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FORMULATION AND CHARACTERIZATION OF DICLOFENAC SODIUM LOADED PLGA NANOPARTICLES FOR INFLAMMATORY DISEASES AND *IN-VIVO* HET-CAM ANALYSIS

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Abstract

The development of a new drug active substance is not only time-consuming and expensive, but also a chain of operations that often fails. However, increasing the bioavailability, effectiveness, safety, or targeting the drugs used in clinic by various methods, such as nanoparticles (NPs), may be a more effective way of using them in clinic. In addition, nanoparticle formulations are becoming increasingly popular in modern medical treatments. Angiogenesis, formation of new capillaries from a pre-existing one, fundamentally occurs in physiological processes such as wound healing, embryogenesis and menstrual cycle, also has a vital role in pathology of cancer, psoriasis, diabetic retinopathy and chronic inflammation. The Hen‟s Egg Test on the Chorioallantoic Membrane (HET-CAM) assay is a useful, well established and animal alternative *in vivo* procedure for evaluation of anti-inflammatory potentials and anti-irritant properties of nano drug delivery systems. In this study, diclofenac sodium (DS) loaded PLGA NPs were prepared and characterized. The particle size (PS) of DS-loaded PLGA NPs was between 114.7 and 124.8 nm and all NPs were monodisperse with negative zeta potential values. The encapsulation efficiency was in range of 41.4-77.8%. *In vitro* dissolution studies of NPs showed up to 24 h of DS release after the first 3 h of burst effect. The 3 h burst effect and 24 h release kinetics studied with DDSolver were found to be predominantly driven not only by one mechanism, by a combined mechanism of Fickian and non-Fickian. Solid state structures of formulations were clarified by DSC and FT-IR analysis. PS, EE% and release rates were found to be affected by the amount of DS added to the formulations. Increasing the amount of DS added to the formulations increased PS, while the EE% decreased. The release rates were affected by PS and the formulation with the lowest PS value showed slower release. The anti-inflammatory

activity of optimum formulation (NP-1) was examined using *in vivo* HET- CAM assay. The anti-inflammatory activity results indicated that NP-1 coded NP formulation showed significantly good anti-inflammatory potential at low dose. As a result, a low dose high anti-inflammatory effect was achieved with the NP structure of DS. To the best of our knowledge this is the first study on *in vivo* anti-inflammatory activities of DS loaded PLGA NPs.

Keywords: Anti-inflammatory; Diclofenac sodium; Drug delivery System; HET-CAM assay; Nanomedicine; Nanoparticle; PLGA

1. Introduction

Inflammation is the immune system‟s response to destructive stimuli, such as damaged cells, pathogens, irradiation or toxic compounds and acts by removing injurious stimuli and beginning the healing process of human body. These factors may induce acute and/or chronic inflammatory responses in liver, heart, kidney, pancreas, lung, brain, reproductive system and intestinal tract, potentially leading to tissue damage or disease (Chen et al., 2018). Chronic inflammation is also referred as slow, long-term inflammation lasting for prolonged periods of several months to years. Mostly, the extent and effects of chronic inflammation vary with the cause of the injury and the ability of the body to repair and overcome the damage (Pahwa et al., 2019). Cancer, nerve damage and arthritis generally cause chronic inflammatory pain. Chronic inflammation and chronic pain may have a severe effect on a person‟s life and society when not treated efficiently. Although pharmacological therapies are available for the treatment of chronic pain and inflammation, they are limited by unacceptable side effects or short-term efficacy and in most cases, efficacy is achieved by high dosing (Su et al., 2014). Chronic pain and inflammation are generally known as any type of disease that lasts longer than 12 weeks. Acute pain is a normal feeling that warns us against possible injuries, but chronic pain and inflammation are much different. Chronic pain and inflammation usually last for months or longer. Chronic regional pain and inflammation are present in 20 to 25% of the population and chronic widespread pain and inflammation are present in approximately 10% of the population (Öztürk et al., 2019a). Diclofenac sodium (DS) is a widely prescribed non-steroidal anti-inflammatory drug (NSAID) for inflammation and pain that acts as a competitive and irreversible inhibitor of the enzyme prostatin synthase (Liang et al., 2019).

Nanomedicine applications and research around the world are rapidly increasing with the promise of targeted and effective drug delivery. Nanomedicines eliminate the shortcomings of traditional treatment. Nanomedicines provide site-specific drug delivery, reduced side effects and better treatment results. The development of suitable and biocompatible drug delivery vehicles is a prerequisite that has been successfully achieved by using simple and functionalized nanoparticles (NPs), liposomes, dendrimers and mesoporous particles etc. (Singh et al., 2019). Several approaches can be used to manufacture NPs such as salting-out, solvent evaporation, supercritical fluid technology, micro-emulsion, mini-emulsion, surfactant-free emulsion and interfacial polymerization (Öztürk et al. 2019b). The most common technique to produce NPs involves evaporation of a polymer emulsion to obtain NPs less than 500 nm in size (Piñón-Segundo et al., 2012). Single oil (o) in water (w) emulsions (o/w) were originally used in this technique, at the present time the use of double emulsions or multiple emulsions is very frequent, essentially, w/o/w emulsions, symbolized as w1/o/w2. When double w1/o/w2 emulsions are employed, the drug dissolves in water (aqueous phase, w1) and the polymer dissolves in an organic solvent (organic phase, o) (acetone, ethyl acetate etc.). Both phases are emulsified and this primary emulsion (w1/o) is then slowly dispersed in a second aqueous phase containing a stabilizing agent (external water phase, w2) (polyvinyl alcohol, Pluronic etc.). These methods use high-speed homogenization or sonication. Finally, the organic solvent is eliminated under reduced pressure or by continuous magnetic stirring in order to form NPs (Vauthier and Bouchemal, 2009; Rao and Geckeler, 2011; Piñón-Segundo et al., 2012). One of the most important nanocapsulation strategies of hydrophilic drugs in polymer-based NPs is „Double

Emulsification Solvent Evaporation‟ technique (Vrignaud et al., 2011). Poly (lactic-*co*-glycolic acid) (PLGA) is a United States Food and Drug Administration (FDA) approved, biocompatible and biodegradable copolymer that is widely used as a matrix for NPs. (Öztürk et al., 2019c). PLGA is also widely used in NP production by „Double Emulsification Solvent Evaporation‟ technique (Öztürk et al., 2019d). In this study, we aimed to develop a drug delivery system with low oral dose and high anti-inflammatory activity. For this purpose, we prepared three different DS-loaded NPs by double emulsification solvent evaporation technique using PLGA polymer. The prepared NPs were examined in detail by important parameters for pharmaceutical technology and pharmaceutical nanotechnology such as particle size, poly dispersity index, zeta potential, encapsulation efficiency, dissolution, release kinetics, DSC and FT-IR analysis. Following the selection of the optimum formulation, the optimum DS loaded PLGA NP has been investigated for its anti-inflammatory and irritancy potentials using *in vivo* HET- CAM assay. To the best of our knowledge this is the first report on the *in vivo* anti-inflammatory evaluation of DS loaded PLGA NP using *in vivo* HET-CAM assay.

2. Materials and methods

Diclofenac sodium was a kind gift from Loba chem private limited, Mumbai, India. Resomer® RG 502 H [Poly(d, l-lactide-*co*-glycolide), acid-terminated, lactide:glycolide 50:50, Mw: 7.000–17.000] and Polyvinyl alcohol (PVA) were purchased from Sigma-Aldrich, Mumbai, India. Acetone was purchased from Merck, New Delhi, India. Deionized and filtered water was used in all experiments, Mumbai, India. Agar and Sodium Dodecyl Sulfate (SDS) were purchased from New Delhi, India. The fertilized eggs used in the HET-CAM test were obtained from NCL Pune, India. All other chemicals used were of analytical grade.

Characterization of PLGA nanoparticles

Particle size, polydispersity index and zeta potential

The particle size (PS) and polydispersity index (PDI) of the NPs was measured by dynamic light scattering (DLS) using Zetasizer. Zeta potential (ZP) of the NPs was also measured using Zetasizer. PS and PDI of NPs were measured by dispersing the formulation in distilled water. ZP values were determined using disposable folded capillary zeta cell, at 25°C room temperature and diluted with distilled water. Each sample was measured three times and the average values and standard deviation of the measurements were calculated.

Encapsulation efficiency

The encapsulation efficiency (EE%) of PLGA NPs was evaluated by coordinated extraction of DS from NPs. Accurately weighed 5 mg of NPs were dissolved in ethyl acetate. The final samples were filtered through 0.45 μm membrane filters and analyzed using UV/Visible spectrophotometer (Shimadzu UV VIS 160) at 276 nm (Gouda et al., 2013; Dhokale et al., 2016). Each experiment was repeated three times for statistical analysis. The average values and standard deviation of the measurements were calculated. The EE % of NPs was calculated by Equation 1 (Eq. 1) (Öztürk et al., 2019d; Öztürk et al., 2019e).

Release kinetics

The drug release data were computed using DDSolver, an Excel-plugin module and the resultant data were fitted to different kinetic models. DDSolver computer program was used to shorten the calculation time, eliminate calculation errors and determine the correct release profile (Zhang et al., 2010). After obtaining the release profiles, data were transferred to the DDSolver program to determine the six most important and popular criteria: coefficient of determination, adjusted coefficient of determination, Akaike Information Criterion (AIC), Model Selection Criterion (MSC), n (release exponent) and β (an indicator of the mechanism of transport of a drug through the polymer matrix). The highest R2, R2adjusted and MSC values and the lowest AIC values were used for

evaluating First order, Higuchi, Korsmeyer-Peppas, Hixson-Crowell, Hopfenberg, Peppas-Sahlin and Weibull models. Finally, release differences of DS and prepared NPs were evaluated by DDSolver according to the „Similarity Factor‟ (*f2*) (Zhang et al., 2010; Öztürk and Kıyan, 2020).

Solid state characterization of PLGA nanoparticles

Thermal analysis

The physical states of NPs were characterized by differential scanning calorimetry (DSC). Aluminum crucibles with 5 mg samples were analyzed under nitrogen gas (50 mL.min-1) and heating rate of 10 ° C.min-1 at a temperature range of 30 and 300 ° C. Pure DS and blank formulation were also analyzed and were used as references.

FT-IR analysis

FT-IR spectra of NPs were recorded using Shimadzu IR Prestige-21 at the wavelength range of 4000–500 cm-1. Pure DS and blank formulation were also analyzed and were used as references.

In vivo anti-inflammatory activity

Preparation of Test Samples

NP-1 (equivalent DS 20 µg/pellet), NP-blank (Blank formulation without DS, 50 µg/pellet), and standard DS (50 µg/pellet), SDS (50 µg/pellet) were dissolved/suspended in a 2.5% (w/v) agarose solution. For ease of application of the pellets of these solutions (10µL) were prepared and applied dropwise on circular stainless steel supports of 5 mm diameter and cooled to room temperature for solidification and applied on to the chick chorioallantoic membrane (CAM).

In vivo HET-CAM assay

The method was performed as described in the previous work of us (Öztürk and Kıyan, 2020). The previously incubated fertilized Hen‟s eggs for 72 h at 36.5°C and a relative humidity of 80% were positioned in a horizontal position and rotated several times. The eggs were opened on the snub side and furthermore 10-15 mL of albumin were aspirated from them. The eggs were traced with a scalpel at the two third of the height (from the pointed side) and after that the shells were removed with forceps. The cavity was covered with film and they were incubated at 36.5°C at a relative humidity of 80% for further 72 h. Pellets (1 pellet/egg) were placed on the CAM which had approximately a diameter of 2 cm. One
further day later the eggs were evaluated under the stereo-microscope. 10-15 eggs were utilized for each test compound. Anti-inflammatory effects were evaluated using a scoring system (Table 2) and followed the conversion of the score index in the proportional inhibition of inflammation (Table 3).

Statistical analysis

Microsoft Excel and DDSolver were employed for mathematical analysis. All data obtained were evaluated statistically (one way ANOVA test, only two way ANOVA test for dissolution test results) using GraphPad Prism version 7.0. All measurements were done for at least three replicates. In all studies, $p<0.05$ was considered to be of statistical significance. The results were each presented as Mean ± SD.

3. Results and Discussions

The development of a new drug active substance is not only time-consuming and expensive but also a chain of operations that often fails. However, increasing the bioavailability, effectiveness, safety, or targeting the drugs used in clinic by various methods, such as nanoparticles (NPs), may be a more effective way of using them in clinic. Many strategies such as personalized drug therapy, NP-based drug delivery systems, drug conjugates, therapeutic drug monitoring, stimulant-sensitive targeted therapy have been extensively studied by the investigators (Öztürk and Aygül, 2020). The fact that drug active substances are so expensive pushes many researchers into new drug delivery systems.

Although the development of new drug active substances is advanced, the popularity of some old drug active substances still continues and DS is an example with its optimum properties. When you write „DS NPs‟ on Google Scholar search engine, many studies stand out about DS loaded nanomedicine/NPs even in 2020 (Lu et al., 2020; Zuppolini et al., 2020; Wang et al., 2020; Cesari et al., 2020). Because of their biocompatibility and biodegradability, polymeric NPs, such as PLGA NPs, are widely used to develop new drug delivery systems of various drugs. PLGA is widely used in drug delivery for a variety of applications. Examples of these different applications of PLGA have entered the literature.

The study of intestinal organoids containing PLGA NP for the treatment of inflammatory bowel diseases has been concluded to be a suitable system for inflammatory bowel diseases (Davoudi et al., 2018). In another study, hydroxyapatite-PLGA NP colloidal gels were designed as promising systems for injectable filling and regeneration of bone tissue (Wang et al., 2013). In another study, a colloidal gel from the alginate- chitosan-PLGA complex was used to deliver the Ac-PLP-BPINH2-2 peptide in a controlled release manner as a vaccine-like therapeutic to suppress experimental autoimmune encephalomyelitis in the mouse model and successful results were obtained (Büyüktimkin et al., 2012). The same research team also developed that insulin and glucose-specific enzymes can be transported with dextran NPs (Gu et al., 2013). In another study, PLGA-chitosan/PLGA-alginate nanoparticle was used as biodegradable colloidal gels to seeding human umbilical cord mesenchymal stem cells. All the results of study indicated the potential application of the biodegradable colloidal gels as an injectable scaffold in tissue engineering and drug release (Wang et al., 2011). In a different study, cohesive dexamethasone loaded colloidal gels made by mixing oppositely-charged PLGA NPs were investigated as potential bone defect fillers. This study demonstrated that dexamethasone loaded PLGA colloidal gels were osteoconductive fillers capable of controlled release for the repair of rat cranial bone defect (Wang et al., 2010). PLGA has also been approved by the FDA as a drug delivery vehicle (Roberts et al., 2020). To date, some DS loaded PLGA NP studies have been introduced to the literature (Chavanpatil et al., 2007; Agnihotri and Vavia, 2009; Çetin et al., 2010; Khanal et al., 2016; Espanol et al., 2016; Dalpiaz et al., 2016). However, PLGA type, production/preparation conditions, surface active agent ratios and DS ratios used in this study were not observed in any study. Such differences indicate the innovation of the study in terms of pharmaceutical technology and formulation. However, the most important innovative aspect is the evaluation of anti-inflammatory activity of the prepared NP system by HET-CAM assay.

Preparation of PLGA nanoparticles

This work describes a method to encapsulate DS using a double emulsification solvent evaporation technique. Many studies have been successful at encapsulating hydrophilic and hydrophobic drugs using PLGA, while keeping PS at minimum (Zhang et al., 2013; Chereddy et al., 2014; Öztürk et al., 2019d). While there are several techniques to prepare NPs, one of the most common ways is the emulsion-solvent evaporation technique (Makadia and Siegel, 2011). Hydrophobic drugs are generally encapsulated via a single emulsion process (o/w), while hydrophilic drugs use a double emulsion (w/o/w) technique (Roberts et al., 2020). Double emulsification solvent evaporation technique is preferred since DS is the one with the highest water solubility in diclofenac derivatives (Fini et al., 2012; Wu et al., 2020). There are many parameters that affect the PLGA based NP properties and these parameters have been optimized in our laboratory with various studies (Öztürk et al., 2019e; Öztürk et al., 2019d). In this study, three different concentrations of DS were used in the formulations (NP-1, NP-2, NP-3) for comparison. The importance of this difference has been characterized and discussed in detail.

Characterization of PLGA nanoparticles

Particle size, polydispersity index and zeta potential

DLS have been used for many years in NP research. The determination of PS and PDI by DLS is quite fast, and this technique is applicable to most colloidal dispersions. This technique is popular in academic research and industry (Ullmann et al., 2019). Based on a recent analysis of the files presented to FDA from 1973 to 2015, DLS has been the most recurrently used sizing technique by nanomedicine stakeholders (Caputo et al., 2019). In line with this information; PS and PDI measurements were performed with DLS technique in our study. The PS and PDI of the NPs measured by Zetasizer Nano ZS are presented in Fig. 1. The PS obtained in the NP-Blank formulation was 106.5 nm, while the PS in the DS loaded NPs was in the range of 114.7-124.8 nm. As can be seen from the results, the encapsulation of DS in PLGA NPs increased the PS. In this study, maintaining a constant initial mass of polymer (60 mg), the mass of DS used varied between 5% and 15% in relation to polymer mass. It was observed that the increase in the initial loading of DS increased the average diameter of the NPs. This can be explained by the fact that a greater amount of DS results in a more viscous dispersed phase, making difficult the mutual dispersion of the phases and originating larger NPs. A similar result was obtained in a study examining the effect of formulation variables on PS and PDI values of PLGA NPs containing praziquantel. It has been reported that PS increases as the amount of praziquantel added to the formulation increases (Mainardes and Evangelista, 2005). When the NP-1, NP-2 and NP-3 coded formulations were compared statistically with NP-Blank, statistical differences were observed at different levels. The statistical difference between the blank formulation and NP-1, NP-2 and NP-3 coded formulations are $p<0.05$ (Significant difference), $p<0.01$ (Very significant difference) and $p<0.001$ (Extremely significant difference), respectively (Fig. 2). The average PDI of blank NP was 0.135, whereas the average PDI of DS loaded PLGA NPs was between 0.113 and 0.147 (Fig. 1). The PDI, which is a ratio that gives information about the homogeneity of the given NP system, reflects the quality of the NP dispersion within the range 0.0–1.0. PDI values <0.1 indicate the highest quality of dispersion. Most authors recognize PDI values <0.3 as optimum; however, values <0.5 are also acceptable (Şenel and Öztürk, 2019). According to the literature, it can be said that a monodisperse NPs are produced in this study. Statistically, there was no significant difference between the PDI values of the formulations ($p>0.05$). PS distribution graphs of formulations are given in Fig.3. As can be seen from the graphs, all formulations showed in a single peak proving the quality distribution of PSs of NPs. Colloidal stability was analyzed by measuring the ZP of the NPs (Fig. 1). Statistically, there was no significant difference between the ZP values of the formulations ($p>0.05$). The encapsulation of DS has not affected the ZP values of NPs. PLGA NPs were normally negatively charged considering that they are associated with a stable colloid nature. The negative ZP can be explained by residual PVA still present on the particle‟s surface even after three washing and which affects the number of carboxylate group endings (de Jesus Gomes et al. 2006). Another important explanation is that PLGA in neutral medium has negative surface potential attributed to terminal carboxyl groups, which can be confirmed by negative ZP obtained in PLGA NPs (de Lima et al., 2018). A colloidal system having ±30 mV as the ZP value is considered a stable formulation if dispersed as a colloidal dispersion in a liquid. ZPs between -5.0 and -15.0 mV are in the limited flocculation zone; and the maximum flocculation zone between -5.0 and -3.0 mV was reported earlier (Öztürk et al., 2019c; Öztürk et al., 2019f). When all the results are examined, it is seen that ZP values aren‟t at the limit of flocculation. This shows the stability of all PLGA NPs prepared.

Dissolution

The dialysis bag method was chosen to investigate DS release from three different PLGA NP formulation in SIF pH 6.8 containing. Dissolution profile of pure DS and all PLGA NP formulations are shown in Fig.5. The statistical comparison of the dissolution study results is given in Table 5. At the end of the 3rd h, pure DS reached 99.27%±0.31 cumulative release rate. NP-1, NP-2 and NP-3 coded NP formulation‟ reached 51.2%±1.7, 46.6%±3.3 and 48.3%±1.8, cumulative release rates at the end of the 3rd h, respectively. The cumulative release of DS from NP-1, NP-2 and NP-3 was

found to be 88.48%±2.69, 94.88%±2.55 and 98.32%±1.44 at 24 h, respectively. In Fig. 5., pure DS powder exhibited a rapid release of 99% of drug within 3h, whereas the release profiles of PLGA NP indicated a biphasic pattern with a burst release during the first 3 h, followed by a sustained release over 24 h. A fundamental understanding of the *in vivo* phenomenon of PLGA biodegradation is important because it determines the rate and mechanism of the release of drugs. The drug is either dispersed throughout the polymeric matrix or encapsulated in the hydrophobic NP core. The release of a therapeutic agent from NPs has usually been shown to be biphasic, initially by diffusion through the polymer matrix and later by diffusion of the therapeutic agent and degradation of the polymer matrix itself (Dinavard et al., 2011). Examining Fig. 5., rapid release followed by slow release. It can be said that it is a biphasic release. The PLGA degradation process is affected by several factors such as the NP preparation technique, the presence of low molecular weight compounds, NP size, the intrinsic properties of the PLGA (molecular weight, chemical structure, hydrophobicity, crystallinity, and glass transition temperature), physicochemical parameters (pH, temperature and ionic strength of the environment), site of implantation, and mechanism of hydrolysis (Jain, 2000; Dinavard et al., 2011; MartínBanderas et al. 2012). DS release rate from NPs within the first 3 h and 24 hours was observed as NP-3>NP-2>NP-1. When the dissolution results in this study were examined, it was concluded that PS was the most important parameter affecting the dissolution results. As can be seen in Fig. 5., there was an inverse relationship between release rate and PS. The smallest DS loaded NP formulation prepared in this study is NP-1 coded formulation. The NP-1 coded formulation showed slower and less release than other formulations. This has been explained by other researchers (Berkland et al., 2003; Martín-Banderas et al. 2012). These researcher verified that large microspheres/NPs degrade more quickly than small microspheres/NPs, probably because of an increased accumulation of the acidic products of PLG/PLGA hydrolysis in large microspheres/NPs. Hydrolysis creates acids, which catalyze hydrolysis. This autocatalytic process is known to cause faster degradation at the center of the PLGA matrix than at the surface. This effect becomes more pronounced with increasing dimensions of the NP system (Fredenberg et al., 2011).

Solid state characterization of PLGA nanoparticles

Thermal analysis

The DSC thermogram of DS, Blank NP and drug-loaded NPs obtained under nitrogen atmosphere are displayed in Fig. 8. In DSC thermogram of DS (Fig. 8a), endothermic and exothermic peak were observed at 286.00°C and 293.59°C indicating its crystalline nature. These complex thermal analysis peaks of DS were found to be consistent with the literature (Tudja et al., 2001). No melting point was observed in Blank NP (Fig. 8b), because PLGA appears amorphous in nature (Mainardes et al., 2006). No thermal analysis peaks of DS were observed in DS loaded PLGA NPs (Fig. 8c, 8d, 8e). According to the literature, it can be interpreted that DS in the NPs are either in an amorphous, disordered crystalline phase or in solid solute (Musumeci et al., 2006; Sanna et al., 2011).

FT-IR analysis

The FT-IR spectra of the DS, blank NP and DS loaded PLGA NPs are portrayed in Fig. 9. The FT-IR spectra of DS (Fig. 9a) exhibited distinctive peaks at around 3380.00 cm-1 due to NH stretching of the secondary amine, 1573.91 cm-1 owing to –C=O stretching of the carboxyl ion and at 744.52 cm-1 because of C-Cl stretching (Aielo et al., 2014). The FT-IR spectra of pure DS also showed that the peaks between 1000.00 cm-1 and 1350.00 cm-1 correspond to the CN group; while the peaks at 1498.69 cm-1 corresponding to C═C group (da Silva et al., 2015). When examine FT-IR spectrum of Blank NP exhibited molecular vibrations of PLGAs functional groups as shown in Fig. 9b. Carbonyl groups gave intense bands at 1755.22 cm-1 due to stretching vibration present in both monomers while medium density bands between around 1380.00 and 1130.00 cm-1 were respectively attributed to asymmetric and symmetric C-C(=O)-O stretches. Bands in those regions are distinctive bands often used for the characterization of esters (Elmaskaya et al., 2019). The blank NP formulation, FT-IR spectrum identical to PLGA, showed that preparation ingredients and formulation

parameters did not alter polymer structure (Yenilmez et al., 2011). The intensity of some peaks of DS decreased and distinctive peaks of DS were not seen in the spectra of all DS loaded PLGA NP formulations indicating the molecular dispersion of DS in the polymeric matrix which was supported by the DSC results. As a result, it can be concluded that PLGA masked the peaks of DS, which is frequently encountered in PLGA NP studies (Gamisans et al., 1999; Vega et al., 2008). This situation confirmed encapsulation of DS within the polymeric structure (Öztürk and Kıyan, 2020).

In vivo anti-inflammatory activity effects of diclofenac sodium and nanoparticles on the CAM

The anti-inflammatory activity of NP-blank, NP-1 and DS were examined using the *in vivo* HET-CAM assay. NP-1 was chosen as the optimum formulation since it has a high EE% value. For the assay, SDS was used to cause irritation and hemorrhage on the CAM. The anti-inflammatory results were summarized in Table 7. For the evaluation of the anti-inflammatory effect, a semi-quantitative score system was used as shown in Table 2 and a score index described in Table 3 (Öztürk and Kıyan, 2020). A characteristic strongly vascularized granuloma which was induced by SDS was observed on the CAM with star-like capillaries surrounding the pellet (Fig. 10). If SDS was applied together with the anti-inflammatory test compounds, normalization of the membrane irritation is observed (Fig. 10). According to the stereomicroscopic evaluations (Fig. 10) NP-Blank coded formulation showed uncertain anti-inflammatory effect with the inhibition of (40.6%±0.2) whereas DS loaded PLGA NP (NP-1) (equivalent DS 20 µg/pellet) showed good anti-inflammatory activity (72.8%±0.3) when compared to the well-known anti-inflammatory agent, DS with the concentration of 50 µg/pellet (83.52%±0.1). However, all tested samples showed no embryotoxic effect at the tested concentrations. As the anti-inflammatory results are discussed, DS loaded PLGA NP including DS lower dosage as 20 µg/pellet showed good anti-inflammatory potential with the inhibition value of 72.8%±0.3 when compared with the known good anti-inflammatory drug DS (50 µg/pellet, 83.52%±0.1). Therefore we can say we achieved to obtain a good anti-inflammatory effect at lower dosage as targeted due to the low PS of NP-1 and also resulted as the increase of permeation and absorption to the CAM (Conte et al., 2017). Anti-inflammatory, anticancer, anti-microbial effects and oral bioavailbility enhanced with NP structure have been reported in the literature before (Ravindran et al., 2010; Singh and Pai, 2014; Öztürk et al., 2019c). The literature data shows that nowadays there is an increasing demand for the research on ocular tolerance, toxicity, irritation potency and anti-inflammatory effects of nano-drug delivery systems. Previously, Gupta et al. reported the ocular tolerance of biodegradable levofloxacin loaded PLGA NPs was evaluated by using *in vivo* HET-CAM test (Gupta et al., 2011). Ocular irritancy of Chitosan-coated PLGA NPs of bevacizumab targeting the retina on the CAM was investigated by Pandit et al., 2017. The other previous work on the toxicity and irritation potentials of voriconazole solid lipid NP on the CAM surface has been reported by Kumar and Sinha at 2016. We also reported the antiinflammatory activity of dexketoprofen trometamol loaded different molecular weight chitosan NPs by using *in vivo* HET-CAM assay in our latest study (Öztürk and Kıyan, 2020). Therapeutic angiogenesis which means anti-angiogenic therapy with the targets of prevention and inhibition of neovascularization for treatment of such pathologies as cancer and chronic inflammation looks like a promising approach to provide new thoughts and leads for the improvement of new anti-tumor or anti-inflammatory drugs which are more effective but less toxic at low dosage (Griffioen and Molema, 2000; Bikfalvi et al., 2011). To the best of our knowledge this is the first study on the *in vivo* anti-inflammatory activities of DS loaded PLGA NPs.

4. Summary and conclusions

In this study, NPs were prepared with three different drug: polymer ratio. We have successfully formulated DS loaded PLGA NPs by double emulsification solvent evaporation technique. The NPs prepared were characterized by analyzes such as particle size, polydispersity index, zeta potential,

encapsulation efficiency, dissolution, release kinetic which are important in terms of pharmaceutical technology and pharmaceutical nanotechnology. In addition, solid state characterization of NPs was made by DSC and FTIR analysis. Particle size values of DS loaded PLGA NPs were in the range of 114.7-124.8 nm. It was observed that the increase in the initial loading of DS in formulation process increased the average diameter of the NPs. In the results of encapsulation efficiency, the opposite situation was encountered. However, it was concluded that NP-1 was quite good at encapsulation efficiency result (77.8%), therefore was chosen as the optimum. All NP formulations prepared released DS for up to 24 h. In the release of DS from NPs, first the burst effect was observed and then it released more slowly. As a result, the release of DS from NPs showed a biphasic profile. The most important thing in dissolution results is that particle size was the most important parameter affecting the dissolution results. An inverse relationship was found between release rate and particle size. Korsmeyer-Peppas, Peppas– Sahlin and Weibull models were found to be the most suitable models for DS release from all NPs. This has been interpreted as the DS release from NPs is predominantly not only by one mechanism, but also by a combined mechanism of Fickian and non-Fickian release. The antiinflammatory activity of NP-1 was examined using the *in vivo* HET- CAM assay. The antiinflammatory activity results indicated that DS loaded PLGA NP formulation (NP-1) showed significantly good anti-inflammatory potantial (when compared to the standard antiinflammatory DS at low dose. In conclusion, this study shows that DS-loaded PLGA NPs with good anti-inflammatory potential may be a new promising nanoformulation for the treatment of inflammation.

Declaration of competing interest

The author declares no conflict of interest, financial or otherwise.

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Tables

Table 1. Formulation ingredients

Formulation code	**PLGA**a	**DS**a
NP-Blank a	60 mg	-
NP-1	60 mg	3 mg
NP-2	60 mg	6 mg
NP-3	60 mg	9 mg

a PLGA: Resomer RG 502 H, **DS:** diclofenac sodium, **Blank:** formulation without diclofenac sodium.

Table 2. The Semi-quantative score system of anti-inflammatory effect on CAM after treatment

Category	**Type**	**Effects observed on CAM after treatment**
1	Irritated	The granuloma is strongly vascularized. A network of capillaries is formed starlike around the granuloma.
2	Weakly irritated	The granuloma is poorly vascularized. A thin network of capillaries is formed starlike around the granuloma.
3	Weakly normalized	The granuloma is somewhat smaller than in category 1 and 2 and only poorly vascularized. The starlike network of vessels is hardly recognizable.
4	Normalized	No granuloma or only a kind of scar can be observed (if the granuloma regresses a non-vascularized scar is left). The network of vessels is normal (as the control)

Table 3. The score index in the proportional inhibition of inflammation

Inhibition (%)	**Anti-inflammatory Effect**
≤ 40	No anti-inflammatory effect
40-55	Uncertain anti-inflammatory effect 55-70 Weak anti-inflammatory effect
70-85	Good anti-inflammatory effect
> 85	Strong anti-inflammatory effect

Table 4. The *in vivo* anti-inflammatory effects of DS-loaded NPs on the CAM

Test compound	DS concentration (μg/pellet)	Average inhibitiona (%)	Anti-inflammatory effect
NP-1	20	72.8 ± 0.3	Good
NP-Blank	-	40.6 ± 0.2	Uncertain
DS	50	83.52 ± 0.1	Good
SDS	-	34.6 ± 0.1	Inactive
Agar	-	-	Inactive

a Values are mean of average score ± Standard deviation (n = 15 for the test substances and n = 10 for standards). *p<0.05 compared with SDS.

Figures

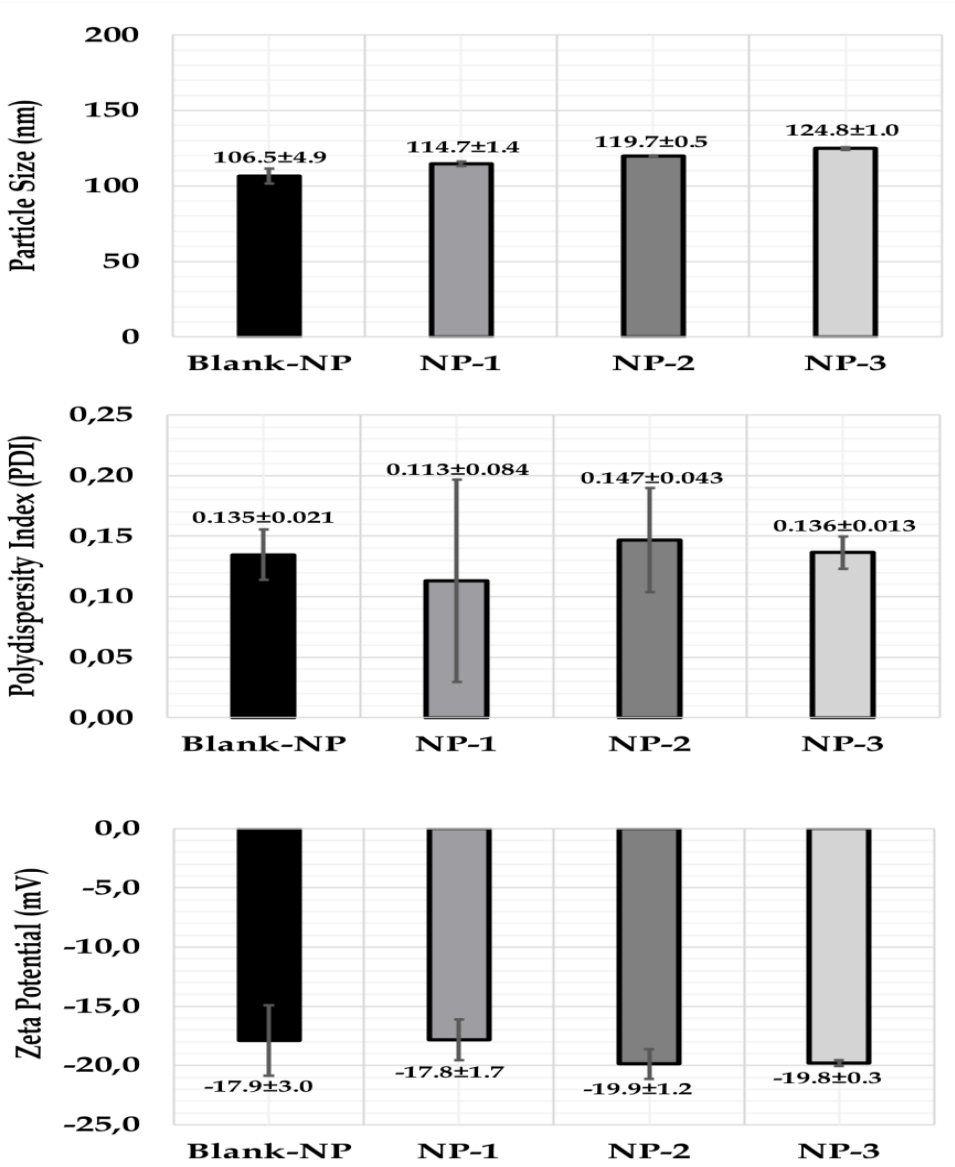

Fig. 1. Particle Size, Poly Dispersity Index and Zeta Potential Results

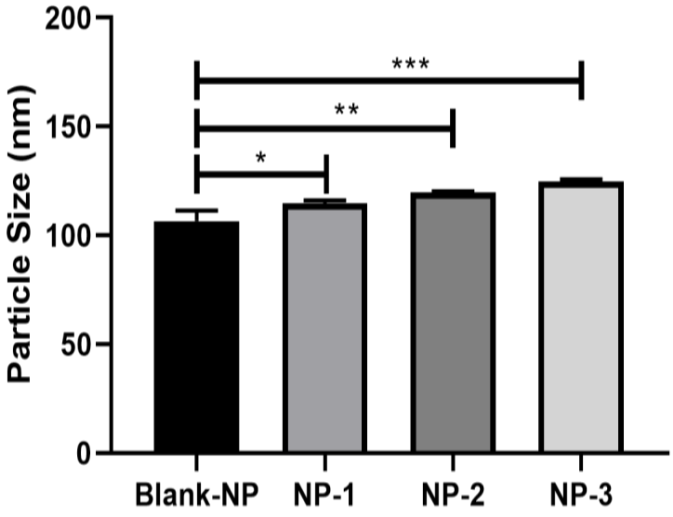

Fig. 2. Statistical analysis results of particle size.

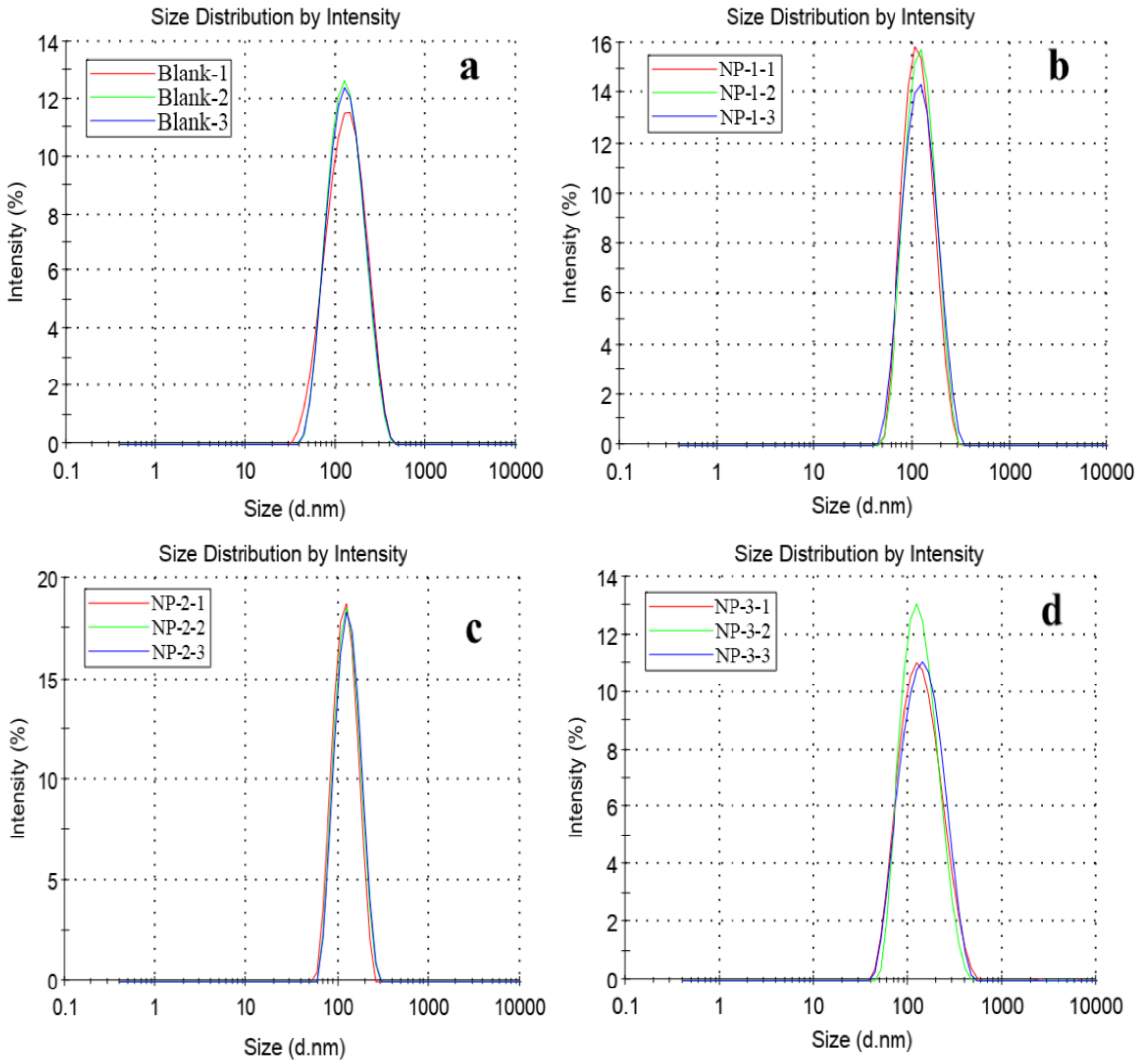

Fig. 3. Particle size distribution curves of nanoparticle formulations a: NP-Blank b: NP-1 c: NP-2 d: NP-3.

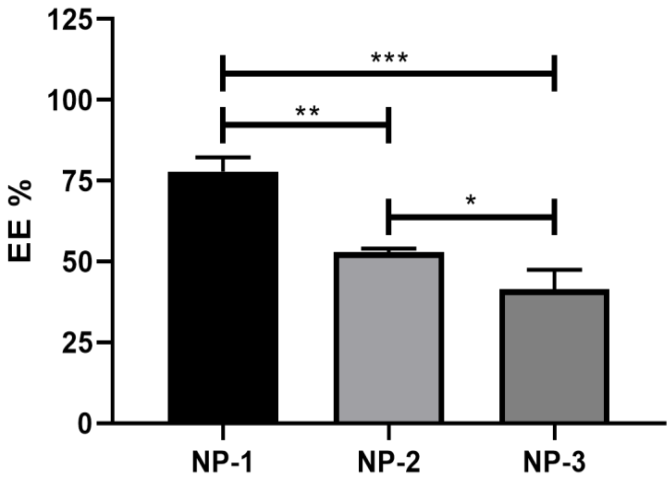

Fig. 4. Statistical analysis results of encapsulation efficiency.

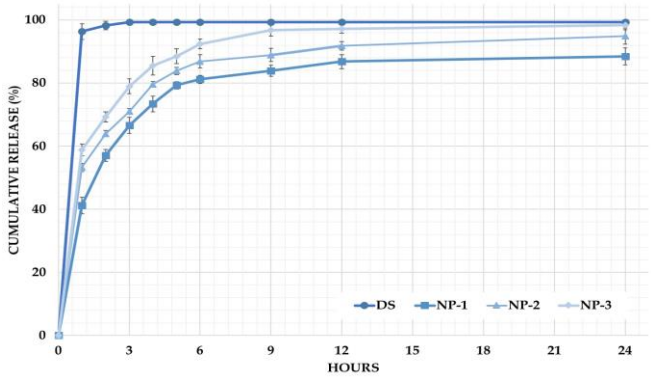

Fig. 5. Dissolution profile of pure diclofenac sodium and diclofenac sodium from nanoparticles

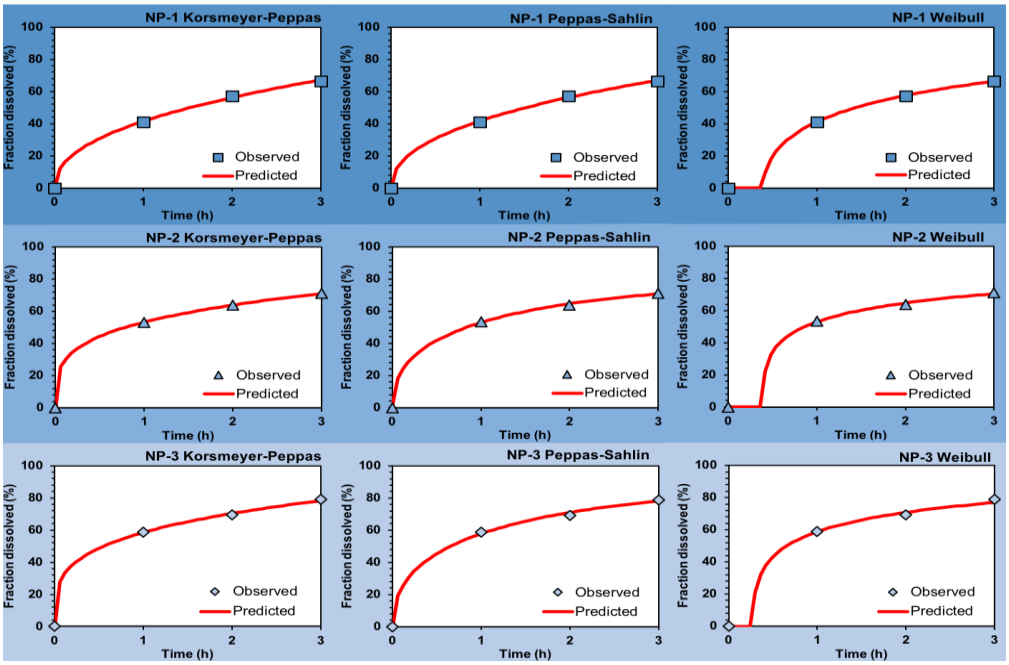

Fig. 6. Release kinetics results received automatically from the DDSolver program for 3 h

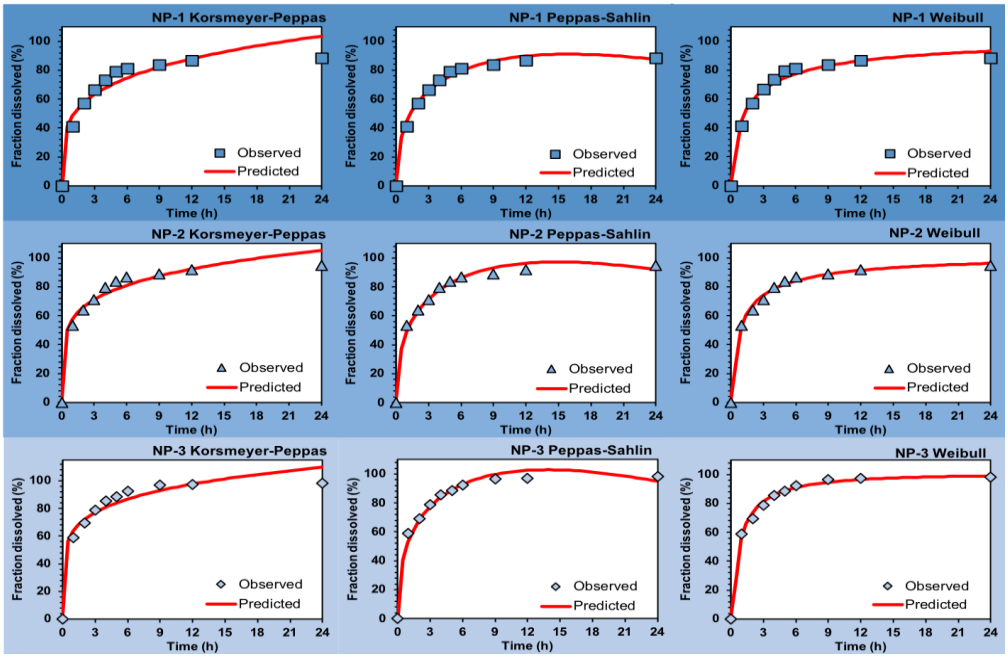

Fig. 7. Release kinetics results received automatically from the DDSolver program for 24 h**.**

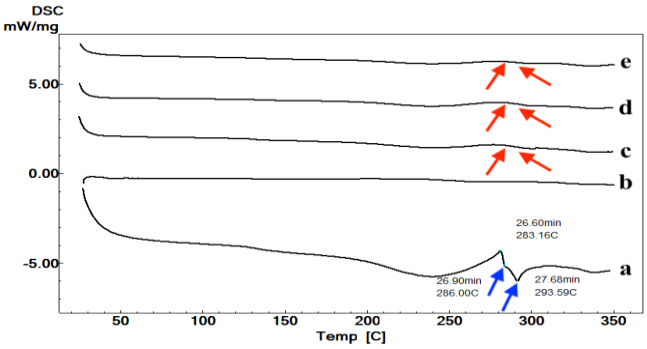

Fig. 8. DSC curves of diclofenac sodium and nanoparticles a: DS b: NP-Blank c: NP-1 d: NP-2 e: NP

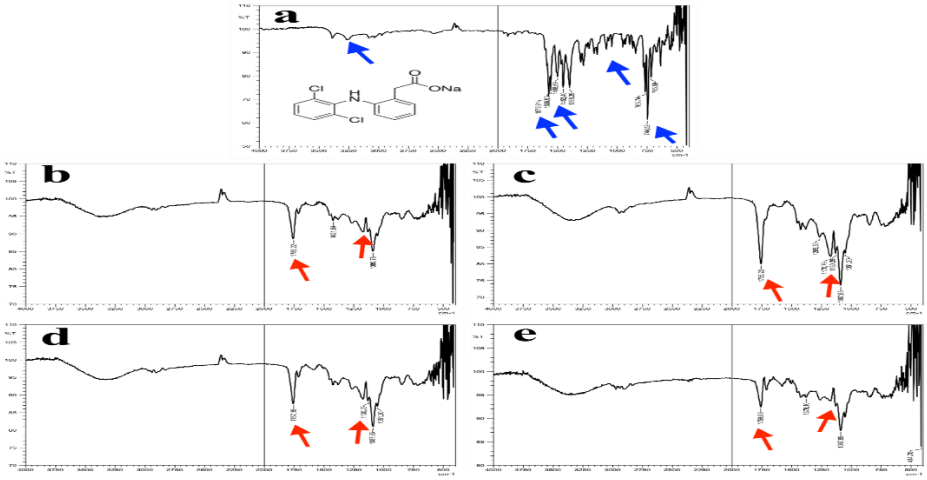

Fig. 9. FT-IR spectrum of diclofenac sodium and nanoparticles a: DS b: NP-Blank c: NP-1 d: NP-2 e: NP-3

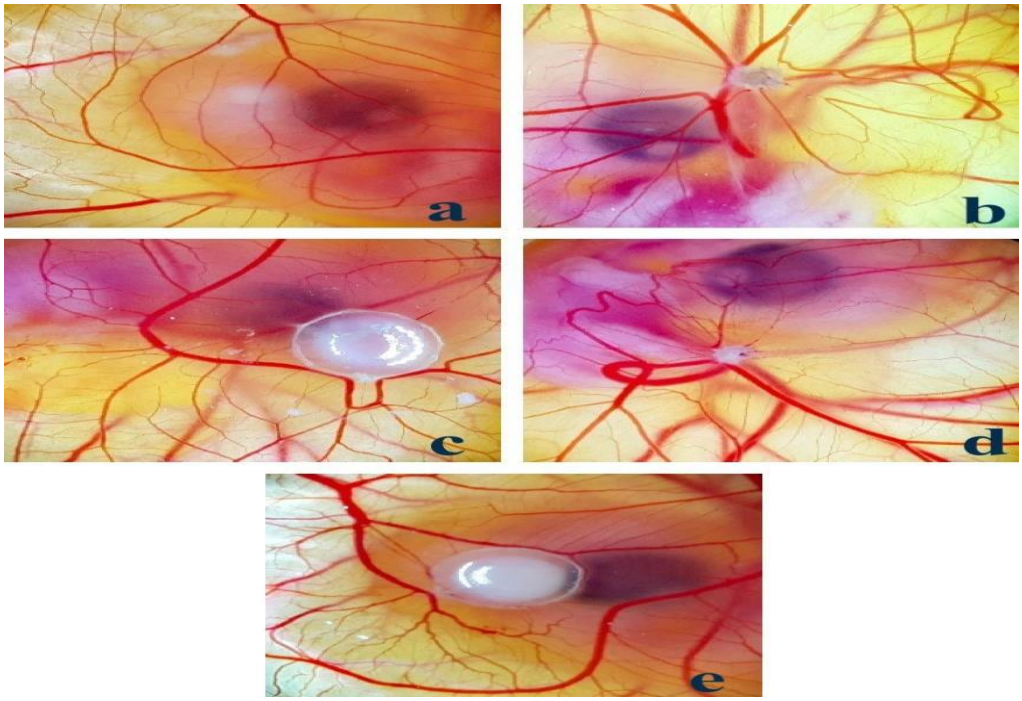

Fig. 10. The in vivo anti-inflammatory effects of DT-loaded NPs on the CAM a: CAM treated with Agar b: irritant and inflammatory effect of SDS c: Good anti-inflammatory effect of DS d: Uncertain anti-inflammatory effect of NP blank e: Good anti-inflammatory effect of treatment with DS loaded PLGA NP.

GRAPHICAL ABSTRACT