4.1 INTRODUCTION

In both developing and developed countries, there is an increase in the number of reported cases of cancer. The report issued by the World Health Organization says that the number of new cases of cancer is over 10 million each year and annual deaths caused by cancer are over 6 million, whereas recent world statistics indicate that the number of new cancer cases will be more than 15 million in 2020. (Stewart & Weihues, 2003) Osteosarcoma is known as primary malignant tumor in especially in children and adolescents. It is the eighth most common cancer in children (Ottaviani & Jaffe, 2009). The major characteristics of osteosarcoma is an unregulated proliferation of osteoid-producing mesenchymal cells (Huvos, 1991). Recently, neoadjuvant and adjuvant chemotherapy along with surgery has increased the survival rate by 5 years for localized disease by more than 60% as compared to surgery alone (Anninga et. al., 2011; Longhi et. al., 2006) However, the 5-year survival rate is below 30% in patients with metastasis continue to have poor prognosis. When chemotherapy was not introduced, the survival rate of patients having osteosarcoma was only 15-20%, regardless of adequate local control (Jain & Kapoor, 2016). Recently, a study was also conducted in India which concluded the two-year progression free survival of 70% for patients having non-metastatic osteosarcoma (Bajpai et. al., 2014). The effective chemotherapy regimens currently utilized against osteosarcoma also have damaging effects on normal cells which result in acute life-threatening complications. Currently, in chemotherapeutic treatment of osteosarcoma combinations of high doses of cisplatin (Benassi et. al., 2007), with methotrexate (Bacci et. al., 1990; Saeter et. al., 1991) ifosfamide and doxorubicin (Ferguson & Goorin, 2001) have shown a significant improvement in
the survival rate. Recently, in the treatment of osteosarcoma EURAMOS study concluded that all the patients received neoadjuvant chemotherapy: 2 blocks of methotrexate, doxorubicin, and cisplatin (MAP) chemotherapy for almost ten weeks and followed by surgery. Before surgery, right responding patients continued on MAP therapy for 28 weeks and no further therapy with pegylated interferon and maintenance required. This study also revealed that addition of etoposide to adjuvant chemotherapy in poor responders did not improve their survival. Also, the addition of pegylated interferon maintenance was useless in good responders (Bielack et. al., 2015). However, there are various side effects associated with these anticancer drugs due to nonspecific uptakes such as necessitating the use of high dosages, the poor blood supply in the case of osteosarcoma (Roche et. al., 2013), secondary malignancies and drug resistance phenotype (Paulussen et. al., 2001).

Cisplatin drug is one the most effective and potent drug against cancers including osteosarcoma and lung cancer. Cisplatin/CDDP is a platinum-based chemotherapy drug which forms platinum complexes in cells. Eventually, these complexes bind and result in cross-linking of DNA finally triggering apoptosis. Its side effects include diarrhea, loss of appetite, nausea, vomiting, and loss of taste may occur. Nausea and vomiting can be quite persistent and severe. (Nishiyama et. al., 2003; Kikkawa et. al., 2008).

Methotrexate is a folate antagonist used as a potent anticancer drug. The anticancerous effect of methotrexate is due to the inhibition of DHFR, which results in a deficiency of purines and thymidylate which ultimate causes decrease in repair and synthesis of DNA and cellular replication. Its damaging effects include
azotemia, bleeding of the stomach or intestine, decreased blood platelets, decreased white blood cells and stomach or intestinal ulcer.

Doxorubicin is an anthracycline drug which has been used in the treatment of cancers. It acts in the cancer cells by two methods 1) Free radicals generation and their damage to DNA, proteins and cell membrane and 2) It interrupt topoisomerase II-mediated DNA repair and intercalates into DNA. Its side effects include cardiomyopathy, secondary malignancies extravasation and tissue necrosis.

Therefore, new approaches are needed since tumors are believed to reoccur in a short time after the first chemotherapy regimen (Riehle et. al., 2011) and these chemotherapy drugs have multiple severe side effects on the body. It is necessary that new approaches such as to destroy at the level of individual cancer cells so that the spreading and progression of cancer cells will be reduced in the body. In newer therapies, some of the agents were tested include Denosumab (human monoclonal antibody against RANKL) (Roth et. al., 2014; Beristain et. al., 2012) and also, Oferibulin (a microtubule inhibitor) (Kolb et. al., 2013).

Recently, engineered nanoparticles become a significant tool in the treatment of cancer. They have the ability to either enhance the delivery of drugs, or uptake by target cells and/or reduce the toxic effect of the free drug to other organs. In particular, gold nanoparticles have attracted significant attention in targeted drug delivery (Zavaletaa et. al., 2009; Dreaden et. al., 2009; Giljohann et. al., 2009; Dhar et. al., 2009), diagnosis of disease (Wang et. al., 2005; G. V. Maltzahn et. al., 2009) and monitoring surgical procedures (Jokerst et. al., 2011; Qian et. al., 2008; Jung et. al., 2011) due to their unique combination of physical, chemical, electronic and optical properties. In the area of nanomedicine targeted drug delivery has been the
main goal to achieve and gold nanoparticles playing a very significant role to achieve that purpose as they can be easily synthesized, functionalized and they are also biocompatible. Gold nanoparticles one of the most studied preparations, due to their perceived bio-compatibility and tunable surface chemistry highlighted by the relative ease with which additional functional groups can be conjugated (Nicol et. al., 2015). Gold nanoparticles have a large surface to volume ratio, which offers an ample number of drug molecules being delivered by these nanoparticles. Gold nanoparticles owing to their large size, preferentially accumulate at the tumor sites and in inflamed tissues. It is due to the characteristically defective architecture of the blood vessels that provide nutrients and oxygen to these tissues (Yuan et. al., 1995). Nanotechnology offers novel ways to deliver anticancer drugs to individual cancer cells and also capable of the detection of cancer (Riehle et. al., 2011).

From past ten years, the increase of nanomaterials in targeted drug delivery to enhance the efficacy of treatment has been widely considered as a potential strategy. There has been various nanomaterial such as nanorods, liposomes, polymers, etc. used for this purpose. In recent years, for the treatment of osteosarcoma different nanomaterials conjugated with the chemotherapy drugs such as LDH conjugated with methotrexate (Oh et. al., 2011), polymerized liposome nanoparticles conjugated with doxorubicin and calcium phosphate and lipid-modified dextran nanoparticles conjugated with cisplatin and doxorubicin respectively (Barroug et. al., 2004; Susa et. al., 2009) have been used, which proved the increase in efficacy of the drugs. The nanoparticles, which have been used in targeted drug delivery systems are being synthesized by various chemical and physical routes. The biological methods utilized for the synthesis of nanoparticles
have various advantages such as they are cheap, simple and yield nanoparticles at physiological pH and room temperature. Nanoparticles synthesis by biological route have high stability, monodispersity and can be obtained in large quantities (Ahmad et. al., 2007; Shankar et. al., 2004; Bansal et. al., 2006).

In this study, we synthesized monodispersed gold nanoparticles (G-BNPs) using bromelain (a naturally occurring cysteine protease) which was used as reducing as well as a capping agent. The anticancerous drugs cisplatin, methotrexate, doxorubicin and in a combination of cisplatin+doxorubicin and cisplatin+doxorubicin+methotrexate were used to conjugate with gold nanoparticles to deliver it specifically to the site directed delivery with significant patient compliance. The characterization of G-BNPs and G-BNPs conjugated with cisplatin (G-B-CNPs), doxorubicin (G-B-DNPs), methotrexate (G-B-MNPs) and in a combination of cisplatin+doxorubicin (G-B-CDMNPs), cisplatin+ doxorubicin+ methotrexate (G-B-CDMNPs) were done by UV-VIS spectroscopy, TEM, FTIR and Zeta potential. Furthermore, the cytotoxic effect of G-BNPs and G-B-CNPs, G-B-DNPs, G-B-MNPs and in a combination of G-B-C-DNPs, G-B-C-D-MNPs were checked on osteosarcoma cell lines Saos-2 and MG-63 and also on primary osteoblast cells.
4.2 MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
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<tr>
<td>H[AuCl₄] (gold salt)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Bromelain</td>
<td>Merck</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Sigma-Aldrich</td>
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<td>Doxorubicin</td>
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<td>Cisplatin</td>
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<tr>
<td>Ethanol</td>
<td>Merck</td>
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<tr>
<td>Milli-Q water</td>
<td>Millipore unit</td>
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<td>Na₂HPO₄</td>
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4.2.1 In vitro Synthesis of G-BNPs by using bromelain as reducing and capping agent

In vitro synthesis of G-BNPs was done by taking reaction mixtures of 3ml, each containing 1mM H[AuCl₄] (prepared in 50 mM Phosphate buffer) and 1mg/ml freshly prepared bromelain. The reaction mixture was incubated at 40°C for 48hrs. A separate reaction was performed in the absence of bromelain was used as a control. The sample was removed at regular intervals and analyzed by UV-Vis spectroscopy to confirm the formation of nanoparticles. On completion of the reaction, the unbound bromelain was removed by treating with 50% v/v of ethanol, nanoparticles
were collected by centrifugation (30,000g, 30min.), washed twice with milli-Q water and used for further characterization.

4.2.2 Bioconjugation of G-BNPs with anticancerous drugs cisplatin, doxorubicin, methotrexate and in a combination of Cisplatin+Doxorubicin, Cisplatin+ Doxorubicin+ Methotrexate.

In vitro synthesized gold nanoparticles were bioconjugated to anticancerous drug cisplatin, doxorubicin, and methotrexate and also, in the mix with cisplatin+doxorubicin and cisplatin+ doxorubicin+ methotrexate. The free amino group of all the anticancerous drugs binds with free amino acid as well as carboxylate group present on bromelain by using the activator 1-Ethyl-3-(3-dimethyl) carbodiimide (EDC) (Timkovich, 1977; Hermanson, 1996). The 5ml reaction mixture containing 50mM HEPES buffer, 250 µg Cisplatin, doxorubicin, methotrexate when conjugated individually, 200 µg in combination of cisplatin +doxorubicin (100 µg cisplatin + 100 µg doxorubicin) and 240 µg in combination of cisplatin +doxorubicin + methotrexate (80 µg cisplatin+ 80 µg methotrexate + 80 µg doxorubicin) and 1mM of G-BNPs with 5mM EDC, in aliquots was used. The reaction was performed at 30°C for 3hours.

4.2.3 Determination of loading efficiency (LE) of cisplatin, methotrexate, doxorubicin and (cisplatin+doxorubicin) and (cisplatin+doxorubicin +methotrexate) on G-BNPs by UV-Vis spectrophotometric method

The percentage loading of cisplatin, methotrexate and doxorubicin were estimated by putting the values of A and B in Eq. (4.1). The standard curve for
cisplatin, methotrexate and doxorubicin were drawn by using absorbance at 300nm, 303nm, and 481nm respectively.

In the case of conjugation of G-BNPs with a mixture of drugs such as in G-B-C-DNPs and G-B-C-D-MNPs, the loading efficiency of each drug calculated one by one using Eq. (1), the absorbance of each drug determined at their respective OD, and finally added together to know the total percentage loading of drugs on G-BNPs.

\[
\% \text{Loading of drugs on G-BNPs} = \frac{A-B}{A} \times 100 \quad \text{Eq. (4.1).}
\]

Where A is the absorbance of total drug added into G-BNPs, B is the absorbance of the drug after conjugation in the supernatant of G-B-CNPs, G-B-MNPs, G-B-DNPs, G-B-C-DNPs and G-B-C-D-MNPs and both A and B were measured at the respective O.D. of each drug (Joshi et al., 2011).

The standard curve of cisplatin, methotrexate, and doxorubicin at absorbance 300nm, 303nm and 481nm was established respectively, and unbound drug was calculated from the standard curve. The amount of bioconjugated drugs was calculated by subtracting unbound drug from the total amount of drug added. The exact amount of bioconjugated drugs was calculated using equation Eq. (4.2). (Khan et al., 2015)

\[
\% \text{Bioconjugation} = \frac{\text{Amount of drug bioconjugated}}{\text{Total drug added}} \times 100 \quad \text{Eq. (4.2).}
\]
4.2.4 Characterization of *In vitro* synthesized G-BNPs and G-B-CNPs, G-B-MNPs, G-B-DNPs, G-B-C-DNPs and G-B-C-D-MNPs by various techniques

4.2.4.1 UV-Vis spectroscopy

UV-Vis spectrophotometry measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC), operated at a resolution of 1 nm.

4.2.4.2 Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) was done on Tecnai\textsuperscript{TM} G\textsuperscript{2} Spirit BioTWIN FEI Company operated at an accelerating voltage of 80kV. Samples were prepared by drying a drop of G-BNPs and conjugated G-B-CNPs, G-B-MNPs, G-B-DNPs, G-B-CDNPs, and G-B-CDMNP solution on carbon coated TEM copper grids followed by measurements on (TEM).

4.2.4.3 Dynamic Light Scattering (DLS)

The mean particle size of G-BNPs and conjugated G-B-CNPs, G-B-MNPs, G-B-DNPs, G-B-CDNPs, and G-B-CDMNP were measured by a dynamic light scattering (DLS) particle size analyzer (Zetasizer Nano-ZS, Model ZEN3600, Malvern Instrument Ltd, Malvern, UK). The samples were taken in a DTS0112-low volume disposable sizing cuvette of 1.5 ml. The sample powder was diluted to a concentration of 0.5% (w/v) in deionized water and sonicated for 1 min before measurement. Mean particle size was the average of triplicate measurements for a single sample.
4.2.4.4 Zeta potential

The surface charge of G-BNPs and conjugated G-B-CNPs, G-B-MNPs, G-B-DNPs, G-B-C-DNPs, and G-B-C-D-MNP were measured using a Zetasizer Nano-ZS, Model ZEN3600 (Malvern Instrument Ltd, Malvern, UK).

4.2.4.5 FTIR Spectroscopy

The confirmation of binding and secondary structure of bromelain at the surface of GNPs and subsequently, bioconjugated G-B-CNPs, G-B-MNPs, G-B-DNPs, G-B-C-DNPs, and G-B-C-D-MNP were done by Fourier transform infrared spectroscopy (FTIR). A bromelain-Au film on a Si(111) substrate was prepared by placing a drop of the G-BNPs/G-B-CNPs /G-B-MNPs/ G-B-DNPs/G-B-C-DNPs/G-B-C-D-MNP solutions on a Si(111) substrate and evaporation of water were performed by gentle heating and FTIR spectra of the film were recorded on a Shimadzu FTIR-8201 PC instrument operated in the diffuse reflectance mode at a resolution of 4 cm$^{-1}$. To obtain good signal-to-noise ratios, 256 scans of the bioconjugate film were taken in the range 400-4000 cm$^{-1}$.

4.2.4.6 Nuclear magnetic resonance (NMR)

All spectra were collected on a Bruker 800-MHz NMR spectrometer equipped with a triple-resonance TCI cryogenic probe. For 2D DOSY $^1$HNMR, stimulated echo bipolar gradient pulse experiments were used with a pulse delay of 5 ms after each gradient, a pulse field gradient length of 2.2 ms and with 15relaxation decay Chemical shift (d, ppm) and diffusion coefficient (m$^2$ s$^{-1}$) was plotted against log concentration (molar) (Wu et. al., 1995).
4.2.5 Cell culture

The human osteosarcoma cell lines Saos-2, and MG-63 were obtained from National Centre for Cell Science (NCCS), Pune, India and primary rat osteoblasts were isolated from neonatal rat calvaria by enzymatic digestion. Saos-2, MG-63 and primary osteoblast were grown as monolayer in MacCoy’S, EMEM and MEM media, respectively, supplemented with 10% fetal bovine serum and 1% antibiotic which contains 10,000 units of penicillin, 10mg streptomycin and 25µg amphotericin B in an incubator with a humidified atmosphere containing 5% CO₂ at 37°C. Stocks were maintained in 75 cm² tissue culture flask.

4.2.5.1 Cell viability assay

Saos-2 cells, MG-63, and primary osteoblast cells were plated in 96 well plate at a density of 1×10⁴ cells per well and incubated for 24 hrs in a humidified 5% CO₂ incubator at 37°C. After 24 hrs, the cells were treated with G-BNPs, G-B-CNPs, G-B-MNPs, pure cisplatin and methotrexate as positive control at (20, 10, 5, 2.5, 1.25 µg/ml), and G-B-DNPS, G-B-C-DNPs, G-B-C-D-MNPs and pure doxorubicin, pure cisplatin and pure methotrexate served as positive control at (1, 0.5, 0.25, 0.125, 0.0625 µg/ml) and with concentration in triplicates, and incubated for 48 hrs. After incubation, media was discarded and 50 µl MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide] (5mg/ml in PBS), was added to each well. The plate was incubated for 4 hrs in 5% CO₂ incubator. The resulting formazan crystals were solubilized in 150µl DMSO (Dimethyl sulfoxide). The quantification of reduced MTT was performed by measuring the optical densities at a wavelength of 570 nm with a reference filter of 655 nm using an ELISA reader [(Microplate
Reader (BIORAD-680)]. Percentage inhibition of the cells was calculated using the formula \( X = 100 - \left( \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \right) \times 100; \) where \( X \) is percentage inhibition, \( A_{\text{test}} \) is the absorbance of the test sample, \( A_{\text{blank}} \) is the absorbance of blank and \( A_{\text{control}} \) is the absorbance of the control sample. The IC\textsubscript{50} value was calculated by fitting the data using ORIGIN 6.1. (Mossman, 1983)

4.2.5.2 Measurement of cytomorphological changes in Saos-2, MG-63 and primary osteoblast

Saos-2, MG-63, and primary osteoblast cells were pre-treated with different concentration of G-BNPs and G-B-CNPs, G-B-MNPs, G-B-DNPs, G-B-CDNPs and G-B-CDMNP, also with pure cisplatin, doxorubicin and methotrexate incubated for 48 hrs at 37°C in 5% CO\textsubscript{2} atmosphere. After the incubation of cells, the gross morphological changes in the cells were observed under an inverted phase contrast microscope (Nikon ECLIPSE Ti-S, Nikon Corporation, Tokyo Japan).

4.3 RESULTS AND DISCUSSION

4.3.1 Synthesis of G-BNPs

When bromelain, a cysteine protease, was incubated with 1mM H[Aucl\textsubscript{4}], at temperature 40°C for 48 hrs, resulted in the synthesis of gold nanoparticles with monodispersity and high stability. The synthesis of G-BNPs was confirmed by a change in color from light yellow to characteristic ruby red color after incubation with bromelain. This change in color appears due to surface plasmon resonance of G-BNPs. Further, it was confirmed by UV-Visible spectroscopy, and surface plasmon resonance band appears at 527nm corresponds to the plasmon band of G-BNPs (Fig. 4.1A) (Mulvaney, 1996). The synthesized G-BNPs are stabilized by the
capping agent due to the presence of functional groups present on cysteine amino acids of the active site of bromelain which makes the nanoparticles stable by preventing them from aggregation. Most of the hydrophilic moieties but not all such as hydroxyl/carboxylate/amino group present on protein will be involved in the bonding with nanoparticles during the process of capping.

4.3.1.1 Characterization of G-BNPs

High-resolution images of G-BNPs were acquired using TEM and their average size were estimated to be ~16nm (Fig. 4.1C). Their shapes were found to be spherical and uniformly distributed under TEM. G-BNPs were found to be highly stable with a negative charge, and their zeta potential was found to be -10.1mV. (Fig. 4.1B) In DLS, the hydrodynamic radius of G-BNPs was estimated 72.99nm (Fig.4.1D).

Figure 4.1 Characterization of G-BNPs under (A) UV-Visible spectra (B) DLS (C) Zeta Potential (D) TEM
4.3.2 Bioconjugation of anticancerous cisplatin drug with G-BNPs (G-B-CNPs)

The synthesized G-BNPs was conjugated with anticancerous drug cisplatin using carboxylate group of bromelain exposed over the surface of NPs with the amino group of cisplatin drug. The surface plasmon resonance absorption spectra of cisplatin drug and G-B-CNPs are plotted in comparison to G-BNPs (Fig. 4.3A) and surface plasmon resonance absorption band of conjugated NPs was observed at 529nm with a decrease in intensity and a significant broadening compared to the absorption band for G-BNPs at 527nm. The broadening of plasmon band and a decrease in intensity is due to the attachment of the cisplatin drug to G-BNPs (Fig. 4.3A). The surface plasmon resonance phenomenon explains the significant change in absorption intensity and full-width half maximum of absorption band by the attachment of any ligand on the surface (Chakraborti et. al., 2012)

![Chemical Formula: Cl₂H₆N₂Pt](image)

Figure. 4.2 Structure of cisplatin
Figure 4.3 Schematic representation of bromelain assisted synthesis of gold nanoparticles conjugated with anticancerous drug cisplatin to combat Saos-2 and MG-63 bone cancer cells and no change in primary osteoblast.

4.3.2.1 Characterization of G-B-CNPs

High-resolution images of G-B-CNPs were acquired using TEM and their average sizes were estimated ~28 nm (Fig. 4.4D). Their shapes were found to be spherical and uniformly distributed under TEM. G-B-CNPs were found to be highly stable with a negative charge and their zeta potential was -8.97 mV. (Fig. 4.4C). DLS measured the hydrodynamic radius of G-B-CNPs and found to be 100.8 nm. (Fig. 4B).
Figure 4.4 Characterization of G-B-CNPs under (A) UV-Visible spectra (B) Size distribution (DLS) (C) Zeta potential (D) Transmission Electron Microscopy
4.3.2.2 FTIR Spectroscopy of G-BNPs and G-B-CNPs

The FTIR spectroscopy was used to detect the capping agent present at the surface of G-BNPs and their bioconjugation with cisplatin. The spectra showed the peaks at 3436.73 cm\(^{-1}\), 1641.35 cm\(^{-1}\) and 1452 cm\(^{-1}\) correspond to –OH stretching, amide-I, and amide–II respectively. (Fig. 4.5A) These functional groups confirm the presence of protein (Bromelain) on the surface of G-BNPs. Further, spectra showing peaks at 3500-3000 cm\(^{-1}\), 1647 cm\(^{-1}\), 1463.03 cm\(^{-1}\), 1045.5 cm\(^{-1}\) represent –NH stretch of the peptide bond, amide I, amide II and –CN for aliphatic amines respectively (Fig. 4.5B) These functional groups confirm the bioconjugation of cisplatin with bromelain present at the surface of G-BNPs.

4.3.2.3. NMR Spectroscopy

Further to characterize the conjugation of G-BCNPs, diffusion-ordered spectroscopy (DOSY) \(^1\)H NMR was done to determine the change in diffusion on formation G-BCNPs. As size increases the diffusion coefficient decreases due to a slower rate of diffusion of bigger particles, indicating aggregate formation (Soong et. al., 2008; Cohen et. al., 2005; Macchioni et. al., 2008). We observed a decrease in diffusion coefficient from 9.082 m\(^2\)s\(^{-1}\) to 8.980 m\(^2\)s\(^{-1}\) as drug conjugated to pure G-BNPs (Fig. 4.5 C). This confirms the change in the size of G-BNPs after attachment of drug molecule on the surface, as also shown by TEM.

4.3.2.4 Drug loading efficiency

The percentage loading of G-BNPs has been calculated by using Eq. (4.1). It was found to be ~77% indicating efficient binding of the drug to G-BNPs. The values of A and B were obtained as 0.070 and 0.016 respectively and substituted in
Eq. (4.1). The quantitative estimation of cisplatin conjugated G-BNPs was also determined by using UV-Vis spectroscopy (Fig. 4.5 D). The absorbance of the pure cisplatin drug was observed at 300nm at five different concentrations (50, 100, 150, 200, 250 µg/ml) and a standard curve was drawn. The amount of bioconjugated drugs was calculated by subtracting unbound drug from the total amount of drug added. The exact amount of drug conjugated to G-BNPs was found to be ~77% revealing efficient binding of cisplatin with G-BNPs (Fig. 4.5D)

Figure 4.5 (A) FTIR spectra of G-BNPs (B) FTIR spectra of Cisplatin conjugated G-B-CNPs (C) 2D DOSY spectrum showing diffusion coefficients of G-BNPs and G-B-CNPs (D) Quantitative estimation of cisplatin conjugated G-BNPs determined by UV-Vis spectroscopy
4.3.2.5 Anticancer studies of G-BNPs and Cisplatin conjugated G-B-CNPs (*In vitro*)

4.3.2.5.1 Cell viability

The G-B-CNPs showed a highly significant cytotoxic effect on Saos-2 and MG-63, which is higher than pure cisplatin drug. The percentage inhibition on Saos-2 and MG-63 was found to be increased in a dose-dependent manner. The IC$_{50}$ values of cisplatin conjugated G-BNPs (i.e. G-B-CNPs) on Saos-2 and MG-63 were found to be 4.51 µg/ml and 3.2 µg/ml respectively which were lower than IC$_{50}$ values of pure cisplatin on Saos-2 and MG-63, which were found to be 8.25 µg/ml and 5.42 µg/ml respectively. G-BNPs have also shown cytotoxic activity on Saos-2 and MG-63 but with IC$_{50}$ values 17.3 µg/ml and 15.67 µg/ml respectively which is much higher than allowed or permitted limit (Patra *et. al.*, 2008; Paciotti *et. al.*, 2004; Hassan, 1985). The cytotoxic effect of G-B-CNPs was also checked on primary osteoblast, and it was found to be insignificant even at a higher concentration up to 20 µg/ml. (Fig. 4.6 A, 4.6B, 4.6 C)

4.3.2.5.1 Measurement of cytomorphological changes on Saos-2, MG-63, and primary osteoblast

The morphological changes in Saos-2, MG-63 and primary osteoblast cells incubated with G-BNPs, G-B-CNPs, and cisplatin, were shown by phase contrast microscopy (Fig. 4.7). The figures of control cells and primary osteoblast indicatethat there were no distinct or remarkable changes in morphology of cells and cells were well spread after 48hrs of incubation (Fig. 4.7A, 4.7 E, 4.7I). However, significant changes were observed in cells treated with G-B-CNPs (Fig. 4.7C, 4.7G, 4.7J) and positive control cisplatin. (Fig. 4.7D, 4.7H). After 48 hrs of exposure, the
cells become irregular, shrink, necrotic and detached from the surface of wells. Also, some cells maintained an intact plasma membrane, showing that apoptosis had started. The pure G-BNPs also induces shrinkage and apoptosis of the cells, but at a much higher concentration as compared to G-B-CNPs (Fig. 4.7B, 4.7F).

Figure 4.6 The cytotoxicity study of G-BNPs, G-B-CNPs on (A) Saos-2 (B) MG-63 and (C) primary osteoblast cells. G-B-CNPs inhibited cell growth significantly of Saos-2 and MG-63 with IC$_{50}$ values 4.51µg/ml, 3.2µg/ml respectively and IC$_{50}$ values of pure cisplatin on Saos-2 and MG-63 were found to be 8.25 µg/ml, 5.42 µg/ml respectively but did not affect primary osteoblast cells significantly, while G-BNPs had also shown cytotoxic activity on Saos-2 and MG-63 but with IC$_{50}$ values of 17.3 µg/ml and 15.67 µg/ml respectively. All the data were expressed in mean ± SD of three experiments.
Figure 4.7. Images showing Saos-2 cells (A) Control (B) treated with G-BNPs (C) treated with G-B-CNPs (D) treated with pure cisplatin. Also, MG-63 cells (E) Control (F) treated with G-BNPs (G) treated with G-B-CNPs (H) treated with pure cisplatin, and also, Primary osteoblast cells (I) Control (J) treated with G-B-CNPs, under phase contrast microscope after 48hrs of treatment.

4.3.3 Bioconjugation of anticancerous methotrexate drug with G-BNPs (G-B-MNPs)

The surface plasmon absorption spectra of G-B-MNPs and pure methotrexate were plotted in comparison to G-BNPs (Fig. 4.9A). Methotrexate drug shows characteristic peaks at 243nm and 306nm when dissolved in 0.1N HCl. The surface plasmon band of G-B-MNPs appeared at 530nm with a decrease in intensity as compared to G-BNPs plasmon band at 527nm. The shift in maximum absorption, a
decrease in intensity and broadening of plasmon band could be explained by the conjugation of methotrexate on the surface of G-BNPs with protein molecules.

**Figure 4.8 Structure of methotrexate**

### 4.3.3.1 Characterization of G-B-MNPs with DLS, Zeta potential, and TEM

In DLS technique, the hydrodynamic diameter of NPs appears much larger than its size appears in TEM images. The reason behind this is in the fact that the TEM micrographs give the projected area diameter of particles while DLS estimates the hydrodynamic radius of the NPs. (Berne & Pecora, 2000). Eventually, DLS provides information about the coating material, inorganic core and solvent layer attached to the NPs while TEM only gives information about the inorganic core. Here, the hydrodynamic radius of G-B-MNPs was found to be ~100.6nm (Fig. 4.9 B), while size under TEM micrograph of G-B-MNPs (Fig.4.9 D) revealing the attachment of methotrexate drug to G-BMNPs as the particles size increased in comparison to G-BNPs was found to be ~24nm (Fig. 4.9 D) Zeta potential of G-B-MNPs (Fig. 4.9C) was found to be -22.5mV which is much higher than the zeta potential of G-BNPs.
(-10.1mV) (Fig. 4.1B), negative potential indicates stability of conjugated nanoparticles.

Figure 4.9 Characterization of G-B-MNPs under (A) UV-Visible spectra (B) Size distribution (DLS) (C) Zeta potential (D) Transmission Electron Microscopy.

4.3.3.2 FTIR Spectroscopy of G-BNPs and G-BMNPs

FTIR spectra used to confirm the chemical interactions between G-BNPs and methotrexate drug molecules. The spectra of G-B-MNPs exhibited absorption peaks at 3437cm^{-1}, 2986.38cm^{-1}, 1635 cm^{-1}, 1452 cm^{-1}, 1406 cm^{-1}, 1226.63 cm^{-1}, 1076.90 cm^{-1}, 1048 cm^{-1}, and 688 cm^{-1}. The peak located at 3437.59cm^{-1} can be attributed to
the -NH stretching vibration of secondary amides. The peak at 2986.38 cm\(^{-1}\) confirms the presence of -CH\(_3\) (alkanes) groups. The additional bands appear on the spectra of G-B-MNPs (Fig. 4.10 A) compared to G-BNPs (Fig. 4.5A) are 1226 cm\(^{-1}\) (C-O-C) stretching, 1406 cm\(^{-1}\) (C-C) stretching, 1452 cm\(^{-1}\) (amide II), and blue shift at 1635.06 cm\(^{-1}\) (amide I) due to conjugation of methotrexate to G-BNPs.

\subsection*{4.3.3.3 Drug loading efficiency}

The percentage loading of G-BMNPs has been calculated by using Eq. (4.1). It was found to be \(~81.5\%\) indicating efficient binding of the drug to G-BNPs. The values of A and B were obtained as 1.846 and 0.340 respectively and substituted in Eq. (4.1).

The quantitative estimation of methotrexate conjugated G-BNPs was also determined by other method using UV-Vis spectroscopy (Fig. 4.10B). The absorbance of the pure methotrexate drug was observed at 303 nm at five different concentrations (50, 100, 150, 200, 250 µg/ml) and a standard curve was drawn. The amount of bioconjugated drugs was calculated by subtracting unbound drug from the total amount of drug added. The exact amount of drug conjugated to G-BNPs was found to be \(~81\%\) revealing efficient binding of methotrexate with G-BNPs.
Figure 4.10 (A) FTIR spectra of methotrexate conjugated G-BNPs (B) Quantitative estimation of methotrexate conjugated G-BNPs determined by UV-Vis spectroscopy
4.3.3.4 Anticancer studies of G-BNPs and G-BMNPs (*In vitro*)

4.3.3.4.1 Cell viability

The G-B-MNPs showed a highly significant cytotoxic effect on Saos-2 and MG-63, which is higher than pure methotrexate drug. The percentage inhibition on Saos-2 and MG-63 was found to be increased in a dose-dependent manner. The IC$_{50}$ values of G-B-MNPs on Saos-2 and MG-63 were found to be 4.65 µg/ml and 1.98 µg/ml respectively which were lower than IC$_{50}$ values of pure methotrexate on Saos-2 and MG-63, which were found to be 10.47 µg/ml and 10.6 µg/ml respectively. G-BNPs have also shown cytotoxic activity on Saos-2 and MG-63 but with IC$_{50}$ values 16.12 µg/ml and 16.79 µg/ml respectively which is much higher than allowed or permitted limit (Danieland & Astruc, 2004; Dobrovolskaiaand & McNeil, 2007; Hainfeld *et. al.*, 2006). The cytotoxic effect of G-B-MNPs was also checked on primary osteoblast, and it was found to be insignificant even at a higher concentration up to 20 µg/ml. (Fig. 4.11 A, 4.11B, 4.11 C).

4.3.3.4.2 Measurement of cytomorphological changes on Saos-2, MG-63, and primary osteoblast

The morphological changes in Saos-2, MG-63 and primary osteoblast cells incubated with G-BNPs, G-B-MNPs, and pure methotrexate were shown by phase contrast microscopy (Fig. 4.12). The figures of control cells and primary osteoblast demonstratethat there were no distinct or remarkable changes in morphology of cells and cells were well spread after 48hrs of incubation (Fig. 4.12A, 4.12 E, 4.12I). However, significant changes were observed in cells treated with G-B-MNPs (Fig. 4.12C, 4.12G, 4.12 J) while positive control methotrexate also showed a majordifference in shapes in cells. (Fig. 4.12D, 4.12H). After 48 hrs. of exposure the
cells become irregular, shrink, necrotic and detached from the surface of wells. Also, some cells maintained an intact plasma membrane, showing that apoptosis had started. The pure G-BNPs also induces shrinkage and apoptosis of the cells, but at a much higher concentration as compared to G-B-MNPs (Fig. 4.12B, 4.12F).

Figure 4.11 The Cytotoxicity (dose dependent) study of G-BNPs, G-B-MNPs on (A) Saos-2 (B) MG-63 and (C) primary osteoblast cells. G-B-MNPs inhibited cell growth significantly of Saos-2 and MG-63 with IC50 values 4.65µg/ml, 1.98 µg/ml respectively and IC50 values of pure methotrexate on Saos-2 and MG-63 were found to be 10.47 µg/ml and 10.6 µg/ml respectively but did not affect primary osteoblast cells significantly, while G-BNPs had also shown cytotoxic activity on Saos-2 and MG-63 but with IC50 values of 16.12 µg/ml and 16.79 µg/ml respectively. All the data were expressed in mean ± SD of three experiment.
Figure 4.12. Images showing Saos-2 cells (A) Control (B) treated with G-BNPs (C) treated with G-B-MNPs (D) treated with pure methotrexate. Also, MG-63 cells (E) Control (F) treated with G-BNPs (G) treated with G-B-MNPs (H) treated with pure methotrexate, and also, Primary osteoblast cells (I) Control (J) treated with G-B-MNPs, under phase contrast microscope after 48hrs of treatment.

4.3.4 Bioconjugation of anticancerous doxorubicin drug with G-BNPs (G-B-DNPs)

G-B-DNPs synthesis has been confirmed by their representative surface plasmon absorption band measured by UV-Vis spectroscopy. The SPR absorption band of pure doxorubicin, doxorubicin conjugate G-BNPs (G-B-DNPs) and G-BNPs are plotted in Fig 14 A. Doxorubicin have a free amino group and hydroxyl group
which may get attached to the carboxylate group of bromelain exposed over the surface of NPs. (Fig.4.13). The SPR absorption band for pure doxorubicin appears at 481nm, which is its characteristic peak (Manocha and Margaritis, 2010). The SPR absorption band for G-B-DNPs appears at 530nm, (Fig.4.14A) with a decrease in intensity and significant broadening compared to the plasmon band for G-BNPs at 527nm.

![Figure 4.13 Structure of doxorubicin](image)

4.3.4.1 Characterization of G-B-DNPs with TEM, DLS, and Zeta potential

The hydrodynamic diameter of G-B-DNPs was found to be 94.82nm in DLS (Fig 4.14B). This large diameter is due to the binding of solvent to the surface of G-B-DNPs. The average size of G-B-DNPs was estimated 25nm to 30nm (Fig. 4.14D) by high-resolution images of G-B-DNPs using TEM. The particles were found uniformly distributed and spherical in shape under TEM. The stability of G-B-DNPs was confirmed by zeta potential and found to be -19.5mV. (Fig. 4.14 C).
4.3.4.2 FTIR Spectroscopy of G-BNPs and G-BDNPs

FTIR spectra were used to investigate the intermolecular interaction between doxorubicin and G-BNPs. The FTIR spectra of pure doxorubicin show peak located at 3459 cm\(^{-1}\) due to (-NH stretching) vibrations for primary amine structures. Pure doxorubicin also shows peak 1636 cm\(^{-1}\) which is due to (N-H) (Kayal et. al., 2010). The spectra of G-B-DNPs illustrates the band at 1752 cm\(^{-1}\) and 1565 cm\(^{-1}\) can be
attributed to the -C=O anhydride stretching vibrations (Fig. 4.15 A). The broad band at 3510 cm\(^{-1}\) can be due to N-H and C-H stretching of doxorubicin and nanoparticles. The shifting of the peak for N-H and C-H as compared to G-BNPs spectra indicates the intermolecular interaction between doxorubicin and G-BNPs (Fig 4.5A). The additional peaks appear in the spectra of G-B-DNPs are 3082 cm\(^{-1}\) (aliphatic C-H) stretching, 1289 cm\(^{-1}\) (C-O-C) stretching, 1416 cm\(^{-1}\) (C-C) stretching, 1585 cm\(^{-1}\) (-CONH) bending, 988 cm\(^{-1}\) (C-O) stretching due to conjugation doxorubicin to G-BNPs (Kayal et. al., 2010).

4.3.4.3 Drug loading efficiency

The percentage loading of G-BDNPs has also been calculated by using Eq. (4.1). It was found to be ~72.9% indicating efficient binding of the drug to G-BNPs. The values of A and B were obtained as 0.037 and 0.007 respectively and substituted in Eq. (4.1).

The quantitative estimation of cisplatin conjugated G-BNPs was also determined by other method using UV-Vis spectroscopy (Fig. 4.15 B). The absorbance of the pure cisplatin drug was observed at 481nm at five different concentrations (50, 100, 150, 200, 250 µg/ml) and a standard curve was drawn. The amount of bioconjugated drugs was calculated by subtracting unbound drug from the total amount of drug added. The exact amount of drug conjugated to G-BNPs was found to be ~73% revealing efficient binding of doxorubicin with G-BNPs.
Figure 4.15 (A) FTIR spectra of G-B- DNPs (B) Quantitative estimation of doxorubicin conjugated G-BNPs determined by UV-Vis spectroscopy.
4.3.4.4 Anticancer studies of G-BNPs and G-BDNPs (*In vitro*)

4.3.4.4.1 Cell viability

Cytotoxic studies of G-BNPs, G-B-DNPs, and pure doxorubicin were evaluated on MG-63 and Saos-2 osteosarcoma cells. The G-B-DNPs effect was also observed on primary osteoblast cells using an MTT assay (Fig. 4.16). The analyzed results revealed good effects with IC$_{50}$ values of G-B-DNPs lower than pure doxorubicin and found to be 0.088 µg/ml and 0.125 µg/ml on MG-63 and Saos-2 cells, respectively. G-B-DNPs showed no significant toxicity on primary osteoblast cells. The IC$_{50}$ values of pure doxorubicin on MG-63 and Saos-2 were found to be 0.131µg/ml and 0.143µg/ml respectively. G-BNPs treated MG-63 and Saos-2 cells showed no significant toxicity. The increased cytotoxicity of G-B-DNPs may be due to efficient transport of doxorubicin by G-BNPs through an endocytosis mechanism compared to the pure doxorubicin passive diffusion mechanism into osteosarcoma cells (Yoo et. al., 2000).

4.3.4.4.2 Cytomorphological changes in G-B-DNPs and G-BNPs treated MG-63, Saos-2, and primary osteoblast cells

Saos-2 and MG-63 cells were treated with G-BDNP, G-BNPs and pure doxorubicin for a period of 48 hrs, and cytomorphological observations (Fig 4.17) revealed that after 48 hrs of treatment most of the cells treated with G-B-DNPs and pure doxorubicin changed their normal shape, and many cells were suspended in culture media, while others have shown blabbing characteristic which was visible. The number of control cells was much higher than the treated cells. G-BNPs showed no significant changes in cell shape on MG-63 and Saos-2 cells. G-B-DNPs also showed no significant effect on primary osteoblast morphology.
Figure 4.16 The Cytotoxicity (dose dependent) study of G-BNPs, G-B-DNPs on (A) Saos-2 (B) MG-63 and (C) primary osteoblast cells. G-B-DNPs inhibited cell growth significantly of Saos-2 and MG-63 with IC$_{50}$ values 0.125µg/ml, 0.088µg/ml respectively and IC$_{50}$ values of pure doxorubicin on Saos-2 and MG-63 were found to be 0.143 µg/ml and 0.131µg/ml respectively but did not affect primary osteoblast cells significantly, while G-BNPs had also shown no cytotoxic activity on Saos-2 and MG-63. All the data were expressed in mean ± SD of three experiment.
Figure 4.17 Images showing Saos-2 cells (A) Control (B) treated with G-BNPs (C) treated with G-B-DNPs (D) treated with pure doxorubicin. Also, MG-63 cells (E) Control (F) treated with G-BNPs (G) treated with G-B-DNPs (H) treated with pure doxorubicin, and also, Primary osteoblast cells (I) Control (J) treated with G-B-DNPs, under phase contrast microscope after 48hrs of treatment.
4.3.5 Bioconjugation of anticancerous drug cisplatin + doxorubicin drug with G-BNPs (G-B-C-DNPs)

The spectral studies of G-BNPs showed an intense peak at 527nm (fig. 4.18A). The SPR absorption band of G-B-C-DNPs and G-BNPs were plotted (Fig.4.18A). G-B-C-DNPs have shown a decrease in intensity of peak with a noticeable red shift from 527nm to 531nm (Fig. 5.18A) in comparison with G-BNPs. The red or blue shift in SPR showing a change in the surface chemistry of G-BNPs due to conjugation of drugs (cisplatin + doxorubicin) on their surface.

4.3.5.1 Characterization of G-B-C-DNPs with TEM, DLS, and Zeta potential

TEM micrographs (Fig. 4.18D) confirmed the increase in size in comparison to G-BNPs and found to be ~ 31nm. TEM images confirm the conjugated nanoparticles were spherical in shape and monodispersed and uniformly distributed. Further, DLS was conducted to estimate the hydrodynamic radius of G-B-C-DNPs and was found to be 99.45nm (Fig. 4.18 B) The zeta potential of G-B-C-DNPs was found to be -24.3 (Fig. 4.18C). The negative charge on nanoparticles may be due to the functional groups present in the amino acids of bromelain, which confirms the high stability of G-B-C-DNPs.
4.3.5.2 FTIR Spectroscopy

FTIR spectroscopy was employed to evaluate the presence of cisplatin and doxorubicin on the surface of G-BNPs. The spectra obtained from G-B-C-DNPs was compared to spectra of G-BNPs and pure cisplatin and doxorubicin. The broad band contour which appears in the range 3600-3000 cm\(^{-1}\) due to the \(-\text{NH}\) stretch of a peptide bond. The spectra also showed a peak shift of carboxylic stretch (C=O) from 1641 to 1614 cm\(^{-1}\) suggested the intermolecular interactions between drugs and
G-BNPs (Fig. 4.19 A and Fig. 4.5 A) The characteristic peak of cisplatin from 1600-1500 cm\(^{-1}\) (asymmetric amine bending) and 1300-1200 cm\(^{-1}\) (symmetric amine bending) were also observed in the spectra (Kumar & Jaikumar, 2011). The emergence of new peaks at 1285 cm\(^{-1}\) (C-O-C stretching), 1407 cm\(^{-1}\) (C-C stretching), 1442 cm\(^{-1}\) (coupling of C-N and N-H), 982 cm\(^{-1}\) (C-O stretching) can be attributed to conjugation of doxorubicin (Kayal et al., 2010) and cisplatin conjugation to G-BNPs.

### 4.3.5.3 Drug loading efficiency

The percentage loading of cisplatin on G-B-C-DNPs was calculated by using Eq. (4.1). It was found to be \(\sim47.5\%\) indicating efficient binding of the drug to G-BNPs. The values of A and B were obtained as 0.040 and 0.021 respectively and substituted in Eq. (4.1).

The percentage loading of doxorubicin on G-B-C-DNPs was calculated by using Eq. (4.1). Percentage loading found to be \(\sim34.4\%\) indicating efficient binding of the drug to G-BNPs. The values of A and B were obtained as 0.090 and 0.050 respectively and substituted in Eq. (4.1). Therefore, a total bioconjugated drug to G-BNPs was found to be \(\sim81.9\%\) by adding the percentage loading of cisplatin and doxorubicin.

The standard curve of pure cisplatin and doxorubicin drug was established at 300 nm and 481 nm respectively, and unbound drug was measured from the standard curve. The amount of bioconjugated drugs was calculated by subtracting unbound drug from the total amount of drug added. The exact amount of bioconjugated drugs was calculated using Eq. (4.2) and was found to be 81\%. (Fig. 4.19B)
Figure 4.19 (A) FTIR spectra of G-B-C-DNPs (B) Quantitative estimation of cisplatin and doxorubicin conjugated G-BNPs determined by UV-Vis spectroscopy.
4.3.5.3 Anticancer studies of G-BNPs and G-B-C-DNPs (*In vitro*)

4.3.5.3.1 Cell viability

Cytotoxic studies of G-BNPs, G-B-C-DNPs, and pure doxorubicin, pure cisplatin were evaluated on MG-63 and Saos-2 osteosarcoma cells. The G-B-C-DNPs effect was also observed on primary osteoblast cells using an MTT assay at different concentrations (1, 0.5, 0.25, 0.125, 0.0625 µg/ml) (Fig. 4.20). The analyzed results revealed good effects with IC₅₀ values of G-B-C-DNPs over pure doxorubicin and pure cisplatin which were found to be 0.071 µg/ml and 0.09 µg/ml on MG-63 and Saos-2 cells, respectively (Fig. 4.20 A, B). G-B-C-DNPs showed no significant toxicity on primary osteoblast cells (Fig. 4.20 C). The IC₅₀ values of pure doxorubicin on MG-63 and Saos-2 were found to be 0.144µg/ml and 0.177µg/ml respectively (Fig 4.20 A, B). G-BNPs and pure cisplatin treated MG-63 and Saos-2 cells showed no significant toxicity at low concentrations. The cytotoxic effect of G-B-C-DNPs on cell lines was dose dependent.

4.3.5.3.2 Cytomorphological changes in G-B-C-DNPs and G-BNPs treated MG-63, Saos-2, and primary osteoblast cells

Saos-2 and MG-63 cells were treated with G-BC-DNP, G-BNPs, pure doxorubicin and cisplatin for 48 hrs. and their cytomorphological observations (Fig 4.21) revealed that after 48 hrs. of treatment most of the cells treated with G-B-C-DNPs (Fig. 4.21 B, G and pure doxorubicin (fig. 4.21 C, H) changed their normal shape, and many cells detached from substratum came into suspended culture media, while others have shown blabbing characteristic which was visible. The number of control cells was much higher than the treated cells (Fig. 4.21 A, F, K). G-BNPs and pure cisplatin showed no significant change in cell shape on MG-63 and Saos-2 cells
G-B-C-DNPs also showed no significant effect on primary osteoblast morphology (Fig. 4.21 L).

Figure 4.20 The Cytotoxicity (dose dependent) study of G-BNPs, G-B-C-DNPs on (A) Saos-2 (B) MG-63 and (C) primary osteoblast cells. G-B-C-DNPs inhibited cell growth significantly of Saos-2 and MG-63 with IC$_{50}$ values 0.09µg/ml, 0.07µg/ml respectively and IC$_{50}$ values of pure doxorubicin on Saos-2 and MG-63 were found to be 0.177 µg/ml and 0.144µg/ml respectively but did not affect primary osteoblast cells significantly, while G-BNPs and pure cisplatin had also shown no cytotoxic activity on Saos-2 and MG-63. All the data were expressed in mean ± SD of three experiment.
Figure 4.21 Images showing MG-63 cells (A) Control (B) treated with G-B-C-DNPs (C) treated with pure doxorubicin, (D) treated with G-BNPs (E) treated with pure cisplatin also, Saos-2 cells (F) Control (G) treated with G-B-C-DNPs (H) treated with pure doxorubicin (H), treated with G-BNPs (I) treated with pure cisplatin and also, Primary osteoblast cells (K) Control, (L) treated with G-B-C-DNPs, under phase contrast microscope after 48hrs of treatment.
4.3.6 Bioconjugation of anticancerous drug cisplatin + doxorubicin + methotrexate drug with G-BNPs (G-B-C-D-MNPs)

The plasmon absorbance band peak of G-BNPs shows a red shift and peak shifted from 527nm to 530nm after the conjugation of a mixture of drugs i.e. cisplatin, doxorubicin, and methotrexate (Fig. 4.22A). This red shift in plasmon absorbance is attributed to the presence of drugs on the surface of G-BNPs.

4.3.6.1 Characterization of G-B-C-D-MNPs with TEM, DLS, and Zeta potential

DLS measured the number and size distribution of G-B-C-D-MNPs particles. The size of G-B-C-D-MNPs was in the range of 20–32 nm estimated from TEM images. (Fig. 4.22B, 4.22D), whereas the hydrodynamic size of the G-BNPs is 72.99 nm, and the size increased to 100.1 nm after bioconjugation in G-B-C-D-MNPs. The noticeable change in hydrodynamic size and the reason could be the presence of peptide molecules capped on the surface of the G–BNPs. The DLS measurements (Fig. 4.22 D) indicate a monodisperse G-B-C-D-MNPs in the aqueous solvent (Aryal et. al., 2006). Similarly, the zeta potential of the G-B-C-D-MNPs (−16.97) is larger than that of G-BNPs alone (−10.1 mV), indicating a greater stability of these nanoparticles than the G-B-C-D-MNPs.
4.3.6.2 FTIR Spectroscopy

The spectra of G-B-C-D-MNPs showing peaks at 3438 cm\(^{-1}\), 1634 cm\(^{-1}\), 1462 cm\(^{-1}\), 1045 cm\(^{-1}\), 988 cm\(^{-1}\) confirming –NH stretch of the peptide bond, amide I and amide, –CN for aliphatic amines and ¬C-O stretching respectively (Fig 4.23 A). These functional groups appear in the FTIR spectra confirms the conjugation of cisplatin/doxorubicin/methotrexate with a bromelain present at the surface of the G-BNPs.
4.3.6.3 Drug loading efficiency

The percentage loading of cisplatin on G-B-C-D-MNPs was calculated by using Eq. (1). The calculated percentage loading was found to be ~30% indicating efficient binding of the drug to G-BNPs. The values of A and B were obtained as 0.020 and 0.014 respectively and substituted in Eq. (4.1).

The percentage loading of doxorubicin on G-B-C-D-MNPs has been calculated by using Eq. (4.1). The calculated percentage loading was found to be ~24.5% indicating efficient binding of the drug to G-BNPs. The values of A and B were obtained as 0.011 and 0.008 respectively and substituted in Eq. (4.1).

The percentage loading of methotrexate on G-B-C-D-MNPs has been calculated by using Eq. (4.1) moreover, found to be ~25 % indicating efficient binding of the drug to G-BNPs. The values of A and B were obtained as 0.704 and 0.528 respectively and substituted in Eq. (4.1). Therefore, a total bioconjugated drug to G-BNPs was found to be 79.5% by adding the percentage loading of cisplatin and doxorubicin.

The standard curve of pure cisplatin, methotrexate and doxorubicin drug was established at 300 nm, 303nm and 481nm respectively, and unbound drug was calculated from the standard curve. The amount of bioconjugated anticancerous drug was calculated by subtracting unbound drug from the total amount of drug added. The exact amount of conjugated drug was calculated usingEq. (4.2) and was found to be ~80% (Fig. 4.23 B).
Figure 4.23 (A) FTIR spectra of G-B-C-D-MNPs (B) UV-Vis spectra of pure cisplatin, doxorubicin and methotrexate drugs
4.3.6.4 Anticancer studies of G-BNPs and G-B-C-D-MNPs (*In vitro*)

4.3.6.4.1 Cell viability

Cytotoxic studies of G-BNPs, G-B-C-D-MNPs, pure doxorubicin, pure cisplatin and pure methotrexate were evaluated on MG-63 and Saos-2 osteosarcoma cells. The G-B-C-D-MNPs effect was also observed on primary osteoblast cells using an MTT assay (Fig. 4.24). The analyzed results revealed enhanced effects of G-B-C-D-MNPs with improved IC$_{50}$, which were found lower than pure cisplatin, doxorubicin, and methotrexate and found to be 0.179 µg/ml and 0.134 µg/ml on MG-63 and Saos-2 cells, respectively. G-B-C-D-MNPs showed no significant toxicity on primary osteoblast cells. The IC$_{50}$ values of pure doxorubicin on MG-63 and Saos-2 were found to be 0.210µg/ml and 0.189µg/ml respectively. G-BNPs, pure cisplatin, and methotrexate treated MG-63 and Saos-2 cells showed no significant toxicity.

4.3.6.4.2 Cytomorphological changes in G-B-C-D-MNPs and G-BNPs treated MG-63, Saos-2, and primary osteoblast cells

Saos-2 and MG-63 cells were treated with G-B-C-D-MNPs, G-BNPs, pure cisplatin, pure methotrexate and pure doxorubicin for a period of 48 hrs, and their cytomorphological observations (Fig 4.25) revealed that after 48 hrs of treatment, most of the cells treated with G-B-C-D-MNPs and pure doxorubicin changed their normal morphology and could not attach to the substratum and hence, floated with suspended media, while others have shown blabbing characteristic which was clearly visible. The number of control cells was much higher than the treated cells. G-BNPs, pure cisplatin, and methotrexates showed no significant change in cell shape on MG-63 and Saos-2 cells. G-B-C-D-MNPs also showed no significant effect on primary
osteoblast morphology pure cisplatin had also shown no cytotoxic activity on Saos-2 and MG-63. All the data were expressed in mean ± SD of three experiments. The inhibitory concentrations of conjugated G-BNPs are summarized in table 4.1.

Figure 4.24 The Cytotoxicity (dose dependent) study of G-BNPs, G-B-C-D-MNPs on (A) Saos-2 (B) MG-63 and (C) primary osteoblast cells. G-B-CDMNPs inhibited cell growth significantly of Saos-2 and MG-63 with IC₅₀ values 0.134µg/ml, 0.179µg/ml, respectively and IC₅₀ values of pure doxorubicin on Saos-2 and MG-63 were found to be 0.189 µg/ml and 0.210µg/ml, respectively
Figure 4.25 Images showing MG-63 cells (A) Control (B) treated with G-B-C-D-MNPs (C) treated with pure doxorubicin, (D) treated with pure methotrexate (E) treated with pure G-BNPs (F) treated with pure cisplatin also, Saos-2 cells (G) Control (H) treated with G-B-C-D-MNPs (I) treated with pure doxorubicin (J), treated with pure methotrexate (K) treated with G-BNPs (L) treated with pure cisplatin also, primary osteoblast cells (M Control, (N) treated with G-B-C-D-MNPs, under phase contrast microscope after 48hrs of treatment.
Table 4.1 Inhibitory concentration values of G-BNPs, G-BNPs conjugated anticancerous drugs as compared to pure drugs. (N.S- Non Significant; N.T- Non Tested)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Nanoparticles/ Pure Drugs</th>
<th>MG-63 (IC\textsubscript{50} - µg/ml)</th>
<th>Saos-2 (IC\textsubscript{50} - µg/ml)</th>
<th>Primary osteoblast (IC\textsubscript{50} - µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>G-B-CNPs</td>
<td>3.2</td>
<td>4.51</td>
<td>N.S</td>
</tr>
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<td></td>
<td>G-BNPs</td>
<td>15.67</td>
<td>17.3</td>
<td>N.T</td>
</tr>
<tr>
<td></td>
<td>Pure cisplatin</td>
<td>5.42</td>
<td>8.25</td>
<td>N.T</td>
</tr>
<tr>
<td>2.</td>
<td>G-B-MNPs</td>
<td>1.98</td>
<td>4.65</td>
<td>N.S</td>
</tr>
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<td></td>
<td>G-BNPs</td>
<td>16.79</td>
<td>16.12</td>
<td>N.T</td>
</tr>
<tr>
<td></td>
<td>Pure methotrexate</td>
<td>10.6</td>
<td>10.47</td>
<td>N.T</td>
</tr>
<tr>
<td>3.</td>
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<td>0.125</td>
<td>N.S</td>
</tr>
<tr>
<td></td>
<td>G-BNPs</td>
<td>N.S</td>
<td>N.S</td>
<td>N.T</td>
</tr>
<tr>
<td></td>
<td>Pure doxorubicin</td>
<td>0.131</td>
<td>0.143</td>
<td>N.T</td>
</tr>
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<td>4.</td>
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<td>0.09</td>
<td>N.S</td>
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<td></td>
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<td>N.S</td>
<td>N.S</td>
<td>N.T</td>
</tr>
<tr>
<td></td>
<td>Pure cisplatin</td>
<td>N.S</td>
<td>N.S</td>
<td>N.T</td>
</tr>
<tr>
<td></td>
<td>Pure doxorubicin</td>
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<td>0.177</td>
<td>N.T</td>
</tr>
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<td>5.</td>
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<td>0.134</td>
<td>N.S</td>
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<tr>
<td></td>
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<td>N.S</td>
<td>N.S</td>
<td>N.T</td>
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<tr>
<td></td>
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<td>N.S</td>
<td>N.S</td>
<td>N.T</td>
</tr>
<tr>
<td></td>
<td>Pure doxorubicin</td>
<td>0.210</td>
<td>0.189</td>
<td>N.T</td>
</tr>
<tr>
<td></td>
<td>Pure methotrexate</td>
<td>N.S</td>
<td>N.S</td>
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</tbody>
</table>
Table 4.2. Summarized characterization of G-BNPs and bioconjugated GBNPs.

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Zeta potential (mV)</th>
<th>DLS (nm)</th>
<th>TEM (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-BNPs</td>
<td>-10.1</td>
<td>72.99</td>
<td>16</td>
</tr>
<tr>
<td>G-B-CNPs</td>
<td>-8.97</td>
<td>106.8</td>
<td>28</td>
</tr>
<tr>
<td>G-B-MNPs</td>
<td>-22.5</td>
<td>100.6</td>
<td>24</td>
</tr>
<tr>
<td>G-B-DNPs</td>
<td>-19.5</td>
<td>94.82</td>
<td>30</td>
</tr>
<tr>
<td>G-B-C-DNPs</td>
<td>-24.3</td>
<td>99.45</td>
<td>31</td>
</tr>
<tr>
<td>G-B-C-D-MNPs</td>
<td>-16.9</td>
<td>100.1</td>
<td>30</td>
</tr>
</tbody>
</table>

4.4 DISCUSSION

In this study, a novel method is used to synthesize enzyme (Bromelain-Cysteine Protease) mediated nanoparticles for targeted drug delivery for cisplatin, doxorubicin, and methotrexate. These anticancerous drugs have been widely used as powerful therapeutic agents against numerous solid tumors including bone and lung tumors because it is strongly potent but highly toxic (Ski et al., 2007). However, therapeutic applications of these drugs have been restricted due to its nonspecific toxicity and severe side effects such as acute nephrotoxicity and chronic neurotoxicity (Cha et al., 2006). They are chemically unstable, sparingly soluble in water, having low lipophilicity and targets healthy cells also, with tumor (Mantri et al., 2007).

Furthermore, single drug based chemotherapy strategy remains unsatisfactory due to drug resistance mechanism along with the complex microenvironment of
cancerous cells (Woodcock et al., 2011). Therefore, combinatorial therapy has been considered as a promising treatment method to minimize the side-effects and improve the therapeutic efficiency of drugs (Sun et al., 2011). In combination therapy, anticancerous drugs believed to act synergistically towards suppression of cancer cells. The adaptation of cancer cells can be delayed by administration of chemotherapeutic drugs with different molecular pathways and further reduce the mutations in cancer cells. Anticancerous drugs with similar molecular pathways could act synergistically towards higher target selectivity and better therapeutic efficiency (Mauceri et al., 1998; Lane et al., 2006). The administration of anticancerous drugs in combination did not result in improved therapeutic efficacy, despite the significance of combination therapy (Hu et al., 2010). The diversity in biodistribution, pharmacokinetics and membrane transport mechanism of two anticancer drugs would result in extreme difficulty in dosing and poor accumulation in cancerous cells (Lehar et al., 2009). Therefore, it is necessary to entrap/conjugate multiple anticancerous drugs to a single nanocarrier so that it can help in the release of anticancerous drug molecules in a predetermined manner.

Further, tumor cells may develop intrinsic resistance to these drugs due to heavy doses. To increase the therapeutic efficacy of these drugs and to alleviate the limitations, cisplatin, methotrexate and doxorubicin were bioconjugated with bromelain encapsulated gold nanoparticles, a cysteine protease, because targeted drug delivery has the advantage of low toxicity, few side effects, and enhanced therapeutic efficacy. Nanoparticles with a hydrophilic surface can evade the recognition by the reticuloendothelial system (RES) because surface functionalization with bromelain makes G-BNPs hydrophilic in nature due to the
presence of $\text{–NH}_2$/-COOH/-OH functional groups in the amino acids of bromelain. Certain functional groups (especially with partial negative charge) interact with the surface of GNPs while other protrude outside towards the solvent due to their charges. Also, NPs due to their small sizes (< 200nm) can abscend from RES and circulate in the bloodstream for a prolonged period than conventional drugs and hence, can accumulate in solid tumors by enhanced permeation and retention EPR effect (Jellinek., 1995). The bioconjugation will also block the functional groups responsible for non-specific interaction because these group will be involved in the covalent bonding for bioconjugation between drug and NPs.

Bromelain can be absorbed in human intestines without degradation and without losing its biological activity, and it is well tolerated in high doses (10g/kg) for prolonged periods of therapy (Taussig et. al., 1975). The anticancerous activity of bromelain is attributed predominantly to its protease component. Bromelain is well known to increase neutrophil activity, downregulate NF-$\kappa$B, Cox-2, PGE-2 (Kalra et. al., 2008; Huang et. al., 2008; Hou et. al., 2006) and TGF-$\beta$ (Massague, 2008); upregulate p53 and Bax (Kalra et. al., 2008); decrease the activity of cell survival regulators such as Akt, ErK and deactivate Akt- dependent Pro-apoptotic regulator FOXO3A (Juhasz et. al., 2008) It also reduces expression of CD44 on the surface of tumor cells (Hale et. al., 2002). Due to bioconjugation between $\text{–COOH}$ group of bromelain and $\text{–NH}_2$ group of cisplatin, doxorubicin, methotrexate few combos could be produced having cisplatin+doxorubicin and cisplatin+ doxorubicin+ methotrexate. The bioconjugations were performed by a coupling agent EDC and their confirmation was done by UV and TEM. There will be either red shift or blue shift in the UV- Visible spectra for conjugated NPs with a decrease
in intensity (Fig. 4.4A, 4.9A, 4.14A, 4.18A, 4.22A) and TEM shows the increase in size with diminished sharpness in the picture (Fig. 4.4D, 4.9D, 4.14D, 4.18D, 4.22D) which is another proof of bioconjugations (Khan et. al., 2015). G-B-CNPs, G-B-MNPs, G-B-DNPs, G-B-CDNPs and G-B-CDMNPs combos showed profoundly enhanced activity against MG-63 and Saos-2 osteosarcoma cell lines in comparison to pure cisplatin, doxorubicin, and methotrexate drugs alone and showed the minimal cytotoxic effect on primary osteoblast cells. It has been reported that colloidal gold nanoparticles within the size range of 3-100 nm do not show significant toxicity, provided that the threshold not exceed a value of the order of $10^{12}$ particles/ml. The most efficient cellular uptake of GNPs was observed with particles ranging from 20nm to 50nm. These bioconjugates were found to be highly effective because they lie in the best range by size, and they will reduce the dose by half where side effects will also be reduced and hence, patient compliance.

The well-known mode of action of cisplatin as anticancer agent is the formation of covalent bonds between DNA purine bases (N7 position) and platinum atom of cisplatin, which forms Intra and inter strands crosslinks and eventually trigger several signal transduction pathways which includes p53, p73, MAPK (Mitogen-activated Protein Kinases) and ATR (Ataxia telangiectasia and Rad3-related protein), finally lead to activation of apoptosis (Wang & Lippard, 2005; Siddik, 2003; Callejo et. al., 2015).

Methotrexate mechanism of action as anticancer drug involves inhibition of Dihydrofolate reductase (DHFR) enzyme. Methotrexate enters inside the cells through facilitated diffusion and an active transport mechanism, and get converted by an enzyme folpoly glutamyl synthase into poly glutamyl synthase into
polyglutamate methotrexate (Cutolo et al., 2001; Tian & Cronsten, 2007; Fotoohi & Albertoni, 2008). DHFR and various other enzymes such as thymidylate synthase (TS) and phosphoribosyl aminoimidazole carboxamide formyl transferase (AICAR) get reversibly inhibited by polyglutamate methotrexate. DHFR inhibition affects the folic acid reduction and culminates in the lack of 5, 10 methylenetetrahydrofolate (coenzyme in thymidine biosynthesis). The inhibition of purine synthesis and dTMP induces cell death induced by methotrexate (McGuire, 2003). Recently, various non-DHFR mediated methotrexate effects have been discovered which can be used as new strategies in the treatment of osteosarcoma (Martin et al., 2016).

The mode of action of doxorubicin as an anticancer agent is not yet fully understood, although it is a widely used anticancer drug. Doxorubicin is known as topoisomerase II α poison (DeVita et al., 2001; Burden & Osheroff, 1998). The unwinding of DNA for replication and transcription catalyzed by topoisomerase family enzymes, which involves cleavage process of one strand of double-stranded DNA. The intermediate forms in this cleavage process known as "cleavable complex." Doxorubicin basically poisons the formation of a cleavable complex which results in a DNA double strand break. (Tewey et al., 1984; Liu et al., 1983). The failure in the repair of DNA double-strand break induces apoptosis. There are various other mechanisms have been studied which are involved in action of doxorubicin as anticancer agent (Gewirtz, 1999; Minotti et al., 2004) such as generation of free radicals (Feinstein et al., 1993), inhibition of RNA and DNA synthesis (Munger et al., 1988; Di Marco et al., 1965) and, synthesis of formaldehyde-mediated doxorubicin-DNA adducts (Cutts et al., 2005; Cutts et al., 2005)
Additionally, cisplatin, doxorubicin, methotrexate, and bromelain will work synergistically as bromelain stimulates immune cytotoxicity of cancer patient-derived immune cells and initiates an intracellular cascade that negatively regulates inflammation-induced NF-κβ activation and their target gene whereas these drugs will bind to the nucleic acid of target cells. It is assumed that bromelain will reduce the rate of formation of cancer cells while drugs will kill cancer cells.

4.5 CONCLUSION

We have synthesized gold nanoparticles (G-BNPs) by using a very simple approach utilizing bromelain (cysteine-protease), as both the capping and reducing agents. These G-BNPs further conjugated with anticancerous drugs such as doxorubicin (G-B-DNPs), cisplatin (G-B-CNPs), methotrexate (G-B-MNPs) and also in a mixture of cisplatin+doxorubicin (G-B-C-DNPs) and cisplatin+doxorubicin+methotrexate (G-B-C-D-MNPs) and the size range of ~ 20-30nm. We utilized UV-Vis spectroscopy, TEM, DLS, zeta potential to characterize these nanoparticles. FTIR spectroscopy used to evaluate the chemical interactions between drugs and G-BNPs. Drug loading efficiency was calculated by UV-Visible spectroscopy method. To investigate the cancer therapeutic effect of conjugated G-BNPs, we applied MTT assay test. The results showed that conjugated drugs of small in size exhibited significantly high cytotoxic effect in osteosarcoma cells (Saos-2 and MG-63) compared to the cytotoxic effects of equal doses of free cisplatin, methotrexate, and doxorubicin and insignificant effect on primary osteoblast cells; these results suggested that G-BNPs conjugated with anticancerous drugs have an enhanced cytotoxic effects.