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Therapeutic Potential of Neem Synthesized Silver Nanoparticles on Human Gastric Cancer Cells *in Vitro*

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Abstract

Nanotechnology has shown significant promise in development of drugs and drug delivery systems that can overcome all limitations and address urgent needs to improve efficacy of diagnosis and therapy of various diseases including cancer. The functionalization with neem compounds as synthesis and capping agent had shown very high anticancer activities against Gastric cancer cells *in vitro*. The biochemical factors like albumin, glucose, and DNA concentrations were modulated along with Protease inhibitor and Catalase activates, the various cancer specific proteins like p53, GRD 70 - 78 kDa and other proteins of sizes 35 - 40 kDa corresponding to H+K+ATPase protein etc. The apoptic activity and antiproliferative activity were demonstrated with Gastric cancer cells *in vitro*.

Keywords

Gastric Cancer, Nanotherapy, Silver Nanoparticles, Neem Compounds, *In Vitro* Cancer Treatment, Biochemical Changes in Nanotreatment

1. Introduction

Cancer is a molecularly heterogeneous hyperproliferative disorder marked by metastasis into the vital organs of the body through invasion and angiogenesis. Gastric cancer remains one of the most common cancers worldwide and is typically associated with late-stage diagnosis and high mortality. According to the World Health Organization, 800,000 cancer-related deaths are caused by stomach cancer each year globally [1]. It is the fourth most common cancer worldwide, but the second leading cause of cancer-related deaths in the world.

Cancer therapies are currently limited to surgery, radiation and chemotherapy. All three methods risk damage to normal tissues or incomplete eradication of the cancer. Improved insights into the etiology of cancer have led

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to the identification of several novel and highly promising classes of anticancer therapeutics, such as growth factor receptor inhibitors, proteasome inhibitors and anti-angiogenic agents etc. [2]-[11]. Nanotherapies are increasing in importance as vehicles for antineoplastic agents because of their potential for targeting and multifunctionality, the multiple hallmarks of cancer pathogenesis, cellular and molecular alterations and associated targeted therapies [12]-[18].

The development of stimuli-responsive nanomaterials for cancer treatment has been developed [19]-[22]. Surface-enhanced Raman scattering, photoacoustic imaging in lymphangiography [23], photodynamic therapy (PDT) [24] and photothermal therapy (PTT) [25] have been actively investigated as applications in nanomedicine.

However, designing adequate therapies is difficult because of the complexity of cancer biology and the vast heterogeneity of tumors. Only a small fraction of tumor cells is highly sensitive to therapy, and even those cells can develop resistance and progress into a more aggressive disease. The aim of our research program is to develop new Np for therapeutic interventions, and to further enhance of tumor therapy and reduce of clinically relevant side-effects. Molecular and genetic analysis allows physicians to detect, classify, monitor and treat cancer more effectively.

Our results of comparative biochemical screening of green synthesized silver nanoparticles may provide the scientific reality for an optimized therapeutic application and it may also provide the basis to find new template structures for the development of next-generation drugs for patients with resistance to the first generation drugs.

2. Methods

2.1. Synthesis and Characterization of Ag-Nps

One pot green Synthesis of silver nanoparticles (Ag-nps) using Neem leaf extract was done following the method of Kiruba *et al.* [26] The Ag-nps were primarily characterized by UV-visible spectroscopy, Atomic Force Microscopy and FTIR.

2.2. Cell Culture

Human gastric cancer cells AGS were kindly provided by Dr. Kumaresan, SBS, MKU University, and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. Cells were grown to confluence at 37˚C and 5% CO_2 atmosphere. All experiments were performed in 6-well plates, unless stated otherwise. Cells were seeded onto the plates at a density of 1 × 106 cells per well and incubated for 24 h prior to the experiments. The cells were washed with (phosphate buffered saline, pH 7.4) PBS and incubated in fresh medium containing different concentrations of Ag-nps suspended in water.

2.3. *In Vitro* Cell Viability/Cytotoxicity Assay

To evaluate the cytotoxicity of the Ag-nps, one hundred microliters of AGS cell suspension was dispersed in a 96-well plate, giving a concentration of 5000 cells/well. The plate was pre-incubated for 24 hours in a humidified incubator (37˚C, 5% CO_2), after which 10 µl of various concentrations of Ag-Np were added into the culture media in the plate. After the plate was incubated for a further 24 hours, Cells were harvested and Trypan blue was mixed. Then the blue stained dead cells were counted to see the cytotoxicity and viability. The dye exclusion test is used to determine the number of viable cells present in a cell suspension. Besides, the trypan blue stain is considered as a simple way to evaluate cell membrane integrity and thus assesses cell proliferation or death.

2.4. Invasion Assay

The invasive potential of tumor cells was determined with an *in vitro* invasion assay. Briefly, cells were tested for their ability to penetrate the intestine in organ culture A suspension of tumor cells (1×10^6) in DMEM containing 2% Rhodamine B. After 48 hrs of incubation at 37˚C in 95% air and 5% CO_2, Then the organ culture media was removed and washed and treated with Ag Nps in medium only for experimental plate and plain medium in control plate. Rhodomine staining was analyzed and photographed under an Olympus Fluoview FV

1000 Laser confocal microscope using the 380 nm excitation 560 nm emission.

Antiproliferative efficacy on AGS cell line was determined using Ag-Np-Rhodamin B method after 48 hrs treatment. The fluorescence was measured in spectroflurimeter.

2.5. Biochemical Analysis

For all biochemical tests, following *in vitro* culture for 24 h, the gastric cancer cells, a total amount of 1 × 106, were grown in serum free medium Minimal essential medium without antibiotics with or without Ag-Np were collected, lysed and used for biochemical assays. Estimation of glucose was done following the method of King & Garner [27], The entire DNA was extracted using lysis buffer, phenol chloroform extraction and alcohol precipitation The concentration of the DNA was estimated by reading the absorbance at 260 and 280 nm using the UV spectrophotometer. The methyl orange method of Bracken and Klotz [28] was used for the estimation of albumin. The absorbance of the solution measured photometrically at 480 nm. Catalase activity was estimated by reacting with H_2O_2 measuring the absorbance at 240 nm [29]. Trypsin inhibitor assay was measured using trypsin as substrate in buffer phosphate buffer pH 7.6. The precipitate was pelleted and the absorbance was measured at 410 nm. Trypsin inhibitor activity was represented as unit of tryps in utilized.

2.6. SDS-PAGE

Samples containing 25 mg of protein from homogenized gastric cancer cells with and without nanotreatment were analyzed by SDS-PAGE (12.5%) under reducing conditions according to Laemmli [30].

2.7. Statistics

The results were determined by three independent experiments but with pooled samples.

3. Results and Discussion

Nanotechnology has shown significant promise in development of drugs and drug delivery systems that can overcome all limitations and improve efficacy of diagnosis and therapy of various diseases [31] [32]. Nanotherapies, as carriers for antineoplastic agents with potential for targeting, and multifunctionality are increasing [12]-[18]. Phytochemicals which exhibit anti-carcinogenesis by affecting a spectrum of different cellular signaling pathways have been well recognized in the scientific literature [33] [34].

Nanoparticles functionalized with anticancer phytochemicals, molecular and genetic analysis would help to treat cancer more preciously. Hao *et al.* [35] have reported neem components as potential agents for cancer prevention and treatment. Preliminary experiments with neem synthesized silver nanoparticles (Ag-Np) were performed against gastric cancer cells AGS *in vitro* to study the toxicity and efficiency.

Colloidal Ag-NPs were prepared using Neem leaves to add drug effect to silver nanoparticles following the modified methods of [26] [36] [37]. The color change from yellow to brown suggested the formation of Ag-Nps. Studies indicated that the reducing phytochemicals in the neem (Azadirachta indica) leaf consisted mainly of terpenoids, nimbin and quercetin which served as capping and stabilizing agents in addition to reduction [36] [37].

A strong and broad surface plasmon peak was observed at 420 nm for the Ag-NPs prepared (**Figure 1**) and the particles were well dispersed without aggregation. The diameter by the spectral response of silver nanoparticles was approximately 20 nm which was confirmed by AFM picture (**Figure 2**). Observation of the strong surface plasmon peak has been well known in the case of silver nanoparticles over a wide size range of 2 - 100 nm [26] [38] [39].

Fourier transform infrared spectroscopy (FTIR), of synthesized silver nanoparticles is depicted in **Figure 3**. The broad band corresponding to the presence of the phenolic −OH occurs at 3600 - 3200 cm^{-1}, maybe due to the polyphenols present in the plant extract. The activated neem leaves consist of mainly three dissimilar kinds of phenolic compounds such as 4-chlorophenol (4-CP), 4-nitrophenol (4-NP) [35]. The peaks at 1635 cm^{-1} and 2073 cm^{-1} indicated the presence of aromatic ring C=C stretching alkyne bonds respectively. These bands denote stretching vibrational bands responsible for compounds like flavonoids and terpenoids [35] [40] adsorbed on the surface which are very abundant in Neem plant, while nanoparticles bond shows strong peak at 600 cm^{-1}.

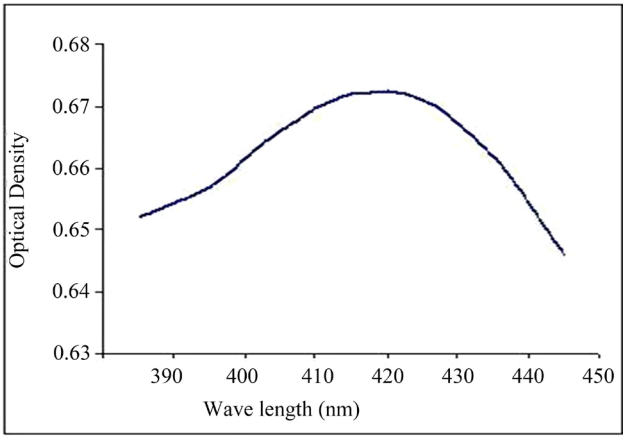

Figure 1. Optical density pattern of neem synthesized silver nanoparticles.

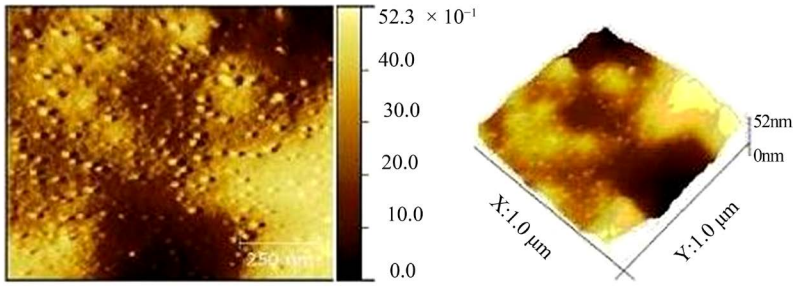

Figure 2. AFM pattern of neem synthesized silver nanoparticles.

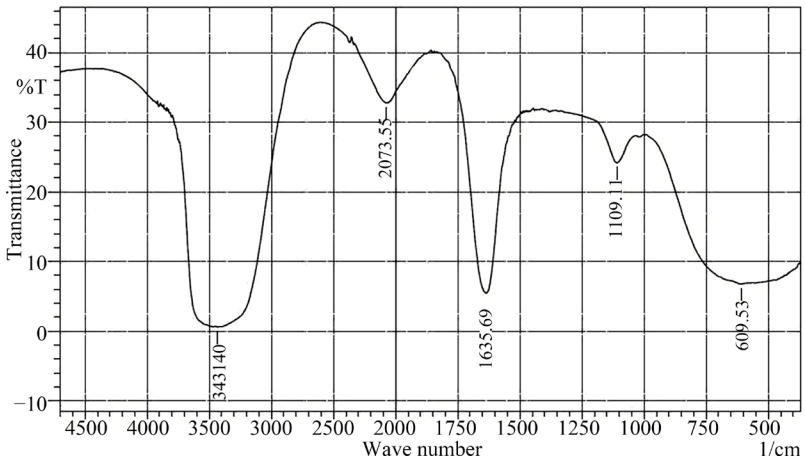

Figure 3. FTIR pattern of neem synthesized silver nanoparticles.

The FTIR spectrum of the un-reacted Azadirachta indica extract showed bands at 1742 and 1636 cm^{-1}. The first band is characteristic of stretching vibrations of the carbonyl functional group in ketones, aldehydes and carboxylic acids. The second absorption at 1636 cm^{-1} corresponded to the amide I band. The intense broad absorbance at 3412 cm^{-1} is attributed to the O-H stretching modes of vibration in hydroxyl functional group in alcohols and N-H stretching vibrations in amides and amines. Moreover, the 1059 cm^{-1} band can be assigned to C-O stretching vibrations. The absorption peak at 2930 cm^{-1} corresponded to C-H stretching vibration modes in the hydrocarbon chains. The main difference between both spectra was that the treated extract exhibits peaks of less intensity for the amide band [35] [40].

In *in vitro* Ag Np viability and antiproliferative analysis (**Figure 4**), these nanoparticles had major effects on

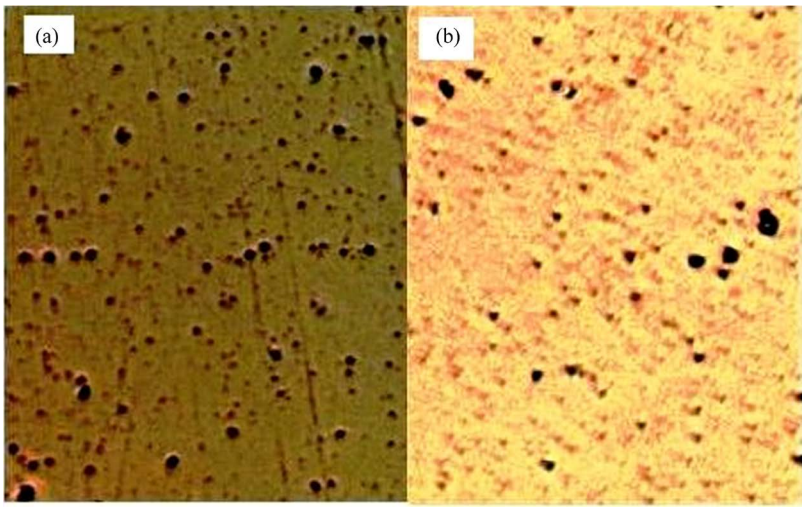

Figure 4. Phase contrast microscopic picture of gastric cancer cells *in vitro* (a) untreated gastric cells (b) Silver nanoparticle treated gastric cancer cells.

the proliferation of Gastric Cancer cells and significantly decreased the viability to 5% - 10%, suggesting good cytotoxicity and antitumor activity. But normal cells treated with Ag-Nps showed no toxicity as observed by our earlier studies as well [26] [38] [39] [41].

Investigation of the antiproliferative effect of Ag-Np in the *in vitro* AGS model system confirmed that the Ag-NP could modulate the sensitivity of the gastric cancer cells. Ag-Np induces cytotoxicity selectively in tumor cells indicating induction of apoptosis. Various nanoparticles were reported to suppress the growth and proliferation of a wide variety of tumor cell lines of different tissue origins [42] [43]. Apoptosis helps to establish a natural balance between cell death and cell renewal in mature animals by destroying excess, damaged, or abnormal cells.

The attachment of nanoparticles to the cell membrane caused aggregation of envelope protein precursors causing dissipation of the protein motive force. Silver nanoparticles also exhibited destabilization of the outer membrane and rupture of the plasma membrane thereby causing depletion of intracellular ATP and rupturing of cell membrane which may lead to cell death. It was also proposed that oxygen associated with silver reacts with the sulphydral (-S-H) groups on the cell membrane to form R-S-S-R bonds causing inhibition of respiration resulting in cell death [44]-[47].

In addition, the anti-proliferative and apoptosis-inducing effects of neem components in which the Ag-nps were prepared are tumor selective as the effects on normal cells are significantly weaker [35].

Over the past decades, albumin has emerged as a versatile carrier for therapeutic and diagnostic agents, primarily for diagnosing and treating diabetes, cancer, rheumatoid arthritis and infectious diseases. Hence in order to understand the role of albumin in cancer therapy, the concentration of albumin in Ag np treated and un treated gastric cancer cells *in vitro* were estimated (**Figure 5**) Serum free minimal essential medium was used for culturing the cells and the whole lysate was used for analysis.

It has also been shown that there is an increase in the albumin flux across the capillary wall, from the intravascular into the extravascular compartments, in patients with cancer and sepsis [48]. There may have been alterations in the rates of albumin turnover with either a decreased or decreased synthesis [49]. Fleck *et al.* [50] have shown that the most important factor in altering serum albumin concentrations is the rate of exchange between blood and the extravascular space. They calculated that this rate of exchange is more than ten times the rate of synthesis and breakdown and suggested variation based on the stage of the tumour, the patient's age, the degree of tumour differentiation [48]-[53]. Those patients with low concentrations of C-reactive protein low concentrations of interleukin 6 (a key cytokine in the induction of hepatic synthesis of acute phase proteins) and higher serum albumin concentrations are more likely to respond to treatment and have a more prolonged survival [54] [55]. Alternatively, tumour necrosis factor may increase the permeability of the microvasculature, thus allowing an increased trans-capilliary passage of albumin [56] and hence a lowering of the serum albumin concentrations.

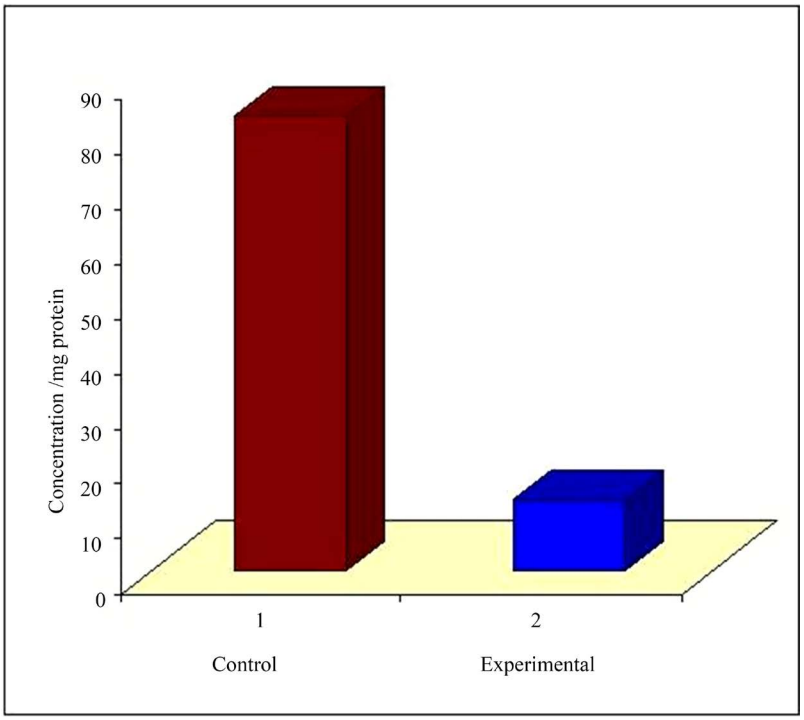

Figure 5. Concentration of albumin in control gastri cancer cells and silver nanoparticle treated cells.

Most human tumors display some forms of genomic instability, including DNA sequence alterations, chromosomal rearrangements, aneuploidy or gene amplifications. These alterations have the potential to affect the function of cell growth-related genes, such as proto oncogenes and tumor suppressor genes, which are associated with the malignant transformation of cells. **Figure 6** shows the DNA content of the Ag np treated and untreated Gastric cancer cells *in vitro*.

Apoptosis is the most important pathway through which many compounds exert their antitumor effects. It has been shown that rhein can induce apoptosis by increasing nuclear condensation and DNA fragmentation [57], activating caspase-8, -9, and -3 [57], increasing the levels of Fas, p53, p21, and Bax, but decreasing the levels of Bcl-2 [58].

The reduction in the DNA level may be due to. the damage in cell function and development which includes oxidative modification of proteins to generate protein radicals [59], initiation of lipid peroxidation [60]-[62], DNA-strand breaks, modification to nucleic acids [63], modulation of gene expression through activation of redox-sensitive transcription factors [64] [65] and modulation of inflammatory responses through signal transduction [66], leading to cell death and genotoxic effects [67]-[72]. The gastric mucosal integrity is maintained through a balance between the proliferation and apoptosis of mucosal cells. DNA damage derived from oxidative stress is another tumorigenic factor attributed to H. pylori infection [73].

Vitamin C is capable of inducing gastric cancer cell growth inhibition, which may be related to the effects on cell protein and DNA synthesis. Extracts of neem has natural substances such as limonin, azadirachtin, kaemferole, beta-carotene and ascorbic acid. In addition to combating oxidative damage in the body, these phytochemicals can help enhance the immune system, reduce inflammation, and interfere with the growth of cancer cells [74]-[77].

The glucose concentration of the Ag-Np treated Gastric cells was three fold higher than the untreated Gastric cancer cells *in vitro* (**Figure 7**). The interactions between cancerous cells and tumor microenvironment during the courses of multistep tumorigenesis play a critical role in modulation of tumor growth, metabolism and metastasis to distant sites [78]-[80].

Enhanced glucose utilization is a prominent and fundamental change in many tumors irrespective of their histological origin and the nature of mutations, first observed by [81]. The extent of increase in glucose utilization measured by FDG-PET has been correlated with the degree of malignancy in some of the tumors [82].

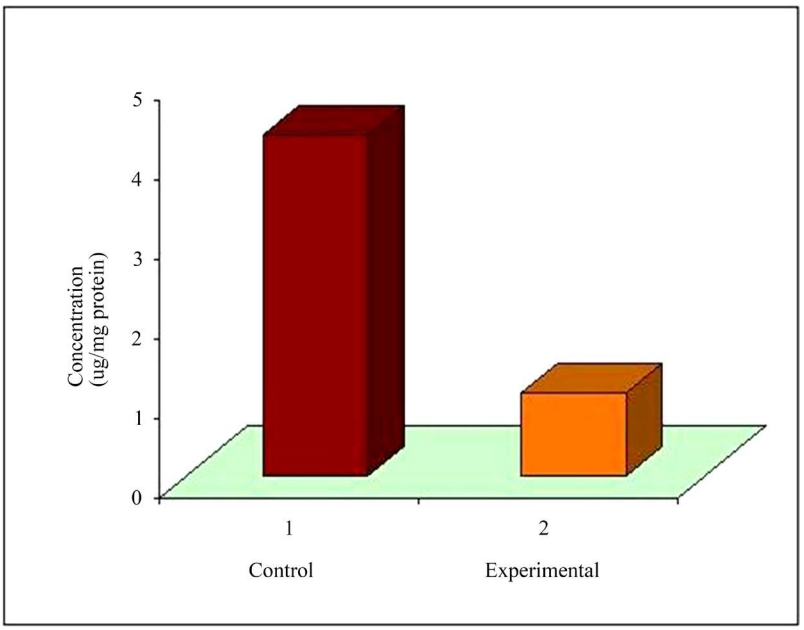

Figure 6. DNA concentration of silver nanoparticle untreated and treated gastric cancer cells.

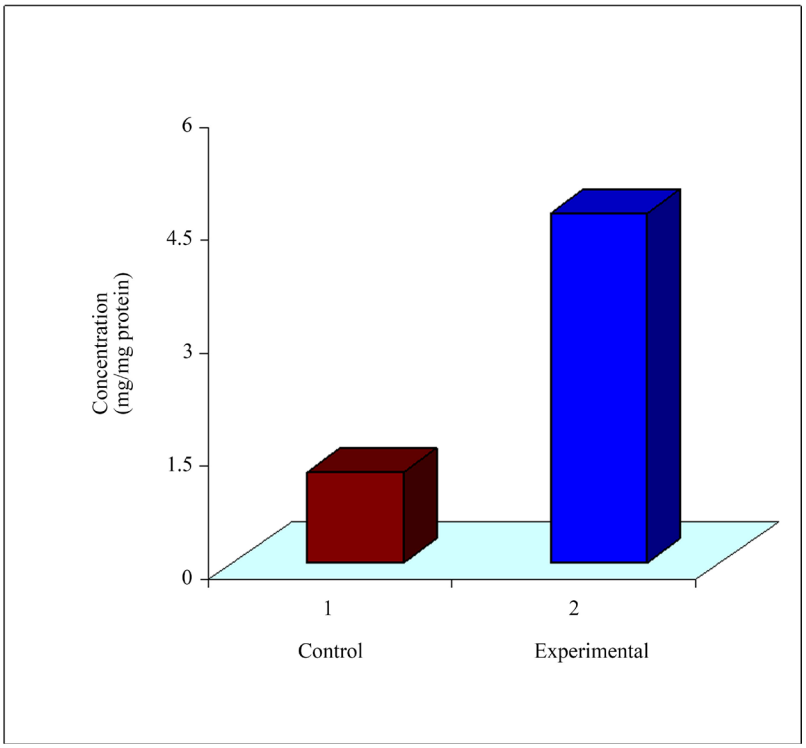

Figure 7. Concentration of glucose in control gastric cancer cells and silver nanoparticle treated cells.

Glucose utilization is also inversely correlated with treatment response in a number of tumors, while changes in tumor glucose utilization during the first weeks of chemotherapy are significantly correlated with patient outcome [83] [84]. Therefore, glucose utilization appears to be a useful metabolic marker for diagnosis, prognosis and prediction of tumor response to a variety of therapies [85].

It was reported that oxidative stress and reactive oxygen species (ROS) were found to be crucial in a variety of diseases such as diabetes, cancer etc. [86]. Catalase is a heme enzyme that has a predominant role in controlling hydrogen peroxide concentration in human cells, by converting H_2O_2 into H_2O and O_2. With superoxide dismutase (SOD) and glutathione peroxidase, catalase constitutes a primary defense against oxidative stress and may provide resistance to the effects of radiation and chemotherapy [87].

To know the effect of Ag-nps in oxidative stress, the catalse activity was measured. As shown in **Figure 8**, the catalase activity was lower in Ag-nps treated cancer cells than the untreated control gastric cancer cells *in vitro* since the antioxidant enzymes are inducible, the levels of the antioxidant enzymes reflect the levels of their substrates, the active oxygen species [88] [89]. Reactive oxygen species (ROS) synthesis in gastric cells [90] [91], and ROS enhances the expression of oncogenes, stimulates cell proliferation and plays an important role in all stages of carcinogenesis [92]. NF-kB was also involved in oxidativestress- mediated cell injury. A variety of antioxidants have been demonstrated to inhibit the activation of NF-κB [93], and micromolar concentrations of H_2O_2 could activate NF-κB, suggesting that reactive oxygen may act as a second messenger in the activation of transcription factor NF-κB [94]. The suppression of NF-κB signaling pathway is, at least partially, involved in the anticancer functions of neem components [35].

Proteases from all catalytic classes positively or negatively affect cancer progression and metastasis through complex and highly regulated processes that involve cleavage of cell adhesion molecules, growth factors, cytokines, or kinases [95]-[99]. The relationship between serum tumor-associated trypsin inhibitor levels with gastri cancer cells with and without Ag Np treatment was studied *in vitro*. **Figure 9** shows more than two fold increased level of protease inhibitor.

The results of this study indicated that trypsin could be considered as a growth factor and the high expression of trypsin inhibitor unravel a new mechanism whereby serine proteases control colon tumours. They are also involved in tissue remodeling during development and in tissue penetration as they induce the migration of monocytes and cancer cells [100]. The lysosomal cysteine proteases, such as cathepsins B, H, and L, are broadly distributed in tissues and believed to be responsible for a major proportion of normal protein turnover and pathological processes.

Upregulation of the protease inhibitor, contributes to cell proliferation inhibition in gastric cancer [101]-[103]. Tumour-associated trypsin inhibitor expression has been associated with impaired survival in several forms of cancer [104]-[106], but not in gastric cancer, where it is believed to have a natural function of protecting the mucosa from proteolytic degradation [107]-[111]. The protease-activated receptor-2 (PAR-2) and trypsin play a

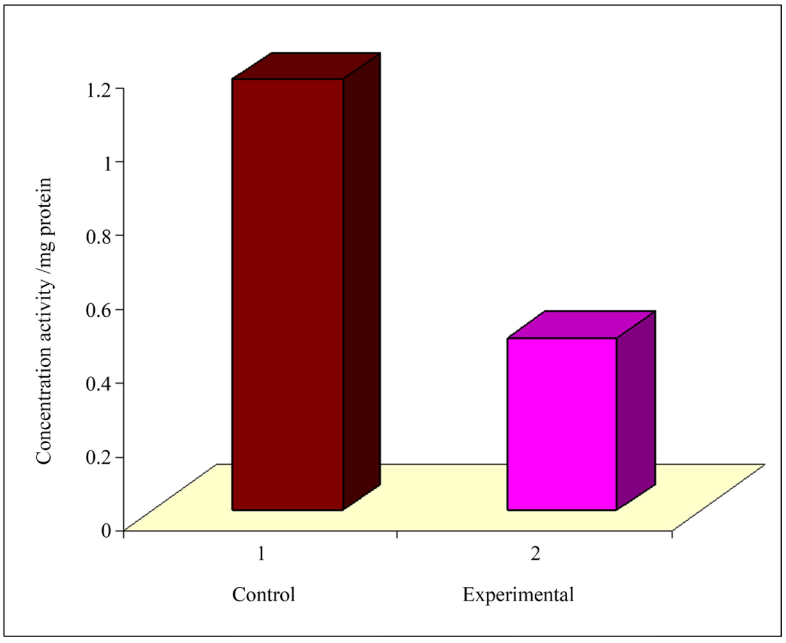

Figure 8. Estimation of catalase activity in control gastric cancer cells and in silver nanopartice treated experimental cells.

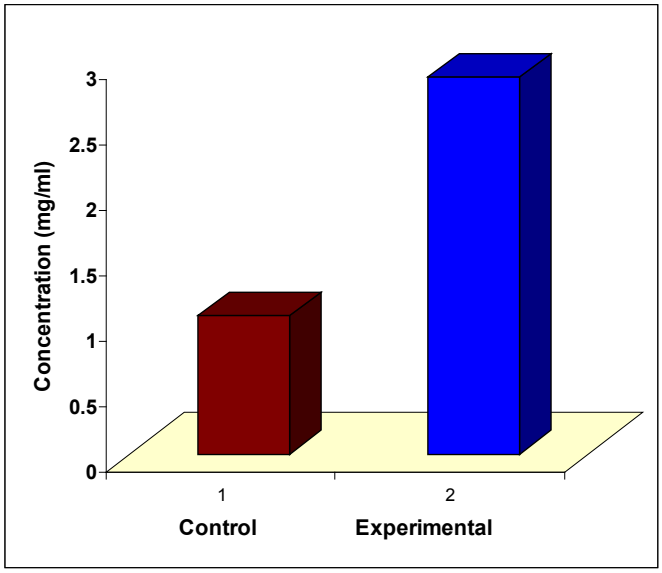

Figure 9. Trypsin inhibitor concentration in Silver nanoparticle untreated and treated gastric cancer cells.

role in cell proliferation in human colon cancer cell lines [112].

Stomach cancer cell lines frequently secreted active trypsin, suggesting that they produced an endogenous activator of trypsinogen, most likely enterokinase. Trypsin (ogen) was frequently expressed at high levels in stomach and colon cancers, but scarcely in breast cancers. In the stomach cancers, the trypsin immunoreactivity was higher. These results support the hypothesis that tumor-derived trypsin is involved in the malignant growth of tumor cells, especially stomach cancer cells [113]. And hence, the level of trypsin inhibitor was found to be high in Ag-Np treated gastric cancer cells.

Knowledge about cancer biomarkers will provide great opportunities for improving the management of cancer patients by enhancing the efficiency of detection and efficacy of treatment. Emerging evidence indicates that most tumor-associated biomarkers are cellular proteins whose aberrant regulation of function could be linked to malignancy [114] [115].

The protein profile of untreated and Ag Np treated Gastric cancer cells were analyzed on 12.5% PAGE and the results are shown in **Figure 10.** The highly expressed proteins of molecular weight 78, 66, 53, 50, 40, 29, 25, 23 and other minor peptides were found to be in high concentration in control untreated gastric cancer cells.

Many stomach, colon, and breast cancer cell lines secreted trypsinogens-1 and/or -2, as well as an unidentified serine proteinase of about 70 kDa, into culture medium. These results support the hypothesis that tumor-derived trypsin is involved in the malignant growth of tumor cells, especially stomach cancer cells [113]. HSPA5 (heat shock 70 kDa protein and glucose-regulated protein 78 kDa) gene is expressed in all nucleated cells, in particular in thyroid-, lung-, smooth muscle-, liver-, and various cells of the immune system [116]. Glucose regulated protein 78 (GRP78) is overexpressed in colorectal carcinoma and regulates colorectal carcinoma cell growth and apoptosis. The HER2 receptor belongs to the epidermal growth factor (EGF) receptor (EGFR) family of tyrosine kinase receptors expressed by a variety of tumor cell lines that appear to drive tumorigenic pathways, including proliferation, invasion, adhesion, and metastatic spread [117] [118]. The 78 - 70 kDa, 25 and 23 kDa protein observed in control untreated gastric cancer cells may be the 70-kDa serine proteinases. 25- and 23-kDa active trypsin observed in various human cancer cell lines [113] p53 protein a tumor suppressor and transcription factor is a 53-kDa protein present in humans and is encoded by the TP53 gene It is a critical regulator in many cellular processes, including cell signal transduction, cellular response to DNA damage, genomic stability, cell cycle control, and apoptosis When tumors develop, point mutations at the TP53 gene can lead to overexpression of p53 proteins, which contribute to continuous cell division and canceration. Overexpression of p53 has been reported in 60% of laryngeal carcinomas, 37% of hypopharyngeal carcinomas, and 52% of tongue carcinomas. With the mortality and disintegration of tumor cells, p53 protein released from cancer cells will enter into the circulation.

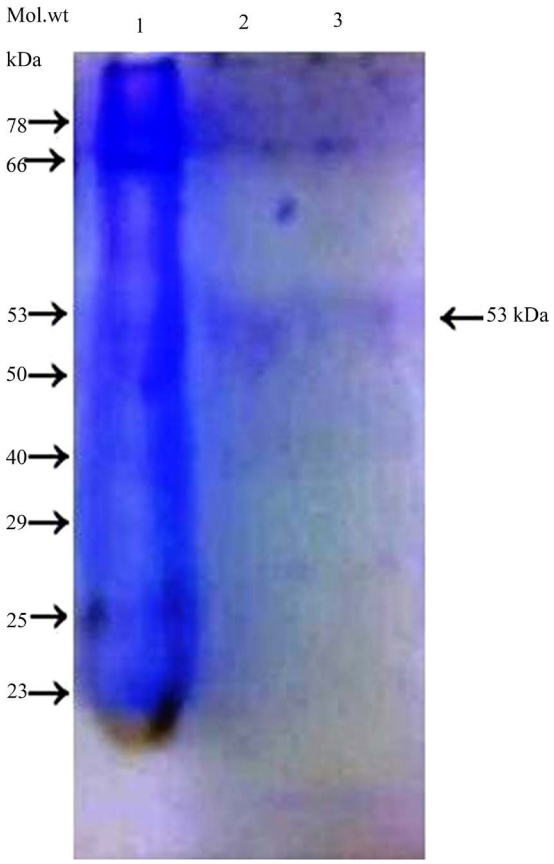

Figure 10. 12.5% SDS-PAGE showing the protein profile of gastric cancer cells (lane 1) and silver nanoparticle treated cells with different concentrations (lanes 2 & 3).

Although p53 is not a typical cancer-specific antigen, its central role in the control of cell growth and apoptosis and frequent mutations in tumours make p53 a unique target for cancer therapy [119]. Curcumin down-regulates the expression of p53 as well as the survival genes egr-1, c-myc, and bcl-XL in B cells [120].

The 40 - 50 kDa glycoprotein was consistently expressed in the intestinal type carcinoma. An albumin associated 40 - 50 kDa glycoprotein was previously shown in mucus gels in gastric cancer. Secreted gastric mucins are large O-glycosylated proteins of crude mucus gels identified as α-1-Acid Glycoprotein which are aberrantly expressed in malignancy [121]. The H+/K+-ATPase enzyme with subunits 35 kDa and 114 kDa of gastric parietal cells exchanges luminal K+ for cytoplasmic H+ and is a specialized proton pump primarily responsible for gastric acidification, leading to the development of gastric enterochromaffin-like (ECL) cell carcinoids in rats [122].

Cancer markers CA 27 - 29 are found on Cancers of the colon, stomach, kidney, lung, ovary, pancreas, uterus, and liver may also raise CA 27 - 29 levels. Noncancerous conditions associated with this substance are first trimester pregnancy, endometriosis, ovarian cysts, benign breast disease, kidney disease and liver disease [119].

The suppression of NF-κB signaling pathway is, at least partially, involved in the anticancer functions of neem components adsorbed with additive effect of Ag-Np Importantly, the anti-proliferative and apoptosis-inducing effects of neem components are tumor selective as the effects on normal cells are significantly weaker. In addition, neem extracts sensitize cancer cells to immunotherapy and radiotherapy, and enhance the efficacy of certain cancer chemotherapeutic agents [35].

To evaluate the effect of Ag Np viability/cytotoxicity assay was done using dissected bit of mice intestine *in vitro* as described in methods and were analysed using confocal microscopy (**Figure 11**). Representative images were selected from the results of one set experiment among three experiments. Higher apoptosis rate, was detected in nanotreated compared with control gut tissue co-cultured with Gastric cancer cells. The gastric mucosal

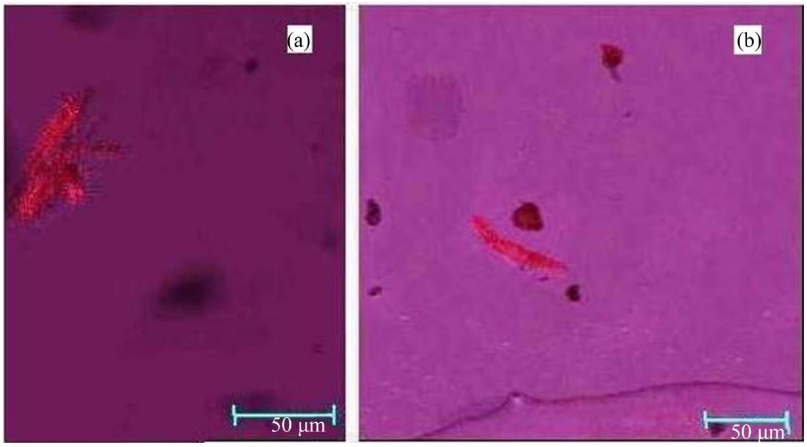

Figure 11. Invasion assay of control and silver nanoparticles treated Rhodamine B labeled gastric cancer cells infected mice intestine in organ culture Confocal microscopic view.

integrity is maintained through a balance between the proliferation and apoptosis of mucosal cells.

Chitosan/heparin nanoparticle-encapsulated CdtB preferentially inhibited the proliferation of cells derived from gastric cancer. Treatment of cells with nanoparticle-encapsulated CdtB enhanced cell-cycle arrest at G2/M, followed by apoptosis. Moreover, our data showed that the mechanism for nanoparticle-encapsulated CdtB-induced cell death was mediated by ATM-dependent DNA damage checkpoint responses [18].

The glandular organization of this tissue, is also critical to its role as a barrier to a range of environmental noxious and immunogenic molecules [123]-[125], During an established infection, the vast majority of H. pylori cells (about 70%) are found in the mucus layer of the superficial gastric mucosa, either motile or adhered to the heavily glycosylated secreted mucins.

Most stomach cancers are adenocarcinomas, which develop in the cells of the mucosa. However, stomach cancer can develop anywhere in the organ and spread to other parts of the body by growing beyond the stomach wall, entering the bloodstream or reaching the lymphatic system.

Gastric cancer cells labeled with rhodamine b was added to mice intestine in organ culture and one set was treated with nanoparticles and the other set served as control (**Figure 12**) The fluoresence spectrometric analysis revealed the reduction in fluorescence and very less accumulation of Ag-nps and less invasion of gastric cancer cells revealing the therapeutic potential of Ag np (**Figure 12**).

The nanoparticle localisation in intestine cultured with and without Gastric cancer cells by the enhanced permeability and retention effect. Ag-nps preferentially accumulated in the tumour mass by extravasation through the fenestrated tumour interstitium Tumor cells, Kupffer cells, and mononuclear phagocyte system have higher phagocytotic rates for uptaking nanoparticles than other tissue cells. Therefore, the Ag-nps could be targeted to tumor, the liver, or spleen [126].

Figure 13 shows the comparison of various factors in gastric cancer cells treated with Ag-nps. Except glucose and antitrypsin concentrations (4 fold and 3 fold respectively) all other biochemical and molecular factors were down regulated in Ag Np treated gastric cancer cells compared to the un treated control gastric cancer cells. Albumin concentration was reduced 6 folds. DNA concentration and catalase activity were down regulated 4 folds and 3 folds respectively (**Figure 13**).

Mechanisms underlying this fundamental alterations in metabolism during carcinogenesis include mutations in the mitochondrial DNA resulting in functional impairment, oncogenic transformation linked upregulation of glycolysis, enhanced expression of metabolic enzymes and adaptation to the hypoxic tumour micro-milieu in case of solid tumours [81]. These abnormalities, which include telomerase activation, genetic instability, and abnormalities in oncogenes, tumor suppressor genes, cell-cycle regulators, cell adhesion molecules, and DNA repair genes, could be effective markers in the molecular diagnosis of gastric cancer.

Apart from being an excellent anti-bacterial agent, Ag-nps had anti-inflammatory properties. The potential anti-inflammatory action of silver nanoparticles has been suggested in various studies described previously (26, 38, 39, 41). Others have also demonstrated the anti-inflammatory effects of silver nanoparticles using a porcine

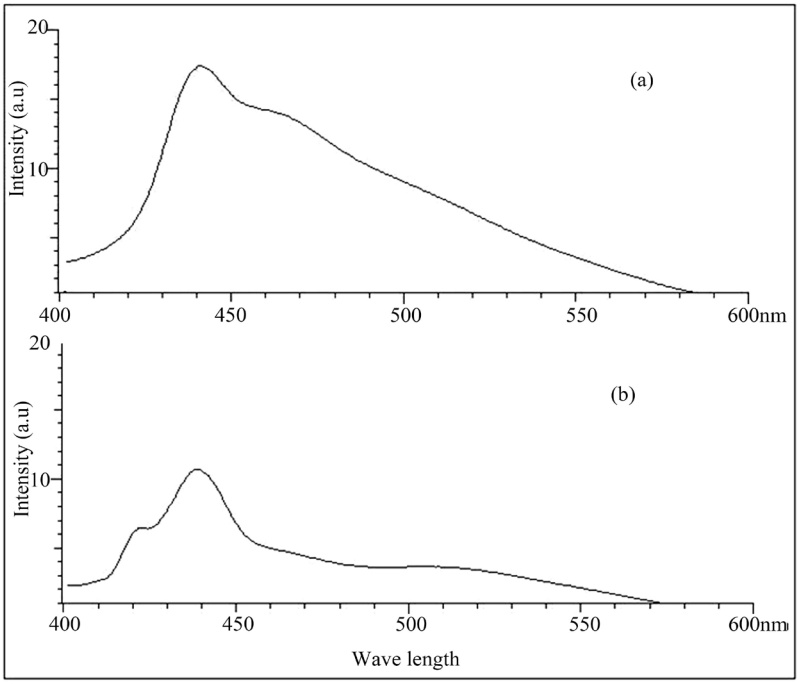

Figure 12. Invasion assay-Fluorescence spectroscopic pattern of Rhodomin B treated control gastric cells (a) and silver nanoparticles treated gastric cancer cells (b) in organ culture.

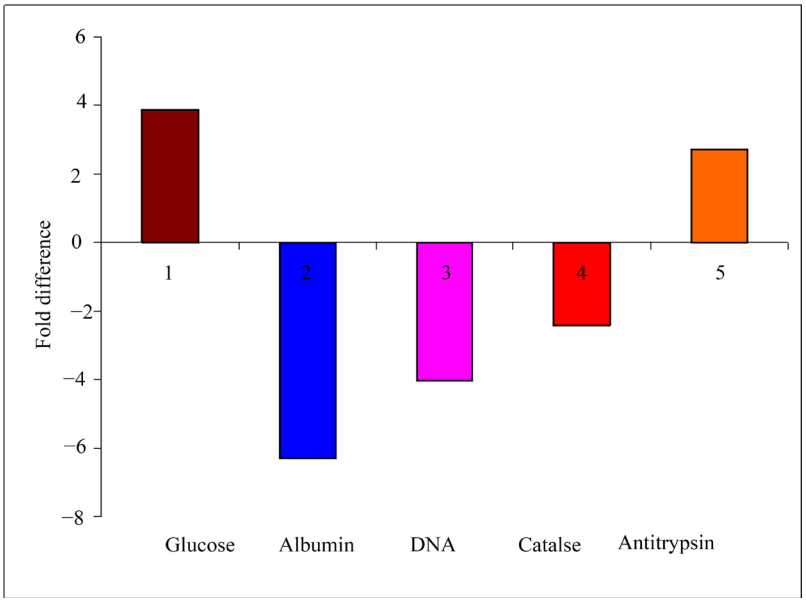

Figure 13. Comparison of various biochemical parameters showing fold difference silver nanoparticle treated gastric cancer cells with un treated control gastric cancer cells.

model of contact dermatitis [127] and in a rat model of ulcerative colitis [128]. Proteins, known as matrix metalloproteinases (MMPs), help cancer cells escape their original locations by cutting through proteins of the extracellular matrix, which normally holds cells in place [129].

Circulating tumour DNA (ctDNA) as a noninvasive modality to assess evolution of solid malignancies, this is DNA originating from cancer cells, carrying tumour-specific genomic alterations, that is present as short cell-

free fragments in body fluids such as blood plasma [130].

Active oxygen species pose a severe threat to cells, and are probably responsible for cellular damage, tissue damage, DNA modifications, and many human diseases [131]. Antioxidant enzymes are the superoxide dismutases (SOD), catalases (CAT), and peroxidases, of which glutathione peroxidase (GPx) appears to be the most important in mammalian cells. free radicals, particularly oxygen radicals, play an important role in the complex course of multistep carcinogenesis. Much of the evidence (**Figure 13**) shows that antioxidants scavenge free radicals directly, or interfere with the generation of free radicals-mediated events, inhibit the neoplastic process [132]-[135]. Overproduction of ROS can induce oxidative stress, resulting in DNA-strand breaks, modification to nucleic acids [49] [63], modulation of gene expression through activation of redox-sensitive transcription factors [64] [65], and modulation of inflammatory responses through signal transduction [66], leading to cell death and genotoxic effects [67] [69].

The protease-activated receptor-2 (PAR-2) and trypsin play a role in cell proliferation in human colon cancer cell lines [112]. Cysteine proteases are released as a response to several normal and pathological processes, including inflammation and tumorigenesis [103] and their proteolytic activities are regulated by potent cystatin inhibitors. Cystatins play a role in the protection of tissues from inappropriate proteolysis, and thus the control of protease activity by cystatins is essential to organisms.

Similar relationship between serum tumor-associated trypsin inhibitor levels and clinicopathological parameters in patients with gastric cancer was reported by Kemik *et al.* [136] Recent exploitation of apoptosis pathways towards re-instating apoptosis induction via caspase re-activation has provided new molecular platforms for the development of therapeutic strategies effective against advanced prostate cancer as well as other solid tumors [137].

Apoptotic cell death induced by Poncirin in AGS cells was mediated by Fas death receptor followed by the caspase-dependent extrinsic apoptosis pathway [138]. In several previous studies, it was found that some phytochemicals induce apoptosis by alteration of MMP in various cancer cells [139] [140].

It can be deduced that upon the microenvironmental stress, such as hypoxia, glucose deprivation and inflammation, the intracellular induced- or extracellular secreted-GRP78 is able to inhibit the function of p53 protein, facilitating genome instability and the related mutations (**Figure 11**).

Glucose regulated protein GRP78 can promote the unfolded or misfolded proteins return to normal conformation, and then protect cells by suppressing oxidative damage and stabilizing calcium homeostasis [72] [116] [134] [141].

Curcumin treatment impairs both Wnt signaling and cell-cell adhesion pathways, resulting in G2/M phase arrest and apoptosis in HCT-116 cells. Curcumin preferentially arrested cells in the G2/S phase of the cell cycle [142] [143].

Poor pharmacokinetic and biodistributional profile upon intravenous administration are the important drawbacks with these second-generation anticancer agents as with the first-generation DNA-damaging counterparts. Multifunctional nano formulations aim to improve the balance between the efficacy and toxicity of systemic anticancer therapy. The currently approved nanoparticle systems have in some cases improved the therapeutic index of drugs by reducing drug toxicity or enhancing drug efficacy. The next generation of nanoparticle systems may have targeting ligands such as antibodies, peptides, or aptamers, which may further improve their efficacy or reduce their toxicities [12] [144] [145].

Gold nanostars (GNSs), as one kind of emerging nanomaterial, have been actively investigated as an application in nanomedicine, including surface-enhanced Raman scattering, photoacoustic imaging in lymphangiography [23] photodynamic therapy (PDT) [33], and photothermal therapy (PTT) [1] [25] [34] [146] [147].

Extracts from the neem tree are packed with beneficial natural substances such as limonin, azadirachtin, kaemferole, beta-carotene and ascorbic acid. In addition to combating oxidative damage in the body, these hytochemicals can help enhance the immune system, reduce inflammation, and interfere with the growth of cancer cells. Neem leaf extracts can cause apoptosis to suppress the proliferation of leukemia and melanoma cell lines [35].

Silver nanoparticles functionalized with anticancer neem phytochemicals would help to treat cancer more preciously in addition to the bactericidal effect, their unique physical, chemical properties, and ease of synthesis and surface modification, biodistribution and biosafety [26] [38] [39] [41]. Ag Nps hold the most promise for achieving optimal targeting all cancers including brain cancer as they can bypass the BBB and improve the distribution within a brain [148]-[150]. Multifunctional therapeutics where a nanoparticle serves as a platform to

facilitate its specific targeting to cancer cells and delivery of a potent treatment, minimizing the risk to normal tissues over coming all problems of cancer therapy.

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Conflict of Interest

There is no conflict of interest.

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