MATERIALS & METHODS

Materials Equipments & Software's used:

Materials used:

Sr.No.	Material	Company
1.	Paclitaxel	Emcure Pharmaceuticals Pvt.Ltd., Pune
2.	Genistein	Swapanroop Drugs Pvt. Ltd.
3.	Tristearin	Sigma Aldrich, India
4.	D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS)	Sigma Aldrich, India
5.	Glycerol tristearate	TCI Chemicals, India
6.	Captex 355	Abitac, USA
7.	Captex 300P	Abitac, USA
8.	Capmul	Abitac, USA
9.	Capmul MCM	Abitac, USA
10.	Miglyol	Chika Pvt. Ltd., Mumbai.
11.	Compritol 888ATo	Gattefosse international, Mumbai.
12.	Dynasan 114, 118	Cremer oleo GmbH, Germany
13.	Imwitor 900K	Cremer oleo GmbH, Germany
14.	Poloxamer 188	BASF, Mumbai.
15.	pluronic 407	BASF, Mumbai.

16.	Solutol HS15	BASF, Mumbai.
17.	PLGA (75:25)	Evonik pharmaceuticals, Mumbai
18.	Heparin sodium inj.	Biological E. Ltd.
19.	Sodium chloride	SD Fine chemicals, India

Equipments used:

Sr. No.	Instruments	Source
1.	Magnetic Stirrer	IKA Germany
2.	Electronic Balance	Dewinter, India
3.	pH meter	IKON Instruments
4.	Digital Optical Microscope	Dewinter, India
5.	Cooling centrifuge	ELTEK, 4100 RCF, India
6.	Particle size analyzer	Beckmen Coulter, USA
7.	Electric oven	Cintex, Mumbai, India
8.	Ultra probe sonicator	UP50H, Hielscher, USA
9.	Microplate absorbance reader	Biorad, USA
10.	FTIR	Shimadzu, Japan
11.	Co_2 incubator	Sanyo, Japan
12.	HPLC	Waters, USA
13.	Deep freezer	Thermoscientific, USA
14.	TEM	TECHNAI-20G^2, Czech Republic
15.	AFM	NT MDT, Russia
16.	Powder XRD	Rigaku, Japan
17.	Vortex mixer	Fisher Scientific, India
18.	Filtration assembly	Millipore, USA
19.	Bath sonicator	Fisher Scientific, India

Software's used:

Sr. No.	Software	Source
1.	Minitab 17	Coventry, UK
2.	Design Expert 10	MN, USA
3.	Graph Pad Prism 5	CA, USA
4.	Origin Pro 8	MA, USA
5.	Compusyn	Biosoft, MO, USA

Preformulation Studies:

HPLC analytical & bioanalytical method Development:

The amount of Gen & PTX (in vitro and in vivo) was measured with the help of HPLC (Waters, USA). HPLC system was consisted of waters 1525 binary HPLC pump (Waters, USA), rheodyne 7725i manual injector (Waters, USA), C18 reverse-phase (4.6 x75 mm; 3.5 µm) Symmetry® C18 column and waters 2998 photodiode array detector (Waters, USA)(11, 46, 47). The mobile phase consisted of Acetonitrile: 2mM Phosphoric acid in Milli-Q water (50:50) and was run at a rate of 1.0 ml/min. Column temperature was maintained at 30±1˚C during the whole process and the peak was detected at 262 nm for Gen & at 227 nm for PTX. HPLC peak area and retention time were integrated by using the Breeze2 software and were utilized for the calculation of drug content at all times. Standard calibration curves in required media were plotted from 500-3000 ng mL^{-1} of Gen & PTX (47, 48) for in vitro samples and from 1000-5000 ng/ml for in vivo samples. Typical validation characteristics (accuracy, precision, specificity, limit of detection, limit of quantification and linearity range) were calculated

in method validation to meet the acceptance criteria described in ICH Q2 (R1) guidelines.

Synergism analysis of ratiometric drug combination:

We used the method given by Chou et al. to evaluate the pharmacological interactions between the drug used in combination (49). In brief, synergism, antagonism and the additive effect of the combinations at different ratios was calculated on the basis of multiple effect equation and was quantified in terms of combination index (CI), where CI>1 indicated antagonism, CI=1 indicates the additive effect of two drugs and CI<1 indicates the synergistic effect of two drugs. To carry out the experiment, drugs were combined in the molar ratios 10:1, 5:1. 1:1, 1:5 & 1:10 and analyzed for cytotoxicity at different F_a values (fraction affected) (0.1-0.9). CI was calculated from data points with F_a >5 by using Compusyn® (Biosoft, Ferguson, MO, USA) software.

Formulation development of Nanostructured Lipid Carriers

Method of preparation:

NLC system was prepared by an emulsification solvent evaporation method (43, 44, 50). Briefly, 187.5 mg of Solid lipid binary mixture (SLB) which comprises of 0.75% for 25 ml total formulation in 60:40; Solid Lipid (SL): Liquid Lipid (LL) i.e.112.5 mg SL (Tristearin)+75 mg LL (Capmul MCM) was dissolved in 7.5 ml of dichloromethane (DCM) along with 10 mg of each drug or their combination (5 mg of each in combination). The surfactant mixture consists of 0.3% w/v (75 mg for 25 ml formulation) TPGS in Milli Q water. Both the aqueous phase and organic phase were kept at 60˚C & 900 RPM for 4 minutes, and the organic phase was added to the aqueous

phase by using high shear IKA T25 digital Ultra turrax homogenizer which was operated at 12500 RPM for 15 minutes. The same temperature (60˚C) was maintained during the addition process. Then, the formed nanoformulation was sonicated for 4 minutes using probe Ultrasonicator which was already set at 40% amplitude on-off cycles. It results in the formation of aqueous colloidal NLC suspension. The formulation was kept for a whole day at room temperature to check any instability and was further characterized for particle size, polydispersity index, zeta potential, surface morphology and entrapment efficiency using suitable techniques.

Optimization of process and product Parameters for Blank NLCs

Risk Assessment Studies: Optimization of different product and process parameters is required for the improvement of the quality of the drug product which requires in-depth knowledge of risk assessment control strategy. The elements of the QbD are:

- Quality target product profile (QTPP) (Table 5.6 that identifies critical quality attributes (CQAs) of the drug products. CQAs are 2 types: Critical material attributes (CMAs) & critical process parameters (CPPs) (Table 5.7 & 5.8.
- Product designing and identification of critical material attributes (CMAs).
- Process designing and identification of critical process parameters (CPPs).
- Process capability and continual improvement (51-53).

The information collected through the above studies will be further used for the development of a validated method of formulation which will be consistent overtimes.

Risk assessment studies help in identification of CMAs & CPPs which significantly affect the product CQAs. Further, failure mode effect analysis (FMEA) was employed to provide ranks to CTQs based on relative effectiveness (Table 5.9. FMEA helps in prioritizing the independent variables before using DoE strategies.

Screening of Factors by Placket Burman Design:

To screen out the important parameters out of large no. of factors, factorial Placket-Burman design was exploited by using Minitab 17 (11, 43). The factors were studied at two levels i.e., low (-1) and high level (+1) respectively. All factors and responses along with their lower and higher levels are described in Table 5.10.

Optimization by Box Behnken methodology:

A QbD approach based on RSM was employed to construct second order polynomial models. A 3 factor, 3 level (3^3) BBD with 16 no. of runs was utilized to investigate the effect of independent variables on responses. Lipid concentration (X1), Surfactant concentration (X2) & amount of organic solvent (X3) were chosen as independent variables based on the preliminary screening studies performed earlier and particles size (Y1), poly dispersity index (Y2) & entrapment efficiency (Y3) were selected as dependable variables based on the requirements of the NLC systems. The variables were varied at 3 different levels i.e -1 (lower level), 0 (medium level) & +1 (higher level). The experimental design was executed by using Design - Expert® software (7.0, Stat Ease Inc., Minneapolis). Data were analyzed using the principles of analysis of variance (ANOVA). Further, optimization was done by employing the desirability approach based numerical optimization. The concept of design space was utilized well

by keeping the responses under constraints and a percentage bias between experimental and practical values of the optimum formulation was calculated.

Formulation development of polymeric nanoparticles

Method of Preparation:

The PLGA (75:25) nanoparticles were formulated by nanoprecipitation method which is also known as solvent displacement method.0.2% (50 mg) of PLGA was added to 5 ml of acetone (semi polar solvent) and was stirred slowly till it completely dissolved. Accurately weighed quantity (10 mg) of each drug & their combination was added to this polymer solution. Simultaneously, the aqueous solution was prepared by the addition of 1% w/v surfactant (Poloxamer 188; 250 mg) to 25 ml of warm water (at 40 ˚C) under constant stirring. Previously formulated organic phase was injected to the magnetically stirred (1200 rpm, at 45˚C) aqueous stabilizer solution through a syringe at a constant flow rate (0.5 ml/minute). Then the prepared emulsion was kept fortnight under constant magnetic stirring to completely evaporate the organic solvent. It resulted in the formation of colloidal nanosuspension. The formulation was kept for whole day at room temperature to check any instability and was further characterized for particle size, poly dispersity index, zeta potential, surface morphology and entrapment efficiency using suitable techniques (8, 9, 23).

Optimization of blank nanoparticles by Box Behnken methodology (BBD):

Response surface methodology (RSM) based QbD approach was utilized to construct second order polynomial models. A 3 factor, 3 level (3^3) BBD with 17 no. of runs was employed to investigate the effect of independent variables on responses. Polymer

concentration (X1), & amount of organic solvent (X2) & Surfactant concentration (X3) were chosen as independent variables based on the literature review and particles size (Y1), poly dispersity index (Y2) & entrapment efficiency (Y3) were selected as dependent variables according to the quality standards of nanoparticles. All the variables were varied at 3 different levels i.e -1 (lower level), 0 (medium level) & +1 (higher level) and the details of the experimental runs were represented in Table number 6. The design was performed with the help of Design - Expert® software (7.0, Stat Ease Inc., Minneapolis). Randomization of the experimental runs was performed to avoid any biasness. The relationship between all the variables and their impact on the responses was predicted by the polynomial equations generated as a result of analysis of each response by using Analysis of variance (ANOVA). Regression coefficient, coefficient of determination and lack of fit were determined to check the adequacy of the data.

Further, optimization was done by employing the desirability approach based numerical optimization. Concept of design space was utilized well by keeping the responses under constrains and percentage biasness between experimental and practical values of the optimum formulation was calculated.

Characterization studies:

Drug Excipient compatibility studies:

Fourier Transformed Infrared studies (FTIR):

The interactions between the drug and the excipients were recognized by taking FTIR spectra of the same. Fourier Transformed Infrared (FTIR-8400S, Shimadzu) was utilized throughout the study. FTIR spectra of drug, each of the component, Physical

mixture (1:1) and formulation was obtained using traditional KBr pellet technique. The samples were prepared by grinding with anhydrous KBr and compressed into pellets by using a hydraulic press. The spectra were measured over the range of 4000- 400 cm^{-1} with a resolution of 4 cm^{-1} for 50 scans.

Differential Scanning Calorimetry (DSC) studies:

Differential scanning calorimetric analysis was performed using DSC 6000 (Pyrix 6, Serial Number: 002082704; Software Version: 11.0.0.0449). DSC studies of individual components including pure drug, polymer, surfactants and physical mixture was performed and compared with the lyophilized formulation. All samples were subjected to heat flow to study the temperature associated changes. Small amount of samples (1-5 mg) were weighed and placed on the aluminum pan and crimped. The samples were heated between from 0.00 °C to 400.00 °C at 10.00 °C/min. Nitrogen gas was introduced immediately at flow rate of 20ml/min(44, 54).

Powder- X Ray Diffractometry (P-XRD):

P-XRD data was obtained to analyze the physical as well as crystallographic changes in samples (Pure drug, polymers and physical mixture) by varying diffraction angle (2Ɵ) between 5 and 70 degree at a scanning speed of 10 degree/min using MiniFlex 300/600 operated at continuous mode.

Particle size and Polydispersity index (PDI):

The mean Particle size and Particle size distribution were determined by Particle size analyzer (Delsa Nano C Beckman Culter).

Zeta Potential:

Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles, and is one of the fundamental parameters known to affect stability. Its measurement brings detailed insight into the causes of dispersion, aggregation or flocculation, and can be applied to improve the formulation of dispersions, emulsions, and suspensions. It was determined by using Particle size analyzer (Delsa Nano C Beckman Cutler).

TEM (Transmission Electron Microscopy):

The surface morphology of the formulation was investigated using transmission electron microscopy. Samples were prepared by placing a drop of formulation which was diluted previously with water, onto a copper grid and kept fortnight for air drying. The air-dried samples were then directly examined under the TEM.

AFM (Atomic Force Microscopy):

To study the surface morphology of the formulation, AFM technique was utilized. Samples were prepared on glass slides by electrospinner technique, diluted formulation was added and the thin film was air dried which were further examined under AFM (AFM NT-MDT, NTEGRA Prima).

Entrapment Efficiency (EE):

Entrapment efficiency is the main parameter to determine the amount of drug that goes inside the nanoparticles. The entrapped drug will show the property of controlled release. We determined the parameter with the help of indirect method and the amount

of drug was analyzed quantitatively by using HPLC (WATERS; Breeze 2 software). Briefly the 1.5 ml of formulation was centrifuged (Eltek Cooling Centrifuge) at 14000 RPM for 15 minutes at temperature 10°C using Nanosep (Pall Corporation, 100 K Omega). The clear liquid was collected from the lower chamber of the tube and was further diluted 375 times with HPLC grade methanol. The samples were filtered through syringe filters (Axiva, PES 0.45 micron) and the peak area was measured against the standard. The entrapment efficiency was expressed as a percentage of the amount of drug entrapped inside the nanoparticles to the total drug added. The total amount of drug in „mg" was calculated by using standard calibration curve.

$$EE = \frac{Total\ drug\ content - free\ drug}{Total\ drug\ content} X100$$

Cumulative Percentage Drug Release (%CDR):

Calculation of % CDR is a determinant of in vitro release behavior of the drug inside the formulation. Dialysis bag diffusion technique was employed to study the release behavior as well as release kinetics of the formulations in comparison to their pure drug suspension. 0.25 % of surfactant was added to the pure drug suspensions. Briefly, 10 ml of formulation and equivalent amount of its corresponding pure drug suspension was added to activated dialysis membrane (8-12 kDa) (Himedia labs, India) molecular weight cut off which was tied from both the ends. The bag was incubated in 50 ml of release medium (PBS 7.4) maintained at 37.5±0.5 °C at 100 rpm. At predetermined times intervals, the whole media was replaced with fresh buffer and the samples were filtered and analyzed by HPLC. Cumulative percentage drug release was calculated. Data was fitted to various kinetic models (zero order, first order, Higuchi kinetics &

Korsmeyer Peppas model) to get the release kinetics. Sink conditions were maintained throughout the release period.

Stability studies and calculation of shelf life:

To get an idea about the performance of the formulation during storage, shelf life studies were performed under stated conditions according to ICH guideline Q1 A(R2) (11, 55). The samples were kept in high-density plastic bottles and were kept at 5±3˚C (refrigeration condition) as well as at 40±2˚C; 75±5% relative humidity (RH) (accelerated studies) and at room temperature (30±2˚C/65±5% RH) for six months. The stored samples were evaluated at specified time periods for changes in the formulation regarding particle size, zeta potential, PDI and % entrapment efficiency. The whole procedure followed was according to ICH Q1 A (R2) guidelines. The shelf life was calculated using Minitab® ver.17.

Hemocompatibility studies:

Evaluation of Heamolysis:

Formulations intended for intravenous administration should be tested for heamolytic potential of the same as the excipients present or drug may cause damage to red blood cells. To asses this, we had used the method mentioned by Vijay Kumar et al.(47). Briefly, fresh human blood was purchased and processed to extract plasma as stated earlier. Study consisted of four groups viz. test formulations, placebo formulations, positive and negative control. RBCs were taken out from the whole blood and washed with equal amount of saline by centrifugation at 1344xg for three times. To resuspend the pellet, normal saline was used and the pellet was diluted 10 times. 10 and 100 µg/ml of each group (Test, Placebo, Positive & Negative control) were taken and mixed with

the erythrocyte suspension upto 1 ml. Positive control (100% lysed erythrocytes) and spontaneous negative control were prepared by diluting an equal volume of erythrocyte suspension with 1% Triton X100 and normal saline respectively. The samples were incubated for 15 minutes and then the aliquots were withdrawn from each sample at predetermined time intervals (0.5, 1,2,4,8 hr). The aliquots withdrawn were centrifuged and supernatants were kept at room temperature for 30 minutes for oxidation of hemoglobin into oxyhemoglobin. The absorbance was measured spectrophotometrically by microplate reader (Biorad, Germany) at 540 nm. The percentage hemolysis was calculated by using the following formula:

$$\%\ Hemolysis = \frac{A_{sample} - A_{spontaneous\ control}}{A_{positive\ control}} \times 100$$

Where Abs_{sample} is the absorbance of the samples (nanoparticles formulations with and without drug), $Abs_{spontaneous\ control}$ is the absorbance of the spontaneous control (0.9% saline solution), and $Abs_{positive\ control}$ is the absorbance of the 100% control (1% Triton X 100). All the samples were analyzed in triplicate batches (n=3).

Platelet aggregation tests:

Platelet aggregation tests were performed to identify the changes in the platelets after treatment with the formulation. Citrated whole blood was incubated with test formulations (10 & 100 µg/ml of the drug), PBS (as a spontaneous control for platelet aggregation), pure drug suspensions and placebo formulations. Method stated by Bender et al.(56) was followed to execute the work. The samples were incubated for 30 min with gentle agitation (400 RPM). The peripheral blood smears were stained with Leishman‟s stain (Span Diagnostic, India) for 5-6 minutes after incubation followed by

rinsing with water which was then allowed to dry and the dried smears were analyzed by an optical microscope in immersion objective. The images were captured using the digital system (Dewinter Trinocular Microscopic Unit, Dewinter Technologies). Platelet aggregation was also evaluated by counting the no. of platelets before and after the addition of formulation to the citrated whole blood by hematological counter (Multisizer 4, Backmann coulter, USA).

In vitro cytotoxicity:

The human ovary cancer PA-1 cell line was grown in Dulbecco‟s modified Eagle Medium (DMEM, Himedia) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin) in a CO_2 incubator at 37 ^oC and 5% CO_2 atmosphere. MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was employed to study the in vitro cytotoxicity of pure drugs, formulation and placebo formulations in PA-1 (Human ovarian cancer cell line). 0.9% normal saline solution equivalent to nanoparticle dispersion was kept as control. Briefly, PA1 cells were seeded onto 96 well microtitre plates at $1x10^4$ cells per well in complete DMEM medium and incubated at 37˚C in humidified CO_2 (5%) incubator for 24h after that they were exposed to fresh DMEM culture medium containing different concentrations of test samples for 72 h under same conditions. After incubation, the medium was replaced with 20 ml of MTT (5mg/mL in PBS) and the cells were incubated for 4 h under same conditions. The culture medium and MTT were washed completely after 4 h and the formed insoluble formazan crystals which were proportional to the number of viable cells were dissolved in 100 ml of dimethyl sulfoxide (DMSO). The plates were agitated for 10 minutes and absorption was

measured at 570 nm using a multimode reader (Synergy H1 hybrid, Biotek, USA).The absorbance of the control cells was used to calculate the % cell viability of the test formulations. The percentage cell viability was calculated by the following equation:

$$Cell\ Viability\ (\%) = \left(\frac{Absorbance\ of\ Test}{Absorbance\ of\ Control}\right) \times 100$$

Data are expressed as mean±S.D. (n=3)

Pharmacokinetic studies:

The protocol of the animal studies was approved by the Central Animal Ethical Committee (CPCSEA) of Banaras Hindu University and the approval No. is **Dean/2016/CAEC/332**. Healthy female wistar rats with average weight of 150-200 g were used for the study which was obtained from Central Animal House, Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi, India. The rats were housed in polypropylene cages over one week before the experiments with 12 h light and dark cycles at ambient temperature; 40-70% relative humidity. Animals were fed over rat chow and water *ad libitum*. The rats were divided into 7 groups with each group containing 6 animals. Group I consisted of 0.9% saline solution as control. Group II : pure PTX in water for injection, Group III: pure Gen in water for injection, Group IV: combination of PTX and Gen in water for injection, Group V: nanoformulation of Gen, Group VI: nanoformulation of PTX & Group VII consisted of combination nanoformulation. The pre sterilized samples were administered intravenously at a dose of 10 mg/kg via tail vein method. The blood samples (200 µl) were collected in heparinized eppendorf tubes from retro orbital cavity at predetermined (0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72 & 96 hrs) time intervals under ether anesthesia.

The collected blood was centrifuged immediately at 2800 RPM; 4 ˚C (Remi centrifuge, India) for 15 minutes to obtain the plasma. Separated plasma was stored at -20˚C till further analysis by HPLC. The liquid- liquid extraction procedure was used for the extraction of the drug from the plasma. Briefly plasma was separated after centrifugation of the heparinized rat blood at 2800 r.p.m for 15 minutes at 4˚C using cooling centrifuge (Eltek India, RC4100F). The plasma was extracted out from the blood and equal amount of acetonitrile (ACN) (HPLC grade) was added to the extracted plasma. The samples were vortexes for 15 minutes to mix the solvent with the plasma gently and after the precipitation of the proteins, the supernatant was withdrawn and was further diluted with HPLC grade dichloromethane (DCM) (amount of DCM equivalent to the total amount of plasma and ACN). The dichloromethane layer was taken out and sample equivalent to 20 µl was injected in the HPLC injector port which was already maintained at previously designed method for the estimation of the drug. Peak area of the chromatogram was used to calculate the amount of drug present in the sample by using Breeze 2 HPLC software. The bioanalytical calibration curve was already prepared by spiking various known concentrations of the drug in plasma. Pharmacokinetic parameters were calculated by using PK solver add in of the Microsoft excel. Non compartmental intravascular analysis was employed for the calculation of parameters (34, 36, 47, 57).

In vivo biochemical estimations:

The protocol of the animal studies was approved by the Central Animal Ethical Committee of the Banaras Hindu University (CPCSEA) **Approval No. Dean/2016/CAEC/332)**. Healthy female wistar rats with average weight of 150-200 g

were used for the study which was obtained from Central Animal House, Institute of Medical Sciences (IMS), Banaras Hindu University (BHU), Varanasi, India. The rats were housed in polypropylene cages over one week before the experiments with 12 h light and dark cycles at ambient temperature; 40-70% relative humidity. Animals were fed over rat chow and water *ad libitum*. These studies were conducted to identify the levels of blood serum parameters for clinical monitoring. The rats were divided into 5 groups and each group consists of 6 rats (n=6). Group I consisted of pure saline (0.9%) solution which was kept as control. Group II: pure PTX in water for injection, Group III: pure drug combination of Gen and PTX in water for injection, Group IV: nanoformulation of PTX and Group V consisted of nanoformulation of combination of both drugs. The samples were administered to overnight fasted rats via tail vein i.v route. Formulation as well as pure drug were given at a dose of 10 mg/kg of the rat weight. The clinical parameters include determination serum level of total bilirubin (TBL), Aspartate aminotransferase (AST or SGOT), alkaline phosphatase (ALP), and alanine transaminase (ALT or SGPT) in order to evaluate liver function. Absolute Neutrophil Count (ANC) was also assessed to evaluate the neutropenia. The blood samples (by retro orbital puncture) were withdrawn from each group after2nd 14th and 28th day of drug administration.

In vivo anticancer activity:

The protocol of the animal studies was approved by the **IAEC** with **approval no. DL/PM/11/17.** Balb/c mice were housed in individually ventilated cage system and fed with standard sterilized diet and sterilized water ad libitum. Animals were maintained in 12-12 light-dark cycle, 25˚C air-conditioned and controlled noise rooms. Balb/c mice of

8-10 weeks were used for the experiment. Briefly ID-8 cells $1x10^5$ (murine epithelial ovarian cancer) cells were injected on the back of the mice and allowed to form tumors. Tumors were minced and re grafted in experimental animals. Administration of test samples was done after the tumor reached a palpable size. Test samples (formulation & pure drug combination suspension) were administered on day 6,12,18,24 & 30th i.v. 1.5 ml (10 mg/kg or 0.25 mg for 25 g mice), doxorubicin (standard) was administered 10 mg/kg i.p. on day 6, 12, 18, 24, 30th & untreated animals received saline 1.5 ml i.v. Tumor volume was measured using a digital vernier caliper (Mitutoyo JAPAN). Tumor volume was calculated by:

$$Volume = \frac{width^2 \times length}{2}$$

Relative Tumor Volume (RTV) $=V_t/V_0$ (V_0: Tumor volume at initial administration, V_t: Tumor volume at each time measurement).

Anti tumor activity was measured by the relative tumor growth rate T/C (%) =TRTV/CRTV *100 (TRTV is the RTV of treatment group and CRTV is the RTV of negative control group).

At the end of the experiment, the animals were sacrificed by cervical dislocation. The animals were dissected and tumors were excised. The excised tumors were immediately imaged.

Statistical analysis:

All the results were expressed as mean ± standard deviation. Results were statistically analyzed by analysis of variance (ANOVA) followed by Bonferroni post-test using

Graph Pad Prism® Ver. 5.Results with $p<0.05$ (95% CI) were considered as statistically significant.