



Preparation, characterization, and anticancer efficacy of novel cobalt oxide nanoparticles conjugated with thiosemicarbazide

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Abstract

Gastric cancer is one of the most common cancers in modern societies. Previous studies have shown that the use of nanoparticle complexes is effective in the treatment of cancer. The aim of this study was to investigate the cytotoxicity and anti-cancer properties of cobalt oxide (Co₃O₄) nanoparticles (NPs) functionalized by glutamic acid (Glu) and conjugated with thiosemicarbazide (TSC) on gastric cancer (AGS) cell line. First, the Co₃O₄@Glu/TSC nanoparticles were synthesized via co-condensation reaction. Fourier-transform infrared (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) tests were performed for identifying the morphology, structure, size and functional groups of produced nanoparticles. MTT assay was also performed to evaluate cytotoxicity effect. Moreover, Annexin V/PI staining with flow cytometry analysis, caspase-3 activation assay, and Hoechst 33258 staining was carried out for evaluating apoptosis. The FTIR results showed that the components of Co₃O₄@Glu/TSC NPs complex were successfully fabricated. Crystallographic structure of Co₃O₄@Glu/TSC NPs was confirmed by XRD patterns. SEM results indicated that the size of the nanoparticles was in the range of 16–40 nm. An EDX spectrum was determined and data explained the existence of cobalt as the prominent element. MTT test results showed that AGS cell life was significantly decreased compared to the control group with increasing concentration of nanoparticles (dose-dependent) ($P < 0.05$), IC₅₀ = 107.5 µg/mL. The results of flow cytometry assay and caspase-3 activity showed that fabricated Co₃O₄@Glu/TSC NPs induced apoptosis in the treated group. Moreover, Co₃O₄@Glu/TSC NPs treated AGS cells indicate an increase in the apoptotic characteristics including nuclear fragmentation. In the current work, the promising cytotoxicity and anti-cancer activities of Co₃O₄@Glu/TSC NPs complex toward gastric cancer (AGS) cell line were showed and it can be suggested for the drug delivery system.

Keywords AGS · Cobalt oxide · Gastric cancer · Glutamic acid · Thiosemicarbazide

Introduction

After cardiovascular disease, cancer is the second leading cause of death in developed countries. Mortality due to the disease has increased gradually in recent years and is considered a serious threat to human health. Gastric cancer is the fourth most prevalent cancer in modern societies and the second leading cause of cancer death (Heise et al. 2009). About 90–95% of gastric cancers are gastric adenocarcinoma. In this case, the cancer spreads to the mucosa-producing cells (the cells that cover the inner lining of the stomach). Gastric cancer is a multifactorial disease that results from a combination of environmental and genetic factors. In this case, helicobacter infection, malnutrition, mutations and polymorphisms in key genes can be mentioned (Ma et al. 2017; Conteduca et al. 2012). The balance

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between proliferation and cell death plays a critical role in physiological homeostasis in tissues. Disruption of this balance will enable the cell to escape the apoptotic stage and lead it to uncontrolled cell proliferation and become cancer-causing. Chromatin condensation, nucleus fragmentation, cell contraction, and ultimately cell death are events that occur during apoptosis (Gonzalez et al. 2002, 2013). Nowadays, nanotechnology using nanoparticles helps to diagnose and treat cancer. In a way, it recognizes and destroys cancer cells (Boyle et al. 2008). Researchers use nanoparticles as a drug to treat malignant cancer cells because they do not have a negative impact on healthy cells and tissues in the body (Mokwena et al. 2018).

Cobalt oxide nanoparticles with Co_3O_4 formula are a mineral compound that has various applications in magnetic and catalytic activities (Ito et al. 2005). It also has antiviral activity and is used for drug delivery into cells and magnetic resonance imaging (MRI) (Liu et al. 2003). Research results have shown that the cellular uptake of Co_3O_4 -NP is high and these nanoparticles can enter cells rapidly and settle into vesicles inside the cytoplasm. Because of these properties, cobalt oxide nanoparticles have attracted researchers' attention for cancer treatment (Papis et al. 2007a, b). Glutamic acid is one of the unnecessary amino acids that is biodegradable and non-toxic to humans and plays an important role in cellular metabolism. Glutamic acid is used as a cancer-fighting compound because it enhances the effect of the anticancer drug and reduces its toxicity against normal cells (Shih et al. 2004). Thiosemicarbazide is one of the most important organic compounds which is very important due to its unique chemical structure and specific biological activities (Kasuga et al. 2001). Thiosemicarbazide has antibacterial, antiviral and antitumor properties (Li et al. 2000; Finch et al. 1999). Thiosemicarbazide can interact with many metal ions and increase their chemical activity. Research has shown that the complexation of ion nanoparticles with thiosemicarbazide can inhibit cancer cells. It also increases the pharmacological effect of metallic ions with thiosemicarbazide (Yildirim et al. 2014; Yang et al. 2004). Our aim in this study was to investigate the anti-apoptotic effect of Co_3O_4 @Glu/TSC NP on gastric cancer cells (AGS).

Materials and methods

Synthesis of Co_3O_4 NPs

300 mg of cobalt salt ($\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) was dissolved in 150 mL distilled water and the pH of the solution was increased to 11 by NaOH and then placed at 80 °C for 2 h. After centrifugation, the precipitate was removed from the solution and washed with ethanol and kept at 70 °C to dry (Yang et al. 2004).

Synthesis of Co_3O_4 @Glu NPs

Co_3O_4 -NPs with 152 mg of glutamic acid was dissolved in 150 mL of distilled water and reached 50 °C and its pH increased to about 11 by adding NaOH. Then the steps continued similar to Co_3O_4 -NP synthesis.

Synthesis of Co_3O_4 @Glu/TSC NPs

Co_3O_4 -NPs were functionalized by glutamic acid (Glu) via co-condensation reaction. In brief, 500 mg of Co_3O_4 @GA-NP was mixed with 200 mg of thiosemicarbazide in 150 ml of ethanol 96% and incubated in an ultrasonic bath for 40 min at 45 °C. The solution was placed on a heater at 40 °C. After 24 h, the precipitate was separated by centrifugation and washed with ethanol at 70 °C to dry.

Characterization of Co_3O_4 @Glu/TSC NPs

Several tests were performed to check the accuracy of the synthesized compounds. In FTIR spectra, the samples of Co_3O_4 @Glu NP and Co_3O_4 @Glu/TSC NP were examined. FTIR spectra of samples in the form of KBr pellets were recorded using a FTIR spectroscopy device (Shimadzu, Japan). X-ray diffraction (XRD) test was performed to investigate the crystal structure of Co_3O_4 @Glu/TSC NP. X-ray powder diffraction (PXRD) data were collected using a Philips X'Pert MPD diffractometer (Co-K α X-radiation, $\lambda = 1.79 \text{ \AA}$). The morphology and elemental composition of the Co_3O_4 @Glu/TSC NPs were analyzed using scanning electron microscopy (SEM) image was taken using an electron microscope and Energy Dispersive X-ray Spectroscopy EDX (FE-SEM, Model: Hitachi S-4500).

Cell line and culture medium

The Gastric adenocarcinoma cell line (AGS) was purchased from the cell bank of Pasteur Institute of Iran. The cells were grown in the DMEM medium supplemented with penicillin/streptomycin at a final concentration of 100 units per ml and 10% fetal bovine serum (FBS) (Gibco, Netherland). Cells were cultured on cell culture plates and incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT assay and cell viability

MTT assay was performed to investigate the effect of Co_3O_4 @Glu/TSC nanoparticles on AGS cells and normal fibroblast cells (L929). By approximate growing cell count, 10^4 cells/100 μL were transferred from cell suspension to 96-well plates and incubated with cell culture conditions.

After 24 h, the culture medium was evacuated and $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ NPs were added to each well at concentrations of 62.5, 125, 250 and 500 $\mu\text{g}/\text{mL}$. A well was considered only with cells treated as the control group. After incubation, 10 μL of MTT solution (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium Bromide) was added to each well at a concentration of 5 mg/mL and incubated for 4 h. 100 μL of DMSO was added to each well. Finally, optical absorption at 570 nm was recorded by ELISA and IC_{50} was calculated using the following formula:

$$(\%) \text{inhibition} = \frac{\text{Abs of Control} - \text{Abs of Test}}{\text{Abs of Control}} \times 100.$$

Analysis of apoptosis/necrosis by flow cytometry

Flow cytometry was used to evaluate apoptosis and necrosis in AGS cells. At this stage, cells were treated with a concentration of 107.5 $\mu\text{g}/\text{mL}$ of $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ NPs in 6-well plates and incubated. After 24 h, cells were separated by trypsin and centrifuged and washed with PBS and added to them 10 μL Annexin-V FLUOS and 5 μL propidium iodide (PI) according to the manufacturer's protocol (Roche, Germany) and then, the numbers of apoptotic and/or necrotic cells were measured by flow cytometry device using PAS machine (Partec, Germany).

Caspase-3 activation assay

Caspase 3 activity was evaluated in AGS cells treated with $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ NPs compared to untreated cells (control group). ApoTarget TM kit was used in this test. 3×10^6 cells/well were cultured in 6 well plates and incubated for 24 h at 37 °C, then treated with 107.5 $\mu\text{g}/\text{mL}$ $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ NPs. One group of untreated cells was considered as the control group. After 24 h, the cells were re-suspended in 50 μL of Cell lysis buffer and incubated on ice for 10 min and centrifuged at 10,000 rpm for 60 s. Then, the dilution of supernatant to a concentration of 100 μL proteins with 50 μL Cell Lysis Buffer was performed. After measuring the protein concentration, the DEVD-pNA (Caspase-3) substrate was added and the samples were read in 400 or 405 nm using a microplate reader.

Hoechst staining

The evaluations of nuclear damage were determined by Hoechst staining. The density of cells 4×10^5 cells/well was grown and treated with IC_{50} concentration of $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ nanoparticles followed by incubation for 24 h. After that, untreated and treated AGS cell line were stained with Hoechst 33258 dye and examined by a fluorescent microscope (Incell Analyser 2000, USA).

Statistical analysis

All steps were repeated three times and the results were reported as mean \pm SD. Data were analyzed using SPSS software. $P < 0.05$ was considered as the level of significance. For comparing the means of control and treated groups, one-way ANOVA test (University Park, USA) used.

Results and discussion

Gastric cancer is one of the most common cancers in modern societies with high mortality rates (Shen et al. 2016). Nowadays, scientists are investigating the effects of different types of nanoparticles on cancer cells with the goal of inhibiting cancer cell growth and treating cancer. Metal nanoparticles have unique properties, including optical and magnetic properties and electrical activity (Riddhipratim et al. 2018; Mandal et al. 2005). Iron, nickel and cobalt nanoparticles were considered in medical biotechnology because of their magnetic properties and new studies have been done to identify their properties. These nanoparticles have been used in the drug delivery and treatment of hyperthermic cancer (Pankhurst et al. 2003). Binding of nanoparticles to the plasma membrane and their uptake by the cell are required for their toxicity process (Papis et al. 2007b). In the present study, we synthesized $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ NPs complex to find novel anticancer compounds and the results of FTIR, XRD, SEM and EDX tests showed that the nanoparticle complex was successfully synthesized. A schematic representation for the fabrication procedure of $\text{Co}_3\text{O}_4@\text{Glu}$ and $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ NPs is indicated in Scheme 1.

The FTIR spectrum results showed that the produced materials were correctly synthesized (Fig. 1a, b). Figure 1a shows the FT-IR spectrum for the $\text{Co}_3\text{O}_4@\text{Glu}$ NPs. The peaks of 3632 cm^{-1} and 2929 cm^{-1} are related to the N–H vibrations and the C–H stretching band. Figure 1b shows the FT-IR spectrum for the $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ NPs. There is a band in area 1340 cm^{-1} that belongs to the C–N functional group. The C=O amide strong band was formed at region 1611 cm^{-1} confirmed that thiosemicarbazide was stabilized on cobalt oxide with glutamic acid. The band between cobalt, glutamic acid and thiosemicarbazide is amide.

The crystal structure of $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ NPs was investigated using the X-ray power diffraction (XRD) method (Fig. 1c). The XRD patterns of the samples were recorded from $2\theta = 10^\circ$ – 95° . The XRD sharp peaks at 2θ values of 85.34° , 82.77° , 81.04° , 72.96° , 68.48° , 60.39° , 44.33° , 37.92° , and 22.07° might be attributed to the structure of $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ nanoparticle.

SEM imaging was performed to investigate the morphology and size of $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ NPs. These nanoparticles were between 16.66 and 40.39 nm (Fig. 2a, b).

Scheme 1 A schematic representation of the synthesis procedure of Co@Glu and Co@Glu/TSC NPs is illustrated

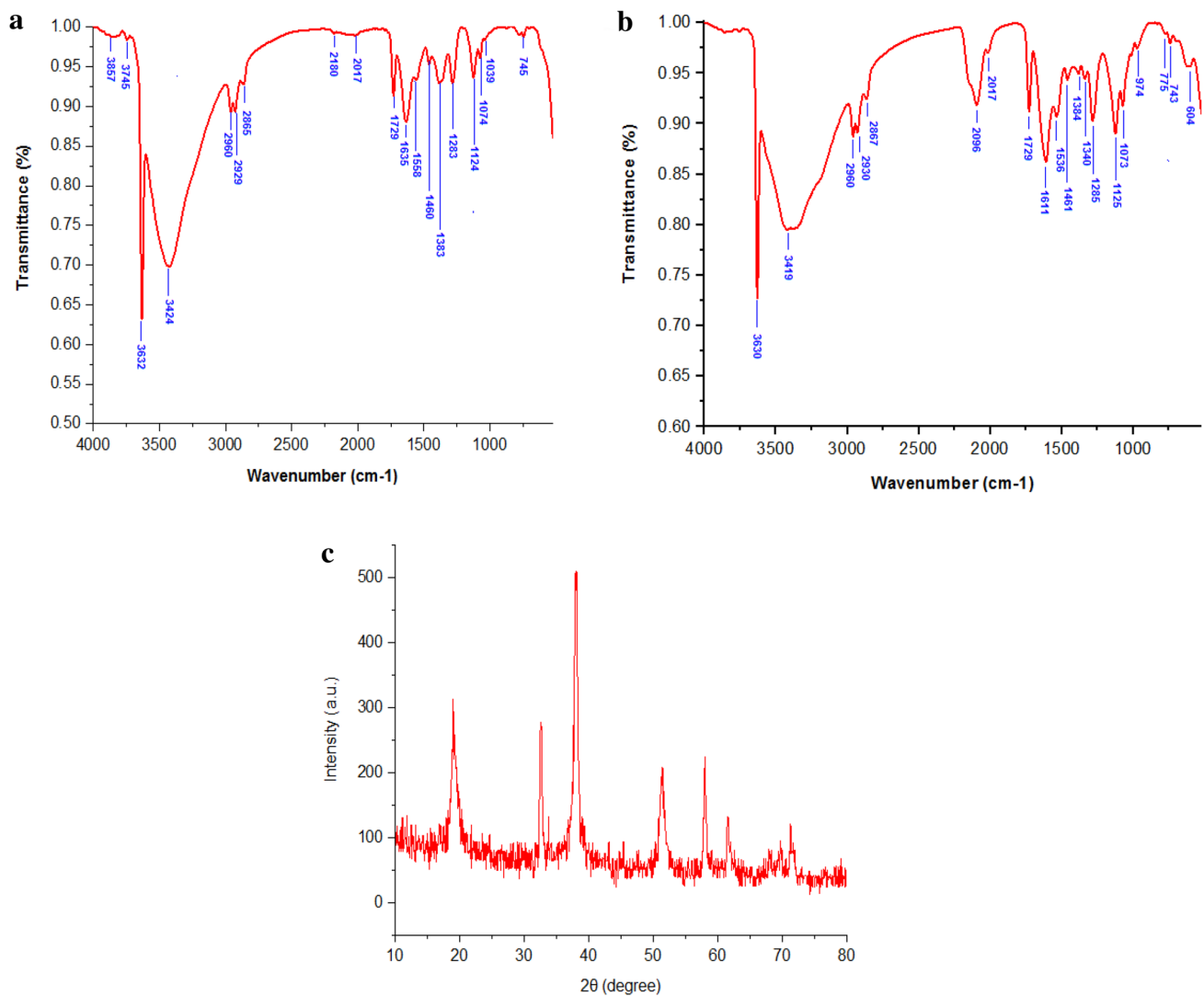
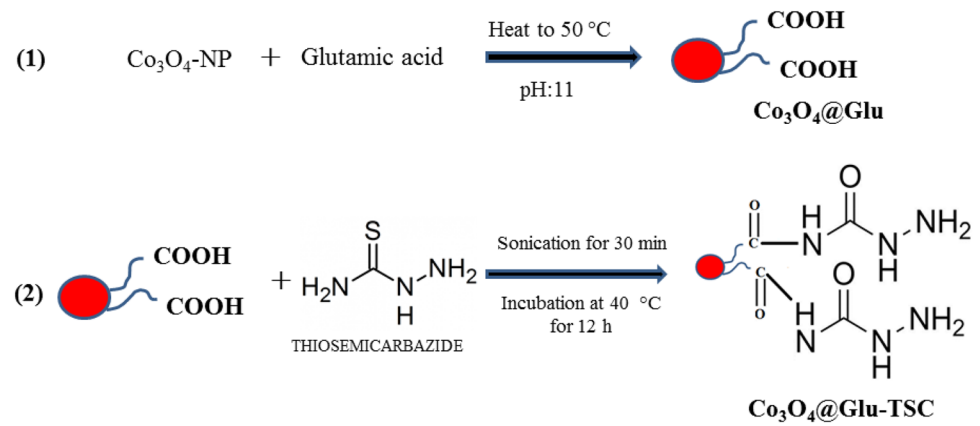


Fig. 1 FT-IR spectra of **a** $\text{Co}_3\text{O}_4\text{@Glu}$ and **b** $\text{Co}_3\text{O}_4\text{@Glu/TSC}$ nanoparticles. The FTIR spectrum result showed that the surface adsorption of functional groups on nanoparticles. The XRD patterns of the synthesized $\text{Co}_3\text{O}_4\text{@Glu/TSC}$ NPs (**c**). In the XRD spectrum, the

peaks at 2θ values of 85.34° , 82.77° , 81.04° , 72.96° , 68.48° , 60.39° , 44.33° , 37.92° , and 22.07° belong to the crystal structure of $\text{Co}_3\text{O}_4\text{@Glu/TSC}$ NPs

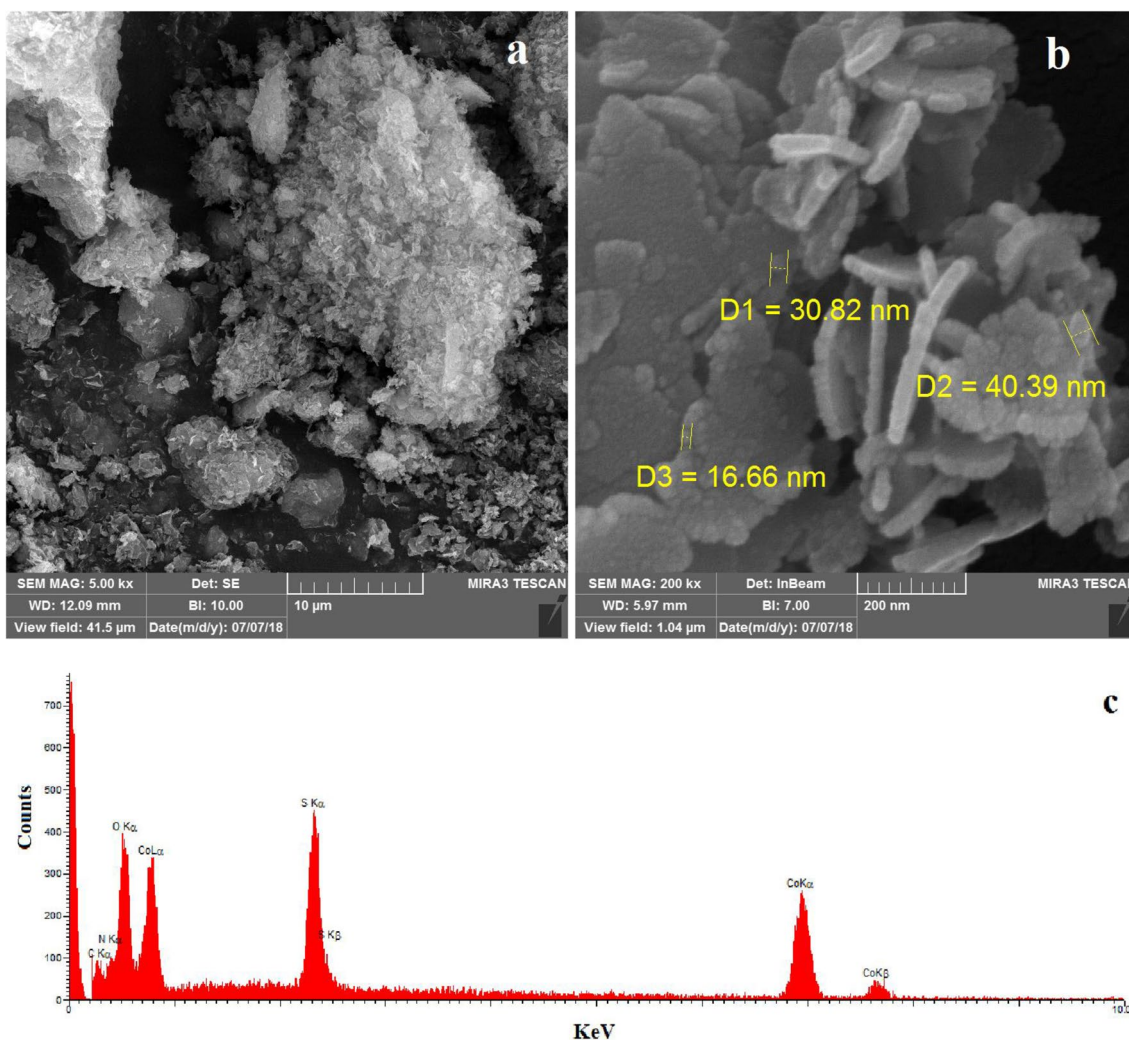


Fig. 2 SEM **a** low and **b** high magnification images of Co@Glu/TSC NPs. These images show that the NPs sizes are between 16.66 and 40.39 nm. The EDX spectrum of Co₃O₄@Glu/TSC NPs (**c**). The

presence of C, N, O, S and Co, as the major constituents, was confirmed by EDX analysis

Elemental composition of Co₃O₄@Glu/TSC-NPs EDX spectra was performed by EDX spectroscopy. The results showed the presence of cobalt, oxygen, nitrogen and sulfur in Co₃O₄@Glu/TSC NPs (Table 1). These elements correspond to the synthesized Co₃O₄@Glu/TSC NPs complex. In the spectrum, each peak represents an element (Fig. 2c). A higher altitude of peak means the greater concentration of the element in the sample.

Subsequently, studies were performed to identify the properties and effects of cobalt oxide nanoparticles on cancer cell lines. For example, Co₃O₄ NPs have toxicity properties and affect on cell viability in hepatocytes (HepG2) and endothelial cells (ECV-304) with depending on concentration and time (Papis et al. 2007b).

Thiosemicarbazide is Schiff based ligands which have gained importance over the decades as potential drug

Table 1 Results of EDS analysis of Co₃O₄@Glu/TSC

Elements	K	Kr	Weight%	Atomic%	ZAF
C	0.0663	0.0323	13.49	22.43	0.2396
N	0.1012	0.0494	18.27	26.03	0.2703
O	0.1646	0.0803	28.69	35.80	0.2798
S	0.1444	0.0704	8.23	5.13	0.8550
Co	0.5235	0.2552	31.31	10.61	0.8150
Total	1.0000	0.4876	100.00	100.00	

The percentage of C, N, O, S and Co in the final product (Co₃O₄@Glu/TSC) is displayed

candidates. When coordinated to metals, they have proved as good anticancer agents. Studies have been performed to identify the function of a complex of thiosemicarbazide

and nanoparticles and to evaluate their effect on cancer cells. For example, the complex of palladium II (PdCl₂) and thiosemicarbazide has cytotoxic effects on the breast (MCF7) and lung (A594) cancer cell lines (Muralisankar et al. 2017). In a research, it was shown that the conjugated thiosemicarbazide with copper induced the apoptosis in HCT116 cells. The copper complex with thiosemicarbazide caused distinct DNA cleavage and Topo II α inhibition unlike that for copper alone. In vivo administration of copper complex with thiosemicarbazide significantly inhibited tumor growth in HCT116 xenografts in nude mice (Palanimuthu et al. 2013).

Anticancer effect of copper (II) complex with a derivative of thiosemicarbazide on colorectal and breast cancer cells was confirmed. Also this complex was a poison inhibitor for human topoisomerase II α (Sandhaus et al. 2016).

The interactions of Fe (II), Fe (III) and Ga (III) with thiosemicarbazone are currently undergoing phase II clinical trials as a chemotherapeutic antitumor agent (Enyedy et al. 2011).

In other study, the zinc (II) and Cd (II) complexes with thiosemicarbazone have been shown to exhibit specific cytotoxic activity against Pam-ras cancer cell line, probably due to cell killing by apoptosis (Sandhaus et al. 1999).

In the present study, MTT assays were performed to investigate cell viability, flow cytometry to investigate percent of apoptosis and necrosis, and caspase-3 activity assay for assessing the potential of Co₃O₄@Glu/TSC NPs in inducing the apoptosis in AGS cancer cell line. The MTT test results for AGS cells and normal fibroblast cells (L929) treated with 0, 62.5, 125, 250, 500 μ g/mL concentrations of Co₃O₄@Glu/TSC NPs are presented in Fig. 3a, b, respectively. The IC₅₀ for AGS cells was 107.5 μ g/mL and for normal cells 350 μ g/mL over 24 h. According to different IC₅₀ values of Co₃O₄@Glu/TSC NP on cancer and normal cell lines, seems that the lethal dose of Co₃O₄@Glu/TSC NP for AGS cancer cell line have a less toxic effect on the normal

cell line. Results for survival, compared with control samples, are presented as mean \pm SD ($P > 0.05$).

The concentration of 500 μ g/mL had a maximum inhibition of cell proliferation and viability compared to the control group. It can be concluded that Co₃O₄@Glu/TSC NPs decrease the viability of AGS cells by dose-dependent manner.

In the MTT assay, Co₃O₄@Glu/TSC NPs affected the viability of AGS cells. Cell viability decreased and apoptosis increased compared to the control group with increasing concentration of nanoparticles.

Flow cytometry with annexin V/PI staining was performed to investigate the occurrence of apoptosis and necrosis in AGS cells treated with Co₃O₄@Glu/TSC NPs compared to untreated cells. Nuclear fragmentation and cancer cell death by nanoparticles are shown in Fig. 4a, b. Data analysis was divided into four regions using software. Q1 region showed necrotic cells, Q2 indicate late apoptotic cells. Q3 showed early apoptotic cells and Q4 region showed healthy cells. Herein, dot plots of flow cytometry indicate 68.7% and 8.85% increases in early and late apoptotic AGS cell line treated with Co₃O₄@Glu/TSC NPs, respectively.

To investigate caspase-3 activity, AGS cells were treated with Co₃O₄@Glu/TSC nanoparticles for 24 h. Caspases play an important role in programmed cell death and apoptosis. Caspase-3 activity test results showed that Co₃O₄@Glu/TSC NPs induced apoptosis by increasing caspase 3 activity compared to the cells of the control group and led cancer cells to death (Fig. 5). Caspase-3 test results on tumor cells treated with silver nanoparticles showed that this nanoparticle was able to inhibit the proliferation of MCF-7 cancer cells (Shandiz et al. 2018). Flow cytometry is a very powerful method for investigating nuclear changes and the occurrence of cell apoptosis. In this study, flow cytometry results showed that the nanoparticles caused the fragmentation of the nucleus in cancer cells and played a key role in apoptosis. In accordance to results of this study, Mahey et al.,

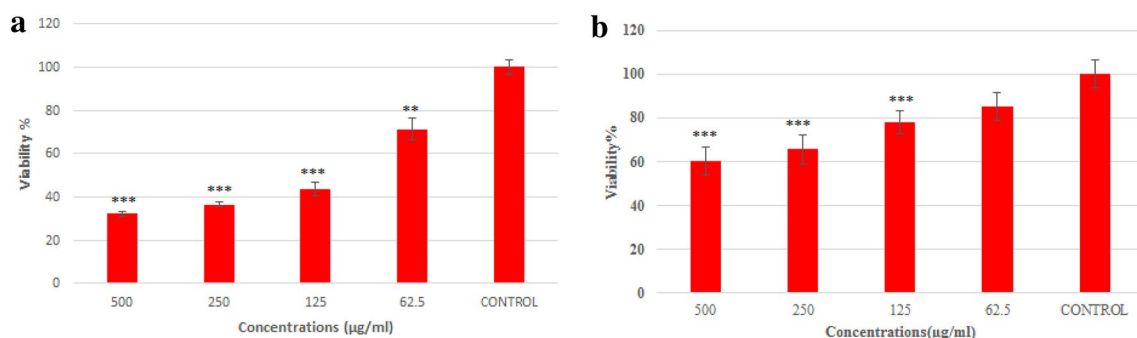


Fig. 3 The survival rate of gastric cancer (a) and normal fibroblast cells lines (b) against serial concentrations of Co@Glu/TSC NPs after 24 h. In concentration of 500 μ g/mL, the percentage of cell viability was lowest in AGS and normal cells. In addition, Co₃O₄@Glu/

TSC NPs decreased the viability of cells by dose-dependent manner. The IC₅₀ for AGS cells is 107.5 μ g/mL and for normal cells 350 μ g/mL. The results are reported compared with control samples ($n = 3$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

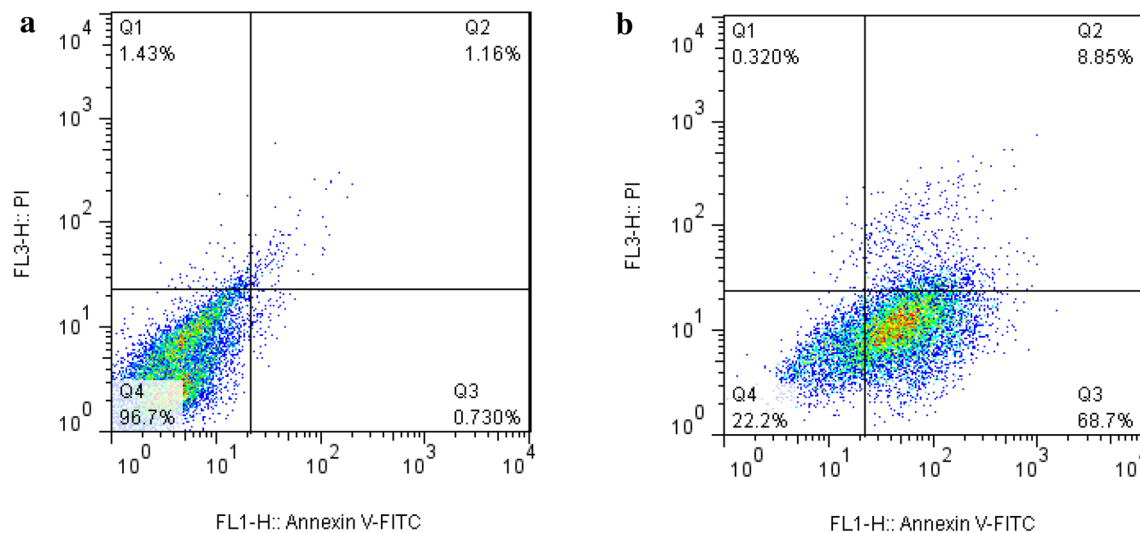


Fig. 4 Detection of apoptosis in AGS cells by flow cytometry. **a** Untreated cells and **b** cells treated with Co_3O_4 @Glu/TSC NPs. Dot plots of flow cytometry indicate 68.7% and 8.85% increases in early and late apoptosis in AGS cell line, respectively

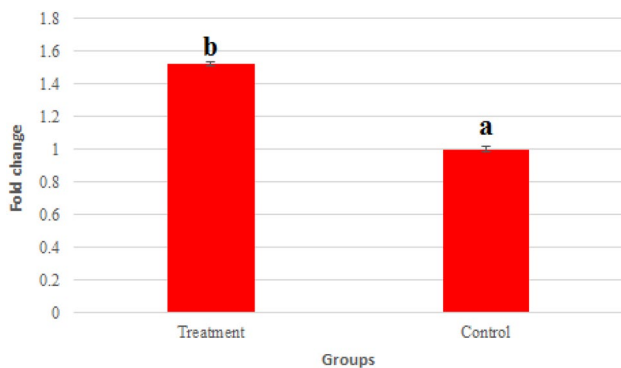


Fig. 5 Caspase-3 activity in cells treated with Co_3O_4 @Glu/TSC NPs and untreated cells (control group). Different letters for each column shows a significant difference (P value < 0.05)

checked out the anti-proliferative and apoptosis-inducing mechanism of three different forms of cobalt i.e., cobaltous ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), macro-Co (II, III) oxide and nano-Co (II, III) oxide for anti-proliferative activity in PC-3 (prostate cancer) cell line. Annexin V/propidium iodide staining confirmed the apoptotic mode of cell death in PC-3 cells and also a dose-dependent increase in caspase activity was observed in PC-3 cells treated with $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (Mahey et al. 2016).

In a research, cobalt-containing cobalamin was capable of killing leukemia cells (HL-60). Also this complex exhibited nano-molar toxicity towards breast, brain, and melanoma cancer cells, however, the IC₅₀ values were tenfold higher than free colchicine (Munteanu and Suntharalingam 2015).

Thamilarasan et al. (2016) showed that the Cobalt (III) complexes including $[\text{Co}(\text{acetylacetonate})(2,2'\text{-bipyridine})(\text{N}_3)_2 \cdot \text{H}_2\text{O}]$ and $[\text{Co}(\text{acetylacetonate})(\text{ethylenediamine})(\text{N}_3)_2]$ had in vitro cytotoxicity on breast cancer cell line (MCF-7)

and suggested that these complexes have the potential to act as effective anticancer drugs.

In another study, cobalt oxide nanoparticles coated with Phosphonomethyl iminodiacetic acid (PMIDA-CoO) increased reactive oxygen species (ROS) in leukemia cells (Jurkat, K562 and KG1A). PMIDA-CoO caused DNA damage and eventually induced apoptosis in leukemic cells by increasing inflammatory cytokines (Chattopadhyay et al. 2015).

Hoechst 33258 staining was explained to evaluate the occurrence of nuclear fragmentation in AGS cells treated with Co_3O_4 @Glu/TSC NPs compared to untreated cells. After exposure to Co_3O_4 @Glu/TSC NPs, it can be indicated that the cell apoptotic morphological changes involved in apoptotic bodies and clumping were observed owing to cell death by Co_3O_4 @Glu/TSC nanoparticles treatment, While, the untreated cells were viable and healthy (Fig. 6).

Conclusion

Results of previous studies have shown that metal nanoparticles with magnetic and fast cellular uptake properties are successful in medical biotechnology and cancer therapy. Separately, each of the Co_3O_4 , thiosemicarbazide and glutamic acid has a little anticancer effect. We synthesized the Co_3O_4 @Glu/TSC nanoparticles complex and investigated its effect on AGS cells and found that this complex inhibits the growth of AGS cancer cells and induces apoptosis in them with their anticancer activity.

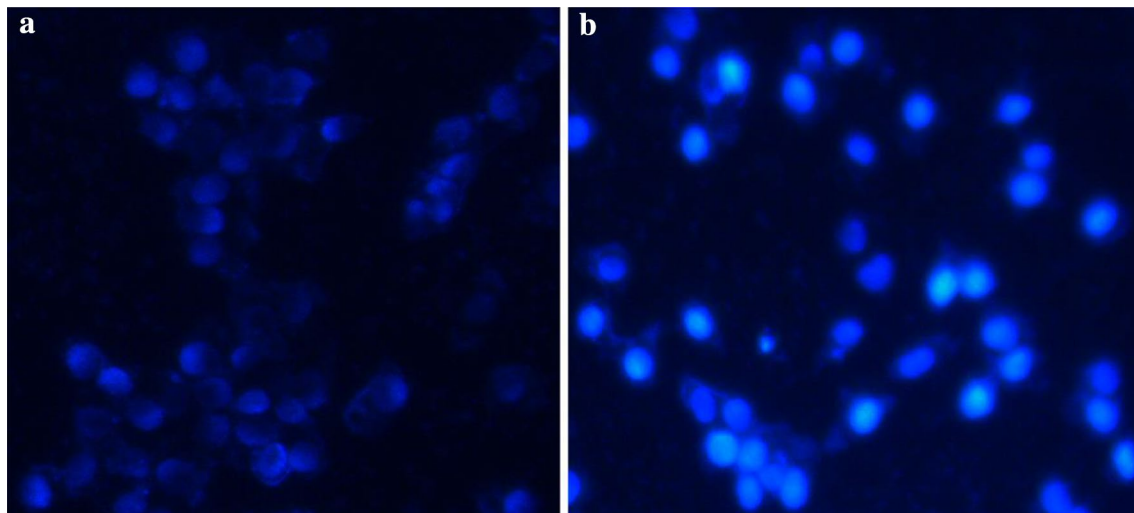


Fig. 6 Fluorescence images of AGS cells which were treated with 107 $\mu\text{g}/\text{ml}$ of $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$ NPs and recognized with Hoechst 33258 staining. **a** Control, **b** the cells treated with $\text{Co}_3\text{O}_4@\text{Glu}/\text{TSC}$

NPs. Control cells indicate normal morphology but treated cells show condensed chromatin and brightly stain

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical statements This article does not contain any studies with human participants or animals performed by any of the authors.

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