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Research Article

Enhancement of *in vitro* anti-inflammatory activity of aqueous leaf extract of *Xanthium strumarium* L. through encapsulation in chitosan nanoparticles

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Abstract

Xanthium strumarium L. (Asteraceae) is a medicinally important plant widely documented for its anti-inflammatory, antirheumatic and anti-rhinitis activities. Chitosan is a noteworthy versatile, biopolymer in drug delivery due to its biocompatibility and efficacy in controlled drug release. The aim of the present investigation was to encapsulate aqueous leaf extract of *X. strumarium* (XS-ALE) using chitosan nanoparticles (CSNPs), determine the *in vitro* drug release kinetics and to evaluate the anti-inflammatory activity using various *in vitro* assays. The XS-ALE encapsulated CSNPs (XS-ALE CSNPs) were synthesized and their entrapment efficiency, drug release percentage, drug release kinetics, various characterization studies such as UV-Vis spectroscopy, FTIR, SEM, zetapotential and anti-inflammatory activity were assessed. The concentration (5µg/mL) with maximum entrapment efficiency percentage (64.80%) was selected for further drug release, characterization and anti-inflammatory assays. *In vitro* drug release studies demonstrated a controlled and sustained drug release of 70.96% within 3.5 hours. The drug release kinetics fit in to the Higuchi model, exhibiting a high correlation coefficient (R^2 = 0.97), which suggests a diffusion-regulated release mechanism. Fourier transform infrared (FTIR) spectral analysis confirmed the presence of characteristic functional groups accountable to both chitosan and the plant extract. XS-ALE CSNPs showed irregular morphology with a positive charge of +32.7 mV. XS-ALE CSNPs showed the highest percentage inhibition for both protein denaturation (79%) and proteinase inhibitory (82.46%) compared to CSNPs and the reference drugs. The outcomes of the study suggested the efficacy of using XS-ALE CSNPs at minimal concentrations as a potent biocompatible and eco-friendly anti-inflammatory agent.

Keywords: *Xanthium strumarium,* chitosan nanoparticles, anti-inflammatory activity, drug release kinetics

1. INTRODUCTION

Nanotechnology deals with the nanometer (nm) sized nanoparticles (NPs), which are distinguishing materials with a characteristic dimension from 1 to 100 nm. Due to their noteworthy structural and physicochemical features, NPs have found applications in various areas such as biology, electronics, cosmetics, medicine, energy, environment and catalysis [1]. Nanoparticles can be prepared from naturally occurring polymers or synthetic polymers. Nanotechnology combined with polymers has recently attracted huge interest in many fields including the pharmaceutical industry and the medical field. Synthesis of nanoparticles using natural polymers provides an eco-friendly process without using organic solvents and higher shear energy. The benefits of polymer based nanoparticles are shielding the drug molecules in the systemic circulation, targeting the drug to the desired site, delivering the drug at sustained rate to the site of action and to discharge the drug at a controlled speed. In general, the active component is dissolved, entrapped/encapsulated, adsorbed or chemically attached in polymer based drug carrier [2].

Chitosan is a natural hydrophilic polymer generally resulting from chitin by alkaline or enzymatic deacetylation process. It is the second most common biopolymer existing on the earth after cellulose and it is found in the exoskeleton of crustaceans such as crabs, lobsters, shrimps and krill. The basic constituents of chitosan such as D-glucosamine and N-acetyl-D-glucosamine connected through β-(1-4) glycosidic bonds which are randomly distributed within the polymer. The cationic feature of chitosan allows it to create electrostatic complexes or multilayer structures with other negatively charged synthetic or natural polymers[3]. Chitosan nanoparticles exhibits many desirable characteristics such as biodegradability, low allergenicity[4], good biocompatibility, higher

encapsulation performance, low cost of manufacturing, renewable, permeation enhancer, pH sensitiveness, safety, improved absorption of drug across the epithelial membrane, controlled release action, bioadhesion and antimicrobial activity. It has been extensively reported for controlled distribution of various active compounds such as drugs, proteins, vitamins and genes [5]. Sodium tripolyphosphate is a commonly used as a crosslinking agent in the ionic gelation method for the synthesis of chitosan nanoparticles or nanochitosan. It reacts with the cationic groups on the surface of the nanoparticles and forms a stable nanoparticle matrix [6].

Inflammation is usually a body response to tissue damage and the reaction of cells toward inflammation which lead to certain pathological expressions characterized by redness, heat, swelling and pain[7]. Inflammation is the second phase of wound healing and arises right after the injury when the injured blood vessels leak extravascular fluid causing localized swelling/inflammation which controls both bleeding and infection. The fluid blister allows healing and repair cells to move to the location of the wound. During the inflammatory phase, damaged cells, pathogens, and bacteria are removed from the wound area. Inflammation is a natural measure of the wound healing progression and only problematic if prolonged for a long time [8]. There are many anti-inflammatory drugs to treat the inflammation which are steroidal or non-steroidal types of anti-inflammatory drugs (NSAIDS). However, various studies imply that these drugs are not free from adverse effects, since they are responsible for gastrointestinal complications such as mucosal damage and bleeding [9]. And also, NSAIDS can also cause acute renal failure [10]. For these reasons, many researchers have displayed their attention on invention of many medicinal plants with anti-inflammatory properties as they have shown reduced side effects [11].

Xanthium strumarium L. belongs to the family Asteraceae and also known as cocklebur or burweed which is commonly found throughout India [12]. It is an annual herb and grows upto 1m in height and shown irregularly toothed three lobed leaves, bur-like flowers and small oblong fruits with surrounded curved hooks. The whole plant has shown various medicinal properties such as anti-inflammatory and anti-nociceptive [13], anti-mitotic [14], diuretic [15], repellent and insecticidal [16], anti-tumour [17,18], anti-microbial [19], anti-fungal [20], anti-trypanosomal [21], anti-oxidant [22], hypoglycemic [23], anti-titussive [24], anti-plasmodial [25]. To the best of our knowledge, there is no report on anti-inflammatory activity of chitosan nanoparticles loaded with leaf aqueous extract of *X. strumarium*. Therefore, the present investigation is aimed to encapsulate *X. strumarium* leaf aqueous extract in nanochitosan and to evaluate its anti-inflammatory potential using some *in vitro* models.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

For this study, medium molecular weight chitosan (CS) (MW=190-310 kDa, degree of deacetylation: 78-85%) was purchased from Sigma-Aldrich. Bovine serum albumin, casein, trypsin, Tris-HCl (MW= 157.6), glacial acetic acid, sodium dihydrogen phosphate, sodium tripolyphosphate were procured from SRL (Sisco Research Laboratories), Mumbai, India.

2.2 Collection, Identification and Plant Extraction

X. strumarium leaves were collected from Bharathiar University, Coimbatore Tamil Nadu, India and authentication of the plant was carried out in the Department of Botany, Bharathiar University. Leaves were collected from mature plant and cut into small pieces and washed under running water to remove adhering rubbles. Then, they were dried under shade and ground into fine powder and stored at 4°C. 10 grams of leaf powder were taken and mixed in 100 ml of distilled water and the mixture was boiled at 80°C for 30 minutes in water bath and cooled at room temperature. Then, the extract i.e. aqueous leaf extract (XS-ALE) was filtered through Whatman No. 1 filter paper and the collected filtrate was dried and stored in refrigerator till further use.

2.3 Synthesis of *X. strumarium* aqueous leaf extract encapsulated chitosan nanoparticles (XS-ALE CSNPs)

XS-ALE CSNPs were prepared according to the ionic gelation method of (Shivangi *et al.* [26]. Briefly, chitosan (2% w/v) was dissolved in dilute acetic acid (1% v/v) overnight at room temperature to obtain 2 mg/2 mL concentration solution and pH was adjusted to 5.0 using 1 M NaOH. Sodium tripolyphosphate (TPP) was dissolved in distilled water to acquire a final concentration of 1 mg/mL. XS-ALE solution was prepared by liquefying the filtrate at a concentration of 1 mg/1 mL. Different concentrations of XS-ALE (5, 10, 15, 20 and 25 µg/ml) were taken and mixed with 1 mL of TPP separately. Next, these mixture were added to 1 ml of chitosan solution to reach a final mass ratio of 3:1 (chitosan:TPP) and then, they were stirred for 45 min. Then, they were centrifuged at 10000 rpm for 15 min and washed twice with de-ionized water. After centrifugation, the supernatants were removed and the dried pellets containing XS-ALE CSNPs were stored in the refrigerator at 4°C for further use.

2.4 Determination of Entrapment Efficiency (EE %)

The amount of XS-ALE encapsulated within the CSNPs was estimated by indirect method, through calculating the amount of unencapsulated drug. After adding the TPP, the mixture was centrifuged at 10000 rpm for 15 min and the clear supernatant containing the free unencapsulated drug was collected, diluted with distilled water and determined spectrophotometrically at 273 nm [27]. The drug i.e. XS-ALE entrapment efficiency in CSNPs was determined using the following equation.

$$EE\% = \frac{(\text{Total amount of XS} - \text{ALE added} - \text{ free XS} - \text{ALE in supernatant})}{\text{Total XS} - \text{ALE added}} \times 100)$$

2.5 Determination of *in vitro* drug release percentage

XS-ALE release percentage from CSNPs was determined

using the technique of dialysis tube analysis (12,000-14,000 molecular weight). In brief, the dialysis membrane was washed with lukewarm double distilled water (70ºC) for 1hr and rinsed thoroughly (thrice) to eliminate glycerin. XS-ALE CSNPs with higher entrapment efficiency (64.80%) was placed in a dialysis bag which was sealed and immersed in 50 mL of phosphate buffer (pH 7.4) at room temperature with stirring at 1000 rpm for 6 h. 3 mL of the solution was withdrawn every half an hour and replaced with an equivalent volume of fresh solution. This process was repeated upto 3.5 hours. The withdrawn samples were analyzed using UV/visible spectroscopy at 265 nm and the amount of XS-ALE release pattern from CSNPs was determined [28]. The drug release percentage was determined by using the following formula.

$$\text{Drug release [\%]} = C(t)/C(0) \times 100$$

where C(t) is the absorbance of XS-ALE CSNPs at 265 nm at time t.

2.6 *In vitro* drug release kinetics

Various release kinetic models such as zero order, first order, Higuchi model and Korsmeyer–Peppas have been used to fit the cumulative *in vitro* drug release data and to describe the drug release kinetics [29]. The best release pattern is explained using the coefficient of determination (R2) value. Model with the highest R2 is considered as the best one [30].

2.7 Characterization of drug loaded chitosan nanoparticles

CSNPs and XS-ALE CSNPs were subjected to UV-vis spectrophotometry and Fourier transform infrared spectroscopy (FTIR) to study optical properties and to identify functional groups, respectively. Crystallinity patterns of CSNPs and XS-ALE CSNPs were determined by X-ray diffraction (Shimadzu LabX- XRD 1600). Particle size, the charge on the surface of the nanoparticles and poly dispersity index (PDI) were measured through Dynamic Light Scattering (DLS) with zetasizer (Malvern analytical, Chennai India). Topography and elemental composition of CSNPs and XS-ALE CSNPs were investigated through Scanning electron microscopy (Quanta 400 ESEM).

2.8 *In vitro* anti-inflammatory activity

2.8.1 Protein denaturation ability

Protein denaturation activity of the CSNPs and XS-ALE CSNPs was analyzed. 5 ml of reaction mixture was comprised of 0.2 ml of eggs albumin, 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of 5µg/ml concentration of both samples. The mixture was incubated at 37ºC for about 15 mins and then heated at 70ºC for 5 mins. After cooling, absorbance was measured at 660 nm. Aspirin was used as reference drug [31]. The percentage inhibition of protein denaturation was calculated by the following formula,

$$Inhibition\ \% = \frac{Absorbance\ of\ control\ -\ Absorbance\ of\ test\ sample}{Absorbance\ of\ control} \times 100)$$

2.8.2 Proteinase inhibitory action

Proteinase inhibitory activity of the CSNPs and XS-ALE CSNPs were quantified according to the method Sakat *et al.*[32] with some modifications. Briefly, the reaction solution (2 ml) consisted of 0.06 mg trypsin, 1 ml of 20 Mm Tris-HCl buffer (pH 7.4) and 1 ml test sample. The solution was incubated at 37ºC for 5 min and then 1 ml of 0.8% (w/v) casein was added and the mixture was further incubated for an additional 20 min. After incubation, 2 ml of 70% per chloric acid was added to terminate the reaction. The mixture was centrifuged and the absorbance of the supernatant was taken at 210 nm. Aspirin was used as reference drug. The percentage inhibition of proteinase activity was calculated as with the following formula,

$$Inhibition\ \% = \frac{Absorbance\ of\ control\ -\ Absorbance\ of\ test\ sample}{Absorbance\ of\ control} \times 100)$$

2.9 Data analysis

Statistical analysis was carried out by applying ANOVA, followed by Duncan's multiple range tests to identify significant differences among means ($P < 0.05$) using SPSS 16.0. All values represent the average of three replicates and are reported as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1 Entrapment efficiency, *in vitro* drug release percentage and kinetics

The entrapment efficiency (EE) of XS-ALE-CSNPs was determined using three replicates (Fig.1). Chitosan nanoparticles displayed a regression in the effectiveness of encapsulation with an increase in the concentrations of ALE. Among various concentrations of ALE (5, 10, 15, 20, 25 µg/ml), the entrapment efficiency was found to be higher (64.80%) at 5 µg/mL. The entrapment efficiency was defined as the ratio of the mass of formulations connected drug to the total mass of the drug [33]. The entrapment efficiency is one of the most important physiochemical features of a drug. It gives an idea about the percentage of drug that is effectively entrapped within nanoparticles [34].

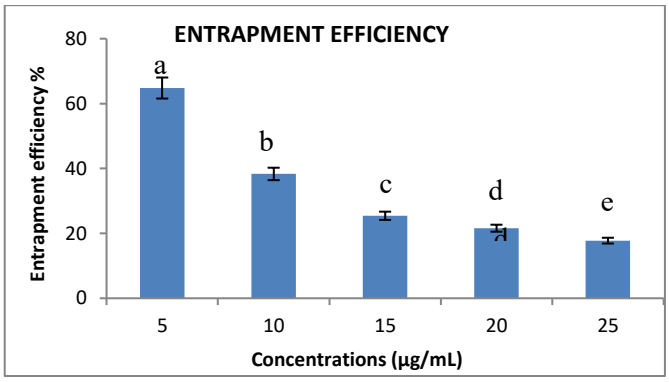


Figure 1: Entrapment efficiency of different concentrations of XS-ALE encapsulated nanochitosan. Data are mean ± Standard deviation of three replicates. Data which are statistically significant (p <0.05) different as stated by Duncan's Multiple Range Test, where, a> b> c> d> e.

The concentration showing higher EE i.e. 5 µg/ml was selected to determine the *in vitro* drug release percentage and also to study the *in vitro* drug release kinetics. The release percentage of XS-ALE from CSNPs steadily increased up to 3.5 hours with a cumulative drug release percentage i.e. 70.96 % then, the release occurred in a sustained manner (Fig. 2). Drug release from chitosan depends upon the degree of cross-linking, morphology, size and density of the particulate system, physiochemical property of the drug, solubility of the drug, adsorption feature of drug, drug diffusion through nanoparticle matrix [35]. Drug release from nanoparticles usually takes place via one or more mechanisms such as surface erosion, diffusion, disintegration or fragmentation [36]. In this study, XS-ALE release from CSNPs occurred in rapid and sustained manners which are the two promising features of chitosan polymer. Generation of initial burst segment due to drug adsorption onto the surface of NPs and subsequent a steady diffusion of the drug from the polymer matrix followed by the breakdown of the carrier matrix are responsible for sustained drug release [37].

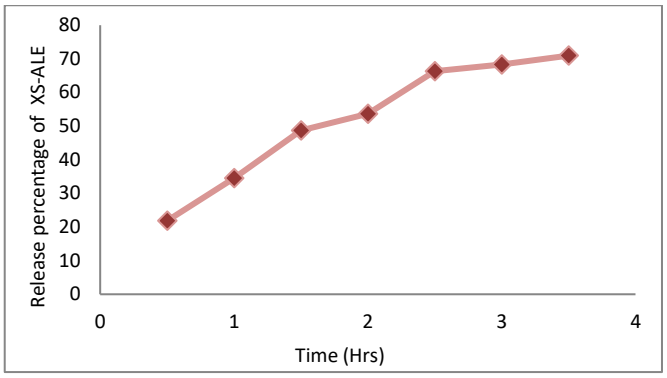


Figure 2: Drug release percentage of XS-ALE CSNPs

In the present study, to determine the kinetic behavior of XS-ALE release from CSNPs, *in vitro* drug release data were fitted into four mathematical models such as zero order, first order, Higuchi model and Korsmeyer–Peppas model (Fig. 3A-D). Among four models, the release of XS-ALE from CSNPs was found to be best fitted into the Higuchi model. The calculated regression coefficient values (R^2) are presented in Table 1.

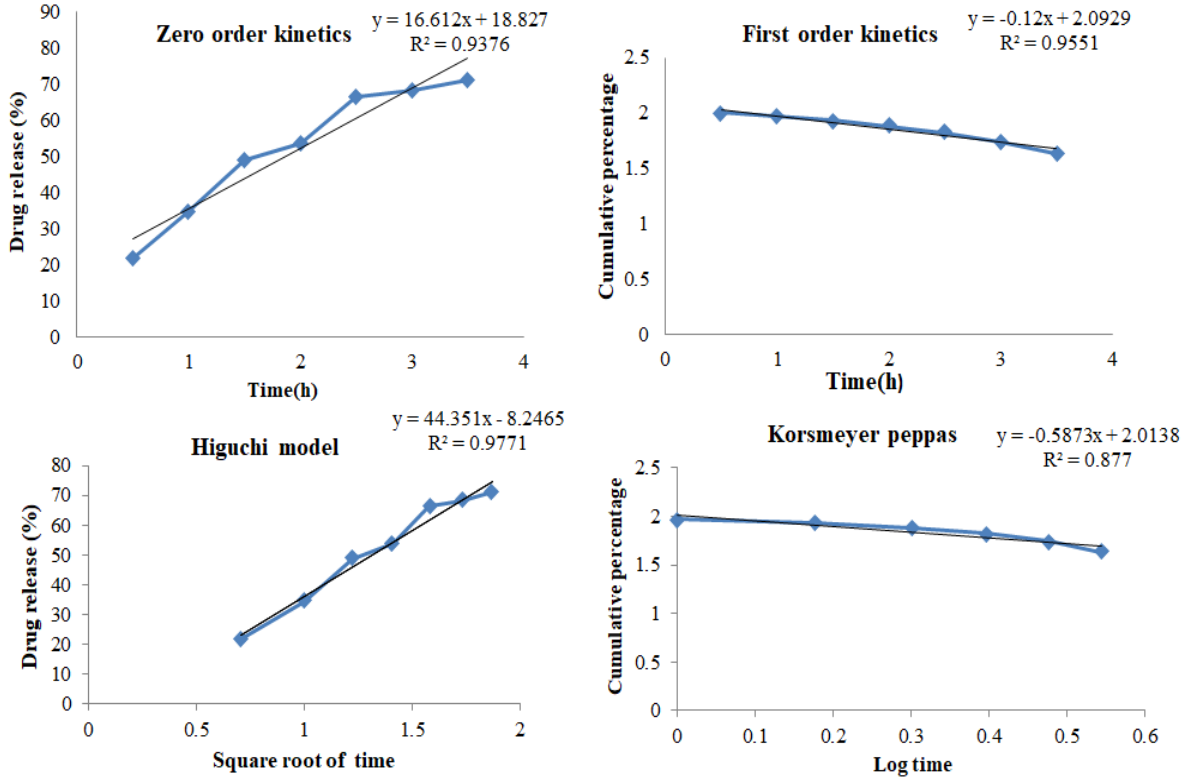


Figure 3: *In vitro* drug release kinetics plots

Table 1: Correlation coefficients and kinetic constants of different kinetics models for XS-ALE CSNPs

Kinetic Models	R^2	Slope	Intercept
Zero order kinetics	0.0937	16.61	18.82
First order kinetics	0.955	-0.12	2.092
Higuchi model	0.977	44.35	-8.246
Korsmeyer-Peppas model	0.877	-0.587	2.013

The successful release behavior of a drug from NPs is very useful in defining its pharmacological effects [38]. The drug release kinetics from NPs is an important characteristic aspect of the formulation. *In vitro* release kinetics data is an important measure for understanding the *in vitro* and *in vivo* relationships [39]. Estimate of drug release by *in vitro* mathematical models not only predicts drug release mechanisms and also helps in the development and controlled release of drugs from formulation [40, 41].

3.2 Characterization of XS-ALE CSNPs

3.2.1 UV Vis Spectroscopy

The concentration showing higher entrapment efficiency i.e. 5 µg/ml was selected for various characterization studies.

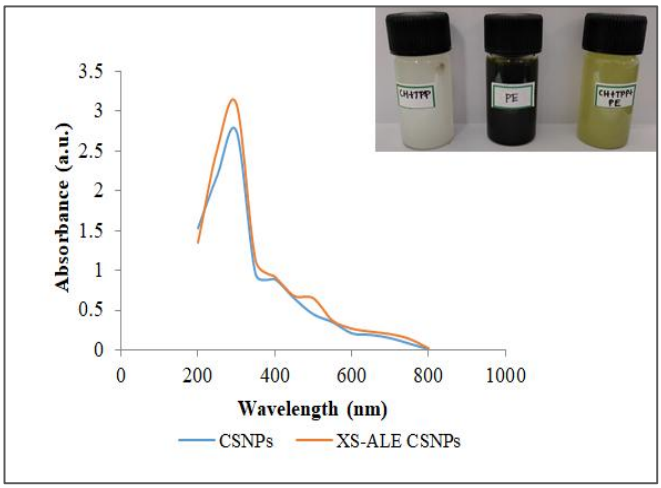


Figure 4: UV absorption spectra of XS-ALE CSNPs

3.2.2 FTIR Spectroscopy

Fourier transform infrared spectroscopy (FTIR) is an analytical technique used to assess variations in biomolecular composition by examining the vibrational characteristics of their chemical functional groups [44]. In this study, the FTIR spectrum obtained for aqueous leaf extract encapsulated nanochitosan functional groups were showed at nine absorption peaks with various intensity peaks such as low, medium and high. FTIR spectroscopy of XS-ALE CSNPs shared some of the functional groups such as C-O, diketones, ester carbonyl and polysaccharides with the chitosan nanoparticles (CSNPs). Functional groups like halogen compound, aromatic ring and phosphine are unique to XS-ALE CSNPs. It may be due to the loading of XS-ALE onto CSNPs. A comparison of the FTIR spectra of CSNPs and XS-ALE CSNPs revealed only minor spectral variations, indicating that XS-ALE was incorporated into the chitosan nanoparticles primarily through physical entrapment rather than chemical interaction, in agreement with the findings reported by Soltanzadeh *et al.* [45].

To identify the absorbance peaks of both CSNPs and XS-ALE CSNPs, a UV/Vis spectrophotometer scans were taken over the wavelength range of 200 to 800 nm. The strong surface plasmon resonance (SPR) centered at 300 nm was ascribed to both CSNPs and XS-ALE CSNPs (Fig. 4.). Optical density (OD) value for XS-ALE CSNPs was found to be 3.09 whereas CSNPs had 2.75. The increased value of OD is due to the encapsulation of XS-ALE with CSNPs which confirmed the formation of nanochitosan encapsulated drug. Similar observation was made by various researchers [42, 43].

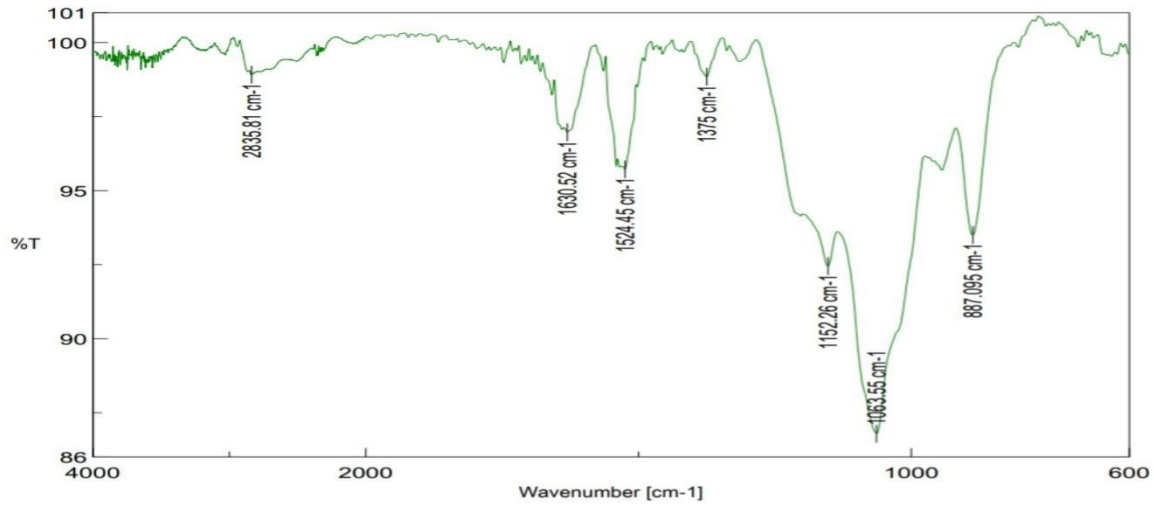


Figure 5: FTIR spectra of CSNPs

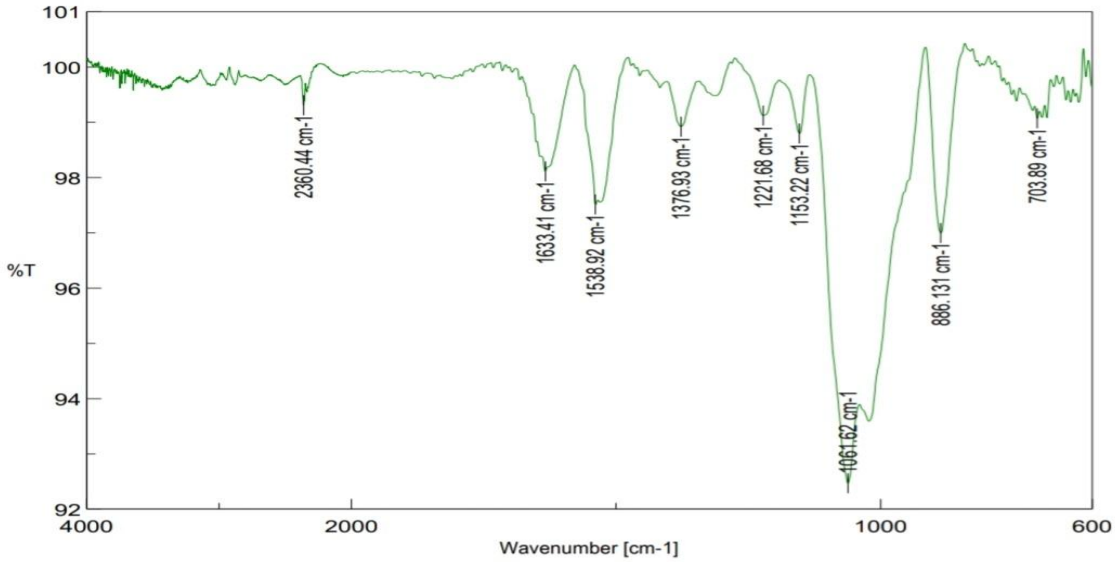


Figure 6: FTIR spectra of XS-ALE CSNPs

3.2.3 SEM analysis

The synthesized chitosan nanoparticles (CSNPs) and aqueous leaf extract encapsulated nanochitosan (XS-ALE CSNPs) were morphologically studied using SEM. Fig. 7 and fig. 8 shows the SEM images of chitosan nanoparticles and aqueous leaf extract loaded nanochitosan at 20 µm scale bar, respectively. The SEM images confirm the formation of chitosan nanoparticles. The observations indicate that the shape of the XS-ALE CSNPs is irregular or agglomerated.

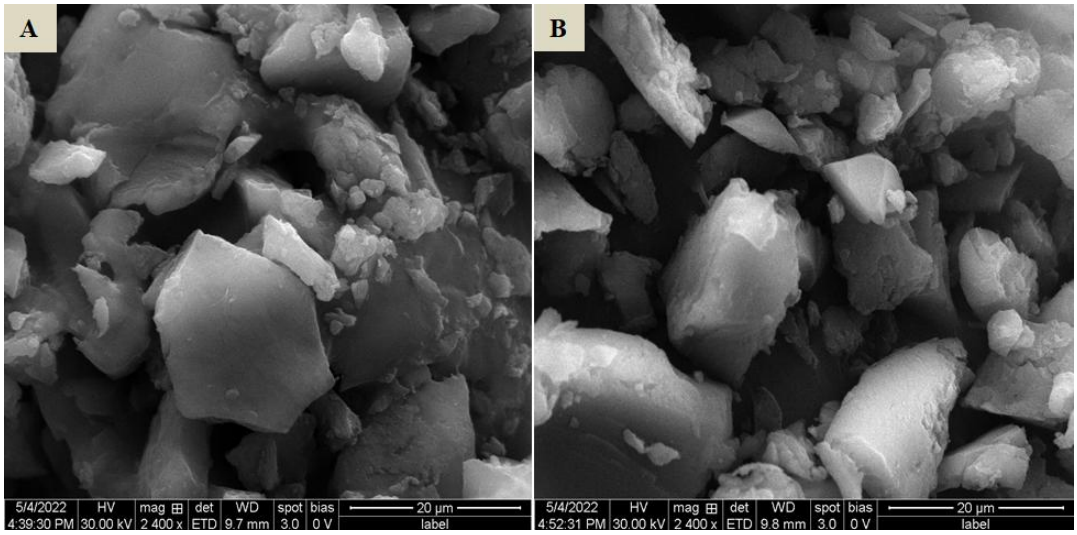


Figure 7: SEM micrograph of A) CSNPs and B) XS-ALE CSNPs

3.2.4 Zeta potential

Zeta potential reflects the surface electrical charge of particles, which is influenced by both the particle composition and the surrounding dispersion medium. It is commonly employed as an indicator of nanoparticle surface characteristics and stability. Zeta potential of CSNPs was found to +26.8 mV. XS-ALE CSNPs showed more charge i.e. +32.7 mV compared to CSNPs (Fig. 8A and B). The strongly positive zeta potential of chitosan nanoparticles facilitates effective interaction with mucosal surfaces. This mucoadhesive property is particularly advantageous for oral and nasal routes of administration, where it enhances the residence time at the absorption site, thereby improving molecular uptake and increasing overall bioavailability [46].

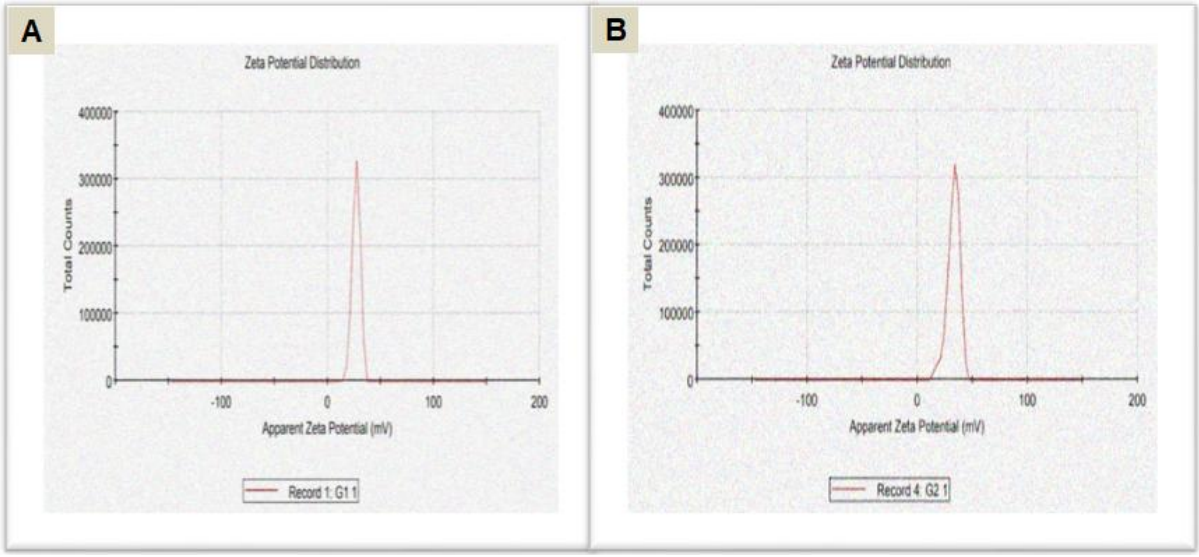


Figure 8: Zeta potential of A) CSNPs and B) XS-ALE CSNPs

3.3 *In vitro* Anti-inflammatory assays

3.3.1 Protein denaturation

Inflammation can be initiated when intracellular proteins lose their native structural integrity. In the present study, *in vitro* anti-inflammatory activity of CSNPs and XS-ALE CSNPs were estimated against denaturation of egg albumin (Fig. 9). Among these, aqueous leaf extract encapsulated chitosan nanoparticles (XS-ALE CSNPs) showed maximum denaturation percentage (79%) compared with reference drug aspirin (68%) at the concentration of 5µg/mL. Our results are in accordance with the findings of Gobalan *et al.* [47] in which *Ocimum tenuiflorum* leaf extract loaded chitosan nanoparticles exhibited maximum denaturation activity at lower concentration. Also this result supports the outcomes of Shafqat *et al.* [48] where ChBetNPs showed higher percentage compared to the CSNPs and standard drug.

3.3.2 Proteinase inhibitory activity

Proteinase inhibitors are essential in limiting the development of numerous inflammation-related pathological disorders [49]. In the present investigation, the *in vitro* anti-inflammatory effect of chitosan nanoparticles (CSNPs) and aqueous leaf extract (XS-ALE CSNPs) encapsulated chitosan nanoparticles were assessed (Fig. 9). Among these samples, aqueous leaf extract encapsulated chitosan nanoparticles (XS-ALE CSNPs) showed maximum inhibitory percentage (82.46%) compared to its reference drug (76.30%) at the concentration of 5µg/mL. Comparable findings have been reported by multiple researchers [50].

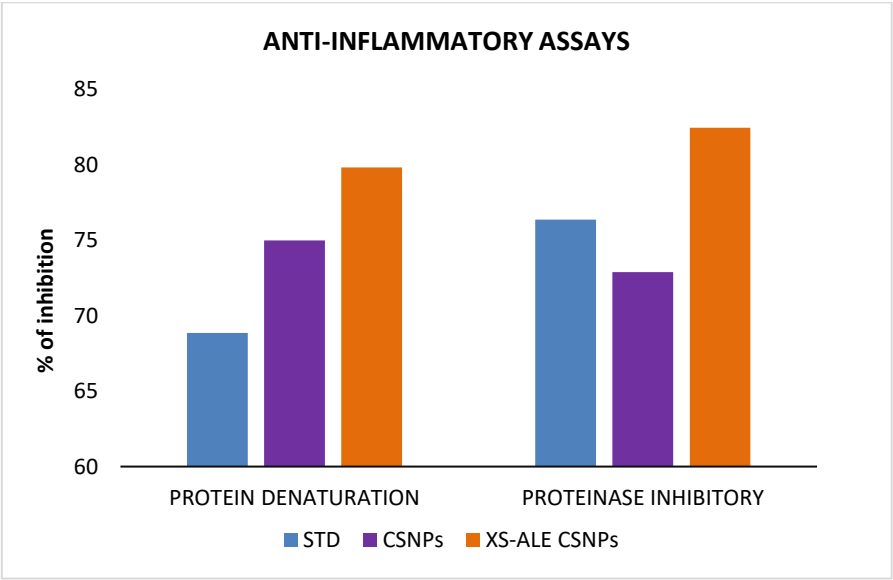


Figure 9*: In vitro* Anti-inflammatory activities of CSNPs and XS-ALE CSNPs

CONCLUSION

Based on the current study, it can be concluded that the aqueous leaf extract encapsulated chitosan nanoparticles at lower concentration (5µg/mL) can be considered as an effective anti-inflammatory drug compared to widely standard drug. This was also supported by the results of the *in vitro* drug release kinetics. However, further cytotoxicity and *in vivo* model studies are necessary for the determination of effective anti-inflammatory potential of the prepared drug.

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Conflicts of interest: The authors declare no conflict of interest.

Data availability: *The data will be made available upon request.*

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