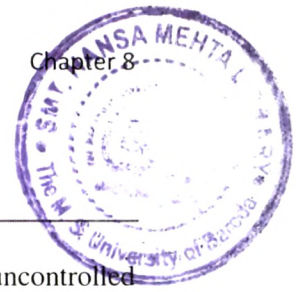
Chapter 8

SUMMARY AND CONCLUSIONS



8. SUMMARY AND CONCLUSIONS

The cancer is one of the leading causes of death in the world, characterized by uncontrolled multiplication and growth of cells. Though advances in the treatment and diagnosis of cancer have improved lifespan and quality of cancer patients, the survival rate is still poor. Treatment related toxicity, development of drug resistance; inadequate target drug delivery and recurrences of disease are the major reasons behind the morbidity and mortality.

Hence the effective therapy for the cancer requires the development of the new strategies to enhance the drug concentrations in cancer regions and facilitate prolonged exposure of cells to the delivered drugs. Many researchers had worked and has been working on nanoparticulate colloidal drug delivery systems to explore the potential of the same in achieving the above mentioned objective.

Tumor angiogenesis inhibition may severely limit the tumor growth as well as metastasis. Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels stimulated by biochemical signals. Drug induced inhibition of angiogenesis is an area of intense research and at least 10000 cancer patients worldwide have received some form of experimental antiangiogenic therapy. Antiangiogenic therapy has been shown to increase the efficacy of classical chemotherapeutic agents in anticancer treatment. Thus combination therapy offers reduced toxic side effects and reduced drug resistance.

This thesis describes our efforts to study the delivery of anticancer-antiangiogenic combination (Etoposide + Quercetin Dihydrate) therapy by incorporating into PLGA (50:50) nanoparticles and also to investigate the antitumour effect in B16F10 melanoma animal model.

Chapter 1 deals with the introduction to the topic of the work done, objectives of the work, and list of materials used in experiments.

Chapter 2 focuses on the literature survey done and the information is presented under the following heads-

Cancer, tumour angiogenesis, antiangiogenic therapy, nanotechnology in cancer and *in vitro* studies on nanoparticles.

This chapter also includes the detailed plan of work.

Chapter 3 discusses in detail the drug profiles of Etoposide and Quercetin Dihydrate. Under this, the description, physicochemical properties, pharmacological properties including indications, pharmacokinetics and toxicology are discussed. Analytical methods were also mentioned for the estimation of drugs.

Chapter 4 explains the analytical methods used in the estimation of drugs.

The spontaneous solubility of Etoposide (ET) and Quercetin Dihydrate (QD) was studied in various solvents. Both drugs exhibited high solubilities in acetone, acetonitrile, DMSO and chloroform.

Analytical methods for the estimation of ET and QD were successfully established. The quantitative evaluation of ET and QD in PLGA nanoparticles and was performed using UV spectroscopic methods. ET and QD have shown absorption maxima at **283.5 nm** and **368.0 nm** with corresponding absorptivities of **4.09 × 10^3** and **21.3 × 10^3 mol^{-1} cm^{-1}** in acetonitrile. The UV spectroscopic methods established for both drugs exhibited good correlation coefficient of **0.999**, thus confirming the linearity of relation between the absorbance and concentration of the drug.

The UV spectroscopic method was set for the estimation of drugs in pH 7.4 phosphate buffer for the purpose of *in vitro* release study. ET and QD have shown absorption maxima at **283.5 nm** and **377.0 nm** with corresponding absorptivities of **3.6 × 10^3** and **18.0 × $10^3$$mol^{-1}$ cm^{-1}** in pH 7.4 phosphate buffer with good correlation coefficient of **0.999**, thus ensuring the linearity of data. The media used for quercetin contains BSA at concentration of 3mg/ml to stabilize the QD solution in pH 7.4 pH phosphate buffer. The effect of BSA on the stability of Quercetin was verified by carrying out experiments with and without addition of BSA to pH 7.4 phosphate buffer.

The methods were validated by carrying out the stability, accuracy and precision studies for the method.

Chapter 5 deals with the preparation and characterization of PLGA nanoparticles of Etoposide (ETN) and Quercetin dihydrate (QDN) . Nanoprecipitation method (Solvent diffusion) is used to prepare the nanoparticles with Poloxamer 407 as surfactant and acetone being used as solvent to dissolve the drugs and the polymer, PLGA. This method was optimized for various formulation parameters such as polymer concentration, surfactant concentration, organic phase volume, aqueous phase volume and theoretical drug loading.

The Entrapment efficiency and the particle size were the response parameters for the optimization process.

The optimized ETN and QDN formulations exhibited mean diameters **153.4±4.2nm** and **148.6±1.6nm** respectively and corresponding drug EEs were found as **63.88±1.5%** and **41.36±3.4%.** The size distribution curves revealed monodisperse unimodal systems which were also reflected by the polydispersity indices (PDI) i.e. **0.058±0.02** for ETN and **0.088±0.03** for QDN.

The characterization of ETN and QDN was done by measuring the zeta potential, TEM and DSC analysis.

The zeta potential of drug free nanoparticles (PN) was on higher negative side **(30.2±0.6)** whereas the incorporation of ET and QD resulted in slight decrease in zeta potentials **(26.4±1.9 for ETN and -27.0±0.7).** The negative zeta potentials are beneficial in drug delivery and prolonging the circulation time property.

The TEM images of ETN and QDN were obtained by staining the nanoparticles with 2% uranyl acetate. The TEM micrographs indicated uniform and spherical shaped, discrete particles without aggregation, and appear to be smooth in surface morphology with the diameters **133.65nm** for **ETN** and **113.14nm** for **QDN.**

The physical state of ET and QD loaded in NPs was investigated by DSC. The DSC thermograms were also obtained for drugs ET and QD, $PLGA_{(50:50)}$ and plain NPs (PN) which served as controls. ETN had not shown exothermic **(206°C)** and endothermic peak **(268°C),** which were exhibited by ET, thus it is concluded that ET formulated in NPs existed as an amorphous state or a solid solution. DSC of QD revealed endotherms at **117.31°C** and **322.32°C,**which were completely disappeared in the thermogram for QDN, thus confirming dispersion of QD throughout the polymer forming high-energy amorphous state.

ETN and QDN were tested for stability by storing them in amber coloured glass vials separately, at refrigerating temperature 2-8°C in dark and at room temperature 25±2°C at room light conditions for a period of 1 and 3 months, followed by evaluation for particle size and drug content parameters. These formulations exhibited good stability properties at refrigerated conditions **(2-8°C)** for a period of **3 months,** which warranted their *in-vitro* and *in-vivo* evaluation.

Chapter 6 describes the *in-vitro* studies carried out on the ETN and QDN. The *in-vitro* studies were done in two parts. Firstly, the *in-vitro* drug release studies from nanoparticles (ETN and QDN) were performed in pH 7.4 phosphate buffer medium by dialysis bag diffusion

technique. A control experiment to determine the release behavior of the free drugs was also performed in the same way using the drug solutions. Nearly 100% of drugs were released from plain drug solutions approximately in 6 h. But only about 40% of drugs released from NPs after 24 hrs (**96.98±2.3% and 87.08±1.9% for ET and QD** respectively from free drug solutions at 6 hrs, **46.69±2.7% and 38.65±2.0% for ET and QD** from nanoparticles at 24 hrs).

Thus pronounced time prolongation of drug release from nanoparticles were observed.

The kinetic analysis of drug release was performed by subjecting the data to dissolution study models. The result of kinetic analysis reveals that the release of ET and QD from free drug solutions followed the first order kinetics and from ETN and QDN followed Higuchi model.

The mechanism of drug release from the nanoparticles was determined using the Korsmeyer-Peppas model. The parameters - release constant (k), release exponent (n), and correlation coefficient were calculated for ETN and QDN. From the release exponent values in the Korsmeyer-Peppas model for the release of ET and QD from their respective nanoparticles , it could be concluded that the mechanism that led to the release of drugs was an anomalous transport (**0.571 for ETN and 0.714 for QDN**).

Secondly, the *in-vitro* cytotoxic activity of the nanoparticle formulations ETN and QDN was investigated in comparison with free drug solutions of ET and QD. The comparison was also made with respect to cytotoxic activity of individual drug and combination of drugs in the form of free drugs as well as nanoparticles. The IC 50 values were found for the formulations as well as the free drug solutions for Cancer cell line A549 (Human lung Adenocarcinoma epithelial cell line). The cell viabilities for predetermined time duration were found by MTT assay method. The results were analysed by student's t-test

IC 50 values at 72 hrs incubation for ETN and QDN were found significantly lower comparable to the reference drugs ET and QD respectively. Combination treatment (EQ) showed higher activity when compared with individual drug treatments. The combination in the form of nanoparticles (EQN) exhibited even higher activity than the plain drug combination (EQ). There was an increase in cytotoxicity in case of ETN and QDN at all time points in comparison to free drug but the effect was non-significant at 24 hrs ($p>0.05$). In the same case, significant ($p<0.01$) differences in cytotoxicities were found at 48 hrs and 72 hrs.

The combination treatment EQ exhibited significantly lower cell viabilities ($p<0.05$) as compared to individual drug treatment. The EQN treatment was found to produce significant improvement in cytotoxicity effect ($p<0.05$) compared with the free drug combination (EQ) except for the results at 24 hrs where the difference is insignificant.

When the comparison is made between the cytotoxic effect of ET with the EQN, extremely significant ($p < 0.001$) enhancement in cytotoxic effect due to EQN is observed at 48 hrs and highly significant ($p < 0.01$) enhancement is observed at 24 and 72hrs.

The greater antiproliferative activity of combination therapy (EQ) may be attributed to additive effect of single drug treatments. The significance of the difference in cytotoxicity effect of individual free drugs and individual nanoparticulate formulation was established at 72 hrs and not at 24 hrs. This may be due to the sustained release of drugs from the nanoparticles which can be well correlated with the results of in-vitro release studies in previous section.

The *in-vitro* cytotoxicity studies concluded that the combination treatment EQN is found to produce far better results as compared to single chemotherapeutic drug treatment i.e. ET alone.

Chapter 7 presents in-vivo studies on ETN and QDN. The comparative study of *in-vivo* biodistribution of NPs as well as free drugs were done by radiolabeling with the ^{99m}Tc by applying direct labeling procedure.

The radiolabeling efficiency and the *in-vitro* serum stability was checked for the ^{99m}Tc-labeled complexes of ET, QD, ETN and QDN by instant ascending thin layer chromatography using acetone as mobile phase. The labelling efficiencies were found to be high for nanoparticulate formulations as compared to the free drug solutions (**97.76% for ET, 88.23% for QD, 99.63% for ETN and 94.12% for QDN**).

Stability of the ^{99m}Tc-labeled complexes of drugs and nanoparticles was determined in vitro in serum by ascending TLC technique at different time points (½, 2, 6, and 24 hrs). The data demonstrated excellent stability of all complexes in serum except for the ^{99m}Tc-labelled QD solution. It exhibited moderate stability in comparison with the other complexes.

Pharmacokinetic studies were conducted by collecting blood samples from the retro-orbital plexus of mice eye at predetermined time point (5, 15, 30, 60, 120, 240, 720, 1440 min)

The blood was weighed and the radioactivity present in the whole blood was assessed.

This study revealed that both nanoparticulate formulations ETN and QDN achieved higher blood concentrations and extended residence times as compared to ET and QD after 2hrs upto 24hrs.

Biodistribution studies of ^{99m}Tc-labeled complexes were carried out in C57BL/6 mice bearing desired size B16F10 tumour at 2, 6, and 24 hrs. Gamma images were taken using single photon emission computerized tomography gamma camera at these time points.

Both free drugs, ET, QD as well as the nanoparticulate formulations, ETN and QDN distributed rapidly to all the organs. At 2 and 6 hrs after injection, no significant difference ($p > 0.01$) in distribution pattern was observed for free drugs and NPs. At 24 hrs, the nanoparticle formulstions ETN and QDN showed significantly higher ($p < 0.05$) concentrations than free drugs thus ensuring the prolonged stay of NPs *in vivo* where as free drugs are cleared rapidly. The overall tumour uptake of nanoparticles was very significantly higher ($p< 0.01$) than the free drug QD and significantly higher ($p < 0.01$) than ET at 6 and 24hrs.

The gamma scintigraphy images of B16F10 melanoma tumour bearing mice after subcutaneous injection of ^{99m}Tc-labelled complexes of ETN and QDN taken at 1hr and 48hrs post injection. This study demonstrates the significant tumour uptake and retention.

The *in-vivo* tumour growth inhibitory activity was assessed using B16F10 melanoma model (21 days protocol) in C57BL/6 mice for nine groups - control (Untreated), PN (Placebo i.e. drug-free nanoparticles), DMSO (20% aqueous solution), ET, QD, EQ, ETN, QDN and EQN. The mean tumour volumes and the mean weight change in mice were recorded for all groups. This study has drawn following conclusions.

Treatment with either placebo or DMSO did not result in significant changes in tumour volumes.

Treatment with ET and ETN formulation resulted in very signigicant ($p < 0.01$) tumour growth inhibition. The nanoparticulate formulation ETN produced significant ($p < 0.05$) tumour growth inhibition as compared to free form of drug ET, thus underlying the usefulness of the nanoparticle drug delivery system in cancer therapy. QD and QDN showed similar results,but the tumour inhibition effect produced by QD is significantly ($p < 0.01$) lower than that produced with ET. Hence Quercetin Dihydrate alone was not able to produce tumour growth inhibition comparable to chemotherapeutic drug ET at the dose level used in the study. The treatment with combination of anticancer and antiangiogenic drugs ET and QD (EQ) produced very significant ($p < 0.01$) tumour growth inhibition as compared with the treatment with any drug alone. The combination treatment in the form of nanoparticles (EQN) resulted in even higher ($p < 0.05$) tumour inhibition than free drugs combination (EQ) as well as individual drug nanoparticle (ETN /QDN). The tumour induction period was maximum **(16 days)** for EQN treatment which is more than double the period that required for the control group **(7 days)**.

The order of tumor growth inhibition activity for various treatments was found as **EQN > EQ > ETN > ET > QDN > QD > Control**.

The ET treatment group led to maximum weight loss of **1.7 gms** which is usually observed with chemotherapeutic drugs. The overall health of the mice of this group was observed to be detoriated in comparison with the other groups. But administering the drug in the form of nanoparticles and adding the antiangiogenic drug QD to therapy lowered this effect to some extent. The positive weight change is minimum for the EQN group while other groups led to higher positive weight change which can be attributed to the higher tumour volumes.

The antiangiogenesis was studied by evaluating the tumour microvessel density. The tumour microsections (5μ) were stained with hematoxilin and eosin and slides were observed under low-power microscopy and the angiogenesis response (tumor microvessel density) was rated. The data was analysed by nonparametric Mann-Whitney rank sum test and Z values determined. Following conclusions are drawn from TMD evaluation.

The control group showed dense or marked microvasculature underlining the role of angiogenesis in the tumor growth. Tumors treated with the QD showed significant reduction in the angiogenesis revealing the QD as potential antiangiogenic agent. Further the antiangiogenesis effect was found significantly improved for the QDN and EQN treatment as compared to plain drug treatments. The Z-values f QD, QDN and EQN were found as **4.16, 4.96 and 4.8** respectively, thus signifying the role of QD as antiangiogenic agent.

The biodistribution studies were successfully carried out and revealed that overall tumour uptake of nanoparticles was very significantly higher than the free drugs ET and QD at 6 and 24hrs.

The tumour growth inhibition studies concluded that the **EQN treatment** was most efficacious as well as safe treatment amongst all the treatment groups.

In this study, The Etoposide and Quercetin Dihydrate loaded nanoparticles were successfully prepared, characterized and evaluated for *in-vitro* drug release and *in-vitro* cytotoxicity studies. The data of the investigation conclusively demonstrates higher tumor uptake of drugs from the nanoparticles. The results also suggest that the long term delivery of these drug nanoparticle combination may lead to effective tumour therapy due to their greater tumour transport and tumour retention properties.The enhanced efficacy of anticancer-antianiogenic drug combination (Etoposide + Quercetin Dihydrate) was verified by simultaneous administration of the combination drugs in B16F10 mouse melanoma model. The role of

Quercetin Dihydrate as an antiangiogenic agent in enhancing the efficacy and lowering the untoward effects of chemotherapy is clearly underlined with our studies. Along with the antiangiogenic activity, Quercetin Dihydrate possesses many other functions like .antioxidation, inhibition of cell cycle at G1 and S phase in vitro, reversal of multidrug resistance, inhibits phosphorylation of protein kinase C (PKC) and tyrosine kinase, blocking of cellular signal transduction, inhibition of the ras cascade, binding to type II estrogen receptors to stimulate growth inhibition/proliferation, inhibition of the mutation of the tumor suppressor protein gene p53, inhibition of matrix metalloproteinases etc. Some of these are just steps in the angiogenesis process. Thus Quercetin Dihydrate can be foreseen as the most capable agent in combination therapy for cancer.

In conclusion the results of this thesis, the combination of Etoposide and Quercetin diydrate in the form of PLGA nanoparticles holds promise for consideration for trials in treating early stage solid tumor cancers such as melanoma, peritoneal cavity tumours, gastric cancers and lung cancers which were predominantly depending on the angiogenesis process to excel to metastatic state.

Thus this nanoparicle formulation treatment in combination (anticancer drug + antiangiogenic agent) can definitely give a new start to fight the dreadful disease cancer and lead to safe and effective chemotherapy showing a ray of hope to cancer patients.