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

Human Journals
Research Article
June 2017 Vol.:9, Issue:3

Anticancer Activity of Cyclophosphamide Nanoparticles against Ehrlich Ascites Carcinoma Cells Bearing Swiss Albino Mice

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals

S.S.Agrawal* and Pallavi Sharma[1]

DPSR University, New Delhi, India.

Submission: 7June 2017
Accepted: 12 June 2017
Published: 25 June 2017

Keywords: Anticancer Activity, Cyclophosphamide, Nanoparticles, Ehrlich Ascites Carcinoma

ABSTRACT

Drug delivery systems such as nanoparticles are used to improve the pharmacological and therapeutic properties of anticancer drugs. In this study, a biodegradable polymer chitosan (CS) was used for delivery of cyclophosphamide (CP). Cyclophosphamide loaded chitosan nanoparticles (CP-CS-NPs) were prepared by ion cross-linking to form a uniform particle size of 186.53 ±1.865 nm with 78±2.67 % entrapment efficiency. The release study data at pH 7.4 showed biphasic release where the first rapid phase of release lasted for 6 hr, followed by a slow diffusion order rate of release for the next 24 hr. Anticancer activity of these nanoparticles was investigated *in-vivo* in Ehrlich ascites carcinoma (EAC) tumour. 2 $\times 10^6$ EAC cells were implanted intraperitoneally (*i.p.*, ascitic tumour) in swiss albino mice. This was assigned as day '0'. After 4 days of incubation, mice with developed ascites tumour were administered orally with nanoparticles at two doses i.e.7.5 mg/kg/d and 10 mg/kg/d for 14 days. On 18th day, animals were sacrificed and blood was collected for estimation of haematological parameters. Ascites fluid was collected for the estimation of tumour volume, cell viability, angiogenic, metastatic and antioxidant parameters. CP-CS-NPs significantly increased total RBCs at a higher dose of 10 mg/kg/d whereas Hb % was increased significantly at both the doses. WBCs were decreased significantly when compared to EAC control group. Furthermore, CP-CS-NPs significantly decreased the tumour volume and cell viability indicating cytotoxic property. Decreased VEGF level and TNF-αand increased IL-12 expression indicated anti-angiogenic potential of CP-nanoparticles. These were also able to increase the antioxidant concentration significantly. All results were dose-dependent when compared to EAC control group. The result suggests that CP-CS-NPs elicit anti-carcinogenic activity and might be a potential candidate for developing multifunctional anti-cancer agent.

INTRODUCTION

Cancer is one of the leading causes of death around the globe. Around 70 percent of the world's death occurs in Asia, Africa and Central and South America due to cancer.[1] 22 million new cancer cases will emerge within the next two decades.[2]Various approaches have been developed in order to curb the havoc created by cancer worldwide. Current cancer treatment regime includes combination of surgery, radiotherapy and chemotherapy. However, treatment poses many limitations of its own. Surgery leads to removal of sizeable tumour or the entire organ. Radiotherapy disrupts the genetic material of the cells surrounding the treatment area, making impossible for healthy cells to grow. Chemotherapeutics killsor slows the growth of healthy cells.Also, occurrence of multidrug resistance (MDR) due to p-glycoprotein action is one of the major threat to treatment plan.[3] Thus novel drug delivery systems (NDDS) such as nanoparticles, liposomes, microspheres etc. have been formulated to circumvent such threats by evading or neutralising p-glycoprotein mediated efflux mechanism.[4] NDDS offers a sustained drug release, thereby reducing frequency of dosing and side effects.[5] Cyclophosphamide is a cytotoxic agent often used to treatretinoblastoma, rhabdomyosarcoma, lymphomas, leukemias, multiple myeloma, neuroblastoma, breast, testicular, endometrial, ovarian, and lung cancers. Traditional dosage forms are encountered with problems like high toxicity and drug leakage before reaching tumours which can be curbed by targeted drug delivery system.[6] However, in this study, we focused on the therapeutic potential of cyclophosphamide when given as chitosan-nanoparticulate dosage form. Nanoparticles are made from different biodegradable and non-biodegradable materials, with dimensions generally less than 500 nm. Nanocarriers used for treatment of a disease have to be biocompatible and nontoxic.[7] Recently, chitosan based nanoparticleshave played a promising role in delivery of cancer chemotherapeutics since chitosan has favourable properties such as mucoadhesivity, biodegradability, low toxicity and notable cell membrane permeability.[8] Commercially, chitosan is formed by deacetylation of chitin. Chitin is a naturally occurring polysaccharide found in exoskeleton of crustaceans such as crabs.[9] Chitosan promotes cross-linkage with various cross-linking agents, such as glutaraldehyde, sodium tripolyphosphate (TPP), genipin etc.Several techniques such as microemulsion,solventevaporation, ionic gelation,etc.have been developed to prepare chitosan nanoparticles.However, for pharmaceutical applications, physical cross-linking utilised in ionic gelation process is more desirable since the cross-linking is reversible and therefore avoid potential toxicity of the reagents.[10] Experimental tumours are extensively

used as models to study anticancer activity. In the current study, Ehrlich ascites carcinoma mouse model has been selected due to resemblance of EAC tumours with human tumours. These undifferentiated tumours have a rapid growth rate and are most sensitive to chemotherapy.

MATERIALS AND METHODS

Materials

Cyclophosphamide (CP) was obtained as a gift sample from Heteroscientific,Hyderabad. Since CP compared to other drugs (viz. vinblastine and methotrexate) increased life span in EAC tumours was used as standard drug.[11] Chitosan (high molecular weight), sodium tripolyphosphate (TPP) and trypan blue were purchased from Sigma-Aldrich (USA). Chloroform, acetonitrile and methanol (HPLC Grade) were purchased from Merck, Mumbai. High-pure water was prepared by using a Millipore Milli'Q'plus purification system. All other chemicals used were of analytical grade.

Preparation of cyclophosphamide loaded chitosan nanoparticles (CP-CS-NPs)

The nanoparticles were prepared by ionic gelation process.Based on literature survey and pilot study done in our laboratory, chitosan and TPP were dissolved at a concentration of 3mg/ml in 1.5% acetic acid solution and 2mg/ml in distilled water respectively. The pH of the resulting solution was adjusted to 6.0 using 20% sodium hydroxide solution. Blank nanoparticles were prepared bydropwise additionof 14ml of sodium tripolyphosphate solution in 35ml of chitosan solution under mechanical stirring (1000 rpm) at room temperature for 60 minutes. The drug loaded nanoparticleswere similarly prepared by using the drug cyclophosphamide (2 mg/ml) in chitosan solution. The resultant nanoparticles were centrifuged at 15000 rpm for 40minutes. The pellet obtained after centrifugation was finally freeze dried and stored in airtight close container.[12] The formation of the particles was a result of the interaction between the negative groups of the TPP and the positively charged amino groups of chitosan (ionic gelation).

Characterization of CP-CS-NPs

Particle size and zeta potential

The mean particle size, size distribution and zeta potential of NPs were obtained by photon correlation spectroscopy (PCS)using a Zetasizer Nano ZS laser particle size analyzer(Malvern Instruments, Malvern, UK). About 1 ml of the suspension was taken in a disposable cuvette and run in triplicates at 25°C.

Transmission electron microscopy (TEM)

The size and shape of the CP-CS-NPs were determined using transmission electron microscopy(H7500, Hitachi Ltd., Tokyo, Japan). The samples were negatively stained with freshly prepared phosphotungstic acid solution (2% adjusted to pH 6.8).

ATR

CP-CS-NP samples were subjected to ATR spectroscopy in attenuated total reflectance spectrophotometer (Bruker, USA) in the range of 4000-600 $cm^{-1.}$ The freeze dried samples were pressed to a pellet to detect any chemical interactions between drug and polymer.

Entrapment efficiency

The entrapment efficiency (EE) of CP was determined by separating supernatant containing free drugfrom nanoparticles bycentrifugation (MPW med instruments, Poland) at 16,000 rpm for 35 min.[13] Then, clear supernatant wasanalysed for the contents of CP by using an already validated HPLC method.[14]LC-2010C HT system equipped with quaternary pump, autosampler unit, online degasser, column oven and photodiode array detector (SPD-M20A)was used. All the data was processed and monitored at LC SOLUTION software provided by Shimadzu, Japan.C18 column (250 ×4.6 mm, 5 µm particle size)(Spincotech Pvt. Ltd.) was used as stationary phase. The mobile phase consisted of 70% phosphate buffer, 25 mM (pH 3.4) and 30% acetonitrile at a flow rate of 1 ml/min and injection volume of 20 µl. The analysis was carried at 30°C and detector wavelength of 195nm. The retention time obtained of cyclophosphamide was at 9.2 minutes. The samples were filtered through a 0.22 µm filter and degassed by ultrasonication (Sonica, model 2200 MH) prior to use. The chromatographic method showed linearity (R^2=0.99) in the range from 80-120µg/ml. Druguntrapped (drug in supernatant) was reported as concentration in µg/ml.All the samples were measured in triplicates. Thepercentage entrapment efficiency was calculated as:

$$\text{Entrapment Efficiency \%} = \frac{\text{Total drug taken- Untrapped drug}}{\text{Total Drug Content}} \times 100$$

In-vitro drug release

The *in vitro* release of drug CP from the nanoformulation was carried out by dialysis technique.CP-CS-NPs were suspended in the dialysis bag containing release medium (Phosphate buffer saline solution, pH 7.4). The dissolution media wascontinuously stirred at 50 rpm and maintained at 37 ±0.5°C. An aliquot of 5 ml sample was withdrawn at various time intervals and subsequently replenishing with equal volumes of fresh media.[15]Isolated samples were filtered through 0.2 micron syringe filter. The detection was carried out using LC-2010C HT system (Shimadzu, Japan) equipped with PDA detector (Spincotech Pvt. Ltd.)at 195 nm.

*In-Vivo*Study

Animals

Swiss female albino mice (*Musmusculus*) of about 4-5 week old with an average body weight of 25-30g were procured and housed in the animal house of the Delhi Institute of Pharmaceutical Sciences and Research(DIPSAR), New Delhi under standard laboratory conditions (26 ±1 °C, 12-h light:12-h dark cycle) with food and water *ad libitum*. The mice were acclimatised to laboratory conditions for 7 days before commencement of experiment. The study and the number of animals used were approved by the Institutional Animal Ethics Committee (IAEC/2015-I/Prot.No. 15).

Tumour cells

The Ehrlich ascites carcinoma (EAC) cells were obtained from Institute of Nuclear Medicine and Allied Sciences (INMAS), New Delhi, India and maintained *in vivo* by weekly intraperitoneal (*i.p.*) passage of cells. Ascitictumour cell suspensions were prepared in phosphate buffer saline (PBS) and cell count was done in a Neubauerhemocytometer using the trypan blue dye exclusion method. Cell viability was always found to be 95% or more.

Ehrlich ascites carcinoma (EAC) model

Mice were randomized and divided into five groups (n=8) each. Mice of all groups except normal control group were inoculated with 2□ ×□10^6 EAC cells (0.1ml/10g mouse/*i.p.*).[16]

This was taken as Day '0'. 4 days of incubation was allowed for the multiplication of cells. Treatment groups were administered orally, with nanoparticles of cyclophosphamidein two doses i.e. 7.5mg/kg/d and 10mg/kg/d from day 4-17. Group of mice serving as positive control were treated with marketed solid formulation of cyclophosphamide, endoxan(CP 10 mg/kg/d), orally.The dosage was selected on the literature survey and pilot study done in our laboratory.[17-19]

Body weight measurement

Mice of all groups were weighed on day 0,2,4,6,8,10,14,16,18. Mean from 7 animals of each group was calculated.

Haematological Parameters

On completion of study, blood was withdrawn by puncturing retroorbital plexus in mice of all groups and haematological parameters such as red blood cell count (RBC), white blood cell count (WBC), haemoglobin (Hb) were analysed. The count was done under microscopeusing Neubauer chamber. Blood plasma was obtained by subjecting blood sample to centrifugation at 2000rpm for 10 minutes.

Estimation of ascites/tumour volume

After completion of study, all mice from each group were sacrificed by CO_2 euthanasia. An incision was made in the abdominal region and ascitic fluid was collected in a measuring cylinder for determining the effect on volume of ascites fluid in mice of treated groups as compared to EAC control group.

Peritoneal angiogenesis

Peritoneal angiogenesis was examined by cutting open the peritoneum of mice from both control and treated groups. The inner lining of the peritoneal cavity was analysed and photographed.[18]

Estimation of cell viability

On 9^{th} and 18^{th} day of study, ascitic fluid was collected and subjected to trypan blue dye exclusion method for estimation of cell viability.[20]The number of viable cells (unstained)

were counted under microscope and average from 5 squares was taken.The cells which took trypan blue were considered non-viable.

Concentration (viable cells/ml)= Average no. of viable cells per square ×Dilution factor $\times 10^{4.}$

Percentage increases in life span

The effect of CP-CS-NPs on percentage increases in life span was calculated on the basis of mortality of the experimental mice.[21]For calculation of mean survival time (MST) and percentage increased life span (%ILS), animals were allowed die naturally.

$$\text{Mean survival time} = \frac{\Sigma \text{ Survival time (days) of each mouse in a group}}{\text{Total number of mice}}$$

$$\%\text{ILS} = \frac{\text{MST of treated group} \times}{\text{MST of control group}} 100$$

Estimation of IL-12, IL-6, TNF-alpha, VEGF, MMP-3 in EAC cells

On day 18 of tumour transplantation, mice bearing EAC tumour were sacrificed and peritoneal lavage was collected and centrifuged at 3500 rpm for 20 minutes.Supernatant was collected and used for cytokine quantification by ELISA for mouse IL-12, IL-6, TNF-alpha, VEGF and MMP-3according to the manufacturer's instructions (Kinesis Dx, USA). Standard curves from each kit were generated by using the reference cytokineconcentrations supplied by the manufacturers. The cytokine concentration in the unknown samples was determined by comparing the absorbance of the samples with the standard curve.

Estimation of Total Antioxidant Capacity (TAC) in Blood plasma

On day 18 of tumour transplantation, mice were sacrificed and blood was collected by puncturing retroorbital plexus. Blood plasma was obtained by subjecting blood sample to centrifugation at 2000rpm for 10 minutes.Standard curve for TAC from kit (Kinesis Dx, USA) was generated by using the referenceconcentrations supplied by the manufacturers. By comparing theabsorbance of the samples with the standard curve, the cytokine concentration in theunknown samples was determined.

Statistical analysis

The results were presented as mean ±standard deviation. Statistical analysis was performed with one-way analysis of variance (ANOVA), student's t-test and statistical significance were designated as $p < 0.05$.

RESULTS

Particle size and zeta potential

The particle size and polydispersity index (PDI) of CP-CS-NPs (n=3) was found to be 183.7±1.865 nm and 0.367±0.150 respectively (fig 1.a.).Due to their small size, these particles are capable of passing through biological barriers and improve delivering drugs to the lesion site, enhancing efficacy. The zeta potential of CP nanoparticles was found to be 34.3±1.85 mV(fig1.b.)

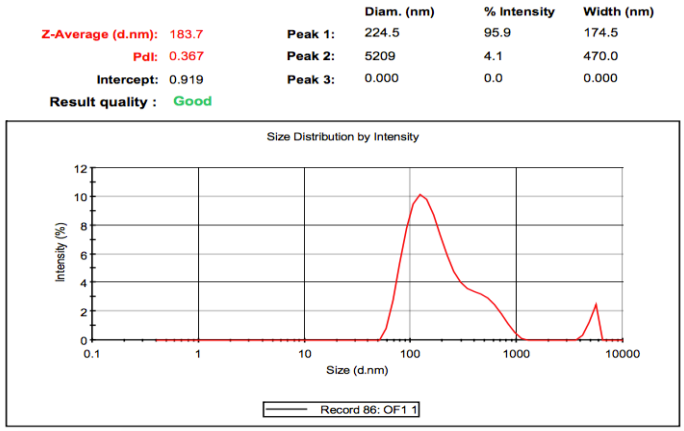

Fig. 1. a. Particlesize of cyclophosphamide nanoparticles

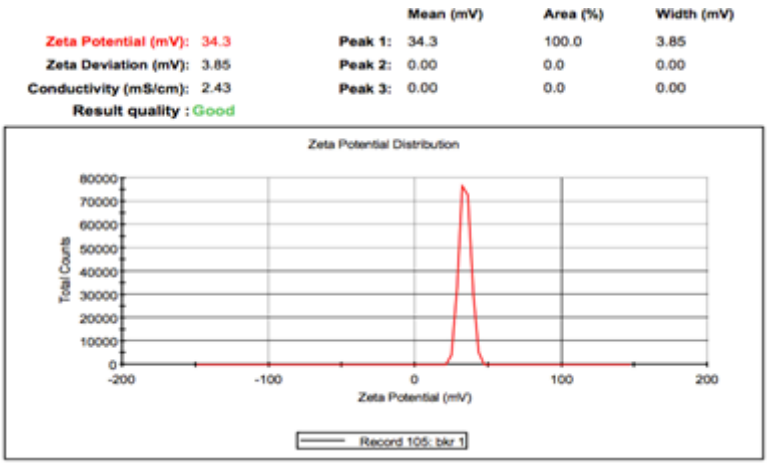

Fig. 1. b. Zeta Potential of cyclophosphamide-chitosan nanoparticles.

Transmission Electron Microscopy (TEM)

The optimised formulation was characterised for its shape and surface morphology by TEM and the results are shown infigure. TEM of CP loaded chitosan nanoparticles (fig. 2) revealed very homogeneous morphology and spherical shape. The size of particles ranged from 180 to 190 nm.

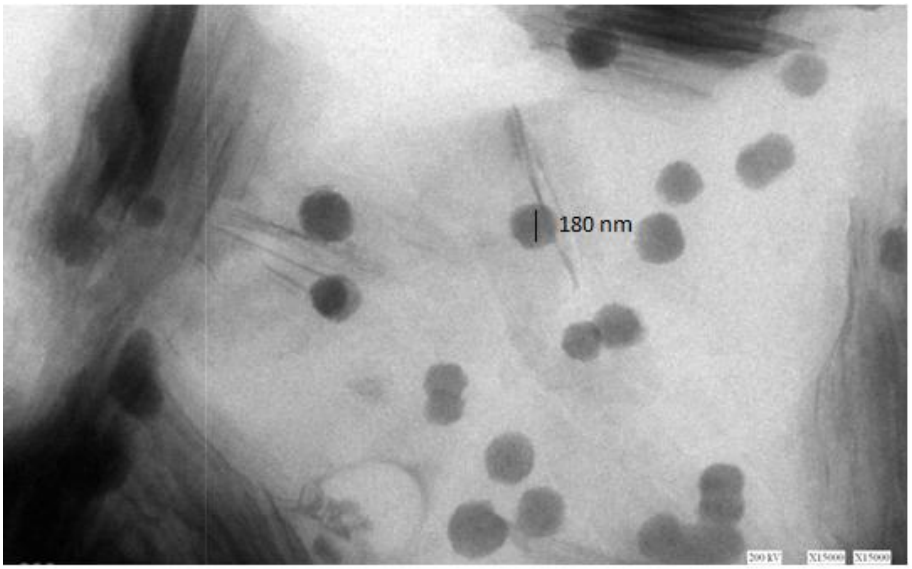

Fig 2. TEM showing the morphology of cyclophosphamide-chitosan nanoparticles.

Attenuated Total Reflectance (ATR)

IR spectra of the liquid nanoparticle formulation and drug CP (fig.3.a. 3.b.) was taken using ATR technique. Chitosan-cyclophosphamide formulation showed a huge curve in the region of 3200-3400 $cm^{-1.}$ This is due to the formulation being aqueous based with band at 3373 cm^{-1}. Another peak at 2890 cm^{-1} obtained in formulation is similar to peak of drug at 2888.38 cm^{-1}due to C-H stretching.[22] A peak at 986.56 cm^{-1} obtained in formulation is similar to peak of drug at 977.85 cm^{-1}.No interaction between drug and other excipients is observed as significant peaks of drug are present in formulation as well.

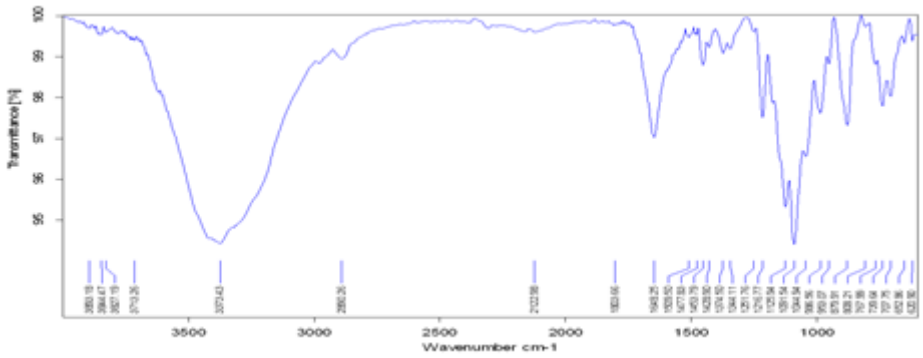

Fig. 3. b. ATR spectra of cyclophosphamide- chitosan nanoparticles.

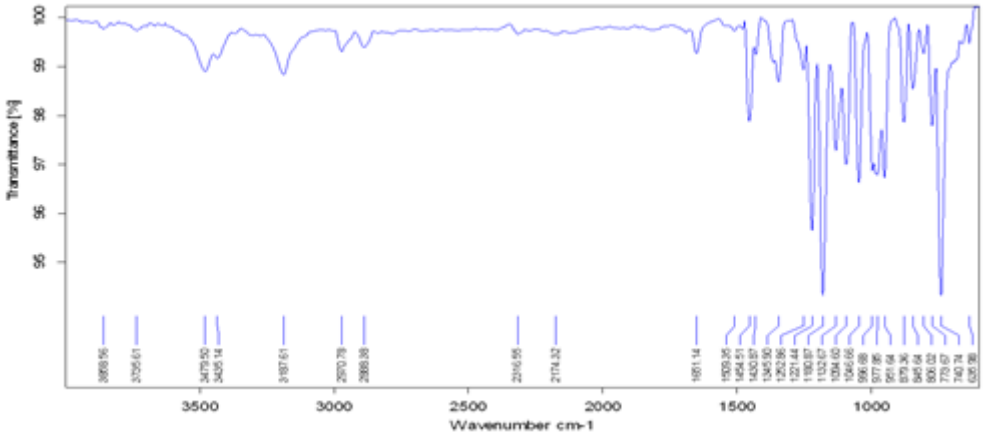

Fig. 3. a. ATR scan of pure drug cyclophosphamide (CP).

Entrapment efficiency

In this study, the drug was incorporated during nanoparticle formation. The entrapment efficiency (EE) is a key indicator of nanoencapsulation process. The EE of formulation was found to be 78±2.6% which can further play a role in optimum delivery of the drug to tumour sites.

In-vitro drug release

The release study data (fig 4.) showed biphasic release where the first rapid phase of release lasted for 6 h, followed by a slow diffusion order rate of release for the next 24 h. The first phase of burst release may be due to dissolution and diffusion of the drug that was poorly entrapped in thechitosan polymer, while the slower and continuous release may be attributed to the diffusion of the drug localised in the core of the formulation.

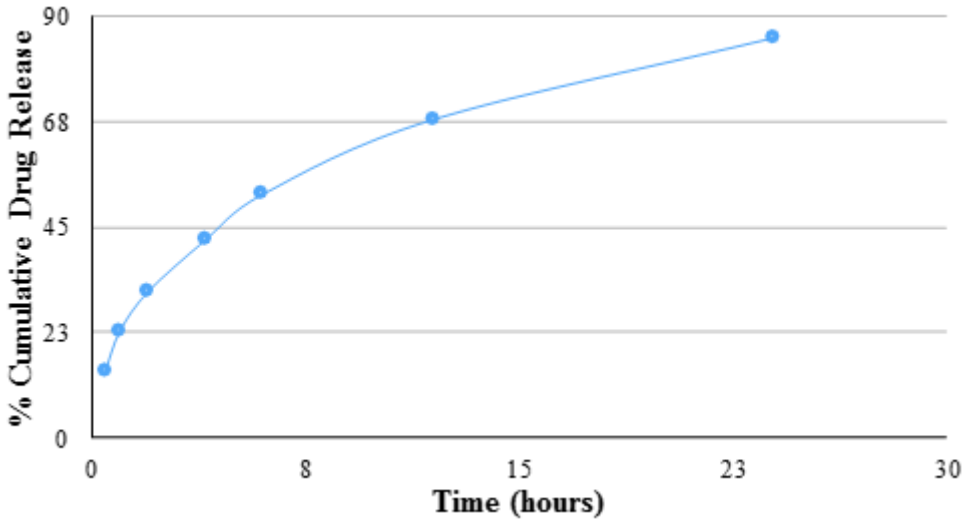

Fig. 4Graph indicating %cumulative drug release from nanoparticles.*In-vivo* Study

Effect on body weight

Body weight is a direct indicator of ascites tumour growth. In EAC bearing mice, due to accumulation of ascites fluid in the peritoneal cavity, the body weight increased significantly (fig.5 a., fig 5.b.)from 32±0.8 g to 48.5±2.5 g after 18 days. CP-CS-NPs treated groupwas able to decrease body weight significantly in all doses as compared to EAC control group.

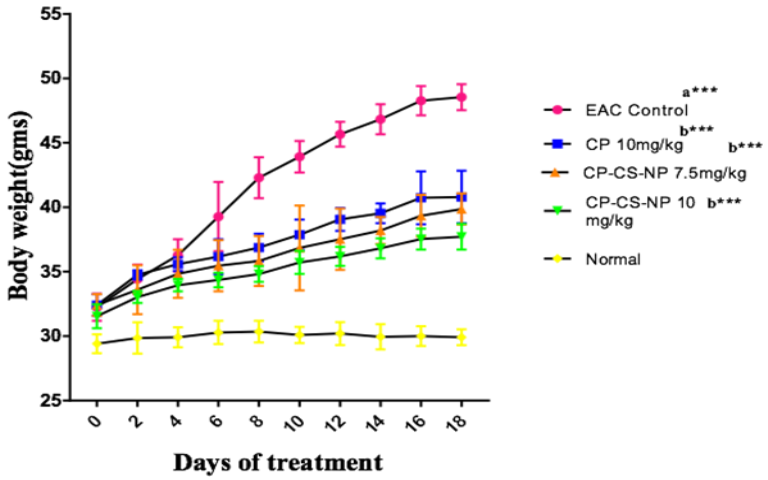

Fig. 5.a.:Effect on body weight of EAC bearingswiss albino mice, significance level[a]p<0.05 vs Normal Group and [b]p<0.05 vs EAC Control. Values are Mean±SD n=7.

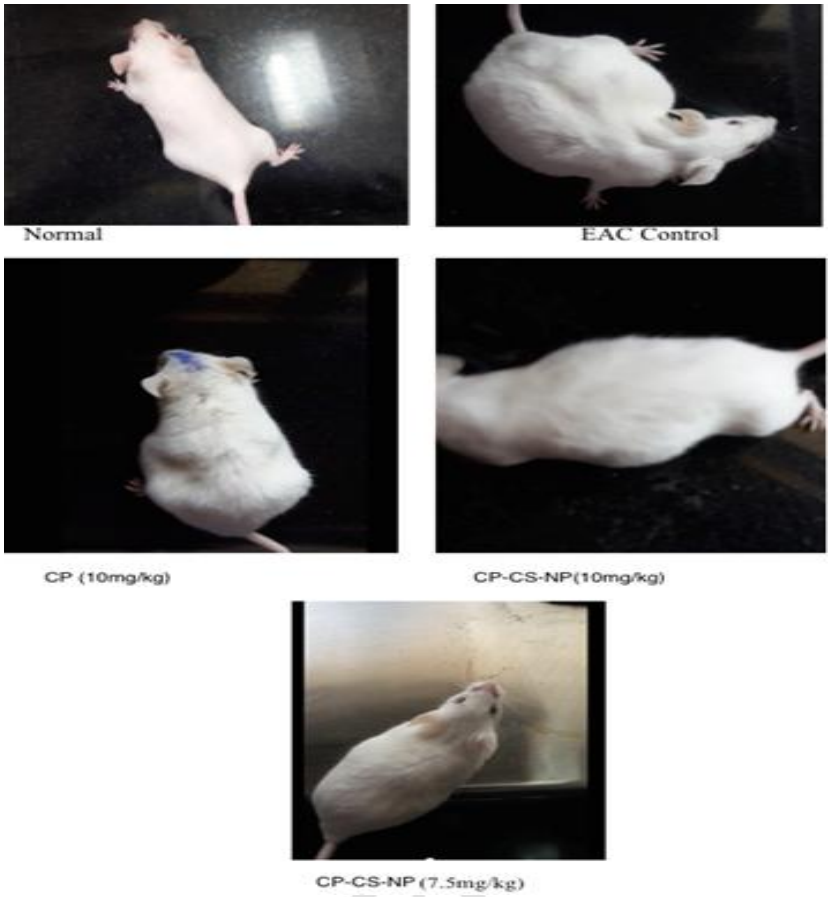

Fig.5.b.:Photographs showing effect on body weight of EAC bearingswiss albino mice as compared to treated groups.

Effect on Haematological parameters

On 18th day, haematological parameters of EAC bearing mice showed significant changes when compared to normal mice.EAC bearing mice showed a decline in RBC and Hb level whereas rise in WBC level ($p<0.05$) during tumour progression. Standard treatment with CP (10mg/kg) and CP-CS-NP (7.5 mg/kg) could not improve RBC count as results were non significant. A higher dose of CP-CS-NPs (10mg/kg) was required to improve the RBC level (table 1fig.6.a-6.c.). Haemoglobin and WBC level ($p<0.05$) were brought to normal with maximum alteration in CP-CS-NP treatment at dose 10mg/kg.

Table 1: Effect on haematological parameters in EAC bearing mice.Significance was [a]p<0.05 vs normal, [b]p<0.05 vs EAC Control, values are Mean ±SD n=7

	Normal	EAC Control	CP (10mg/kg)	CP-CS-NP (7.5mg/kg)	CP-CS-NP (10mg/kg)
RBC (10^9/µl)	4.50±0.46	2.95±0.42^{a***}	3.39±0.19^{ns}	3.72±0.931^{ns}	3.96±0.38^{b**}
WBC (10^6/µl)	7.93±0.86	12.11±0.84^{a***}	10.37±0.48^{b**}	11.1±0.836^{b*}	9.45±0.26^{b***}
Haemoglobin (g%)	13.11±1.92	6.67±0.53^{a***}	8.47±0.34^{b**}	8.48±0.414^{b**}	9.41±0.58^{b***}

Effect on Ascitictumour growth

EAC bearing mice showed a rapid increase in ascitic fluid volume which meets the nutritional requirements for tumour growth. Treatment with nanoparticles showed significant decrease (table 2, fig. 7.a.,7.b.) in the ascitic volume and cell viability and subsequently arresting tumour growth. On 18th day, maximum inhibition was achieved by CP-CS-NPs at the dose of 10mg/kg whereas, at a lower dose of 7.5mg/kg, the reduction was comparable to standard CP (10mg/kg).

Table 2: Effect on ascites/tumour volume and cell viability in EAC bearing mice. Significance was[a]p<0.05 vs normal, [b]p<0.05 vs EAC Control, values are Mean ±SD n=7

Groups	9th day cell viability	18th day cell viability	Ascites/Tumour Volume (ml)
EAC Control	70.25 ±4.52	89.48±0.89	11.021 ±0.57
CP 10mg/kg	60.25±2.05^{a****}	51.14±2.03^{a***}	8.41±0.88^{a***}
CP-CS-NP 7.5mg/kg	54.8±3.48^{a****}	48.42±3.20^{a***}	8.22 ±0.81^{a***}
CP-CS-NP 10mg/kg	50.14±5.29^{a***}	39.77±1.45^{a****}	6.51±0.73^{a****b***}

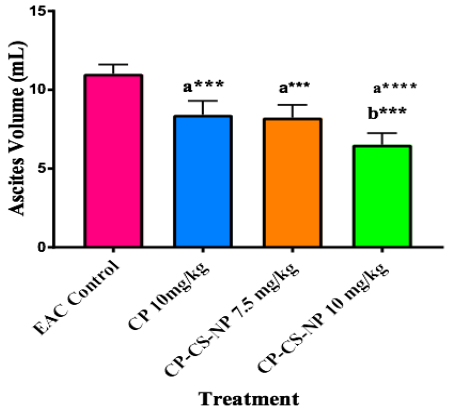

Fig 7.a.: Effect on Ascites/Tumour volume after treatment withCP-CS-NPs (7.5mg/kg and 10 mg/kg). Significance was[a]p<0.05 vs EAC Control, [b]p<0.05 vs CP 10mg/kg, values are Mean ±SD n=7

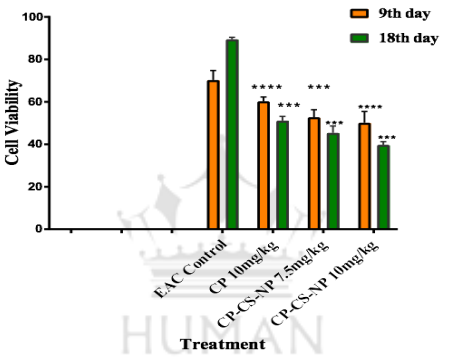

Fig. 7.b.:Effect on cell viability in EAC bearing mice. Significance was [a]p<0.05 vs EAC Control, values are Mean ±SD n=7.

Effect on life span of tumour bearing mice

Percentage increase in life span was calculated on the basis of mortality of the experimental mice in ascites tumour. For determination of mean survival time (MST) and percentage increased life span (%ILS), animals were allowed to progress to a natural death. CP-CS-NPs prolonged the life span of tumour bearing mice from 32.2±3.67 to 44.0±10.22 days (table 3).

Table 3: Showing mean survival time and percentage increase in life span mean±SD, n=8

Groups	Mean Survival Time (Days)	% Increased Life Span (%ILS)
EAC Control	32.2±3.67	-
CP 10mg/kg	40.4±7.88	125.4
CP-CS-NP 7.5mg/kg	42.2±11.47	131
CP-CS-NP 10mg/kg	44.0±10.22	136.6

Effect of CP-CS-NP on angiogenesis in tumour bearing mice

Neovascularization is the critical step in the development of tumours. Increased angiogenesis was seen in peritoneum of EAC bearing mice. Therefore we detect the anti-angiogenic property of CP-nanoparticles against tumour bearing mice. Treatment involving two different doses of CP-CS-NP (7.5 mg/kg and 10 mg/kg) revealed reduction in number of blood vessels on peritoneal wall (fig 8.a.) when compared to EAC control.

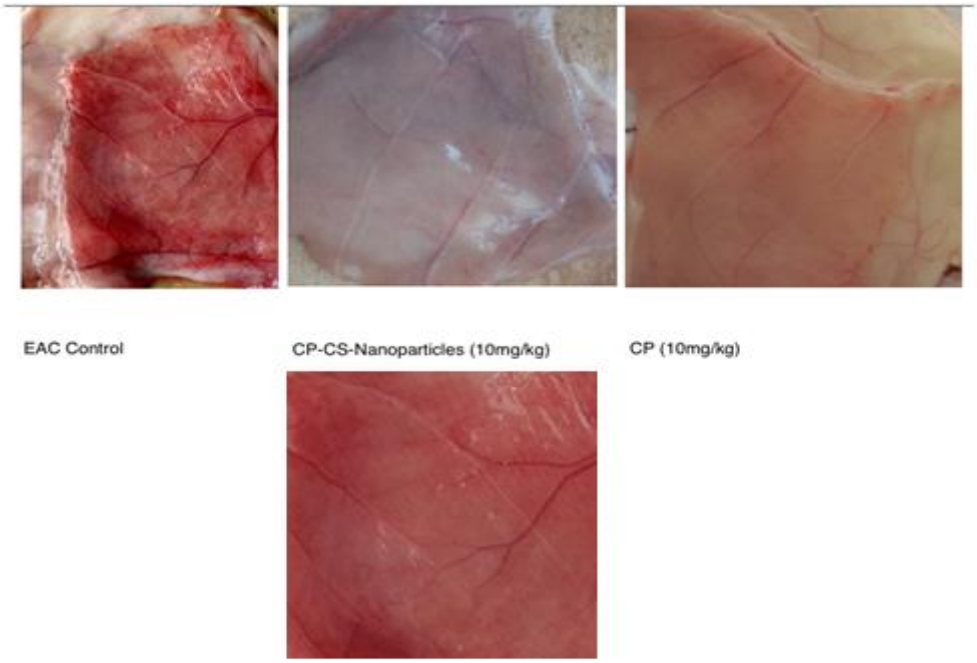

Fig. 8. a. Neovascularization in peritoneal lining of EACbearingswiss albino mice.

Matrix mettaloproteinase-3 (MMP-3) plays a significant role in tumour metastasis and angiogenesis by regulating vascular endothelial growth factor (VEGF).[23] Hence, to further validate anti-angiogenic property of CP-CS-NPs, VEGF (fig 8.b.) and MMP-3 level (fig.8.c.) in ascites secretion were also analysed. The results suggested that CP-CS-NPs decreased VEGF level ($p<0.05$) from 550.043±26.29 pg/ml to 233.33±41.63 pg/ml (table 4) in a dose dependent manner. MMP-3 levels raised in EAC bearing mice were also reduced ($p<0.05$) in a dose dependent manner. Therefore, CP-CS-NPs suppressed tumoral angiogenesis through downregulation of VEGF level.

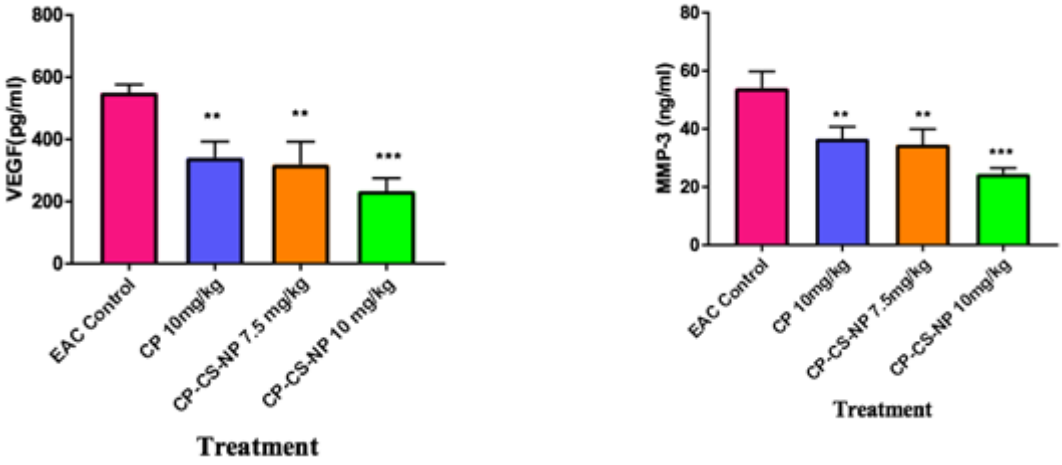

Fig. 8.b., 8.c.: Effect on VEGF (pg/ml) and MMP-3 (ng/ml) after treatment with CP-CS-NP (7.5 mg/kg and 10 mg/kg). Significance was $p<0.05$ vs EAC Control, values are Mean ±SD n=7.

Table 4: Effect on VEGF (pg/ml) and MMP-3 (ng/ml) after treatment with CP-CS-NP (7.5 mg/kg and 10 mg/kg)

Groups	VEGF level(pg/ml)	MMP-3 level (ng/ml)
EAC Control	550.043±26.29	53.98±5.79
CP (10mg/kg)	340.00±52.9	36.55±4.138
CP-CS-NP (7.5mg/kg)	328.33±74.21	35.56±5.351
CP-CS-NP (10mg/kg)	233.33±41.63	24.41±2.088

Effect of CP-CS-NPs on cytokines profile

Several cytokines (IL-12, IL-6, and TNF-α) are critical for tumour growth. Therefore, cytokine levels were estimated in the peritoneal fluid of EAC bearing mice in order to understand affects of CP-CS-NPs in modulating tumour microenvironment. CP-CS-NP treated group decreased the levels of IL-6 and TNF-α ($p < 0.05$) in a dose dependent manner as compared to EAC control group (Fig.9.a.9.b.) (Table 5). CP-CS-NPs increased IL-12 level ($p<0.05$) at dose of 10mg/kg (fig. 9.c.) as compared to standard CP (10mg/kg).

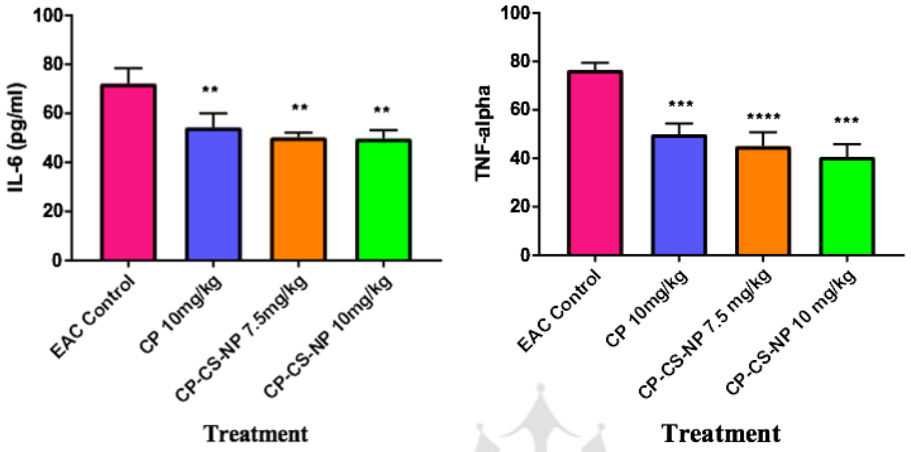

Fig.9.a., 9.b.: Effect on IL-6and TNF-αafter treatment with CP-CS-NP (7.5mg/kg and 10mg/kg). Significance was p<0.05 vs EAC Control, values are Mean ±SD n=7.

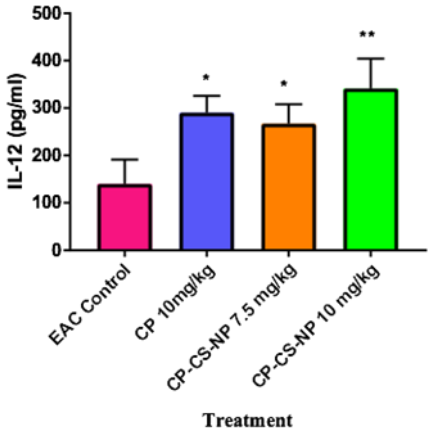

Fig. 9.c.:Effect on IL-12 (pg/ml) after treatment with CP-CS-NP (7.5mg/kg and 10mg/kg). Significance was p<0.05 vs EAC Control, values are Mean ±SD n=7.

Table 5: Effect on IL-6, IL-12 and TNF-α (pg/ml) after treatment with CP-CS-NP (7.5 mg/kg and 10 mg/kg).

Groups	IL-6 level(pg/ml)	IL-12 level(pg/ml)	TNF-alpha level(pg/ml)
EAC Control	72.09±6.340	139.8±51.61	76.47±2.98
CP (10mg/kg)	54.313±5.688	290±36.05	49.92±4.45
CP-CS-NP (7.5mg/kg)	50.24±1.98	276.66±41.6	46.06±5.76
CP-CS-NP (10mg/kg)	49.70±3.539	341.33±62.9	42.21±7.83

Effect of CP-CS-NPs on total antioxidant capacity (TAC)

Oxidative stress is one of the hallmarks of tumour progression and TAC measurement helps in establishing a link between oxidative stress and the severity of the cancer progression in EAC bearing mice.In the present study, treatment with CP-CS-NPsimproved TAC in blood plasma (fig. 10 table 6) from 9.85±1.397 to 18.50±1.138 in a dose dependent manner as compared to EAC control group.

Table 6: Effect on Total antioxidant capacity in blood plasma after treatment with CP-CS-NP (7.5 mg/kg and 10 mg/kg).

Groups	Total antioxidant capacity(µmol/ml)
EAC Control	9.85±1.397
CP(10mg/kg)	15.43±1.447
CP-CS-NP (7.5mg/kg)	14.84±1.961
CP-CS-NP (10mg/kg)	18.50±1.138

DISCUSSION

Cyclophosphamide, an alkylating agent is well known for its anti-cancer activity and is widely used for treating neoplasms. Treatment with anti-cancer drugs has been detrimental to non-cancerous tissues and organs as well causing harmful effects to the patients. To overcome this problem, nanoparticle drug delivery system (DDS) has emerged as the rescuer

for cancer therapeutics. DDS can easily deliver cancer drugs to specific targeted organs infested with cancerous growths or tumours.[24-27]The current study critically investigates the therapeutic potential of cyclophosphamide in the form of chitosan nanoparticles against EAC tumour. Chitosan was selected as the polymer for formulation due to its biocompatibility, bioadhesivity and low toxicity profile.[8] It also exertsantitumour activity by directly interfering with cell metabolism, inhibiting cell growth, and inducing cell apoptosis. The high positive zeta potential (32.03±1.072mV) conferred high stability and internalisation of positively charged nanoparticles, possibly utilizing clathrin-mediated mechanisms.[28]Due to small size of CP-CS-NPs (i.e. 186.53 ±1.865 nm), these particles passed through biological barriers and improved drug delivery to the tumour site. The biphasic release causing initial burst release of drug and subsequent slow and continuous release may have caused increase in blood circulation time of drug.Small size and controlled release diminishes uptake by the reticuloendothelial system (RES), causing effective targeting of the tumour sites through the disorganised and defective vascular architecture in tumour tissue known as enhanced permeability and retention (EPR) effect.[29] Further, entrapment efficiency of CP-CS-NPs was found to be 78±2.6% which play a role in optimum delivery of the drug to tumoursites.*In-vivo*study revealed CP-CS-NPs reduced the tumour burden by decreasing ascitic fluid volume, which serves as a nutritional source for cancer cells.[30] As a result, there was a decrease in body weight of EAC bearing mice. The antitumour cytotoxicity of CP-CS-NPs was associated with an increased life span and decreased ascites cell viability of the EAC bearing female swiss albino mice. To establish efficacy of an anticancer drug, prolonged life span and decreased WBC are taken into account.[31]In cancer chemotherapy,myelosuppression and anaemia due to iron deficiency or haemolytic or myelopathic conditions have been a major problem,[32-34] but it was found that RBC, WBC and haemoglobin levels were brought to normal range significantly by CP-CS-NPs indicating their protective action on haematological system. It has been demonstrated in many *in vivo* studies that angiogenesis is responsible for tumour growth and metastasis. Thus inhibiting angiogenesis, tumour growth and metastatic potential of tumourare diminished.[35-38]MMP-3 plays a crucial role in angiogenic switching by increasing VEGF bioavailability. VEGF is the most potent inducer of tumour angiogenesis.[39,40] MMP-3, VEGF and TNF-αlevelswereanalysed in ascitic fluid of EAC bearing mice and found to be decreased ($p<0.05$) by nanoformulation in a dose dependent manner exerting the anti-angiogenic activity. This result was fortified with the prevention of blood vessel in peritoneal lining of EAC bearingswiss albino mice. IL-12 and TNF-α are the major mediators of inflammatory response.[41]IL-12 increases the level of TNF-

α which in turn encourages the production of IL-12 by signalling to macrophages through CD40. But, TNF-α is also involved in up regulation of TNF-α-receptor 1(TNFR1) which inhibits IL-12 production.[42]Our study showed reduced TNF-α and elevated IL-12 levels in ascitic fluid of CP-CS-NP treated mice. The increased IL-12 levels may be due to negative feedback mechanism of TNF-αsignalling and positive feedback of IFN-γ (initially induced by IL-12 itself during an inflammatory reaction).[43]This elevated IL-12 level may activate the complement cascade to opsonize tumour cells exposing them to cytotoxic action of NK cells.[44] Decrease in TNF-α levels suggests an anti-inflammatory effect which might delay cancer progression.[17] In EAC bearing mice, the elevated cytokines (TNF-α and IL-6) level may be attributed to oxidative stress. This might have initiated the immune response and activated transcriptional factor NFκB which in turn elevated TNF-α levels.[45] Treatment with CP-CS-NPs decreased the TNF-α and IL-6 levels and increased ($p<0.05$) total antioxidant capacity (TAC) of EAC bearing mice in a dose-dependent manner. The antioxidants rejuvenated the scavenging process to remove any ROS or free radical formed during tumour progression. The increased TAC in CP-CS-NPs treated group indicated its antioxidant activity which caused cytotoxicity towards tumour cells.

CONCLUSION

The present investigation suggested that the nanoparticulate delivery system of cyclophosphamide exhibited sustained release properties for 24 hr. This may reduce the tumour burden, frequent dosing and thereby minimise side effects. The *in vivo* study clearly indicated comparable therapeutic potential even when dose was reduced to 7.5mg/kg (25%reduction) than marketed formulation, although needs further validation. In future, work can be done in the directionof formulating these nanoparticulate dosage forms for active targeting to tumour sites by coating them with folic acid and carrying out pharmacokinetic and toxicity studies.

REFERENCES

1. Benelli, G., Iacono, A. L., Canale, A., &Mehlhorn, H. (2016). Mosquito vectors and the spread of cancer: an overlooked connection?*Parasitology Research*, *115*(6), 2131-2137.
2. Murugan, K., Dinesh, D., Kavithaa, K., Paulpandi, M., Ponraj, T., Alsalhi, M. S., & Kumar, S. (2016). Hydrothermal synthesis of titanium dioxide nanoparticles: mosquitocidal potential and anticancer activity on human breast cancer cells (MCF-7). *Parasitology Research*, *115*(3), 1085-1096.
3. Di, C., & Zhao, Y. (2015). Multiple drug resistance due to resistance to stem cells and stem cell treatment progress in cancer (Review). *Experimental and therapeutic medicine*, *9*(2), 289-293.

4. Gao, Y., Chen, Y., Ji, X., He, X., Yin, Q., Zhang, Z., & Li, Y. (2011). Controlled intracellular release of doxorubicin in multidrug-resistant cancer cells by tuning the shell-pore sizes of mesoporous silica nanoparticles. *ACS Nano*, *5*(12), 9788-9798.
5. Tiwari, G., Tiwari, R., Sriwastawa, B., Bhati, L., Pandey, S., Pandey, P., &Bannerjee, S. K. (2012). Drug delivery systems: An updated review. *International Journal of PharmaceuticalInvestigation*, *2*(1), 2.
6. Mousa, S. A., &Bharali, D. J. (2011). Nanotechnology-based detection and targeted therapy in cancer: nano-bio paradigms and applications. *Cancers*, *3*(3), 2888-2903.
7. Jahromi, M. A. M., Karimi, M., Azadmanesh, K., Manesh, H. N., Hassan, Z. M., &Moazzeni, S. M. (2014). The effect of chitosan-tripolyphosphate nanoparticles on maturation and function of dendritic cells. *Comparative Clinical Pathology*, *23*(5), 1421-1427.
8. Nitta, S. K., &Numata, K. (2013). Biopolymer-based nanoparticles for drug/gene delivery and tissue engineering. *International journal of molecular sciences*, *14*(1), 1629-1654.
9. Bansal, V., Sharma, P. K., Sharma, N., Pal, O. P., &Malviya, R. (2011). Applications of chitosan and chitosan derivatives in drug delivery. *Advances in Biological Research*, *5*(1), 28-37.
10. Mitra, A., &Dey, B. (2011). Chitosan Microspheres in Novel Drug Delivery Systems. *Indian Journal of Pharmaceutical Sciences*, *73*(4), 355–366.
11. Fodstad, O., Olsnes, S., &Alexander Pihl, A. (1977). Inhibitory effect of abrin and ricin on the growth of transplantable murine tumors and of abrin on human cancers in nude mice. *Cancer Res.* 37, 4559–4567.
12. Liu, H., &Gao, C. (2009). Preparation and properties of ionically cross-linked chitosan nanoparticles. *Polym. Adv. Technol*, *20*, 613-619.
13. Vimal, S., Majeed, S. A., Taju, G., Nambi, K. S. N., Raj, N. S., Madan, N., ... & Hameed, A. S. (2013). Chitosan tripolyphosphate (CS/TPP) nanoparticles: preparation, characterization and application for gene delivery in shrimp. *ActaTropica*, *128*(3), 486-493.
14. Salgueiro, A., Egea, M. A., Valls, R., Espina, M., & García, M. L. (1999). An inductively coupled plasma method for determination of cyclophosphamide loaded to polymeric systems. *Journal of pharmaceutical and biomedical analysis*, *21*(3), 611-618.
15. Solanki,G., Shah, N., Chauhan, S., Aundhia, C., Javia, A.,&Seth, A.K.(2015). Formulation and evaluation ofcyclophosphamide loaded chitosan nanoparticlesconjugated with folic acid for cancer targeting. *Pharma Science Monitor* 6(1), 171-189.
16. Devbhuti, D., Gupta, J. K., &Devbhuti, P. (2012). Studies on antitumor activity of *Bryophyllumcalycinum*Salisb. against Ehrlich ascites carcinoma in Swiss albino mice. *Journal of PharmaSciTech*, *2*(1), 31-33.
17. Agrawal, S. S., Saraswati, S., Mathur, R., & Pandey, M. (2011). Cytotoxic and antitumor effects of brucine on Ehrlich ascites tumour and human cancer cell line. *Life Sciences*, *89*(5), 147-158.
18. Saraswati, S., Agrawal, S. S., &Alhaider, A. A. (2013). Ursolic acid inhibits tumour angiogenesis and induces apoptosis through mitochondrial-dependent pathway in Ehrlich ascites carcinoma tumour. *Chemico-biological interactions*, *206*(2), 153-165.
19. Agrawal, S. S., Saraswati, S., Mathur, R., & Pandey, M. (2011). Antitumor properties of Boswellic acid against Ehrlich ascites cells bearing mouse. *Food and Chemical Toxicology*, *49*(9), 1924-1934.
20. Strober, W. (2001). Trypan blue exclusion test of cell viability. *Current protocols in immunology*, A3-B.
21. Sur, P., &Ganguly, D. K. (1994). Tea plant root extract (TRE) as an antineoplastic agent. *PlantaMedica*, *60*(02), 106-109.
22. Szymanska-Chargot, M., &Zdunek, A. (2013). Use of FT-IR Spectra and PCA to the Bulk Characterization of Cell Wall Residues of Fruits and Vegetables Along a Fraction Process. *Food Biophysics*, *8*(1), 29–42.
23. Andisheh-Tadbir, A., Khademi, B., Kamali, F., Fattahi, M. J., Malekzadeh, M., &Taghva, M. (2014). Upregulation of serum vascular endothelial growth factor and matrix metalloproteinase-3 in patients with oral squamous cell carcinoma. *Tumor Biology*, *35*(6), 5689-5693.
24. Jabir, N. R., Tabrez, S., Ashraf, G. M., Shakil, S., Damanhouri, G. A., & Kamal, M. A. (2011). Nanotechnology-based approaches in anticancer research. *International journal of nanomedicine*, *7*, 4391-4408.
25. Bolhassani, A., Javanzad, S., Saleh, T., Hashemi, M., Aghasadeghi, M. R., & Sadat, S. M. (2014). Polymeric nanoparticles: potent vectors for vaccine delivery targeting cancer and infectious diseases. *Human vaccines &immunotherapeutics*, *10*(2), 321-332.

26. Slate, D.L., Bruno, N.A., Casey, S.M., Zutshi, N., Garvin, L.J., Wu, H., &Pfister. (1995) J.R. RS-33295-198: a novel, potent modulator of P-glycoprotein-mediated multidrug resistance. *Anticancer Res.*;15:811–814.
27. Eidi, H., Joubert, O., Némos, C., Grandemange, S., Mograbi, B., Foliguet, B., &Rihn, B. H. (2012). Drug delivery by polymeric nanoparticles induces autophagy in macrophages. *International journal of pharmaceutics*, *422*(1), 495-503.
28. Nasti, A., Zaki, N. M., de Leonardis, P., Ungphaiboon, S., Sansongsak, P., Rimoli, M. G., &Tirelli, N. (2009). Chitosan/TPP and chitosan/TPP-hyaluronic acid nanoparticles: systematic optimisation of the preparative process and preliminary biological evaluation. *Pharmaceutical Research*, *26*(8), 1918-1930.
29. Miller, S. M., & Wang, A. Z. (2013). Nanomedicine in chemoradiation. *Therapeutic Delivery*, *4*(2), 239–250. http://doi.org/10.4155/tde.12.147.
30. Patra, S., &Muthuraman, M. S. (2013). Gracilariaedulis extract induces apoptosis and inhibits tumor in Ehrlich Ascites tumor cells in-vivo. *BMC complementary and alternative medicine*, *13*(1), 1.
31. Oberling C, Guerin M. The role of viruses in the production of cancer. Adv Cancer Res 1954;2:353–423.
32. Rim, H. (2012). Chemotherapy-Induced Anemia in Cancer Patients. Journal of Abrasom Cancer Center of the University of Pennsylvania.
33. Kannu, K. D., Rani, K. S., Jothi, R. A., Gowsalya, G. U., &Ramakritinan, C. M. (2014). *In-vivo* anticancer activity of red algae (Gelidielaacerosa and Acanthophoraspicifera). *International Journal of Pharmaceutical Sciences and Research*, *5*(8), 3347.
34. Barrett-Lee, P. J., Ludwig, H., Birgegård, G., Bokemeyer, C., Gascón, P., Kosmidis, P. A. &Schrijvers, D. (2006). Independent risk factors for anemia in cancer patients receiving chemotherapy: results from the European Cancer Anaemia Survey. *Oncology*, *70*(1), 34-48.
35. Benazzi, C., Al-Dissi, A., Chau, C. H., Figg, W. D., Sarli, G., de Oliveira, J. T., & Gärtner, F. (2014). Angiogenesis in spontaneous tumors and implications for comparative tumor biology. *The Scientific World Journal*, *2014*.
36. Joyce, J. A., & Pollard, J. W. (2009). Microenvironmental regulation of metastasis. *Nature Reviews Cancer*, *9*(4), 239-252.
37. Prager, G. W., Poettler, M., Unseld, M., & Zielinski, C. C. (2011). Angiogenesis in cancer: anti-VEGF escape mechanisms. *Translational lung cancer research*, *1*(1), 14-25.
38. Cornet, A., Blacher, S., Tabruyn, S. P., Foidart, J. M., Noël, A., Martial, J. A., &Struman, I. (2007). Inhibition of tumor growth and metastasis establishment by adenovirus-mediated gene transfer delivery of the antiangiogenic factor 16K hPRL. *Molecular Therapy*, *15*(12), 2094-2100.
39. Kessenbrock, K., Plaks, V., &Werb, Z. (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell*, *141*(1), 52-67.
40. Deryugina, E. I., & Quigley, J. P. (2010). Pleiotropic roles of matrix metalloproteinases in tumor angiogenesis: contrasting, overlapping and compensatory functions. *BiochimicaetBiophysicaActa (BBA)-Molecular Cell Research*, *1803*(1), 103-120.
41. Feghali, C. A., & Wright, T. M. (1997). Cytokines in acute and chronic inflammation. *Front Biosci*, *2*(1), d12-d26.
42. Ma, J., 2001. TNF-a and IL-12: a balancing act in macrophage functioning. Microbe. Infect. 3, 121–129.
43. Zitvogel, L., Couderc, B., Mayordomo, J.I., Robbins, P.D., Lotze, M.T., StorkusW, J., 1996. IL-12-engineered dendritic cells serve as effective tumor vaccine adjuvants *in vivo*. Ann. NY Acad. Sci. 795, 284–293.
44. Pio, R., Corrales, L., &Lambris, J. D. (2014). The role of complement in tumor growth. In *Tumor microenvironment and cellular stress* (pp. 229-262). Springer New York.
45. Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: How are they linked? *Free Radical Biology & Medicine*, *49*(11), 1603–1616.