. It inhibits TNF-α and IL-1. APO is also selective inhibitor of another inflammatory enzyme's expression known as COX-2, which makes it a potential alternative of NSAIDs to treat inflammation and pain [7]. Though APO is a potent compound for many ailments but its promising prospective is impeded due to its poor aqueous solubility, limited oral bioavailability (8%), frequent elimination and elevated protein binding [8,9]. Eudragit L100 is an anionic, biocompatible and biodegradable pH sensitive polymer. It exhibits excellent physicochemical properties including sustained drug release and maintaining drug stability [10,11]. Eudragit contains negatively charged carboxyl group [12]. It is soluble at pH above 6.0 [13]. Eudragit L100 gets an advantage of being methacrylate polymer so it has ability to cross multiple layers of skin. To achieve targeted drug delivery of nanoparticle at inflammatory site, eudragit L100 is a competent polymer to work at inflamed pH of 6.8 in course of RA [14,45].

The APO oral delivery has some basic pharmacokinetic limitations, including increased drug degradation and poor solubility profile. It has very low bioavailability which restricts its therapeutic efficacy. The route of administration is significant in the delivery of active pharmaceutical ingredient (API) to the required targeted site. Thus, transdermal drug delivery system (TDDS) serves as an efficient alternative route, which can provide a sustained release of API with improved delivery of drug at the disease site. Additionally, it can overcome adverse effects that come with intravenous and oral routes.

Considering the side effects of administration of APO through oral route, and the advantages of pH-sensitive NP-based carrier system, in the current study, Eudragit L 100 (EL 100) was used to synthesize APO-Nanoparticles. The NPs were loaded into carbopol-934 based hydrogel system for delivery of apocynin. pH responsive biodegradable polymers can sense an environment associated with any pathophysiological circumstance and respond uniquely as comparatively non pH-stimulus responsive polymers. The pH-sensitive polymers form extremely useful nanocarriers-based delivery system having applications in improving solubility profile, enhancing bioavailability and therapeutic efficiency with reduced toxicity and cost effectiveness.

EL 100 was used to its anionic nature, biocompatibility, biodegradability, and pH-responsive profile. This polymer releases drug at pH of 6.8, which correlates to pH at the inflamed site. It exhibits excellent physicochemical properties including sustained drug release and maintaining drug stability [10,11]. Eudragit contains negatively charged carboxyl groups and the carboxyl to Ester ratio in Eudragit L 100 is 1:1. Moreover, EL 100 polymer is studied to release drug in sustained fashion from the nanoparticles and it can be as promising nanocarrier-based delivery system for the management of RA. The selection of a suitable permeation enhancer (PE) is vital to overcome the barrier function of dermal layers [15]. Nanocarrier with good permeation can increase the permeation through stratum corneum. In this study, oleic acid was used as a PE and it works by dissolving sub cutaneous lipids matrix of the skin. Unlike few other PE, oleic acid does not make skin patchy when applied topically [16].

The rationale of selecting carbopol 934 based hydrogel as final dosage form was primarily its hydrophilicity, deformability, and bio adhesion. It is biocompatible and biodegradable as well. This system provides optimal release and to maintains the minimum threshold level [17].

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2. Materials and Methods

2.1. Materials

APO (4-Hydroxy-3-Methoxyacetophenone), tween 80, monobasic potassium dihydrogen phosphate, Complete Freund Adjuvant (CFA), disodium hydrogen phosphate (Na2Hpo4), triethanolamine, carbopol 934, purchased from Sigma Aldrich. Ethanol, oleic acid, sodium hydroxide pellets purchased from Merck Darmstadt, Germany. Sodium chloride (NaCl), potassium chloride (KCL) purchased from BDH laboratory supplies Poole, England, eudragit L 100 (Evonik, Germany), dialysis membrane (Fluka, Hohenbrunn, Germany), ELISA kit (bioscience, Inc., San Diego, CA ELISA kit), Brufen Cream (Abbott Laboratories Pakistan LTD) and hydrochloric acid was obtained from Daejung Chemicals and Materials Co.Ltd.

2.2. Methods

2.2.1. Optimization and Preparation of APO-NPs

Design expert software, version 12 was used to optimize APO-NPs. 14 formulations were generated using box behnken design. Eudragit L 100, tween 80 and APO were the variables and particle size (PS), polydispersity index (PDI), zeta potential (ZP) and entrapment efficiency (EE) were the responses to be evaluated. Modified nanoprecipitation technique was used to prepare formulations. Formulation with maximum desirability was selected as optimized [8,16], as shown in Figure 1 (A). Organic and aqueous phase were prepared first with ratio of 1:2. Organic phase was prepared by dissolving 2.5% eudragit L100 and 6mg of APO in ethanol. Aqueous phase was prepared by obtaining a clear solution of 1.5% tween 80. Organic phase was injected into aqueous phase slowly keeping the stirring speed and injection rate at 1200 rpm and 0.1mL/min respectively. Nano-formulation thus obtained was then subjected to rotary evaporation to evaporate organic phase traces at 45°C temperature and 150 rpm under vacuum. Resulted nano-formulation was then centrifuged at -4°C and 15000 G for 2 hours [18–20].

2.2.2. Physicochemical Characterization of APO-NPs

APO-NPs were evaluated by Zetasizer (Malvern Instrument, Worcestershire, UK) for physicochemical properties like PS, PDI and ZP [21].

Indirect method was adopted to find encapsulation efficiency (EE) of prepared formulations. In this method, all formulations were centrifuged for 2 hr at -4°C and 15000rpm. Supernatant was analyzed through UV-Vis Spectrophotometer (UV-Vis double beam spectrophotometer spectrum 100, Dynamica, Halo DB-20 U). Supernatant was diluted with solvent to obtain absorbance at 275nm [22,23]. To calculate EE (%) equation 1 was used.

$$EE\ \% = (W1 - W2)/W1 \times 100 \quad (1)$$

W1 is the amount of drug initially added
W2 is the amount of drug in supernatant

Compatibility Studies of Nanoparticles

To verify absence of incompatibility between all the ingredients used to formulate optimized nano-formulation, Fourier Transform Infrared spectroscopy (FTIR) (model Carry 630, Agilent technologies) was performed. X-ray Diffraction (XRD) was done to confirm the amorphic or crystalline nature at angle of 20-80 θ. Differential scanning calorimetry (DSC) was performed to determine the thermal behavior [24,25].

2.2.3. Optimization and Preparation of APO-NPs Based Hydrogel

Optimization of Hydrogel

Carbopol-934 hydrogel was prepared in four different concentrations e.g. 0.5%, 1%, 1.5% and 2%. The prepared hydrogels were evaluated based on following parameters: pH, Viscosity, physical appearance and drug content.

Preparation of Hydrogel

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Blank hydrogel (1.5%) was prepared by overnight stirring of carbopol-934 in distilled water. A viscous solution was obtained as shown in Figure 1 (B). 1mL of oleic acid as PE was added and stirred until completely mixed. 1mL of glutaraldehyde is added as cross linker. 1-2 drops of triethanolamine (TEA) were added to obtain desired viscosity of hydrogel. For drug loaded hydrogel, APO-NPs were added into prepared solution and stirred again for 30 minutes before addition of 1ml oleic acid [26–28]. Likely, APO-NPs based hydrogel without PE, Free APO based hydrogel with and without PE were also prepared.

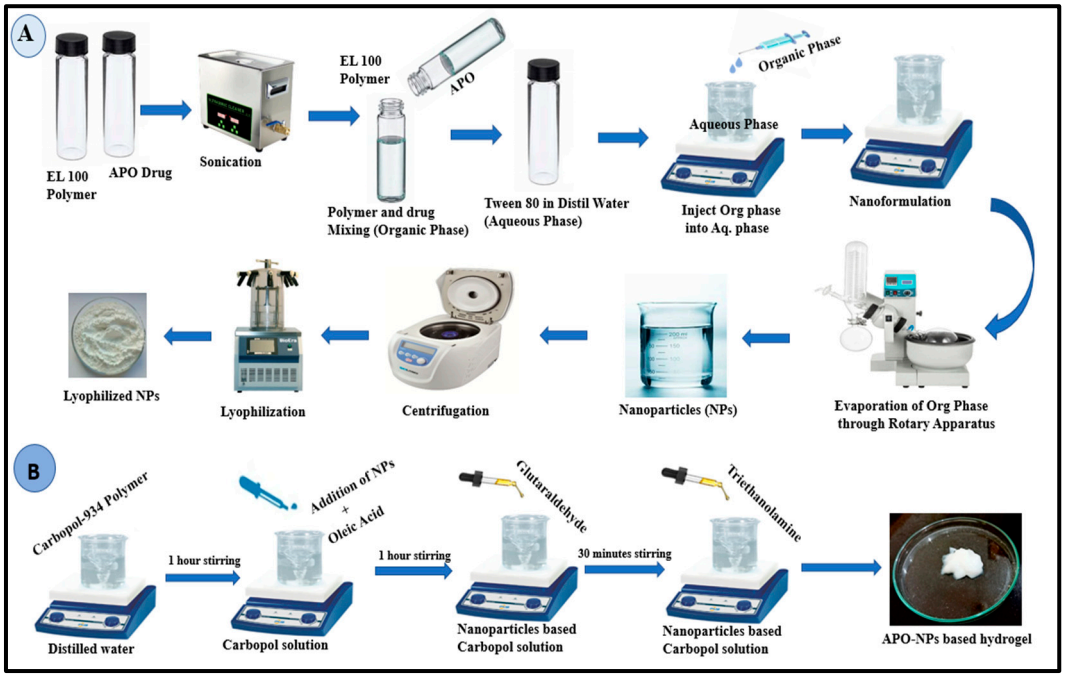

Figure 1. (A) Schematic representation of modified nanoprecipitation method for APO-NPs preparation (B) Preparation of APO-NPs based hydrogel.

2.2.4. Characterization of APO-NPs Based Hydrogel

Physical Appearance and pH

APO-NPs based hydrogel was visually observed for physical appearance. pH hydrogel was evaluated using pH meter (PHS 25CW, Bante instruments, Chicago, USA).

Drug Content

To estimate the amount of APO in prepared NPs based hydrogel, 10mL of phosphate buffer (PBS) (5.5 and 6.8) was used to dissolve 1g of hydrogel at 30°C. Absorbance was checked at 275nm using UV-Vis spectrophotometer in PBS 5.5 and 6.8 as reference individually [29,30]. Equation 2 was used to calculate the drug content:

$$\% \text{ Drug Content} = (\text{Observed amount of drug in formulation})/(\text{Theoretical amount of drug in formulation}) \times 100 \quad (2)$$

Rheology

Rheology of APO-NPs based hydrogel was checked by using Brookfield viscometer (Ametek DV3TRVCCJ0). Viscosity was determined at shear rates 1, 2, 4, 6, 8, 12, 20, 24, 40, 60, 100, 120, and 200 rpm by using spindle CPA 52 Z at 25°C temperature [31,32].

Spreadability

Glass slide method was used to determine spreadibility. A circle of diameter of 1cm was made on the lower slide and 0.5 g hydrogel was placed inside circle. Other slide was placed on it and hydrogel was pressed in between the slides with the help of 500 g weight [33,34]. Spread area of

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hydrogel before and after applied weight was measured and spreadability was calculated using equation 3.

$$Si = (\pi/4) \times d^2 \quad (3)$$

Where Si is the spread area in cm and d is the hydrogel spread diameter and Si is area spread after applied weight.

In-Vitro Drug Release Studies

In-vitro release was performed at two pH e.g., 5.5 (skin) and 6.8 (inflammation site). APO-NPs based hydrogel, free APO based hydrogel and APO dispersion were loaded in dialysis membrane (Fluka, Hohenbrunn, Germany) (individually and then placed in a beaker containing 50mL of PBS of above mention pH. Shaking water bath method was used, set at 37°C ± 0.5°C temperature and 3,035 cycles/min for 48 hours. Samples of 1mL were withdrawn from each beaker after 0.25, 0.5, 1, 2, 4, 6, 12, 24, 36, 48 hrs and replaced with 1mL of respective buffers. Withdrawn samples were analyzed [35,36].

a. Application of kinetic models on drug release statistics

Drug release from the carrier was studied using mathematical models. These included zero order, first order, Higuchi model, Korsmeyer peppas model and Hixson Crowell model. Best fit model with highest regression (R2) value was selected. These models predict possible drug release from nanoparticles as well as NPs based hydrogel [37,38].

Ex-Vivo Permeability Studies

a. Preparation of mice skin for ex-vivo permeation

Healthy mice were euthanized ethically, skin from their abdomen was separated. Hair were removed using a sharp razor and heated up to 60°C to remove the lipid layer. The prepared skin was stored in 10% formalin to avoid its degradation [39].

b. Procedure for ex-vivo permeation study

Permeation of APO through dermal layers was studied using Franz diffusion cell. Keeping the temperature at 37°C ± 0.5°C, study was performed at two different pH (5.5, 6.8) for 24 hr. Cells were filled with PBS of respective pH buffer (5.2 mL). Prepared skin was clamped between donor and receptor chamber. Four different formulations i.e., APO-NPs based hydrogel with and without PE, Free APO based hydrogel with and without PE, were placed on the skin individually. Aliquots of 1mL were withdrawn from receiver compartment of each cell using lumber puncture syringe at dissimilar time slots of 0.5, 1, 2, 4, 6, 8, 12 and 24 hr. Each cell's volume was maintained by fresh respective phosphate buffer. Samples collected were analyzed for APO concentration by using UV-Vis spectrophotometer [40,41].

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In Vivo Studies

a. Animals

For in-vivo study, male BALB/c mice of age six to eight weeks weighing 20 to 30 gm were acquired from National Institute of Health (NIH), Islamabad and housed in laboratory animal house. Standard laboratory conditions of temperature and humidity with consecutive 12 hours light and 12 hours dark cycle were provided. Acclimatization was done for one week and with standard rodent diet and water. All animal studies were approved by Bioethical committee Quaid-i-Azam University, Islamabad, Pakistan, Ref. No: #BEC-FBS-QAU2022-387.

b. Chronic CFA-induced inflammatory pain model

The anti-inflammatory and antioxidant potential effect of formulated pH-sensitive APO-NPs loaded hydrogel was evaluated against the chronic CFA-induced inflammatory pain model. Mice were randomly assorted into five groups, with five mice in each group. The CFA-induced model was employed in mice. Model was induced by injecting CFA (20 µL) into right hind paw [42]. The therapeutic potential of APO-NPs loaded hydrogel on inflammation and pain, and paw edema was evaluated for baseline purpose on day first, (before induction) and then was evaluated at pre-determined time intervals of 14,15,16,17,18,19,20 and 21 days after CFA injection. The experimental animal grouping of the current study is given below;

Group 1: Normal Control (received normal saline)
Group 2: Negative Control Group (received 20 µl CFA, intraplantar with no treatment)
Group 3: Positive Control Group (received 20 µl CFA (i.pl) and administered with the marketed Brufen cream, twice daily on dorsal side
Group 4: Free APO based hydrogel treated Group (received 20 µl CFA (i.pl), and administered with the formulated free APO based hydrogel, applied once daily
Group 5: APO NPs-based hydrogel treated Group (received 20 µl CFA (i.pl), and administered with the formulated APO NPs-based hydrogel, with P.E, applied once daily

c. Organ Sample collection

All the CFA-induced animals were anesthetized with anesthetics agent (i.e., ketamine and xylazine at 16 mg + 60 mg respectively, I.P) at the end of the activity. The blood sample of each experimental group was taken by the cardiac puncture, centrifuged at 4050 RCF at 4ºC for 5 minutes. The sample was used for biochemical assays to investigate potential effect of prepared hydrogel. Similarly, tissues and organs were isolated, washed, and stored in 10% formalin for the histopathological and radiological analysis.

d. Behavioral Parameter Assessment

i) Assessment of paw edema parameter

According to current study design and protocol, the paw edema parameter was assessed by digital thickness gauge meter (No.2046F, Mitutoyo, Kawasaki, Japan) in utilized in chronic pain inflammatory models [43]. As CFA was injected into four groups except of normal group, and the inflammation led to paw swelling and redness which is one of the key parameter assessments of RA model induction. In the chronic study, the paw thickness mice data was taken at day 0 and from 14-21 days after induction using CFA.

ii) Thermal hyperalgesia

Hot-plate method was used to measure the thermal hyperalgesia. In order to perform thermal hyperalgesia, experiment each experimental mice were kept in a beaker, on hot plate with temperature of 50°C ±0.5°C for cut off 1 minute, until they start either paw licking, paw biting and jumping behavior [44].

iii) Arthritis Index

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Arthritic index is a behavioral parameter to measure that RA model either is fully induced in experiment mice or not and the score was given to each experimental mice group are based on the paw thickness by using following arthritis index scoring scale [6,45]. According to that scale the arthritic scoring was done as per mentioned such as the 0 value representing no swelling or inflammation and 1 number illustrated = mild swelling/ redness, 2 digit value is showing erythema with mild edema and 3 number defined the pronounced edematous swelling and 4 explain joint rigidity.

iv) Mechanical allodynia

The Mechanical allodynia parameter for the formulated APO-NPs loaded hydrogel in chronic inflammatory model was measured using Von Frey filament apparatus. In this test, mice were placed on a mesh covering the plastic box, such that dorsal side of paw faces mesh floor. Different sized filaments were used to observe of mechanical pain threshold in mice [46]. Paw withdrawal threshold was estimated by observing the visual response, indicated by flicking and licking of the stimulated paw against the various filament size.

v) Radiological and Histological assessment of mice inflamed paws

The radiological and histological analysis was done to determine the pathological changes ensued in joints during development of CFA induced RA model. The paw samples of all experimental groups were collected from euthanized mice and preserved in 10% formalin. X-ray analysis was to be done examine the degree of inflammation, and bone erosion. Thus, the efficacy of formulated APO-NPs loaded hydrogel was compared with other groups. The right-hand paws were cut vertically and embedded in paraffin wax and stained with hematoxylin-eosin dye. The slides were visualized for any pathological features like infiltration of macrophages, neutrophiles and monocytes, in the stained tissues via microscope.

Stability Studies

To ensure prepared nanoparticles and APO-NPs based hydrogel are stable at different conditions of temperature and humidity, stability studies were performed in accordance with international conference on harmonization (ICH) guidelines. [47]. Stability study was performed at accelerated temperature e.g., 40°C± 2°C and relative humidity e.g., 75% ± 5% for 0, 1, 3 and 6 months. Nanoparticles were analyzed for PS and EE whereas APO-NPs based hydrogel was analyzed for physical appearance (phase separation, color, grittiness), pH and drug content.

3. Results and Discussion

3.1. Optimization of APO NPs and APO Based Hydrogel

3.1.1. Optimization of APO-NPs

14 formulations were generated with different polymer, APO and surfactant concentrations as shown in the Table 1. Formulation number 4 was considered as optimized as it gave optimum PS, PDI, ZP and %EE.

Table 1. Optimization of Hydrogel.

Parameters	Formulation 1 (0.5%)	Formulation 2 (1%)	Formulation 3 (1.5%)	Formulation 4 (2%)
pH	5.6	5.7	5.8	5.6
Viscosity (CPA 52Z, 20rpm)	180	220	260	Thick
Drug content	83.09%	82.9%	84.03	81.9%
Physical appearance	Very Thin, Liquefy time 4 hours	Thin, Liquefy time 9 hours	Uniform, non-gritty	sticky

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By increasing the concentration of eudragit L100 and APO, PS increases (Figure 2A). It can be explained as increase in polymer concentration prompts increase in particles collision during emulsification. This leads to binding of partially formed particles thus increasing particle size. Also, increased polymer concentration leads to enhanced viscosity of organic phase. It impedes the diffusion of organic and aqueous phase which results in the formation of larger particles. However, increase in drug concentration creates more viscous organic phase ultimately more complexes.

However, with increase in tween 80 concentration, PS is reduced. Tween 80 acts as stabilizing agent in course of particles formation. Tween 80 minimizes surface free energy and hinder nucleation [48].

Figure 2B shows the effect of variables on ZP. With increase in concentration of eudragit L100, zeta potential increases. Eudragit L 100 is anionic methacrylate polymer. This imparts more negative charge due to carboxyl group, on the surface of particle making nanoparticles more stable [49].

As APO bears no charge, ZP is reduced due to variation in eudragit L 100 concentration. However, with increase in tween 80 concentration ZP is increased. Tween 80 prevents aggregation of particles thus increasing repulsion between particles [50].

The effect of variables on PDI is shown graphically in Figure 2C. By increasing the concentration of eudragit L100 and APO, PDI also increases. Increase in polymer and drug concentration leads to fusion of particles occurs. Non-uniform particle aggregation results in increased PDI. With increase in concentration of tween 80, poly dispersibility index is reduced due to interfacial tension stabilization [51,52].

The relation between variables and encapsulation efficiency is shown in Figure 2D. By increasing the concentration of eudragit L100, APO and Tween 80, %EE is increased. With increase in eudragit L100 and APO concentration, viscosity of organic phase is increased making nanoparticles to retain more drug. Also, it prevents diffusion of drug from nanoparticles [12,53]. However, increase in tween 80 concentration decreases the surface tension of polymer and increases the encapsulation efficiency [54].

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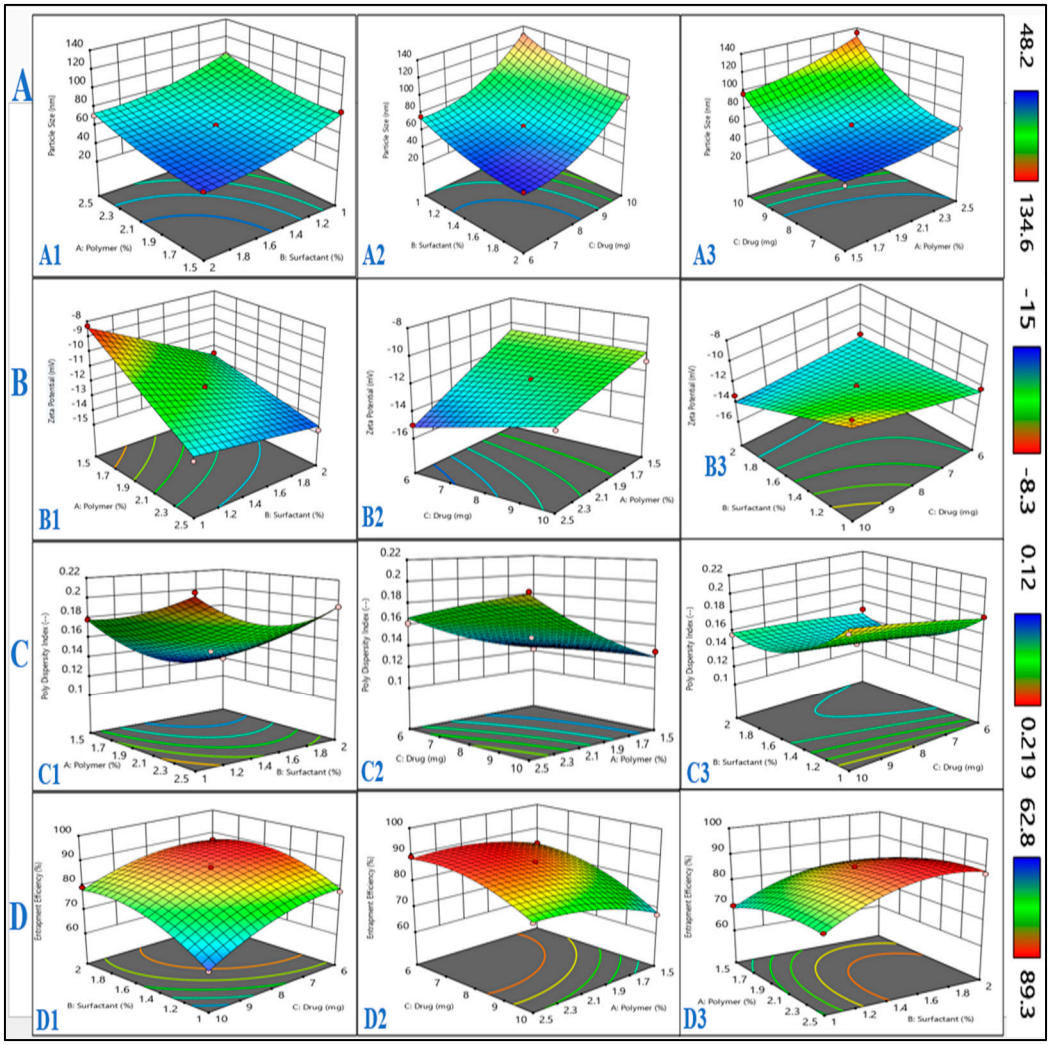

Figure 2. 3D graphs for the effect of three different parameters on PS, ZP, PDI and %EE.(A1) Effect of surfactant and polymer on PS; (A2) Effect of surfactant and drug on PS (A3) Effect of drug and polymer on PS.(B1) Effect of polymer and surfactant on ZP; (B2) Effect of drug and polymer on ZP; (B3) Effect of surfactant and drug on ZP.(C1) Effect of polymer and surfactant on PDI; (C2) Effect of drug and polymer on PDI; (C3) Effect of surfactant and drug on PDI while (D1) Effect of surfactant and drug on EE; (D2) Effect of drug and polymer on EE; (D3) Effect of polymer and surfactant on EE.

3.1.2. Physicochemical Characterization of APO-NPs

Optimized formulation had PS of 63.4mm, 0.161 PDI and -15mV ZP as shown in Figure 3 (A and B). Lowest PS is optimum for delivery of nanoparticles through the skin barrier. PDI less than 0.5 is considered optimum and zeta potential near to ± 30mV gives a stable formulation [55,56]. %EE of optimized formulation was 89.3% ± 0.73.

Compatibility Studies of Nanoparticles

Figure 3C represents the graphs for spectra of APO, eudragit L100, APO-NPs were generated and compared. Figure 3D shows functional group identification. The FTIR spectrum of APO showed the characteristic peaks of functional groups: 3200 (phenyl-OH), 2700 (aromatic –H), 2900 (alkane C-H), and 1400 cm^{-1} (ketone C=O). Eudragit L 100 showed peaks at 3150 (Phenyl-OH), 2850 (alkane-CH), 1450 (Ketone C=O) and 850 cm^{-1} (carbonyl C-O). Whereas same peaks were also appeared in nanoformulation which is an evidence for successful drug loading in nanoformulation without any drug and excipient interaction.

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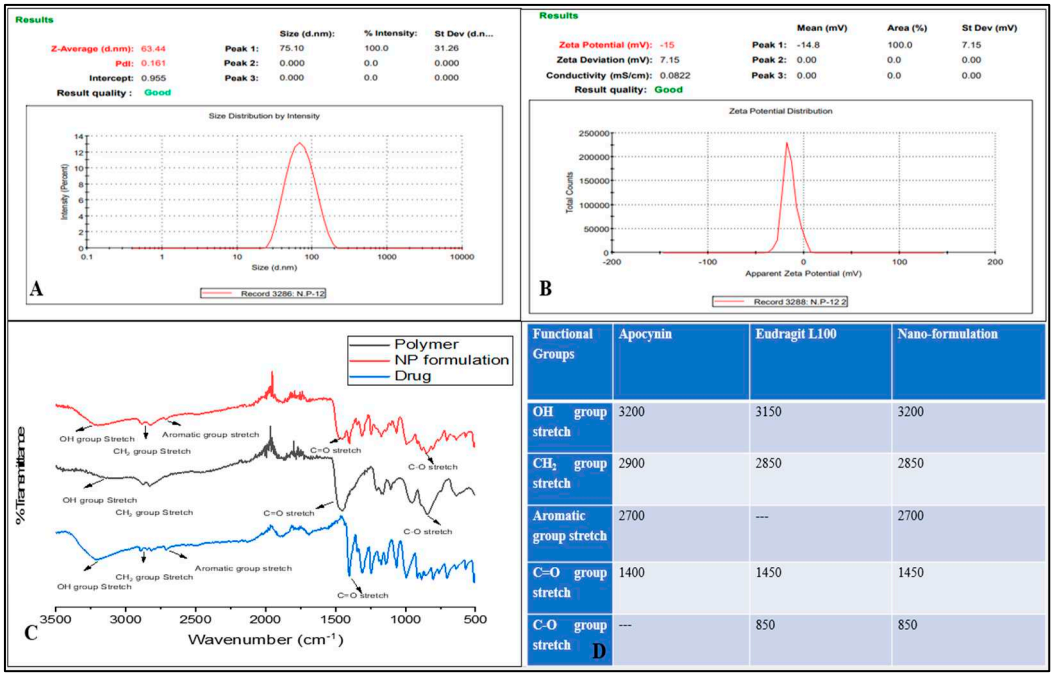

Functional Groups	Apocynin	Eudragit L100	Nano-formulation
OH group stretch	3200	3150	3200
CH_2 group stretch	2900	2850	2850
Aromatic group stretch	2700	---	2700
C=O group stretch	1400	1450	1450
C-O group stretch	---	850	850

Figure 3. Characterization of APO-NPs; (A) PS of APO-NPs (B) ZP of APO-NPs, (C) shows FTIR of drug, polymer and nano formulation spectrum (D) FTIR spectrum peak Interpretation.

APO, eudragit L 100 and physical mixture of APO and eudragit L 100 showed different peaks at angle 2θ. However, nano-formulation did not show any peak from 20-40° which confirms the loading of drug in nanoparticles. Figure 4 (A) showed complete conversion of crystalline nature of pure drug into amorphous form.

APO thermogram in Figure 4 (B) has shown its characteristic endothermic peak at 120 °C whereas Eudragit L 100 showed characteristic endothermic peak at 250 °C. While APO-NPs showed these characteristic peaks but with lower intensity confirming successful loading of drug in nanoparticles.

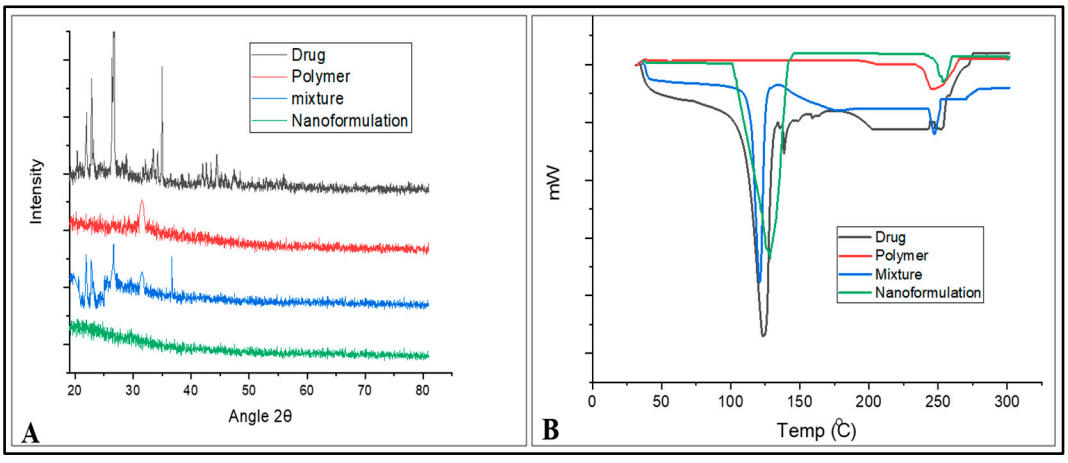

Figure 4. (A) shows XRD of drug, polymer, drug polymer mixture and nano formulation analysis (D) shows DSC of drug, polymer and drug polymer mixture and nano formulation thermograms.

3.2. Characterization of APO-NPs Based Hydrogel

APO-NPs based hydrogel was prepared by the method described above, Figure 1(B).

3.2.1. Physical Appearance and pH

Both blank and APO-NPs based hydrogel were semisolid in nature, smooth in texture, lacking grittiness, easy to apply. However, blank hydrogel appeared as transparent in color whereas APO-NPs based hydrogel appeared as milky with uniform distribution of nanoparticles. While the pH of

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APO-NPs based hydrogel was 5.8±0.01. pH of hydrogel more than pH of skin (5.5) facilitates the intact nature of NP, so that they may not release the drug at skin surface but reach systemic circulation easily.

3.2.2. Drug Content

Amount of APO in hydrogel was calculated as 84.03%±0.69.

3.2.3. Rheology

To find out flow properties of hydrogel, rheology study was performed using spindle CPA 52 Z at different shear rate. Figure 5 (A) shows the rheology of prepared hydrogel showed that it does not follow Newtonian behavior rather it shows pseudoplastic behavior and shear thinning properties. This means viscosity of hydrogel decreased with increase in shear rate. This property for prepared hydrogel is ideal and favors smooth application of hydrogel on skin surface.

3.2.4. Spreadability Studies

Spreadability of APO-NPs based hydrogel was found to be 3.14 cm ± 0.54. This percentage lies in optimum range (3-5 cm) of spreadability.

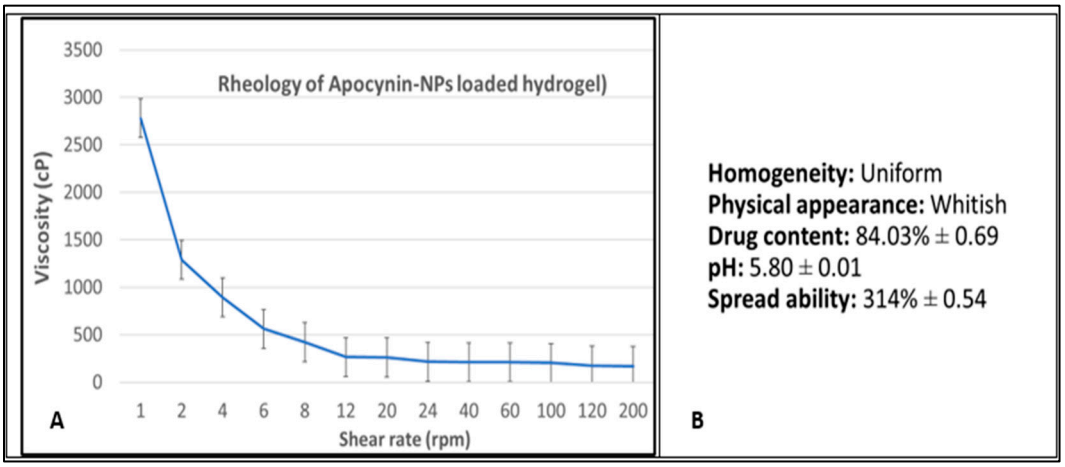

Figure 5. (A) Rheology of APO-NPs based hydrogel (B) shows APO-NPs based hydrogel characterization.

3.2.5. In-Vitro Drug Release

At pH 5.5, drug dispersion showed maximum APO release whereas APO-NPs based hydrogel gave 6.2% release, nano-formulation has given 14.1% and free APO based hydrogel has given 7% drug release as shown Figure 6 (A). However, Figure 6 (B) shows that APO dispersion gave maximum release in first 6 hours at pH 6.8 but APO-NPs based hydrogel gave 81% release by 48 hours. Nano-formulation and free APO based hydrogel gave 98% and 30% APO release, respectively.

a. Application of kinetics models on in-vitro release profile

Kinetic models applied on the release profile of APO, showed that best R2 value i.e., 0.9962 was obtained for korsmeyer peppas model as shown in Table 2. Drug release profile following this model showed drug is released from the carrier through fickian diffusion more than polymeric chain relaxation as value of 'n' is less than 1 (0.186).

Table 2. Optimization of APO-NPs in terms of various independent factors and dependent responses.

Run	Factor 1 EL 100 (%)	Factor 2 Tween 80 (%)	Factor 3 APO (mg)	Response 1 PS (mm)	Response 2 ZP (mV)	Response 3 PDI	Response 4 EE (%)
1	2.5	2	8	71.2	-14.8	0.194	87.3
2	2	1	6	76.16	-12.2	0.179	78
3	2	1.5	8	65.58	-12.9	0.152	86.01

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4	**2.5**	**1.5**	**6**	**63.44**	**-15**	**0.161**	**89.3**
5	1.5	1	8	83.58	-8.3	0.178	69.96
6	2	1	10	128.73	-8.5	0.197	62.8
7	1.5	1.5	10	96.94	-11.2	0.135	67.3
8	2	2	10	98.29	-13.3	0.156	79.3
9	1.5	2	8	51.24	-12.5	0.12	78.8
10	2	2	6	48.61	-12.9	0.151	87
11	2	1.5	8	65.14	-12.1	0.148	87.7
12	2.5	1	8	99.62	-13.6	0.219	76

3.2.6. Ex-Vivo Permeation Studies

Figure 6 (C) shows the ex-vivo permeation studies of APO nanoparticle-based hydrogel with and without permeation enhancer, at pH 5.5 and 6.8. APO-NPs based hydrogel without PE showed less permeation i.e., 195µg/cm2 as compared to APO- NPs based hydrogel with PE i.e., 278µg/cm2 through skin at pH 5.5. Whereas APO-NPs based hydrogel with PE at pH 6.8 showed maximum penetration 1900 µg/cm2. Maximum release at pH 6.8 confirms APO release at inflamed pH due to pH sensitive eudragit L100. 1050 µg/cm2 drug release was observed from APO-NPs based hydrogel without PE at pH 6.8. However, free APO based hydrogel with PE at pH 5.5 and 6.8 showed permeation 110µg/cm2 and 300µg/cm2, respectively.

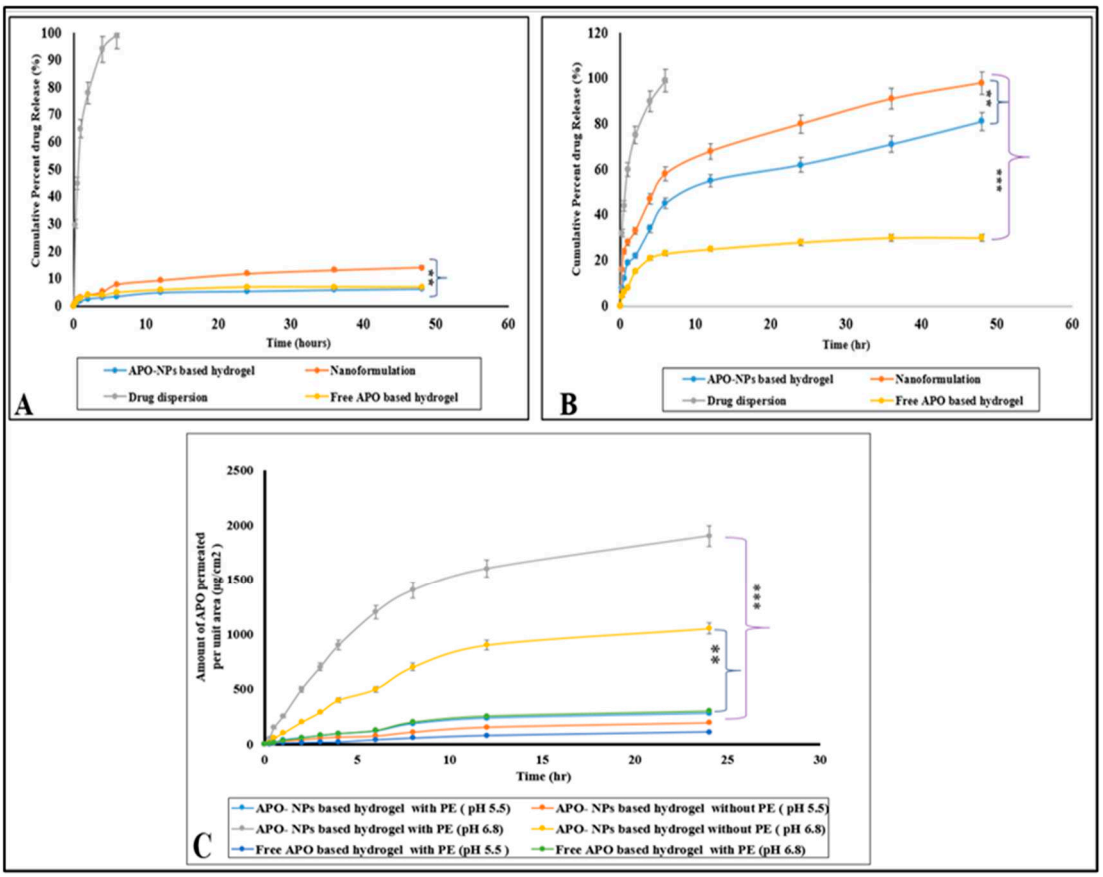

Figure 6. (A) shows In-vitro drug release at pH 5.5 (B) shows In-vitro drug release at pH 6.8 (C) ex-vivo permeation studies at pH 5.5 and 6.8.

3.2.7. In-Vivo Studies

a. Chronic CFA-induced inflammation model

The pathophysiological measurements and clinical scores were made before and after induction. The chronic inflammatory study was designed and conducted for the 21 days as explained in the methodology section. The chronic RA model was developed and different behavioral parameters were observed to find out anti-inflammatory effect of the APO nanocarrier system thus in the start of

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CFA-induced RA model, overall basic initials assessments were made to investigate the progression of the RA disease. Similarly, the post-injection data were also taken to determine the efficacy of the APO-NPs based hydrogel.

b. Effect of treatment groups on paw edema

Higher therapeutic potential of the prepared APO-NPs-based hydrogel system could be due to pH-sensitive based drug release from nanocarrier at the inflamed or damaged sites than that of as compared to other body organs. The physical appearances of experimental mice paws of different groups have been shown below in Figure 7.

The therapeutic effect of the prepared APO-NPs-based hydrogel with permeation enhancers is significantly improved. This is shown by swelling of the paw, severe inflammation clinical scores, intensive erythema, and edema comparison of control, positive and treatment groups. The prepared APO-NPs-based hydrogel showed a significant reduction in swelling and abolished erythema as well as clinical scores ($p<0.001$) and thickness ($p<0.01$) and treatment with the marketed formulation (positive control) also reduced paw edema to some extent when compared to the negative CFA control group. See Figure 8A.

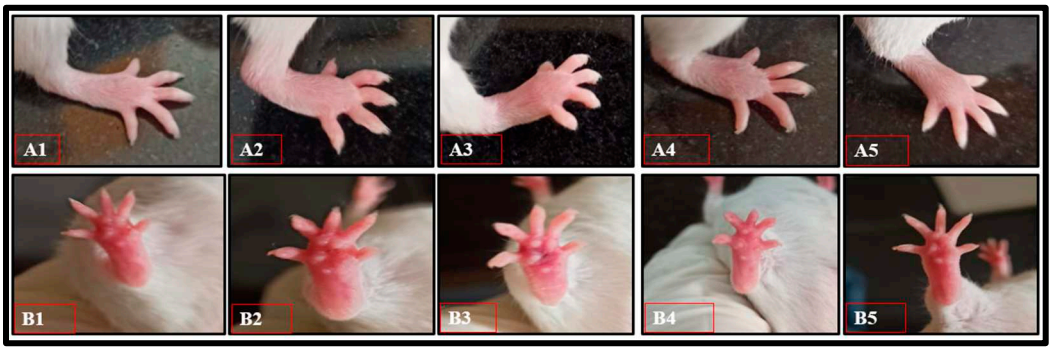

Figure 7. (A) Representing dorsal view of paw and (B) shows plantar view of RA mice. A1 and B1: Normal group; A2 and B2: Negative group; A3 and B3: Positive group; A4 and B4: Free APO treated group; A5 and B5: APO-NPs based hydrogel treated group.

c. Effect of treatment groups on thermal hyperalgesia

The hot plate response was noted higher in CFA-treated group (negative control group) as compared with other treatment groups. Additionally, the efficacy of treatment of APO-NPs based hydrogel was assessed on increased pain threshold against hot plate compared with free APO based hydrogel treated and marketed product treated positive group as illustrated in Figure 8 (B).

d. Effect of treatment groups on mechanical allodynia

Significantly increased the sensitivity towards pain threshold was seen, in inflamed hind paw against painful sensation by touching in von Frey test as shown in Figure 8 (C) below. Paw withdrawal timing was similar in all groups at day 0, while at day 21, the animals that were treated with APO-NPs based hydrogel revealed that the inflammation is significantly reduced and exhibited not only to decrease the sensitivity effect towards allodynic stimuli, but also improved paw withdrawal threshold as compared with positive, negative and control group [45].

e. Effect of treatment groups on arthritic score index

The Arthritic index score representing the severity of the RA disease, the normal group, arthritis score was observed zero while the APO-NPs based hydrogel significantly improved the Arthritic index as compared to the marketed treated cream (positive group) and negative control group which has 3.8 arthritic score as show in Figure 8 (D).

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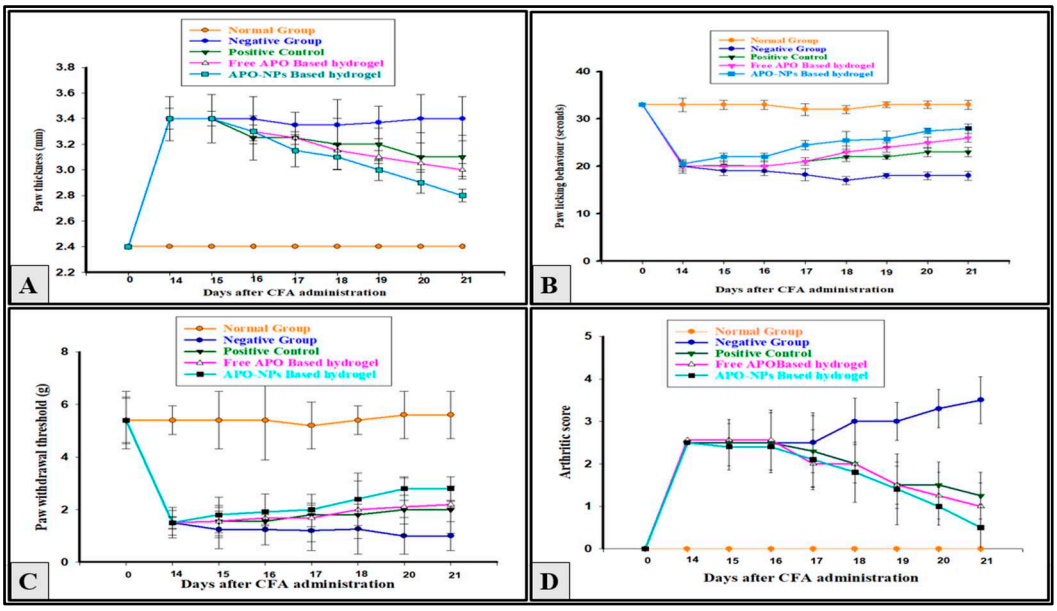

Figure 8. Behavioral parameters in CFA induced RA model (A) effect of treatment groups on paw edema (B) effect of treatment groups on thermal hyperalgesia (C) effect of treatment groups on mechanical allodynia (D) effect of treatment groups on arthritic score index.

f. Radiological analysis and histopathology of paw

The radiological analysis and H and E staining was performed for different group of RA model to observe changes in bones and soft tissues and to determine the therapeutic outcome of prepared hydrogel. The X-Ray imaging of CFA-arthritic control mice paw exhibited bone swollen and erosion as well as cartilage deterioration in comparison to normal group and there are no changes shown in the prepared hydrogel treated group as evident in X-ray images in Figure 9(A). However, the histopathology of CFA-arthritic control mice paw exhibited a significant increase in synovial hyperalgesia, marked increased in infiltration of pro-inflammatory cells into synovial lining, narrowing of the joint space as comparatively of normal group. Although, the APO-NPs based hydrogel exhibited an effective improvement in the histopathology of mice ankle joints, see Figure 9 (9B). Thus, it can be concluded that the formulation of pH-sensitive based polymeric nanoparticles could be a promising potential carrier system for drug delivery of APO at the inflamed joint sites.

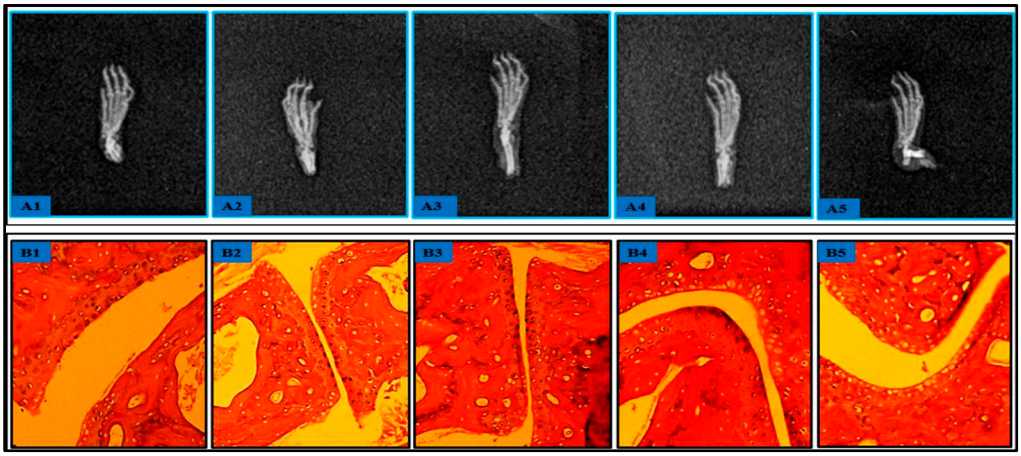

Figure 9. X-ray imaging of mice paw and histopathological analysis of ankle joint (A1 and B1) Normal (A2 and B2) Negative (A3 and B3) Positive (A4 and B4) free APO treated (A5 and B5) APO-NPs based hydrogel treated.

3.2.8. Stability Studies

Table 3 shows the stability study data of APO nanoparticles and APO nanoparticles-based hydrogel. PS was increased due to slight particle aggregation and EE was reduced. Whereas there is

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no significant change in PDI of nanoparticles. These results showed that APO-NPs are stable up to 6 months. There was no observed phase separation and color change in APO-NPs based hydrogel. However, pH of hydrogel was slightly increased, but this increase is not significant whereas drug content was decreased which shows that hydrogel is stable during observed period [57–59].

Table 3. R^2 values for kinetic models.

Zero order	First order	Korsmeyer-peppas	Higuchi	Hixon-Crowell
0.817	0.873	0.996	0.718	0.692

Table 4. Stability studies of APO-NPs and APO-NPs based hydrogel for 6 months.

APO-NPS							APO-NPS hydrogel								
Time (month)	Particle size (nm)			Encapsulation efficiency (%)			Phase separation/color change			pH			Drug content (%)		
Temp ± 2°C	4°C	25°C	40°C	4°C	25°C	40°C	4°C	25°C	40°C	4°C	25°C	40°C	4°C	25°C	40°C
0	63.44	63.44	63.44	89.3	89.3	89.3	NO	NO	NO	5.80±0.1	5.80±0.1	5.80±0.1	84.03	84.03	84.03
1	63.90	64.03	64.2	88.5	88.5	88.5	NO	NO	NO	5.82±0.15	5.82±0.15	5.82±0.2	83.94	83.94	83.94
3	64.21	64.29	64.4	88.40	88.30	88.10	NO	NO	NO	5.84±0.25	5.84±0.25	5.84±0.2	83.02	83.02	83.02
6	64.95	65.05	64.15	87.82	87.53	87.25	NO	NO	NO	5.86±0.12	5.86±0.15	5.86±0.2	82.98	82.98	82.98

4. Conclusions

Then aim of the current research was to prepare APO loaded pH-sensitive nanoparticles based transdermal hydrogel against the management of rheumatoid arthritis (RA). The slight modified nanoprecipitation technique was used to prepared polymeric nanoparticles and then the optimized APO-NPs were successfully loaded into carbopol-934 based hydrogel. The characterization of APO-NPs and APO-NPs based hydrogel has shown a successful polymeric nanoparticle formation and significant results. In-vitro studies showed sustained release of drug at pH 6.8. The ex-vivo studies ensured higher penetration of prepared nanoparticles with permeation enhancer through skin. Stability studies showed that APO-NPs and APO-NPs based hydrogel was stable upon long term storage at various temperature conditions.

The therapeutic efficacy of the hydrogel in chronic CFA-induced inflammatory pain model was confirmed. Moreover, morphological, histopathological and radiological examination of pain were significantly reduced the hind paw edema and enhanced anti-inflammatory activity in the APO-NPs based hydrogel treated group as compared with the free AP-based hydrogel and marketed product treated group.

The results showed that the development of pH-sensitive nanoparticles and site-specific release of APO from APO-NPs based hydrogel. Thus, it is concluded that APO-NPs based hydrogel formulation could serve as a potential nanocarriers system for the management of rheumatoid arthritis and may be used as an alternative to commercial cream.

Author Contributions: "Conceptualization, R.A.; methodology, D.K and R.A.; software, M.B.S; validation, R.A, D.K and I.B.R.; formal analysis A.U.R and I.S.; investigation, K.U.S.; resources, A.U.R. and K.U.S.; data curation, S.U.S and M.B.S.; writing—original draft preparation, R.A and M.B.S.; writing—review and editing, D.K and I.B.R; visualization, N.D and A.N.; supervision, K.U.S.; project administration, F.F.N, S.M and B.K.; funding acquisition, S.Y.A and I.S. All authors have read and agreed to the published version of the manuscript."

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