IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
January 2017 Vol.:8, Issue:2

Evaluation of Anticancer Activity of Anastrozole Loaded Nanoparticle on DMBA Induced Breast Carcinoma Model in Mice

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Submission: 5 January 2017
Accepted: 10 January 2017
Published: 25 January 2017

www.ijppr.humanjournals.com

Keywords: DMBA, chitosan, anastrozole, breast carcinoma, ionic gelation method, immunoassay

ABSTRACT

Breast carcinoma is the most commonly occurring carcinoma comprising almost one-third of all malignancies in females both in the developed and the developing world. This research was aimed at evaluating the effect of anastrozole loaded chitosan nanoparticles on the progression of breast carcinoma in a chemically induced estrogen-dependent breast model in mice. Breast carcinoma was induced by divided doses 4 mg/day for 5 consecutive days of 7, 12-dimethylbenz (a) anthracene in 0.2 ml olive oil. After 12 weeks, Animals were administered with doses of anastrozole loaded chitosan nanoparticles and non-nano (standard) drug, treatment lasted for 4 weeks. An ionic gelation method was used to prepare anastrozole controlled release nanoparticle and their characterization was done by particle size determination, zeta potential measurement, TEM (transmission electron microscopy) analysis and release rates of drug from nanoparticles. Attenuated total refraction infrared spectroscopic analysis was employed to determine any interactions between drug and polymer. During the therapy, the efficacy of prepared nanoparticles was compared with standard drug. Body weight changes, hematological parameter (Hemoglobin level, RBC count, WBC count, Hematocrit value, Platelets Counts), histopathology and immunoassay (VEGF, TNFα) were carried out to compare the efficacy and toxicity associated with nano and non-nano anastrozole formulation in breast carcinogenesis. The results demonstrated that anastrozole nanoparticles with chitosan could be an alternative delivery method for the long-term treatment of breast cancer.

1. INTRODUCTION

Carcinoma is a group of diseases that cause epithelial cells in the body to change and grow out of control. Among women, the 5 most common sites diagnosed with carcinoma were breast, colorectum, lung, cervix, and stomach. Breast carcinoma is the most commonly occurring carcinoma comprising almost one-third of all malignancies in females both in the developed and the developing world (WHO 2014). The number of new cases of female breast carcinoma in 2016 was 125.0 per 100,000 women per year. Approximately 12.4 percent of women will be diagnosed with female breast cancer at some point during their lifetime (NCI, SEER 2016). Mortality rates are highest in the very young (<35yrs) and in the very old (> 75yrs)[3].

The main factor that contributes to breast cancer mortality is the presence of metastasis and development of resistance to chemotherapeutics due to inefficient drug delivery to tumor cells. Owing to these short- comings, there is a need for better therapeutic options which will increase the chances of survival of breast cancer patients with high efficacy for a sufficiently long period of time and to improve the patient compliance. Therefore the development of drug delivery systems by using nanomaterial's as a carrier that is able to modify the biodistribution, tissue uptake and pharmacokinetics of anticancer drugs so that thousands of molecules of a drug deliver to the required site for a longer period.

Anastrozole is a potent and selective non-steroidal aromatase inhibitor. The dose of Anastrozole (Arimidex tablet) is 1 mg orally once a day. For adjuvant treatment of early breast cancer in postmenopausal women, the median duration of therapy is 31 months[4]. To increase patient compliance, a sustained delivery system of Anastrozole could be used. One of the technological resources used to improve the permanence of drugs at the site of action is the use of therapeutic systems prepared using biodegradable polymeric carriers such as nanoparticles. The Nanometric size of these carrier systems allows efficient crossing of biological barriers, amelioration in tissue tolerance, improved cellular uptake, and transport, thus enabling efficient delivery of the therapeutic agents to the Breast carcinoma[5]. Chitosan possesses some ideal properties of a polymeric carrier for nanoparticles such as biocompatibility, biodegradability, positive charge, non-toxicity and low cost. This characteristic employed to prepare cross-linked chitosan nanoparticles.

7, 12-dimethylbenz (a) anthracene (DMBA) is a precarcinogen that can be converted into the ultimate carcinogenic metabolite DMBA 3, 4-dihydrodiol-1,2-epoxide which subsequently bind to adenine and guanine residues of DNA and form adducts, contributing to carcinogenesis[6]. This model induced by DMBA closely resembles human Breast Carcinoma and the histopathological changes to form premalignant and then malignant lesions are alike in many respects to those of human Breast Carcinoma Thus, DMBA-induced mammary gland tumors in mice are a useful tool for investigating the mechanisms of pathogenesis and development of human Breast Carcinoma.

Hence, in the present study, an attempt was made to formulate sustained release dosage form of Anastrozole using biodegradable polymers and evaluate their anticancer activity in 7, 12 Dimethyl Benz Anthracene model in mice.

2. MATERIAL AND METHODS

2.1.Material:

Anastrozole was a gift sample from Cadila healthcare Pvt. Ltd., Ahmedabad. DMBA was purchased from Sigma chemical company (St. Louis, MO, USA). Chitosan, sodium tripolyphosphate (TPP), Glacial acetic acid and other reagents were made available at department through a distributor. All chemicals used were of analytical grade.

2.2.Methods

A. Preparation of Anastrozole loaded chitosan nanoparticles:

Preparation of Anastrozole loaded chitosan nanoparticles (CS) nanoparticles were prepared by the ionic gelation method. The preparation of CS nanoparticles is based on an ionic interaction between positively charged CS solution and negatively charged sodium TPP solution. The accurately weighed amount of Anastrozole was dissolved in a polymeric solution of chitosan in 1% aqueous acetic acid solution and TPP was dissolved in distilled water. Then, 12 mL of sodium TPP solution was dropped into 30 mL CS solution under magnetic stirring (1000 rpm) at room temperature. CS nanoparticles were formed instantaneously. CS nanoparticle suspension was kept stirring for 30 min for further cross-linking of nanoparticles. Finally, CS nanoparticles were collected by centrifugation at 15,000 rpm and freeze-drying at – 20°C for 3 hr.

B. Characterization of Nanoparticle:

B.1. ***Drug entrapment efficiency and loading capacity***: Drug entrapment efficiency was determined by centrifugation method. The redispersed nanoparticles suspension was centrifuged at 15,000 rpm for 30 min at 8°C to separate the free drug in the supernatant. The concentration of Anastrozole in the supernatant was determined by using UV- Visible spectrophotometer at 263 nm after suitable dilution. The drug entrapment efficiency was determined using the relationship given by the following equation.

$$\% \text{ entrapment efficiency: } \frac{W_{\text{Total drug}} - W_{\text{Free drug}}}{W_{\text{Total drug}}} \times 100$$

$$\% \text{ loading capacity: } \frac{\text{Total amount of Anastrozole- free drug}}{\text{Weight of nanoparticles}} \text{ X } 100$$

B.2. Particle size distribution: The particle size is vital because it influences the physicochemical properties and biological fate of the nanoparticles after *in-vivo* administration. The particle size distribution was characterized using polydispersity index (PDI) which is the measure of the size distribution. The particle size distribution of the nanoparticles was determined by laser particle size analyzer (Zetatrac 10.6.2, Microtrac Inc.) using distilled water as a dispersant. The nanoparticle dispersions were added to the sample dispersion unit containing stirrer and stirred to reduce the aggregation of the nanoparticles. The average volume mean particle size was measured after performing the experiment in triplicate.

B.3 Zeta potential: The surface charge determines the interaction of nanoparticles with the cell membrane and this assess the stability of colloidal systems. The zeta potential of drug loaded nanoparticles was measured by Zeta sizer (Zetatrac 10.6.2, Microtrac Inc.). To determine the zeta potential, nanoparticles samples were diluted with KCl (0.1 Mm) and placed in an electrophoretic cell where an electrical field of 15.2 Vcm^{-1} was applied. Each sample was analyzed in triplicate.

B.4 Drug-polymer interaction study: The ATR-IR spectra of pure Anastrozole, chitosan, sodium TPP and mixture of all these three ingredients were recorded to check drug polymer interaction and stability of the drug.

B.5 In-vitro release studies: *In-vitro* release studies were carried out by using dialysis tubes with an artificial membrane. The prepared chitosan nanoparticles were redispersed in 5 ml of phosphate buffer pH 7.4 and subjected to dialysis by immersing the dialysis tube to the receptor compartment containing 50 ml of phosphate buffer pH 7.4. The medium in the receptor was agitated continuously using a magnetic stirrer and the temperature was maintained at 37 ± 0.5°C. 5 ml sample of receptor compartment was taken at various intervals of time over a period of 72 h and each time 5 ml fresh buffer was replaced. The amount of drug released was determined spectrometrically at 263 nm.

B.6 TEM (transmission electron microscopy): For the observation of morphology and size distribution, a drop of the sample solution was placed onto a 400 mesh copper grid coated with carbon. About 1 min after deposition, the grid was tapped with a filter paper to remove surface water. The samples were air dried before measurement. Transmission electron microscopy (TEM) was performed on a TECNAI SPIRIT, model FE1 electron microscope, Netherlands.

C. Experimental Animals, induction of mammary gland tumor and study design: The protocol was approved by the Institutional Animal Ethics Committee (IAEC/2015-I/Protocol No. 16). 40 Swiss albino female mice 20-25gm were divided into 4 groups, obtained from the Animal facility of the Institute were housed under controlled room temperature (21±2 0^0C) with a 12/12 h light/dark cycle. The experimental mice received food and water *ad libitum*. Mammary gland tumor was induced with 5 divided doses of 0.2 ml of 7, 12-dimethylbenz (a) anthracene (DMBA 20 mg/ml) in olive oil. Normal mice were administered with the equal volume of olive oil. DMBA and treatment were given intragastrically by gavage using a cannula fitted with a feeding needle. Each mouse had 6 pairs of mammary glands that were checked by inspection, touching and palpitation.

1. Group PC (Positive Control): Mice treated with olive oil only.
2. Group NC (Negative Control): Mice treated with 20 mg/ml/week of DMBA only.
3. Group MA: Mice treated with 20 mg/ml/week of DMBA+1mg/kg of non nano Anastrozole.
4. Group MB: Mice treated with 20 mg/ml/week of DMBA+ calculated dose of nano anastrozole formulation.

After 12 weeks of DMBA administration, treatment of animals lasted for next 4 weeks. The experimental and control animals were carefully checked and their weight is taken weekly.

To evaluate the insight of tumor composition blood sample was withdrawn from control and treated mice on last day before sacrifice in EDTA tubes from retro-orbital plexus and various hematological parameters such as; hemoglobin % (Hb%), white blood cells (WBC) count, red blood cells (RBC) count, Hematocrit value, blood platelets counts were checked. Mice were sacrificed at the end of the sixteen weeks by CO_2 overdose. The breast tissues from each animal were sliced off and divided into two portions using a surgical blade. One portion was fixed in formalin saline for histology using hematoxylin and eosin staining while the other portion was taken for immunoassay (TNFα, VEGF).

3. RESULTS

Characterisation of Nanoparticles

Anastrozole encapsulation efficiency (EE) and loading capacity (LC).

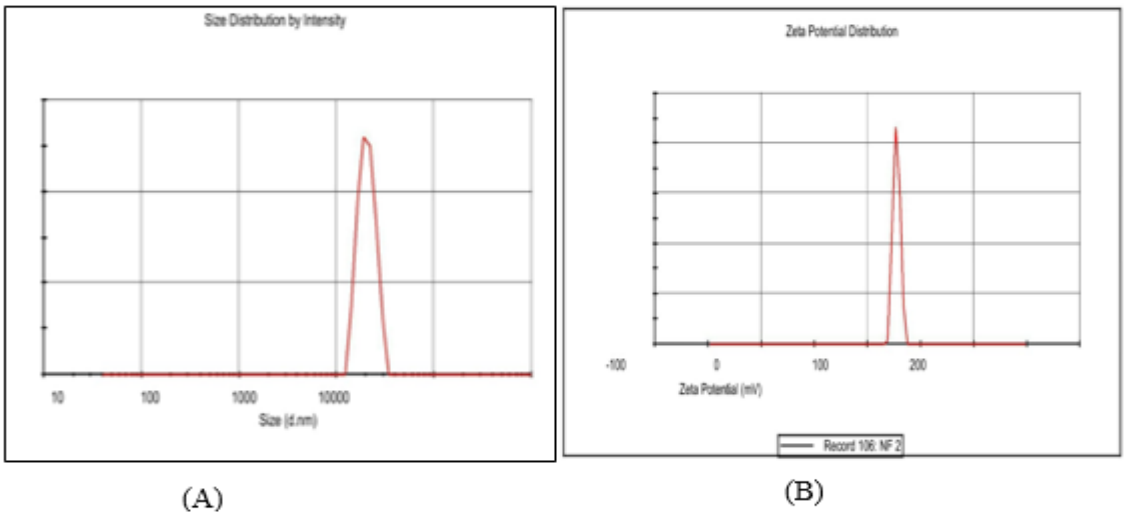
EE of Anastrozole was found to be 82% when the concentration of chitosan used was 1mg/ml. The LC of the nanoparticles was 57%. The percentage yield was calculated to be 68%.

Measurement of Particle Size, Polydispersity, and Zeta Potential:

The average size, measured by Zetasizer, of Anastrozole, loaded chitosan nanoparticles were approximately 263.47 nm. The PDI value was 0.162, thus indicating a narrow and favorable particle size distribution (PDI < 0.1). Zeta potential was 26.267 mV, which shows nanoparticles are positively charged.

Table 1 Particle size, polydispersity index, Zeta potential of anastrozole nanoparticles

Sr. No.	Particle Size (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)
1	259	0.124	27.4
2	264	0.179	24.6
3	268	0.184	26.8
Mean ± SD	263 ± 2.43	0.162±0.45	26.26±1.24



(A) (B)

Fig1. Reports of size distribution using Zetasizer (A) and zeta potential (B) in sample.

Drug-polymer interaction:

The interaction study between the drug and polymer was evaluated using ATR-IR spectrophotometer. There was no significant difference in the IR spectra of pure drug loaded nanoparticles as shown in fig.

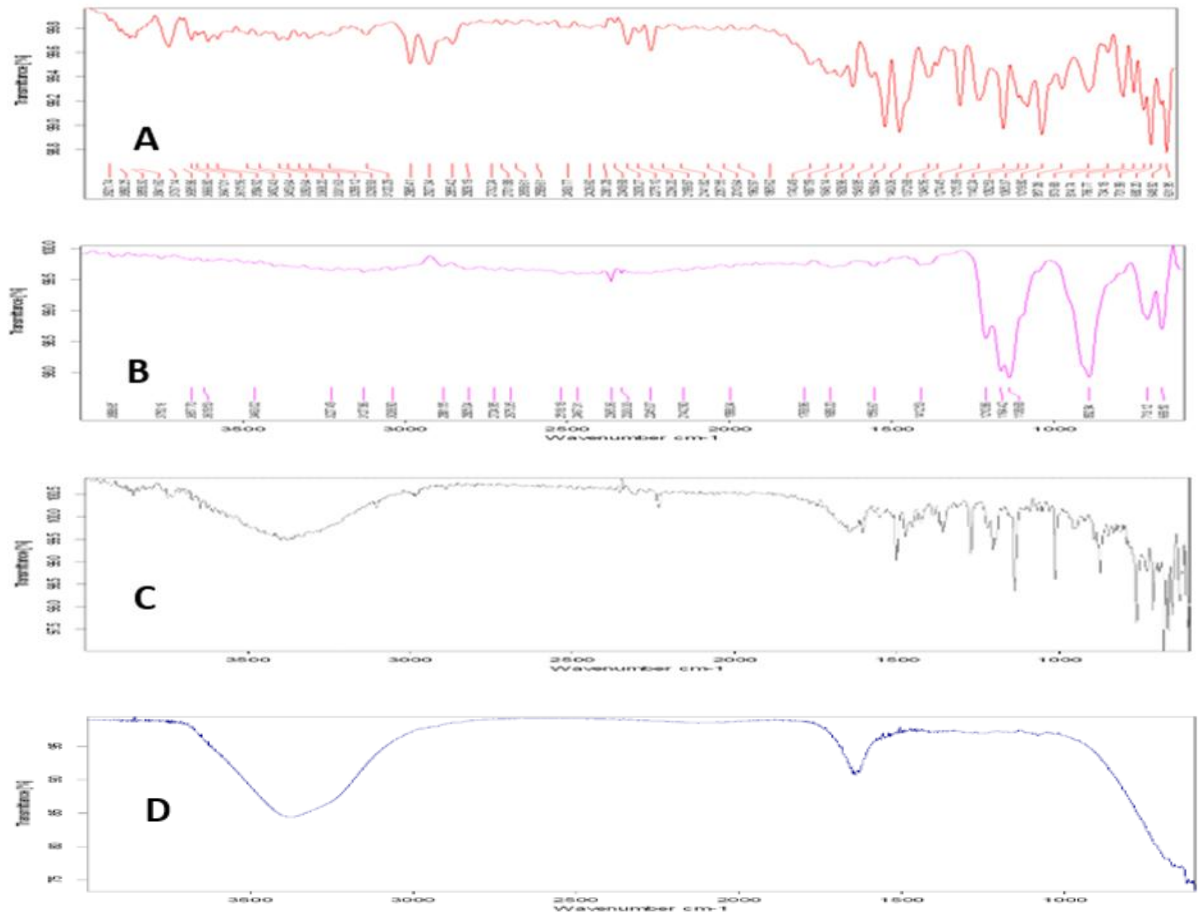


Fig 2.ATR IR Spectrum of A: chitosan (CS), B: Tri-poly phosphate, C: Anastrozole (API), D: CSNPs

Anastrozole Nanoparticles Release Study:

Our observations showed that about 51% of the loaded Anastrozole was released within 12 hours of incubation in PBS. The release profile of Anastrozole loaded nanoparticles exhibits an initial release of about 10% in the first 5 hours followed by a slow release for the subsequent 12 hours.

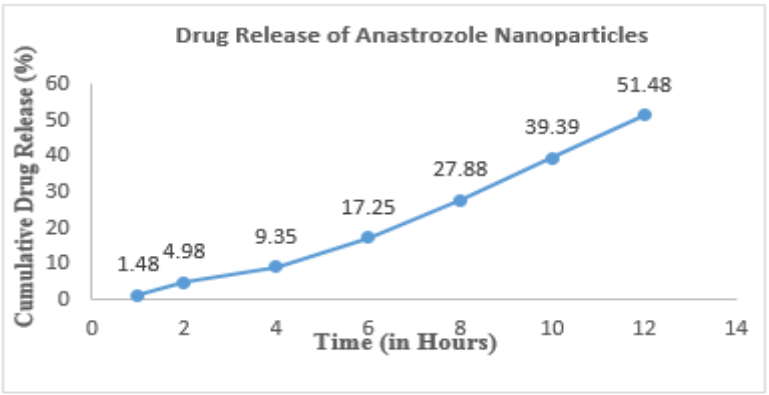


Fig 3 *In-Vitro* Release Profile of Chitosan anastrozole nano-particles (CASNPs)

Transmission electron microscopy (tem)

In the present study, TEM images have shown the morphological properties and surface appearance of nanoparticles. The nanoparticles have nearly spherical shape, smooth surface and size range of about 260-300nm.

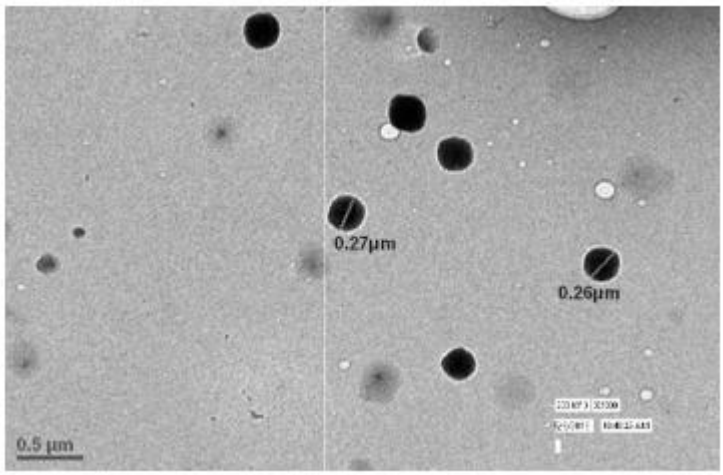


Fig 4. TEM image of CSANPs

Animal Study- Body Weight Changes

The increase in the body weight in DMBA induced tumor was controlled by Nanoformulation and Standard in a dose-dependent manner with significance.

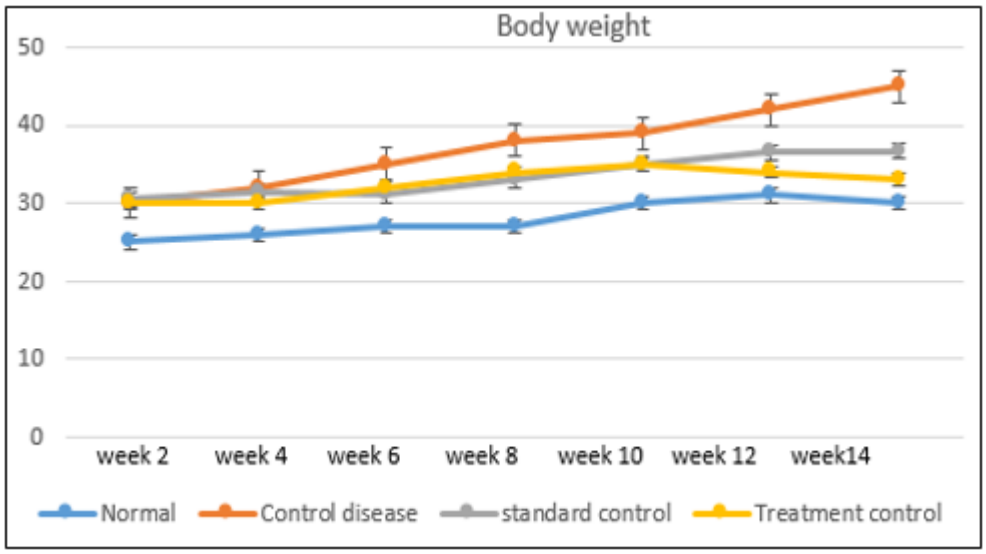


Fig 5.Change in body weight measured every 14th day till the end of the experiment.

Effects of Anastrozole Nanoparticle Formulation on the Survival rate of DMBA-induced cell proliferation in mice: Cancer survival rates or survival statistics tell you the percentage of animals who survive a certain type of cancer for a specific amount of time. Survival response of untreated mice died within 2, 6 and 12 weeks. ANS Nanoformulation treatment enhanced the survival rate even when compared to Disease control.

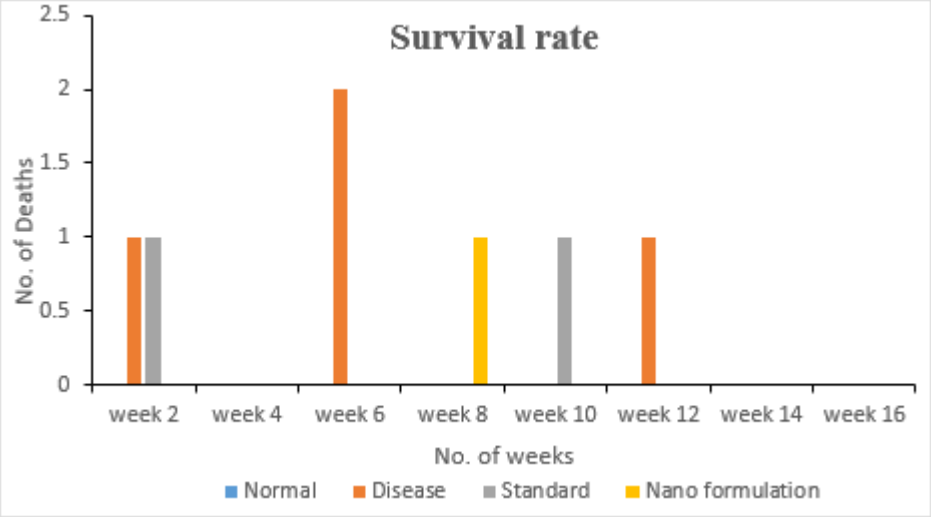


Fig 6. Effects of ANS CNPs Formulation on The Survival rate of DMBA-induced cell proliferation in mice:

Effect of Anastrozole on Hematological parameters:

Hemoglobin, RBC and hematocrit value that generally go down during the progression of the tumor were found to improve in mice treated with Anastrozole Nanoformulation. Anastrozole treatment reduced WBC count compared with the disease control.

Table 2. Effect of Anastrozole on hematological parameters in tumor-bearing mice. Significance was ***p<0.0001

	Normal	**Disease Control**	**Standard Treatment**	**NanoFormulation treatment**
Hemoglobin (g%)	14.05±1.23	8.1±0.08***	7.7±1.01***	10.5±0.6*
RBC (10^6/mm^3)	6.27±0.27	3.29±0.21***	4.04±0.10**	4.73±0.13**
WBC	7.4±0.57	13.33±0.28***	12.13±0.61*	6.42±0.44**
Hematocrit value	57.40±0.9	38.30±0.4***	34.90±1.3***	42.40±0.7***
Platelets Counts(×1000/µl)	1532±3.5	427±6.5***	1186±13.5**	1399±23.5***

Effect of Anastrozole on Angiogenic and anti-inflammatory parameters: Vascular Endothelial Growth Factor (VEGF) and TNFα level were measured using RayBio® RatVEGF and RayBio® Rat TNF-α ELISA kit of Ray Biotech, Inc. as per the manufacturer's instructions respectively. This assay is an *in vitro* enzyme-linked immunosorbent assay.

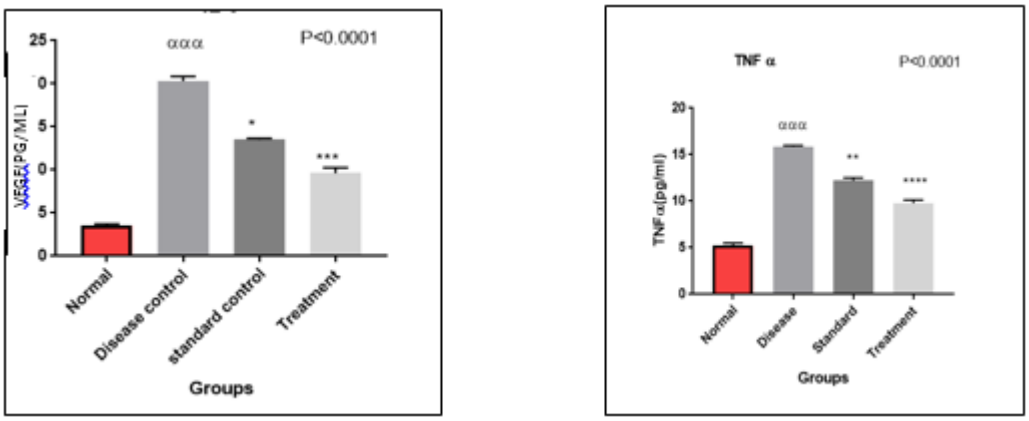


Fig 7. Effect of Anastrozole and its Nanoformulation on VEGF level after 6 weeks evaluated by ELISA. Each value represents mean±S.D. NC, normal; DC, Disease control group; standard Anastrozole group, Treatment with Nanoformulation group. *p<0.0001; *** p<0.01 vs NC.

Fig 7.1 Effect of Anastrozole and its Nanoformulation on TNF-α level after 6 weeks evaluated by ELISA. Each value represents mean±S.D. NC, normal; DC, Disease control group; standard Anastrozole group, Treatment with Nanoformulation group. *p<0.0001; ** p<0.01 vs NC; $^{\alpha}$p<0.0001 vs DC.

Histopathological examination: Breast tissue was immersion fixed in 10% phosphate buffered formalin. These sections were embedded in paraffin, sectioned at 5 µm, and serial sections were stained with hematoxylin eosin (H and E). All histological parameters were quantified by an experienced pathologist blinded to the identity of the sample being examined

Histopathological evaluation of mice breast tissue was conducted according to the pathological diagnostic criteria for human breast tissue.

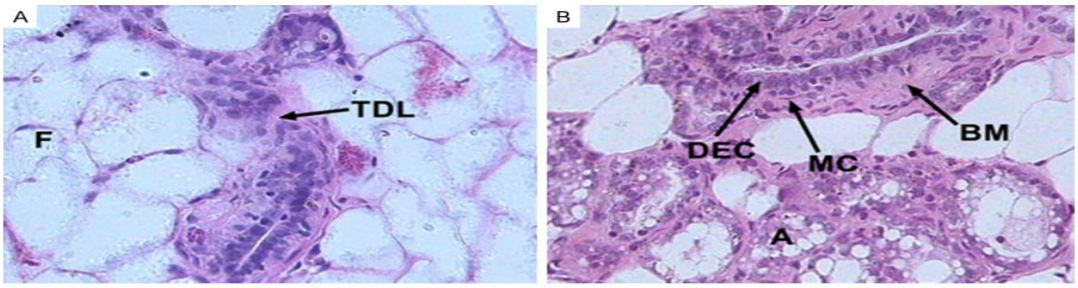


Fig 8. Normal histopathological characteristics of breast tissue in the mice. (A) The rat breast tissues were composed of terminal duct-lobule (TDL) units and fatty tissue (F); each TDL unit included terminal ducts and acini. (B) The terminal duct was composed of ductal epithelial cells (DEC) and myoepithelial cells (MC) surrounded by thick basement membrane (BM). The acini (A) were lined by a single layer of cells that were mainly dark cells with occasional myoepithelial cells (HE, original magnification ×400).

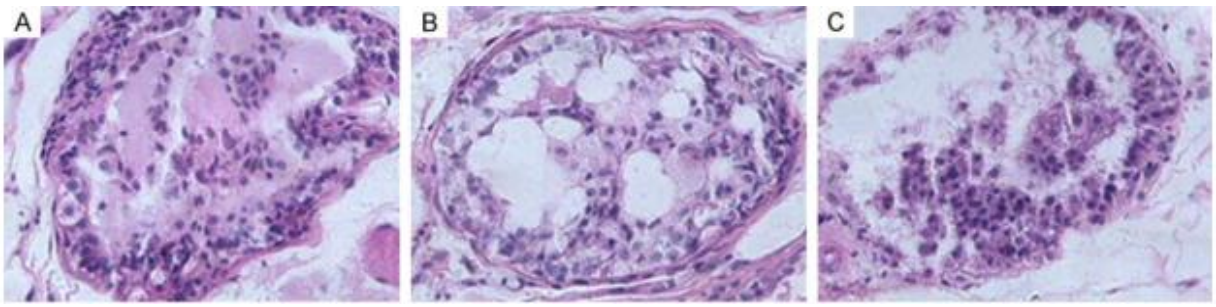
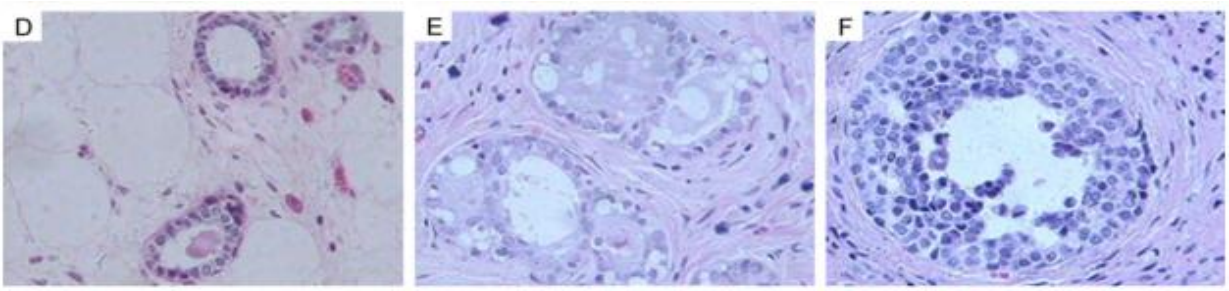
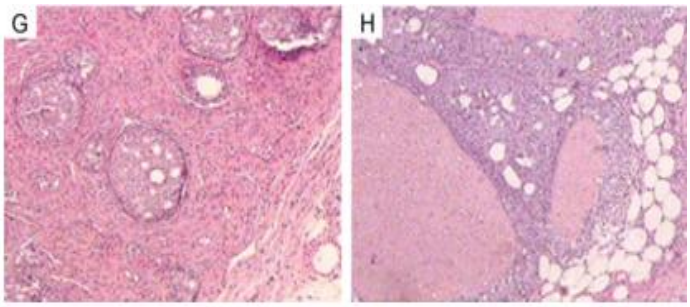


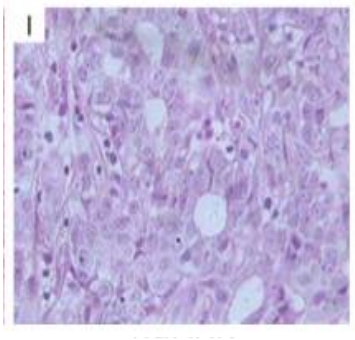
Fig 9. (A-C) UDH (usual ductal hyperplasia was characterized by dilation of the terminal ducts, budding and extension of the ducts to the surrounding fat, irregular curvature of the lumen, the presence of occasional shedding cells and phagocytic histiocytes)tissue in the nanoformulation group showed proliferative cells in a mixed and disorderly pattern.The size of the proliferative cells was similar to that of the normal ductal epithelial cells (A), with a second lumen (B) and papillary structure (C).



(D-F) UDH tissue non nano formulation anastrozole group showed that the ductal epithelial cells were larger than normal cells and had an increased cytoplasm-nucleus ratio. Nuclear basophilicity was enhanced and eosinophilic substances were found in the lumen (D), forming a cross-bridge (E) and cribriform (F) structure.



(G, H) DCIS (ductal carcinoma in situ was characterized by the presence of atypical epithelial cells in the duct, solid or cribriform morphology, and comedo necrosis in the center) tissue in disease control showed that the lumen of the duct was filled with atypical proliferative cells, which formed a cribriform (G) and solid structure with comedo necrosis in the center (H) (HE, original magnification ×100).



(I) IDC (invasive ductal carcinoma was characterized by infiltration of the cancer cells into the interstitial tissues and through the basement membrane of the duct) tissue in diseases control mice showed that the proliferative cells in the DCIS tissue had damaged the basement membrane and then infiltrated into the fibrous connective tissue (HE, original magnification ×400).

DISCUSSION

In the recent years, many nanocarriers such as lipid-based formulations, polymeric micelles, and polymeric nanoparticles have been employed as drug carriers for tumor therapy. We have reported that the polymeric nanoparticles have the ability to increase the loading capacity, drug stability and therapeutic activity of the anticancer drugs. These nanoparticles are talented to accumulate in the tumor tissue, causing a disorganized vascular construction, referred to as the enhanced permeability and retention effect[7].

CSNPs have been accepted as a promising drug delivery system, attributing to the positively charged chitosan polymer[8]**.** Newly, the interest of the CSNPs as antitumor drug carriers has

been increased. It was described that the Chitosan has anticancer activity is due to its interfering with the cell metabolism and inhibition of cell growth CSNPs have been applied in pharmaceutical applications to target cancer cells like 5-fluorouracil

Anastrozole loaded Chitosan nanoparticles were employed as a novel carriers for breast cancer treatment. These nanoparticles were prepared by an ionic gelation method and characterized by ATR-IR. The prepared nanoparticles showed the spherical shape of nanoparticles having an average size of 0.26µm, PDI in the range of 0.359±0.015 and zeta potential between 35.5± 1.9 mV. The ANS entrapment efficiency of CSNPs was in the range of 49.05-72.04%. The *in vitro* release studies showed an initial rapid ANS release up to 6 hrs followed by a slow release ranging from 52 to 92%.

In Our study, we have taken a rapid and reproducible model of breast cancer in which Mammary gland tumor was induced with 5 divided doses of 0.2 ml of 7,12-dimethylbenz(a)anthracene (DMBA 20 mg/ml) in olive oil.

In the current study we investigated the efficacy of ANS nano formulation with the non nano (standard) formulation of ANS by checking Animal blood profile like Hemoglobin (g%), RBC count (10^6/mm^3), WBC count, Platelets Counts (×1000/µl), Body weight change, Effect of Angiogenic parameters (VEGF) and inflammatory parameters (TNF α) were also compared and Histopathological examination also revealed the better results as compared to non nano (standard) formulation.

The present research work has demonstrated a step-by-step approach to problem solving and translation from a theoretical idea to a tangible application.

CONCLUSION

In the present study, we had developed chitosan loaded Anastrozole nanoparticle to assess its comparison with non-nano (standard) formulation of Anastrozole for its efficacy and toxicity. The drug loaded nanoparticle formulation was administered by oral route to increase the therapeutic effect of the drug and to reduce its unwanted side effects.

To study the anti-angiogenic property of Anastrozole in Breast cancer model we adopted DMBA induced mammary gland carcinogenic model in Swiss albino mice. It was found that there is a significant reduction in the body weight of treated mice in this model. The *in vivo* anticancer evaluation showed that chitosan loaded nanoparticle had a comparable tumor

suppressive potential as that of the non-nano (standard) treatment. We also considered the effect of the formulation on hematopoietic system and it was found that nano formulation has more protective action as compared to standard drug

However, extensive studies in terms of chronic toxicity, pharmacokinetic and attachment of more specific targeting molecule are needed before establishing nanoparticle-mediated delivery of this drug.

Furthermore, clinical trials need to be done to characterize and authenticate the use of Anastrozole Nano-formulation, so that its possible usefulness in the Carcinoma can be well determined.

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