Research Article

Impact of Surfactants on Formulation Parameters and *in vitro* Cytotoxicity of Boswellic Acids Loaded Nanoparticles on Human Colon Cancer Cell Lines

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ABSTRACT

Objective: Boswellic acids extracted from the gum resin of plant Boswellia serrata are well reported for anti-inflammatory and anticancer activities. The Present investigation was aimed to optimize the surfactant concentration by formulating a series of Boswellic acids nanoparticles. **Methods:** Nanoparticles were prepared by nanoprecipitation technique using diverse ratios of non-ionic surfactants (Polyvinyl alcohol and Pluronic F-127). Effect of surfactant concentration was studied on size, polydispersity index, zeta potential, entrapment efficiency and loading capacity of nanoparticles. Transmission Electron microscopy was used to examine the effect of surfactant type on surface morphology of developed nanoparticles. In vitro cytotoxicity was evaluated by 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay against colon cancer cells lines (HCT-116). **Results:** Smallest particle size and polydispersity index (189.2 nm, 0.27), high entrapment (49.53 ± 0.5) and loading capacity (23.64 ± 0.43) with spherical shape was obtained in Pluronic F-127 nanoparticles in comparison to Polyvinyl alcohol nanoparticles. Pluronic F-127 nanoparticles showed higher cytotoxicity against colon cancer cell lines with IC_{50} value 21.3 μM having significant cytotoxic effects in comparison with Polyvinyl alcohol (IC_{50} value 23.2 μM) nanoparticles and Boswellia serrata extract (IC_{50} value 30.8 μM). **Conclusion:** It is concluded that the choice of surfactant is a critical parameter in the formulation of nanoparticles.

Key words: Boswellic acids, Nano precipitation, Surface morphology, MTT assay.

INTRODUCTION

Recently, considerable advancements have been made for early diagnostic and treatment strategies in case of human colon cancer, but still, the occurrence rate of this disease is high and the second foremost cause of cancer-related deaths. Human colon cancer is a complex disease that occurs as a result of numerous genomic and epigenetic variations in vital oncogenes and tumor-suppressing genes.[1] Herbal products are standardized formulations of one or more than one plant materials including pharmaceutical excipients in processed, crude form. It is estimated about eighty percent of the world's population, especially in developing countries, depends upon the herbal products for their primary health care needs. Biocompatibility and safety concerns of herbal preparations generally make them preferable over synthetic products and increase public interest in herbal medicines.[2] Currently, number of plants derived anticancer active agents are utilizing in the market and variety of natural agents are still under development that site specifically target signalling along with epigenetic pathways that are leading cause of cancer disease. A variety of anticancer drugs are unmodified natural products derived from natural sources (plants or microorganisms) including semisynthetic derivatives of natural products have established promising platform are paclitaxel (Taxol™), vincristine, vinblastine, bleomycin, doxorubicin, daunorubicin,

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mitomycin, streptozocin, Irinotecan (derived from camptothecin), podophyllotoxin derivatives (etoposide and tenoposide). Boswellic acids (BAs) are pentacyclic triterpenoidal moieties naturally obtained from oleo gum resin of the plant *Boswellia serrata* (family Burseraceae) widely dispersed in India commonly known as Salai Guggul. This plant mainly constitutes of four important BAs: namely α-boswellic acid, β -boswellic acid, 11-keto β- boswellic acid (KBA), and 3-acetyl-11-keto- β –boswellic acid (AKBA).[3] BAs derived from gum resin are reported to have significant pharmacological activities especially anti-inflammatory activity in traditional ayurvedic medicines[4]. BAs also utilized in the treatment of diseases like, arthritic pain[5-7] colitis[8] Crohn's illness[9] and hyperlipidemic management[10] etc. In addition to the above potential therapeutic effects, different research and case studies on brain tumors and leukemic cells revealed that BAs might have some anticancer related effects. Antiproliferative and antitumor activities of BAs were reported by Hoernlein *et al.* (Leukemia cells HL 60) Liu *et al.* (colon cancer HCT-116) Lu *et al.* (Prostate cancer LNCaP and PC-3) Ravanan *et al.* (Lung Cancer A549) Takahashi *et al.* (Colorectal cancer HCT116, HT29, SW480 and SW620), Ranjbarnejad *et al.* (Colon cancer HCT 29 cells) and Neeta and Dureja, 2018 (Melanoma A 375 cancer cells).[1,11-16] Various human and animal models were used to explore the pharmacokinetic data of BAs and concluded the poor oral bioavailability constraints of BAs. It has been well-established fact evidenced by a number of research and clinical studies that the bioavailability constraints of hydrophobic drug moieties can be significantly increased by processing them in a nanoparticle formulation with suitable surface-active agents.[2,11,1]

There is an increasing optimism that nanotechnology, as applied to medicine, will bring significant advances in the diagnosis and treatment of disease. Cancer nanotechnology has been aggressively evaluated and implemented in cancer management and therapeutics, with suggestions that it might lead to major advances in diagnosis, detection, and treatment of the disease. It offers many potential benefits in cancer research ranging from, but not limited to, passive and active targeting, increased solubility/bioavailability, and novel therapies.[17] Nanoparticles [NPs] formulations keep the number of benefits primarily enhanced aqueous solubility of phytochemicals, high drug loading with drug targeting, thereby enhanced absorption, controlling the drug release in apredictable manner. NPs have higher cellular uptake with improved bioavailability, half-life, leads to minimization of therapeutic dose and toxicity of drugs. Developments of nanoparticle formulations involve different types of surfactants. The nonionic surfactants are frequently utilized in oral formulations for bioavailability enhancement of, especially water-insoluble drugs. Nonionic surfactants possess various merits like these agents improves the solubility of water-insoluble drugs, low toxicity to biological membranes. Some nonionic surface active agents are reported to have an effect on drug pharmacokinetic parameters by modifying efflux pumps (P-glycoprotein and/or multi-drug resistance associated proteins).[18] Pluronicspoly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer and PVA are a type of nonionic surface-active agents and possess various applications in several biomedical emerging fields extending from drug delivery and medical imaging particularly Pluronics generally interacted with multidrug-resistant tumors, leading to drastic sensitization in the tumors with improved cancer treatment.[19]

Nanoprecipitation technique is also known as interfacial deposition or solvent displacement. It is widely employed and one of the easiest development technique in the arena of NPs.[20] Nano precipitation method is widely recommended the technique for the entrapment of hydrophobic drug molecules into nanoparticle formulations. Nanoprecipitation is an effective to develop ultra fine nano carriers based on the mechanism of supersaturation produced by mixing solution and anti-solvent. Fonseca *et al.* work demonstration and several other research investigators established a procedure that permits nanoparticles precipitation, by circumventing extensive diffusion of the active molecule along with the evaporating solvents leading to higher drug encapsulations.[21] Nanoprecipitation technique has been investigated earlier by many investigators for the development of nanoparticles of hydrophobic drug molecules (AKBA*, Boswellia serrata* extract, Curcumin, Paclitaxel, KBA).[22-24]

Currently natural phytochemicals are widely used treatment modalities in cancer management. With this perspective BAs were extracted and encapsulated in NPs formulations using Pluronic F127 (PF-127) and polyvinyl alcohol (PVA) as surfactants. The aim of the present study was to optimize surfactant concentration by preparing BAs nanoparticle formulations by nanoprecipitation technique with diverse ratios of non-ionic surfactants. The objective behind the study was to evaluate the influence of surfactant concentration on the shape, size, zeta potential, entrapment efficiency, loading capacity of nanoparticles and *in vitro* cytotoxic effects of BAs on colon cancer cells lines.

MATERIALS AND METHODS

Plant Material

Gum resin of plant *Boswellia serrata* was procured from the local market and authenticated from National Institute of science communication and information resources (NISCAIR) by Dr. H.B. Singh, Chief Scientist and Head, Raw Materials Herbarium and Museum (RHMD), NISCAIR.

Cell Lines and Cell Culture

Colon cancer (HCT-116) cell lines were procured from National cell repository situated at Pune India. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) media accompanied with 10% fetal bovine serum (FBS) and antibiotic solution (1X Penstrip, Invitrogen).

Chemicals and Reagents

Pluronic F-127 and Polyvinyl alcohol were procured from Sigma Aldrich, Germany, Central Drug House, New Delhi respectively. All other chemicals used including the solvents were of analytical grade.

Extraction and Identification of BAsby FTIR and DSC

Gum resins of *Boswellia serrata* were crushed into the smaller size and soaked in methanol for 12 h. Resulting solution was filtered and the filtrate was concentrated to obtain a reddish brown mass. A 3% w/v potassium hydroxide solution was used to basify the mass and continuous stirring was performed to formulate a uniform emulsion. Dichloromethane was used for separation of the lower solvent layer from this emulsion. BAs are found to be present in the upper layer which was then acidified with dilute hydrochloric acid to precipitate the acid content. Centrifugation was performed for the separation of precipitated acids and these acids were washed several times with distilled water. The light yellow powder obtained after drying was stored in desiccators for nanoparticles preparation and further processing.[25] FTIR is one of the commonly used analytical techniques for the identification and characterization of unknown compounds.[26-27] *Boswellia extract* was homogeneously mixed with potassium bromide and the mixture was then compressed into discs by a hydraulic compressor through applying pressure of about 10 tonnes in 2 min. The discs were placed in the infrared light pass and the infrared spectrum was recorded in the region of 400–4000 cm^{-1} was scanned on FTIR Alpha Bruker 1206 0280, Germany.[28] In order to observe crystalline or amorphous nature of the active drug, DSC is the most frequently used technique. The physical state of the extract was observed by DSC instrument (DSC Q10 V9.9., USA) by calibration with Indium as standard. An analysis was carried with respect to the reference as empty pan and samples in the sealed aluminum pan under the nitrogen atmosphere (60 ml/min) and heating at the temperature range of 30ºC to 300ºC.[29]

Preparation of BAs Nanoparticles

In the present study, BAs loaded NPs were prepared by a modified nanoprecipitation method by using PF-127 and PVA as surfactants. Total eight formulations were prepared by varying concentration of these surfactants to optimize the surfactant concentration (Table 1). The powder extract was dissolved in 10 mL of acetone (organic phase).The organic solution was then added dropwise under stirring to an aqueous solution of PF-127 and PVA (30 ml). The colloidal suspension (milky appearance) was obtained and evaporated at room temperature for 8 h to remove the organic solvent. The suspension was centrifuged at 25,000 rpm for 30 min for NPs separation from the unencapsulated drug and remaining surfactants. The obtained product was washed three times with distilled water and resultant NPs sediment was lyophilized for 24 h. The freeze-dried powder was packed in glass vials and stored in desiccators for Furtheranalysis.[30-31]

Physicochemical characterization of NPs

Particle Size and Zeta Potential

Zetasizer was used for the determination of mean particle size and the size distribution of freshly prepared particles by dynamic light scattering principle. Particle size was determined by dissolving 2 mg nanoparticles in 5 ml of distilled water. The sample dilution was filled in a polystyrene cuvette in the hydro dispensing unit and the scan was carried out at 64 runs per sample. After the completion of the scan, the average diameter/scan of all the 64 runs was taken out and recorded

Table 1: Description of formulation batches with variation in surfactants concentration.

Formulation code	*Boswellia* extract (mg)	Organic Phase/ Aqueous phase (mL)	PVA/PF-127 concentration (%)
NP1	200	10/30	0.25
NP2	200	10/30	0.5
NP3	200	10/30	1
NP4	200	10/30	2
NP5	200	10/30	0.25
NP6	200	10/30	0.5
NP7	200	10/30	1
NP8	200	10/30	2

as Z-average. Similarly, zeta potential was determined using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) to check the stability of nano formulation. The electrophoretic mobility of nanoparticles in aqueous suspension was utilized to determine the zeta potential. The nanosuspensions were scanned in clear disposable zetacell with an equilibrium time of 120s.[32]

Entrapment Efficiency and Loading capacity

The BAs entrapment efficiency and loading capacity wereestimated by the direct method. Amount of BAs entrapped in NPs was estimated by adding 5 mg of NPs in 5mL of methanol and vigorously shaken for solubilization of NPs. Binary gradient program was used for the quantitative estimation of BAs in the *Boswellia* extract. The column used was octadecylsilane silica gel C-18 250 x 4.6 mm; 5 μm particles. The mobile phase A was a blend of 0.1: 99.9 v/v (Phosphoric acid: Water) and mobile phase B was having compositions of 0.1: 99.9 v/v (Phosphoric acid: Acetonitrile). Both of the mobile phases were filtered using 0.45 µm membrane filter.[33] The entrapment efficiency and drug loading were calculated according to the following equations:

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Total drug} - \text{Free drug}}{\text{Total drug}} \times 100 \quad \text{eq (1)}$$

$$\text{Loading Capacity (\%)} = \frac{\text{Total drug} - \text{Free drug}}{\text{Nanoparticles weight}} \times 100 \quad \text{eq (2)}$$

Transmission Electron Microscopy

Transmission electron microscopy (Hitachi –H7500, Japan) was used to study the morphology of nanoparticles prepared by different stabilizing agents. Nanoparticles sample was deposited on the copper coated carbon grid. Carbon grid was kept on the wax sheet and excess sample was wipe out and dried for 25-30 min and examined at accelerating voltage of 120 Kb.[34]

Cell Culture and Treatment

Colon (HCT-116) cancer cell lines were grown in DMEM media accompanied with 10% fetal bovine serum (FBS) and antibiotic solution (1X Penstrip, Invitrogen). The cells were incubated at 37°C with 5% CO_2 and 95% humidity conditions. For experiments, cells were seeded in equal numbers after trypan blue cell counting (5,000-8000 cells per well of 96-well plate). Afterwards, cells were washed once with sterile 1X PBS and cultured with serum-free media for 24 h for synchronization. Series of dilution (10, 20, 40, 80, and 160 µg/mL) of supplied samples were dissolved in PBS and DMEM and was added to the plate in triplicates.

MTT Assay

MTT assay was done using 96-well plate; each well was filled by 100 µL media to which cell were treated with the synthetic compounds for 24 h. After 24 h media was discarded and subsequently washed with 1X PBS and were consequently treated with MTT dye (5 mg in 10 mL of 1X PBS) at a concentration of 10 µL per well (and incubated at room temperature in dark for 4 h to allow the formation of formazan crystals. After 4h crystals so formed were dissolved thoroughly using DMSO (100µL). This was followed by readings using microplate reader at 570 nm. The results were then represented as mean ± S.D obtained from three independent experiments.[35]

$$\text{\% cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100 \quad \text{Eq (3)}$$

In order to calculate inhibitory concentration$_{50}$ (IC_{50}) which is representation of the concentration of sample necessary to inhibit 50% of cells was calculated Graph Pad Prism software version 5.

RESULTS AND DISCUSSION

Percentage extractive value of extract was found to be 38.41% and the presence of BAs in the extract was confirmed by FTIR and DSC analysis. The FTIR spectra of the obtained sample showed peaks at 1699 cm^{-1} for C=O stretching of aryl acid, 1648 cyclic ketone, 1453 cm^{-1} for CH_2 scissoring of cyclohexane and 1238 cm^{-1} for C-Co-C skeleton alkyl ketone. The peak observed at 2942 cm^{-1} is assigned for OH stretching of carboxylic acid, 3442 cm^{-1} for OH stretching (bonded) observed when steric hinderance presents polymeric association and 1022 cm^{-1} and 984 cm^{-1} for ring stretching of cyclohexane. Various peaks are depicted in (Figure 1) which revealed the presence of BAs in the gum extract. DSC thermogram showed two main endothermic peaks for BAs at 193°C and 270°C respectively corresponding to their reported melting point (Figure 2).

Particle Size and Zeta Potential

The particle size and zeta potential of different formulations are shown in Table 2. Surfactants have the ability to decrease surface tension between the organic phase droplets and the aqueous dispersion medium. Therefore, increase in surfactant concentration leading to decrease in the nanoparticle size and the amount

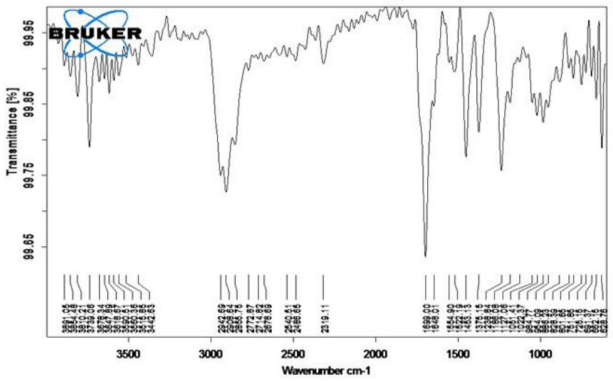

Figure 1: FTIR spectra of *Boswellia* extract.

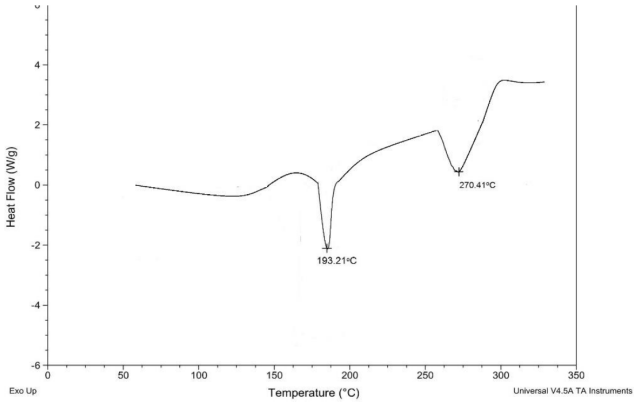

Figure 2: DSC thermogram of *Boswellia* extract.

Table 2: Characterization of nanoparticles prepared by using PF-127 and PVA.

Formulationcode	Size (nm)	PDI	Zeta Potential (mV)	Entrapment Efficiency (%)	Loading capacity (%)
NP1	428.1	0.673	-2.73	32.83±41	17.61±0.37
NP2	382.4	0.666	-4.13	24.54±0.38	14.77±0.04
NP3	331.8	0.423	-5.18	20.49±0.45	11.06±0.13
NP4	363.2	0.606	-5.72	10.3±0.23	5.42±0.39
NP5	238.4	0.514	-8.21	49.53±0.5	23.64±0.43
NP6	203.9	0.351	-10.8	43.63±37	20.53±0.31
NP7	189.2	0.277	-11.5	40.42±33	18.56±0.4
NP8	210.8	0.352	-12.3	25.53±51	15.69±0.2

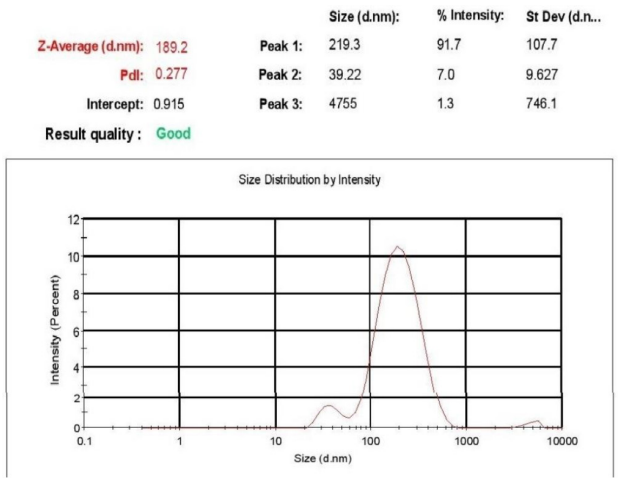

Figure 3: Particle size of NP7 Batch.

of surfactant was varied while the other parameters were kept constant to investigate the surfactant concentration on nanoparticle size. The average size of the nanoparticles was decreased by increasing the amount of surfactant. Obtained data showed (Figure 3) mean diameter 189.2 nm (NP7) of PF-127 encapsulated NPs (polydispersity index of 0.277) with zeta potential of -11.5 mV (Figure 4). PVA NPs (NP1) have highest particle size 428.1 nm with zeta potential -2.73 with less stable NPs preparation in comparison with PF-127 NPs. Surfactants oriented at the interface between the organic solution and the aqueous medium, thereby reducing the interfacial tension and thus increasing the net shear stress. This in fact would promote the formation of small size particles. Higher amount of surfactant resulted in aggregation leading to the formation of large-sized particles while optimum concentration of stabilizer led to the formation of nanoparticles with small size. The particle size is affected by the type of surfactants employed. Described the effect of PVA and PF-127 F68 on the viscosity and particle size of formulations. 37 PVA was responsible for an increase in suspension viscosity, which led to higher particle sizes, while the use of PF-127 did not alter these parameters.

Entrapment Efficiency and Loading Capacity

The effect of different surfactants on entrapment efficiency and loading capacity is given in Table 2. Entrapment efficiency was significantly decreased with

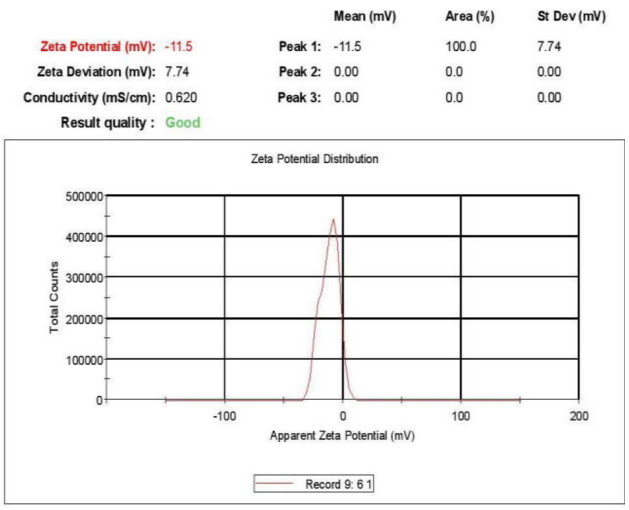

Figure 4: Zeta potential of NP7 Batch.

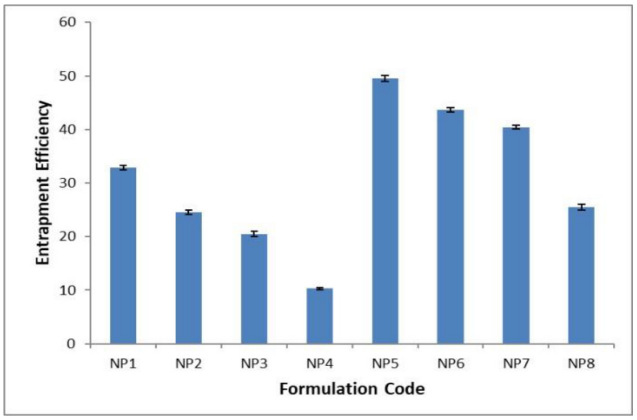

Figure 5: Entrapment efficiency of different Bactes.

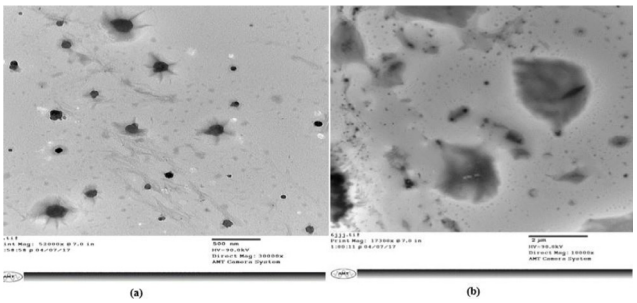

Figure 6: TEM images (a) NP5 and (b) NP1Batch.

increasing amount of surfactant pluronic F-127 from 0.25 to 2%w/v. Higher entrapment and loading capacity was found in NPF5 batch 49.53±0.5 % (Figure 5) and 23.64±0.43 % and NPP4 showed lowest entrapment and loading capacity 10.3±0.23 % 5.42±0.39 respectively.

Transmission Electron Microscopy

TEM analysis of NPs batches NP5 and NP1 was performed as shown in Figure 6 (a) and (b) respectively.

Table 3: Percentage cell viability of different samples against colon cancer cell lines.

Concentration µg/mL	*Boswellia serrata* Extract	PF-127 NP (NP5)	PVA NP (NP1)
10	81.33±0.5	90.33±0.57	92.33±0.5
20	64±1	62±1	69
40	42.33±1.5	37.66±1.1	35±1
80	27.33±1.1	22.33±1.5	26±1
160	15±1	14±1	14±1

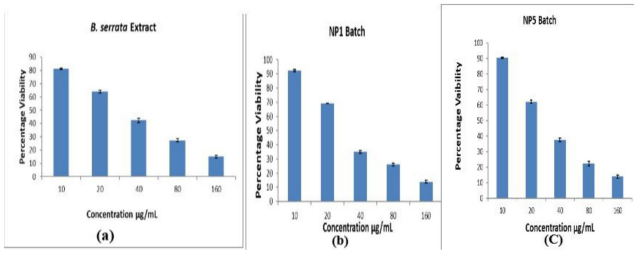

Figure 7: Percentage viability (a): *Boswellia serrata* extract (b)NP1(c) NP5 Batches.

NP5 batch NPswere spherical in shape with the almost smooth surface. No aggregation, separated particles with smooth surfaces confirmed the suitability of PF-127 for preparation of NPs. TEM photomicrograph of the NP1formulation containing PVA showed aggregation of nanoparticles with irregular surfaces.

MTT Assay

Cytotoxic effects of extract and BAs loaded (NP1 and NP5) NPs were evaluated by MTT assay using the Colon (HCT-116) cancer cell lines based on the colorimetric estimation of reduced MTT by mitochondrial succinate dehydrogenase enzyme. The range of concentrations of the samples were 10 to 160 µg/ml and MTT results with percentage viability are shown in Table 3 and Figure 7 which revealed concentration-dependent cytotoxic effects and comparison of viability is depicted in Figure 8. Regression graphs were plotted for calculation of IC_{50} for extract, PF-127 NPs and PVA NPs. PF-127 NPs showed higher cytotoxicity against colon cancer cell lines with IC_{50} value 21.3 µM having significant cytotoxic effects because of small particle size and high entrapment in comparison to PVA with IC_{50} value 23.2 µM NPs and *Boswellia serrata* extract having IC_{50} value 30.8 µM as represented in Figure 9. The higher cytotoxicity of the drug formulated into NPs can be attributed to the combination of different mechanisms. NPs were adsorbed on the cell surface leading to an increase in drug concentration near the cell membrane, thus generating a concentration gradi-

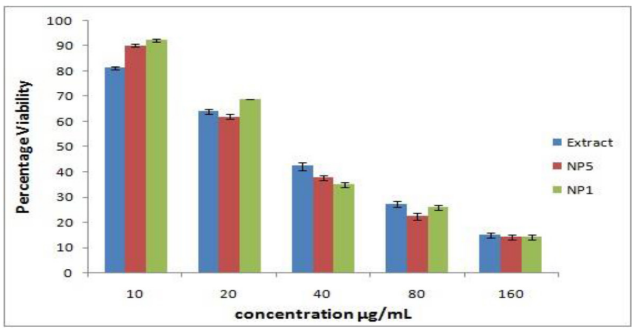

Figure 8: Comparison of viability between different samples.

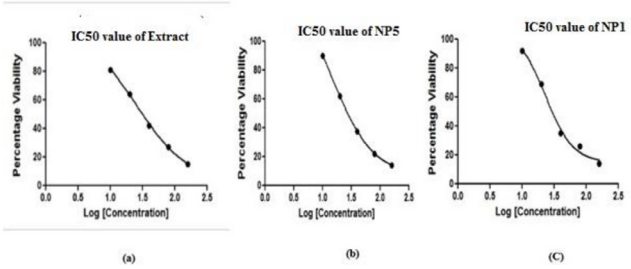

Figure 9: Regression graphs (a): *Boswellia serrata* extract (b) NP5 and (c) NP1 Batch.

ent that promotes the drug influx into the cell. MTT is only step used for investigation of *in vitro* cytotoxicity of the samples against cancer cell lines and give indications that the test samples may have the anticancer potential which can be further confirmed by other assays.

CONCLUSION

Nanotechnology holds effective potential for cancer treatment by means of targeted delivery and gained more attention due to unique accumulation behavior. PF-127 nanoparticle have smaller particle size better entrapment, spherical shape nanoparticles with high zeta potential and cytotoxic potential in comparison with PVA nanoparticles. Development of nanoparticles with suitable surface active agent plays an important role in nanoparticles formulation influencing the cellular internalization as well cellular toxicity.

CONFLICT OF INTEREST

Authors do not have any conflict of interest.

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