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Preparation and Characterization of Cytotoxic Drug-Loaded Gold Nanoparticles

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ABSTRACT

In the present study, we have conjugated methotrexate as a cytotoxic drug with gold nanoparticles modified with 11-mercaptoundecanoic acid to explore their potential application in cancer therapy. The anticancer activity of methotrexate-gold nanoparticle conjugates (GNP-MTX) was demonstrated in MCF-7 breast cancer cells. Gold nanoparticles have been synthesized using tri-sodium citrate as a reducing and stabilizing agent whereas 11-marcaptoundecanoic acid was used as a thiol functionalizing ligand in the aqueous medium. The formation of gold nanoparticles was confirmed from the characteristic surface Plasmon absorption band at 522 nm and the transmission electron microscopy revealed the average particle size to be (~ 20 nm). Methotrexate was conjugated to thiolated gold nanoparticles by using N-hydroxy succinimide (NHS) and N, N-Dicyclohexylcarbo-Di-imide (DCC) conjugation procedure and the binding was analyzed using Fourier transform infrared spectroscopy (FTIR) to investigate the possible chemical interaction between MTX and 11-mercaptoundecanoic acid.

1. INTRODUCTION

Cancer is considered as one of the horrible diseases. Despite the progression in the treatment of cancer, the cure rate for this horrible disease without any side effects is difficult to reach[1]. As surgery and radiation therapy may not always possible at advanced stages of cancer, chemotherapy is commonly preferred. Among various chemotherapeutic agents, methotrexate (MTX) is a preferable anti-metabolite drug. It is used in the treatment of certain cancers like breast, skin and lung cancer. As folate receptors are overexpressed on the surfaces of many cancer cells, MTX is one of the most commonly used chemotherapeutic agent for human malignancies[2]. However, most of the existing chemotherapeutic agents suffer from less selectivity, less solubility and shorter lifetime in blood stream in addition to the multi-drug resistance with the potential risk of side effects. Also, clinical studies have revealed that the curative effect of MTX regarding cancers was limited due to its toxic dose-related side effects and also due to the drug resistance of the tumor cells[3]. Based on these data, various targeted drug carriers have been developed to improve the existing therapeutic efficacy and also to protect the drug from biodegradation before reaching the target cells, as well as to reduce the expected side effects [4]. Furthermore, the nanoparticles mediated drug delivery systems (DDS) with diameter less than 30 nm may be preferred for enhanced permeability and retention effect (EPR) of tumors with reduced side effects[5, 6]. Various functionalized nanoparticles constituting a bio- conjugated assembly with anti-cancer drugs to formulate a targeted DDS have been reported[7].

Considerable research efforts have been directed toward novel MTX delivery systems, such as nanoparticles[2, 8], microspheres[9], liposomes[10] and miscellaneous multiparticulate systems[11]. Among these, nanoparticles seem to be a promising tool for site specific delivery[12], based on the fact that they will release the active pharmacological moiety in tumor cells owing to the enhanced permeability and retention (EPR) effect[13, 14]. Figure (1).

Methotrexate acts as anti-cancer drug through its anti-metabolic effect by Interfering with DNA and RNA growth by substituting for the normal building blocks of RNA and DNA[15]. There are a variety of nanoparticle systems currently being explored for cancer therapeutics[16]. The material properties of each nanoparticle system have been developed to enhance the delivery of drug to tumor, for example, hydrophilic surfaces can be used to provide the nanoparticles with the needed properties for longer circulation times and over positively charged surfaces can enhance endocytosis. The types of nanoparticles currently

used for cancer therapeutic applications include dendrimers[17], liposomes[18], polymeric nanoparticles[19], micelles[20], protein nanoparticles[21] and metallic nanoparticles[22].

Gold nanoparticles (AuNPs) are particularly preferable because of their favorable properties such as non-toxicity[23], ease of functionalization via ligand exchange which enhances the capability of binding with drug molecules[24] and simple fabrication in various sizes and shapes[25].

Conventional techniques for aqueous synthesis of gold nanoparticles involve Turkevitch process of reduction of Au (III) Cl_3 with trisodium citrate. This process gives uniform and fairly spherical particles but proceeds at a very slow rate requiring nearly one hour for complete reduction of Au (III) Cl_3[24].

To examine the cancer therapeutic effect of these nanoparticles, we have used 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay test. The obtained results proved that the MTX-AuNPs of small size (~20nm) exhibited considerably high toxic effects in breast cancer cell lines compared to the effects of equal doses of free MTX. These results suggest that anti-cancer drug-conjugated AuNPs have an enhanced and significant therapeutic effect.

2. MATERIALS AND METHODS

2.1. Materials

Methotrexate ($C_{20}H_{22}N_8O_5$) was obtained from Sandoz Pharmaceutical Company as freeze dried powder. Hydrogen tetrachloraurate trihydrate ($HAuCl_4.3H_2O$) was acquired from across organics, Belgium. Trisodium citrate dihydrate was provided from Fisher Scientific Company, USA. Mercapto Undecanoic Acid (MUA) was purchased from Sigma Aldrich, USA. N-hydroxy succinimide (NHS) and N, N-Dicyclohexylcarbo-Di-imide (DCC) were obtained from Sigma Aldrich, USA.

2.2. Preparation of gold nanoparticles

The gold nanoparticles were prepared through reducing gold (lll) ions with tri-sodium citrate by heating 100 ml distilled water until boiling under reflux, then we have used different concentrations of $HAuCl_4.3H_2O$ and after 15 minutes, 1 ml of aqueous solution of tri-sodium citrate solution was added rapidly. Boiling and stirring were continued for 20 minutes. After

that, we removed the heating mantle and the solution was left with stirring till the solution was cooled down to reach room temperature. We have used different concentrations of citrate and gold solution to investigate the effect of citrate/gold ratio on the size of the nanoparticles. The produced nanoparticles were characterized by UV-Vis spectroscopy, size and zeta potential measurements[26].

2.3. Preparation of MU-MTX conjugates

In a typical experiment we added 1 mole of mercaptoundecanoic acid (MWT: 218.36) to dry di-methylformamide (DMF) then adding 2 moles of N-hydroxy succinimide (NHS) and 2 moles of N,N-Dicyclohexylcarbo-Di-imide (DCC) for the activation of the -COOH group. We have stirred this colloidal dispersion at room temperature for 6-8 hours, then adding1.2 mole of methotrexate solution.

2.4. Characterization

2.4.1. UV-visible spectroscopy of the prepared nanoparticles

Ultraviolet-visible light absorption spectra of the citrate-stabilized GNPs were taken at room temperature using a spectrophotometer (Schimadzu1601-double beam spectrophotometer, Shimadzu Company, Japan).

2.4.2. Size measurement of nanoparticles by photon correlation spectroscopy (PCS)

The size distribution and the zeta-potential of the prepared gold nanoparticles before and after polymer coating were analyzed by photon correlation spectroscopy PCS using Zetasizer ZSNano (Malvern instruments, UK).

2.4.3. Nanoparticles morphological determinations using TEM imaging

The morphology of the prepared gold nanoparticles was determined by TEM imaging. TEM samples were prepared by dropping gold colloids on carbon-coated copper grids followed by natural drying. The samples were observed on a JEM-100CXII transmission electron microscope (JEOL Ltd, Tokyo, Japan).

2.4.4. IR analysis

The IR analysis was carried out using "Nicolet 6700 FT-IR instrument, Thermo-Fischer Scientific Corporation, Madison, USA" Department of Chemistry, Faculty of Science, Assiut University.

2.5. Quantification

The amount of MTX-conjugated to AuNPs (% of loading) was quantified prior to the *in-vitro* study. Two calibration curves were applied individually for the MTX-AuNPs and the free MTX in the solution. In order to quantify the concentration of excess free MTX in the solution after the reaction, the calibration curve based on UV-Vis absorbance measurements of free MTX was constructed. The curve was constructed based on the absorption peak of pure MTX at 382 nm, the absorbance changes with the variation of the MTX solution concentration, the percentage (%) loading of MTX was estimated using equation (1):

$$\% \text{ loading} = (A-B) / B \times 100 \qquad (1)$$

Where, A is absorbance of total MTX added to the GNP solution, and B is the absorbance of unbound MTX in the supernatant of the GNP-MTX solution after centrifugation.

2.6. Cytotoxicity and viability assays

The JAR cell lines were cultured in DMEM/F12 supplemented with 10% fetal bovine serum and antibiotics at 37 ^{0}C with 5% CO_2. Approximately 5000 cells (200 µL of a suspension of 2.5 × 104 cells/mL) were placed in each well of a 96-well culture plate kept for 24 h. For conducting cytotoxicity and viability experiments, serum-free medium was used to avoid the interference of the background absorption. The cancer cells in a serum-free medium were separately treated with reference AuNPs (naked GNPs), free MTX, and MTX-AuNPs. The concentrations of reference AuNPs and MTX-AuNPs were 100µg/ml. After gentle agitation, 100 µL of the culture medium was transferred into a new 96-well culture plate for the MTT assay, 25 µL of 5 mg/mL MTT was added into each well containing treated cells. After 4 hours, the supernatant was discarded and 100 µL of DMSO was added to each well. The mixture was shaken, and the optical density was measured at 570 nm using an ELISA reader scanning multi-well spectrophotometer (Perkin Elmer VICTOR 2, GMI, USA).

3. RESULTS AND DISCUSSION

3.1. Preparation and characterization of gold nanoparticles

3.1.1. Effect of citrate concentrations on the size of the produced nanoparticles

The size of the nanoparticles prepared by the reduction of gold salt normally depends on the type of reducing agent which determines the rate of nucleation and subsequently the particle growth. It was observed that the slow reduction produces large particles, while fast reduction usually produce smaller particles[27]. After addition of tri-sodium citrate to the boiled solution of the gold salt, a bluish color appeared indicating the formation of gold nuclei. A few minutes later, the solution was turned brilliant deep red due to the formation of the nanoparticles. The deep red color of gold nanoparticles in water is due to the strong light absorption in the visible region due to the oscillation of surface electrons after exposure to light. This phenomenon is characteristic for metallic nanoparticles called surface Plasmon resonance (SPR)[28]. Different concentrations of tri-sodium citrate were used. Through increasing the citrate to gold ratio from 10 to 30, the sizes of the particles were reduced from 35 nm to 22 nm. Table (1).

3.1.2. UV-visible spectroscopy of the prepared nanoparticles

Particles synthesized by citrate reduction method can be obtained as monodisperse spheres where the sizes of which are controlled by the initial reagent concentrations and the chosen reaction conditions. The particles in aqueous dispersion are characterized by a deep red color with surface Plasmon resonance peak at about 524 nm as shown in Figure 2.[29]

3.1.3. Size measurement of nanoparticles by photon correlation spectroscopy (PCS)

Many trails were conducted for the preparation of gold nanoparticles in different sizes by altering gold/citrate ratio. Thus, upon increasing the citrate concentration (citrate/gold=30) we have got gold nanoparticles with a small size and acceptable polydispersity (PDI) indices. So, we have considered these results are indicating for high reproducibility of the citrate reduction to prepare the gold nanoparticles. Figure shows the measured size distributions of different batches of the prepared GNPs. The obtained GNPs show a quite narrow monomodal size distribution with only one peak observed in the size distribution curve (Figure 3).

3.1.4. Nanoparticles morphological determinations using TEM imaging

TEM observations were employed to clarify the morphology of formed AuNPs. As seen from the TEM image of AuNPs, almost all of the AuNPs were spherical in shape and well dispersed. These results illustrated the formation of monodispersed and spherical AuNPs. Figure (2).

3.2. Investigation of MU-MTX conjugates by IR analysis

Firstly, it is so necessary to activate the carboxylic group of MUA to render it ready to form amide bond with the primary amine of MTX. Figure (3).

To understand the interaction between MTX and MUA, we carried out FTIR measurements. There is a strong peak is observed at 1708 cm^{-1} due to the C=O stretch of tertiary amide (-CONH-). The weak broad peak around 3361 cm^{-1} is assigned to tertiary amine group of MTX. The observed results clearly suggest that methotrexate is binding to MU–functionalized GNPs through the formation of an amide bond with the carboxyl group of MU as showed in Figure (6).

3.3. Cytotoxicity and viability assays

The *in-vitro* cytotoxicity characteristics of free MTX, naked GNPs and GNPs-MU-MTX against breast cancer cell lines (MCF-7) was determined by MTT assay. Regarding the *in-vitro* cytotoxicity assay, different concentrations of GNPs , free MTX and MTX loaded GNPs were used. The *in-vitro* cytotoxicity characteristics of them against a breast cancer cell line (MCF-7) was determined by MTT assay. Fig. 8 compares the cellular viability between free MTX, free GNPs and MTX loaded GNPs. From the data obtained from the figure (7), it was observed that at concentration above 50 µg/ml of MTX-GNPs exhibited more cellular toxicity than that of naked gold nanoparticles. However, it was found that nanoparticles alone have also produced considerable cytotoxicity at concentration above 80 µg/ml. From the *in-vitro* cytotoxic activity of drug, NPs and MTX-NPs, when the drug was conjugated with the nanoparticles, it was found that inhibitory concentration was decreased compared to the free MTX. The IC_{50} value for (MCF-7) cells was observed and it was found to be decreased from 30.31µg/ml for free MTX to 9.41µg/ml for MTX-NPs after 24 hours of incubation. Figure (4).

Methotrexate is a cytotoxic drug that works by interfering with DNA and RNA growth by substituting for the normal building blocks of RNA and DNA and is commonly used in the treatment of wide range of cancers[(15)].

4. CONCLUSION

In this work, we have demonstrated a proper method for direct synthesis of gold nanoparticles using (tri-sodium citrate reduction method), which gives us monodispersed and small sized gold nanoparticles (~ 20 nm), then making conjugation with methotrexate. From the *in-vitro* study (MTT assay) results, it was found that the methotrexate loaded gold nanoparticles showed enhanced cytotoxicity on a breast cancer cell line (MCF-7) in comparison to free methotrexate and free gold nanoparticles.

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Table (1): Effect of added amounts of sodium citrate on the size and the Surface Plasmon Resonance (SPR) of the synthesized gold nanoparticles

$HAuCl_4.3H_2O$ (mg)	Tri-sodium citrate (mg)	gold/citrate ratio	Size (nm)	SPR maximum (nm)
5	50	1:10	35.14	525.5
5	100	1:20	27.17	524
5	175	1:30	22.19	523

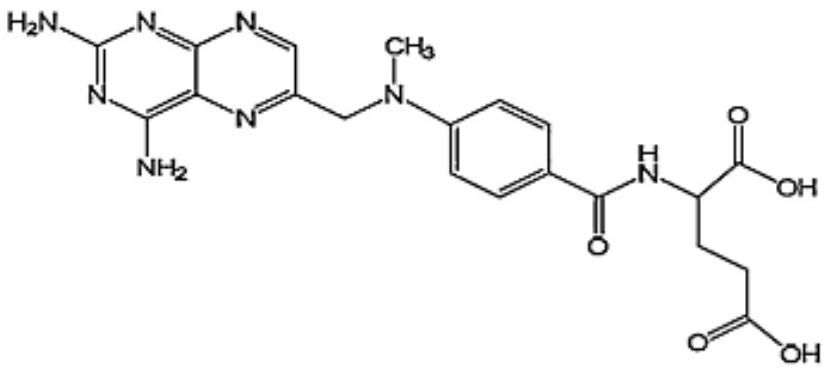

Figure (1).Molecular structure of Methotrexate

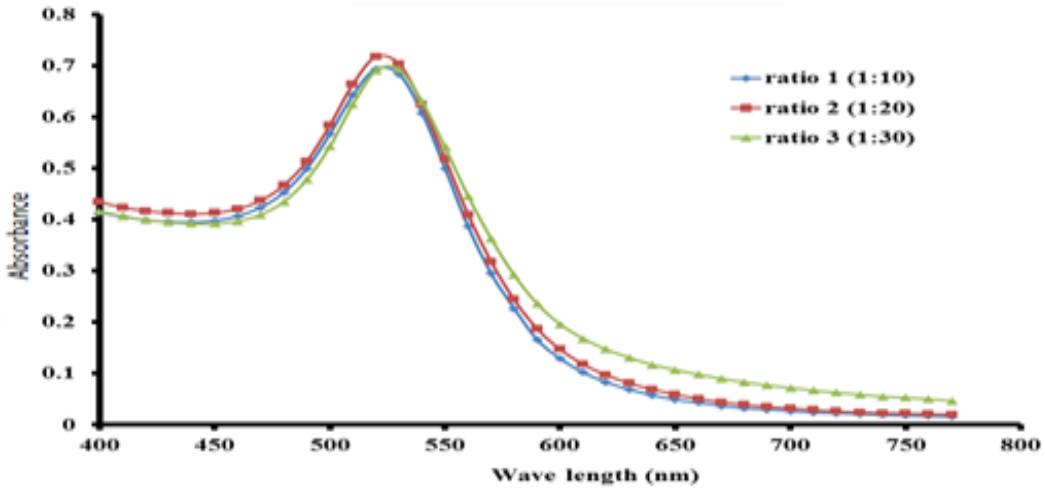

Figure (2). UV-Vis absorption spectra of different gold nanoparticles preparations

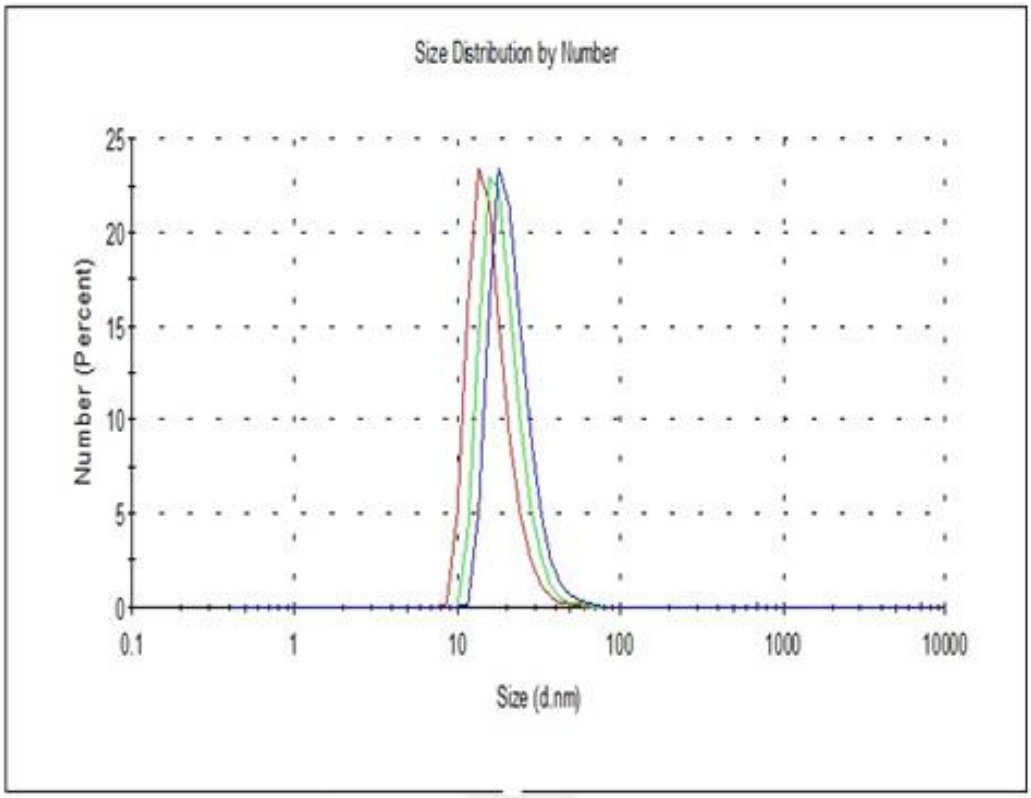

Figure (3).Size distribution of different batches of synthesized gold nanoparticles

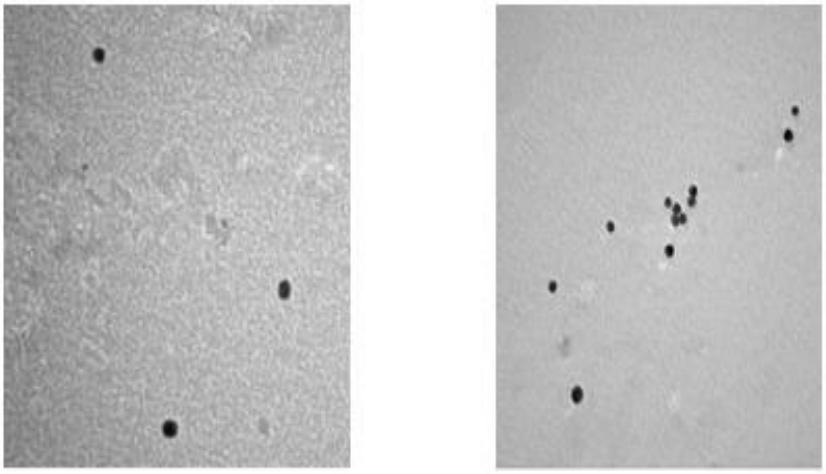

Figure (4).TEM images of gold nanoparticles reduced by trisodium citrate method

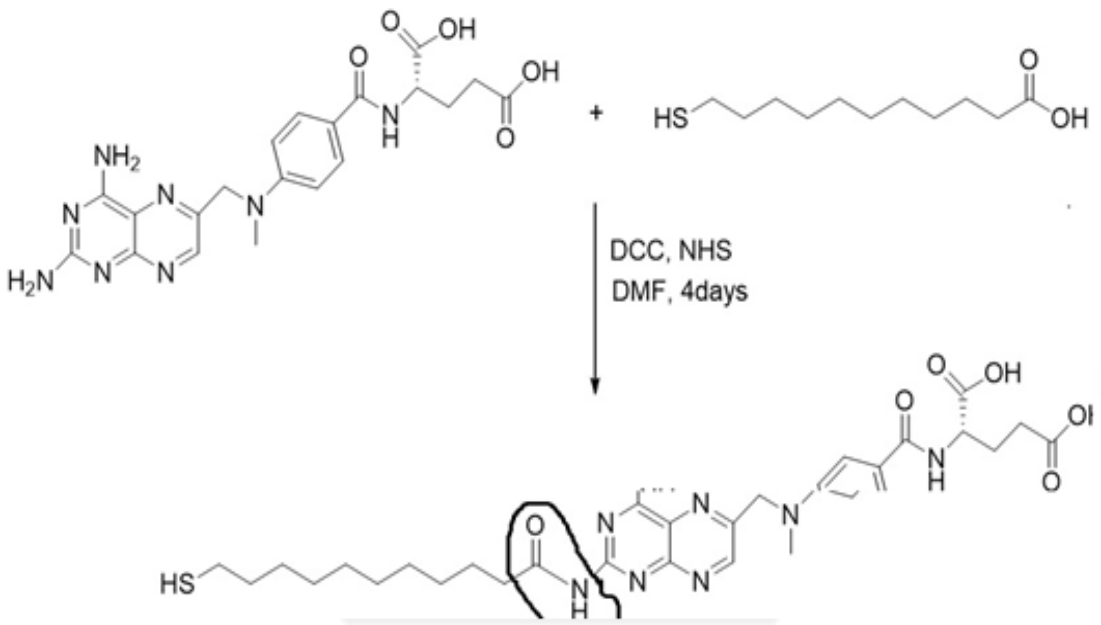

Figure (5). Conjugation between MTX and MUA

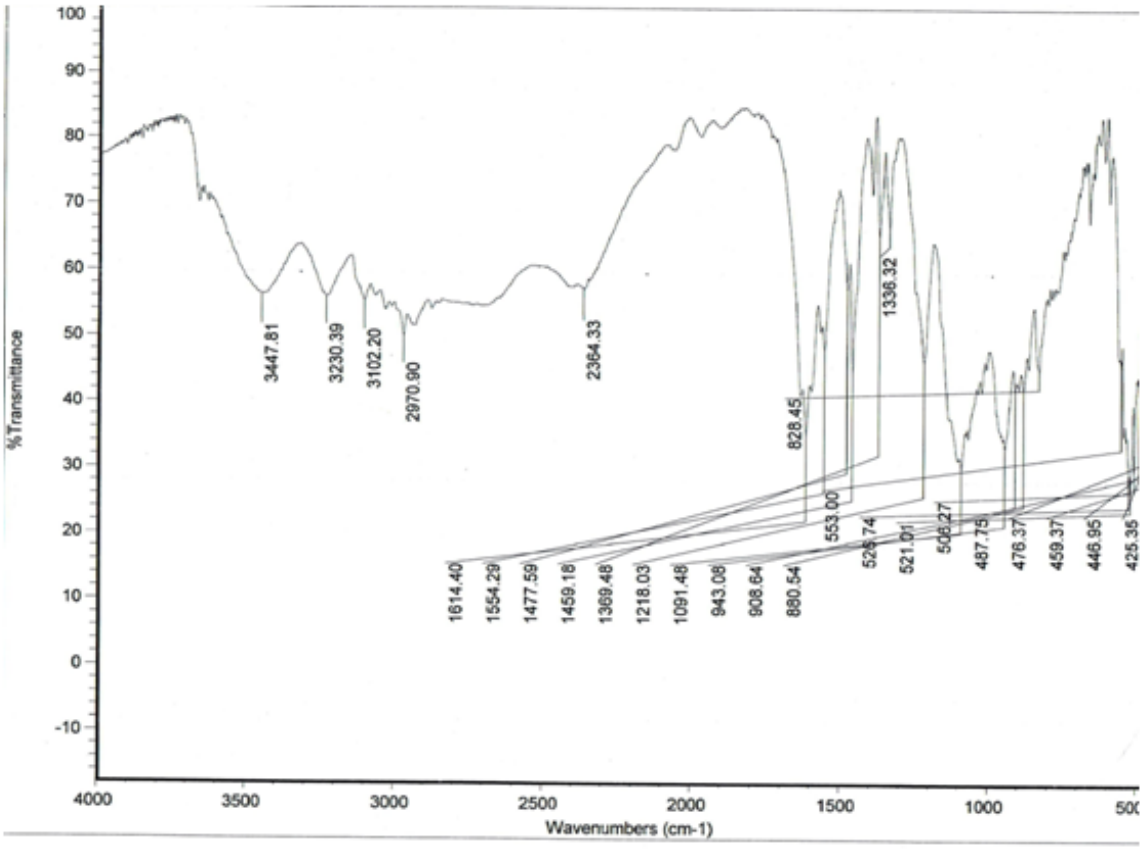

Figure (6). FTIR spectra of MTX and MU- MTX conjugates

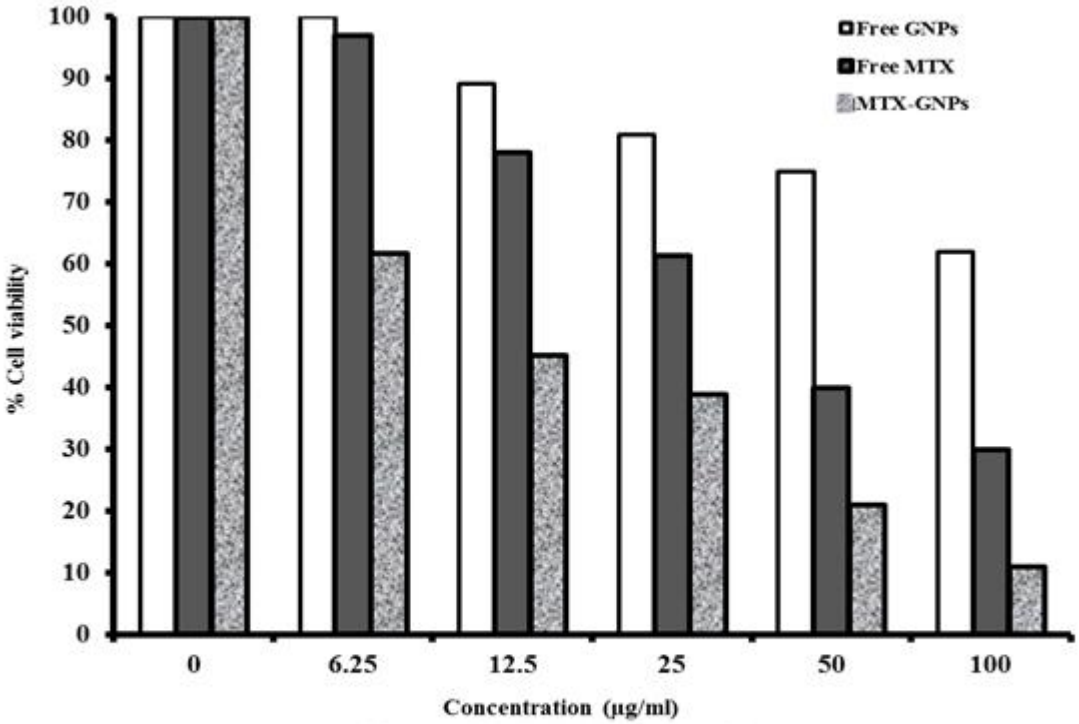

Figure (7): The MTT assay showing the effect of naked GNPs, MTX and MTX loaded GNPs on breast cancer cell line.