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Assessment of the effect of $CoFe_2O_4$@Ag synthesized by *Spirulina platensis* on colon cancer cell line and evaluation the expression of *BAX* and *Bcl-2* genes

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ABSTRACT

Colorectal cancer is a common cancer in both men and women. Colorectal cancer is the third widely diagnosed cancer in the world. The emergence of nanotechnology led the researchers to introduce novel nano-products for cancer diagnosis and treatment. Treating colon cancer has difficulties associated with the inefficiency of adjuvant chemotherapy and the development of metastatic disease. In this work, $CoFe_2O_4$@Ag nanocomposite was synthesized using the *Spirulina platensis* extract and characterized by FT-IR, XRD, SEM, TEM, DLS, Zeta potential, and EDS-mapping analyses. Then, the effect of the nanocomposite on the survival, apoptosis, and expression of the *BAX* and *Bcl-2* genes in colon cancer cells was investigated. The spherical nanocomposite particles were synthesized properly with a size range of 20-40 nm, the zeta potential of -42 mV, and hydrodynamic size of 250 nm. The 50% inhibitory concentration (IC_{50}) of the nanocomposite for normal and colon cancer cells was measured 256 and 65 µg/mL, respectively, indicating the higher susceptibility of cancer cells. Further, treating colon cancer cells with the nanocomposite significantly increased the frequency of apoptotic cells from 4.47 to 65.6%. In addition, the *BAX* and *Bcl-2* genes were up-regulated by 2.7 and 1.25 folds, respectively. According to the results, the $CoFe_2O_4$@Ag could considerably inhibit colon cancer cells through the activation of the pro-apoptotic signaling pathways.

Camera Ready

Introduction

Colon cancer accounts for 6% of the new cancer cases and cancer-associated mortality (Sung et al. 2021). This disease is considered the 6th most prevalent cancer, worldwide and finding novel and efficient chemotherapy agents to combat colon tumors has been the aim of many studies. In early stages, the treatment of colon cancer with efficient

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drugs could reduce the risk of micrometastasis disease and thus, could increase the survival rate of the disease (Fotheringham et al. 2019).

The emergence of nanotechnology resulted in the introduction of a large number of products to be used in biomedical applications, including disease diagnosis and treatment. Metal nanoparticles (NPs) have been extensively studied for their anticancer potential. However, due to the low efficacy and considerable adverse effects, many nanotechnology products fail to reach clinical applications (Awasthi et al. 2018). To overcome these limitations, complex nanostructures with both therapeutic and drug delivery compounds could be prepared.

The preparation of magnetic NPs could facilitate site-directed drug delivery via magnetic forces (Avval et al. 2020). Drug delivery using magnetic nanoparticles could minimize side effects and provide better control for drug delivery to the target site (Tietze et al. 2015). Among magnetic compounds, Iron oxide has been extensively used in biomedical applications, due to its low toxicity and good magnetic features. Cobalt is a chemical element with considerable toxicity for living organisms. However, the conjugation of Co atoms to Iron oxide NPs showed controlled intrinsic cobalt toxicity to be used in cancer treatment (Balakrishnan et al. 2020).

Silver is well-known metal with considerable pharmaceutical features that could be applied in cancer chemotherapy. The anticancer activity of silver atoms is mainly related to the generation of free oxygen radicals and direct interaction with cell components (Guo et al. 2019). Therefore, the conjugation of silver atoms to metal NPs may increase their anticancer potential and provide a more efficient nanocomposite.

Green synthesis of NPs using biological materials is an inexpensive and environmentally friendly approach. *Spirulina platensis* is a blue-green alga with considerable nutritional and medicinal properties. Green synthesis of several NPs by *S. platensis* has been extensively reported (Mahdieh et al. 2012, Govindaraju et al. 2008). Therefore, in the current work, we aimed to fabricate $CoFe_2O_4$@Ag nanocomposite using *S. platensis* and evaluate its anticancer effect on colon cancer cells. Besides, the effect of $CoFe_2O_4$@Ag nanocomposite on the expression of the *BAX* and *Bcl-2* genes was investigated.

Materials and Methods:

Preparation of *S. platensis* extract

At first, 0.5 g of algal dried powder was suspended in 25 mL of deionized water and maintained in a 55 °C water bath for 20 min. Next, the suspension was centrifuged for 10 min (6000 g) and the supernatant was collected and stored at 4 °C.

Synthesis of $CoFe_2O_4$

First, 0.1 g of $CoCl_2.4H_2O$ and 0.4 of $FeCl_3.6H_2O$ were dissolved in 100 mL distilled water. The mixture was heated at 80 °C for 60 min. Then, 10-20 mL of 6M NaOH solution was added and the resulting $CoFe_2O_4$ particles were collected. The NP was washed several times using distilled water and ethanol and finally, dried at 100 °C for 60 min.

Synthesis of $CoFe_2O_4$@Ag

To prepare $CoFe_2O_4$@Ag, 5 mg of $CoFe_2O_4$ was suspended in 100 mL distilled water and 50 mg of $AgNO_3$ was added to the reaction vessel. The mixture was kept at a 55 °C water bath for 45 min with continuous shaking. Then, 20 mL of *S. platensis extract* was added to reduce the silver ions. The mixture was shaken for 24h at 25 °C and the resulting $CoFe_2O_4$@Ag was collected, rinsed several times, and dried (Salehzadeh et al. 2019).

Physicochemical features of the nanocomposite

FT-IR analysis was used to evaluate the functional groups of $CoFe_2O_4$ and $CoFe_2O_4$@Ag nanocomposites using the Perkin Elmer spectrum 100 FT-IR spectrophotometer. The XRD was performed to evaluate the crystalline structure of the $CoFe_2O_4$@Ag (Co-Ka X-radiation, k = 1.79 Å). The size and morphology of the nanocomposite were characterized using the Scanning (TESKAN MIRA 3) and transmission (Zeiss-EM900) electron microscopy. The zeta potential and hydrodynamic size of $CoFe_2O_4$@Ag were measured by a zeta sizer device (Malvern Instruments Ltd, Ver. 6.32) and EDS-mapping was used to evaluate the elemental composition of the nanocomposite.

MTT assay

The cytotoxic activity of $CoFe_2O_4$@Ag for SW480 (colon cancer cells) and HEK-293 (normal human embryonic kidney cells) was studied by the 2-(4,5-dimethythiazol-2-yl) -2,5-diphenyltetrazolium

bromide (MTT) assay, according to the method described by Hosseinkhah et al. (Hosseinkhah et al. 2022). In brief, monolayers of the cells with an approximate density of 1×10^4 cell/well were prepared and treated with a gradient concentration of the nanocomposite (12.5 – 400 µg/mL). After incubation at 37 °C for 24 h, 200 µL of 0.5 mg/mL MTT solution was added to each well. The plate was incubated for an additional 4 h and then, the medium was aspirated and 200 µL DMSO (dimethyl sulfoxide) was added. The plate was shaken for 30 min and then, the optical absorbance (at 590 nm) of each well was measured. The wells that did not receive the nanocomposite were considered control wells. The following formula was used to calculate the 50% inhibitory concentration (IC_{50}) of the nanocomposite (Hosseinkhah et al. 2022):

$$\boldsymbol{Inhibition}\ (\%) = \frac{\boldsymbol{Abs\ of\ control - Abs\ of\ Test}}{\boldsymbol{Abs\ of\ control}} \times \mathbf{100}$$

Flow cytometry Analysis

To investigate the effect of $CoFe_2O_4$@Ag on the frequency of apoptotic cells, a monolayer of colon cancer cell line was prepared and treated with the nanocomposite (at IC_{50} concentration). The control cells were not exposed to the nanocomposite. After incubation for 24 h, the cells were harvested and washed with phosphate-buffered saline (PBS). Then, the cells were subjected to the propidium iodide and Annexin V (Roche, Germany) staining. Finally, the frequency of apoptotic cells was analyzed using a flow cytometry device ((BioRad, USA).

RNA extraction, cDNA synthesis, and quantitative PCR

To evaluate the effect of $CoFe_2O_4$@Ag on the expression of the *BAX* and *Bcl-2* genes, the colon cancer cells were treated with the nanocomposite for 24 h, and then were harvested and subjected to the total RNA extraction using the TriZol reagent (Sigma-Aldrich), according to the manufacturer's instruction. The extracted RNA was treated with DNase I to remove DNA impurities and then, complementary DNA (cDNA) was synthesized by a cDNA synthesis kit (Yekta Tajhiz, Iran). The relative expression of the *BAX* and *Bcl-2* genes in nanocomposite treated and control cells were studied by quantitative polymerase chain reaction (qPCR) assay. The GAPDH was used as internal control genes and the assay was performed using an SYBR Green qPCR kit (Yekta Tajhiz, Iran). The gene amplification was performed using the primers that were presented in Table 1 and interpretation of the results was performed based on the method reported by Pfaffl (Pfaffl 2001).

Statistical analysis

The SPSS. 16.0 was used for the analysis of the results. Significant differences were evaluated by the *one-way ANOVA* and the p-values less than 0.05 were considered statistically significant.

Results:

Physicochemical characteristics of $CoFe_2O_4$@A

The functional groups of $CoFe_2O_4$ and $CoFe_2O_4@A_g$ were compared by FT-IR analysis. The spectrogram of $CoFe_2O_4$ reveals a broad peak at 3443 cm^{-1} that is associated with the stretching vibration of the O-H bond and a peak at 1634 cm^{-1} that is related to the bending vibration of the H-O-H bond. Also, the metal oxide (M-O) bonds at their tetragonal phases caused two peaks at 481 and 571 cm^{-1}. The FT-IR spectrogram of $CoFe_2O_4$@Ag showed a similar pattern. However, upon the conjugation to the Ag atom, the stretching vibration of the O-H bond and bending vibration of the H-O-H bond shifted to 3436 and 1639 cm^{-1}. Also, the stretching vibration of the metal oxide (M-O) bonds shifted to 478 and 595 cm^{-1}. The FT-IR results confirm the proper synthesis of the $CoFe_2O_4$@Ag nanocomposite (Hajializadeh et al. 2022) (Fig. 1).

The XRD analysis was performed to evaluate the crystalline structure of the $CoFe_2O_4$@Ag nanocomposite. According to Fig. 2, the peaks at 2θ of 31.19, 36.56, and 44.29 correspond to (220), (311), and (400) plans of cubic $CoFe_2O_4$ (JCPDS card 073-1701). Also, the peaks at 2θ of 33.11, 43.27, 64.20, 75.47 corresponding to (111), (200), (220), and (311) contribute to the Ag atom (Abbasian and Shafiee Afarani, 2019).

The morphology and size of the prepared $CoFe_2O_4$@Ag nanocomposite were investigated by SEM and TEM. We found that the synthesized nanocomposite was spherical and with low agglomeration. The particles were in the size range of 20-40 nm. Fig. 3 displays the SEM and TEM images of the nanocomposite.

The DLS analysis showed that the average hydrodynamic size of the $CoFe_2O_4$@Ag in an aqueous medium was 250 nm and particles had a proper particle size distribution (Fig. 4). Moreover,

the zeta potential of the nanocomposite was measured -42 mV which indicates proper repulsive force between the particles to avoid particle agglomeration (Fig. 5).

EDS-mapping was performed to analyze the elemental composition of the synthesized nanocomposite. The results showed that the nanocomposite particles contained only Co, Fe, O, and Ag and no elemental impurity was observed (Fig. 6).

Cytotoxicity of $CoFe_2O_4$@Ag for colon cancer and normal human cells

The MTT assay was performed to determine the toxic concentrations of $CoFe_2O_4$@Ag for colon cancer and normal human cells. Our results showed that at the concentration of 50µg/mL and above, the nanocomposite was highly toxic for the colon cancer cells. The highest toxicity was observed at the 400 µg/mL that decreased the relative cell viability to almost 10% and the IC_{50} was calculated 65µg/mL. In contrast, $CoFe_2O_4$@Ag was less toxic for normal human cells with IC_{50} of 256 µg/mL (Fig. 7).

Flow cytometry Analysis

A flow cytometry analysis was performed on nanocomposite treated and control cancer cells to determine the effect of the $CoFe_2O_4$@Ag on the frequency of apoptosis genes. According to the results, treating with the nanocomposite increased the population of apoptotic cells from 4.47 to 65.6 %. In other words, the frequency of the cells with primary and late apoptosis in nanocomposite treated cells was recorded 52.2 and 13.4%, respectively. Also, only 2.84 and 1.63 % of control cells showed primary and late apoptosis, respectively. The results were presented in Fig. 8.

Relative gene expression

The relative expression of the *BAX* and *Bcl-2* genes in $CoFe_2O_4$@Ag treated and control cells were investigated. We found that treating the SW480 cells with the nanocomposite increased the expression of the Bax gene by 2.7 folds, compared with the control cells. Also, the relative expression of *Bcl-2* was increased by 1.25 folds, following the exposure of the cells to the nanocomposite. The ratio of BAX to Bcl-2 expression was equal to 2.16. Fig. 9 presents the relative expression of the studied genes.

Discussion:

Management of stage II colon cancer faces several difficulties. At this stage of the disease, adjuvant chemotherapy aims to eradicate micrometastatic disease to prevent the development of distant metastatic disease (Varghese 2015). However, there are challenges in the management of this disease and about one-third of colorectal cancer cases do not benefit from the current anticancer drugs (Fotheringham et al. 2019). The emergence of nanotechnology provided a novel field in the development of cancer diagnosis and chemotherapy. The preparation of nanotechnology products containing therapeutic and drug delivery compounds could provide more efficient and accurate therapeutic approaches to combat cancers, including colon cancer. Therefore, in the current study, we aimed to synthesize and characterize $CoFe_2O_4$@Ag nanocomposite using a blue-green alga, *S. platensis*, and to investigate the effect of the synthesized nanocomposite on the viability and apoptosis of colon cancer cells.

Physicochemical characterization of the nanocomposite revealed that the particles were properly synthesized and conjugated with Ag atoms. The nanocomposite particles were in the nano-scale size range, with low agglomeration level and without elemental impurities.

Based on the results from the MTT assay, we found that $CoFe_2O_4$@Ag has a considerably higher inhibitory effect on cancer cells than normal human ones. Higher susceptibility of target cancer cells than normal ones is considered a fundamental feature of any anticancer candidate (Double and Bibby 1989). According to the results, it could be predicted that $CoFe_2O_4$@Ag may have a large therapeutic index (the ratio between the toxic and therapeutic doses of a drug), however, further investigations are required. The cytotoxicity of metal NPs is mainly associated with the generation of radical oxygen species (ROS) and oxidative stress that could damage cell structures and thus, inhibit normal cellular functions, including replication of nucleic acids, cell division, and metabolic pathways (Alamer et al. 2021, Ullah et al. 2021). Cancer cells have a normally higher metabolic rate and nutrient demand than healthy cells which result in the higher cell permeability and internalization of extracellular compounds into the cytoplasm (Kettler et al. 2014). The higher susceptibility of the colon cancer cells than normal ones, which was observed in our work, could be

related to the higher permeation of the nanocomposite particles into the cancer cells. Upon the internalization of the particles, the generation of oxidative stress could damage cell components and interrupt cell functions.

Evaluation of the frequency of apoptotic cells in colon cancer and normal cells showed that the exposure to the nanocomposite significantly increased cell apoptosis from 4.47 to 65.6 %. Cell cycle arrest and triggering apoptosis pathways are the major consequences of the damage to cell structures, especially DNA molecules, and blockage of DNA replication (Roos and Kaina 2006).

Following the occurrence of DNA damage and cell cycle arrest, the pro-apoptotic factors including BAX protein are induced by the action of the p53 protein (Roos and Kaina 2006). Our study showed that the transcription level of the *BAX* gene was considerably increased following the exposure of colon cancer cells to the $CoFe_2O_4$@Ag nanocomposite. The activation of Bax results in the increased permeabilization of the mitochondrial outer membrane and subsequently, the initiation of the intrinsic apoptotic signaling pathways (Brentnall et al. 2013). In addition, we found that the expression of the *Bcl-2* gene was slightly increased following the treatment of colon cancer cells with the nanocomposite. The Bcl-2 protein is a pro-survival member of the BCL-2 family which inhibits the oligomerization of BAX and BAK proteins and results in apoptosis inhibition (Czabotar et al. 2014). The *BAX*/*Bcl-2* ratio corresponds with the initiation of the pro-apoptosis or pro-survival pathways in mammalian cells (Perlman et al. 1999). Although the exposure to $CoFe_2O_4$@Ag increased the expression of the *Bcl-2* gene, the higher up-regulation of the *Bax* gene than the *Bcl-2*, could favor the activation of the pro-apoptotic signaling pathways in nanocomposite treated cells.

Conclusion:

In this work, $CoFe_2O_4$@Ag nanocomposite was synthesized using the *S. platensis* extract. Treating the SW480 and HEK293 cells with the nanocomposite indicated the higher susceptibility of the cancer cells to the nanocomposite. Also, $CoFe_2O_4$@Ag caused a significant apoptosis induction among the cancer cells which could be associated with the observed up-regulation of the pro-apoptotic *BAX* gene.

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Conflict of interest: The authors declare that they have no conflict of interest.

Data availability statements:

The datasets generated during the current study are available from the corresponding author on request.

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Table 1. Sequence of the primers used in this study

Primer	Sequence (5'-3')	Product size (bp)	Reference
BAX-forward	TTGCTTCAGGGTTTCATCCA	113	(Xu et al. 2012)
BAX-reverse	AGACACTCGCTCAGCTTCTTG		
Bcl-2-forward	TGGCCAGGGTCAGAGTTAAA	147	(Xu et al. 2012)
Bcl-2-reverse	TGGCCTCTCTTGCGGAGTA		
GAPDH-forward	CCCACTCCTCCACCTTTGAC	75	(Bigdeli et al. 2019)
GAPDH-reverse	CATACCAGGAAATGAGCTTGACAA		

Fig. 2. XRD pattern of $CoFe_2O_4$@Ag nanocomposite

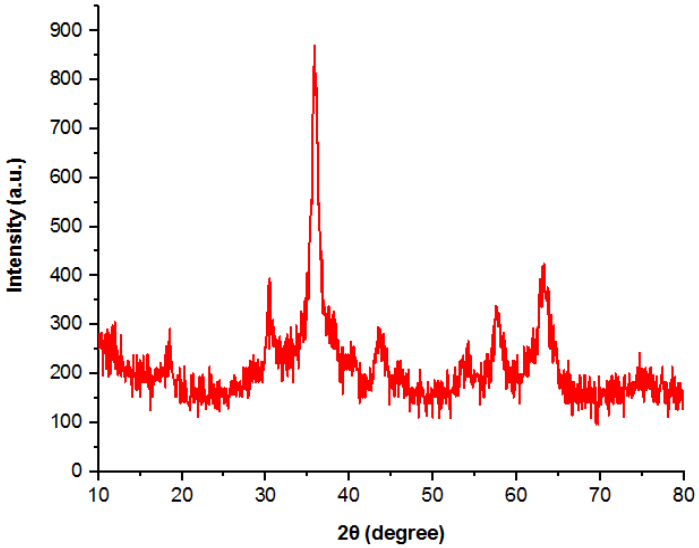
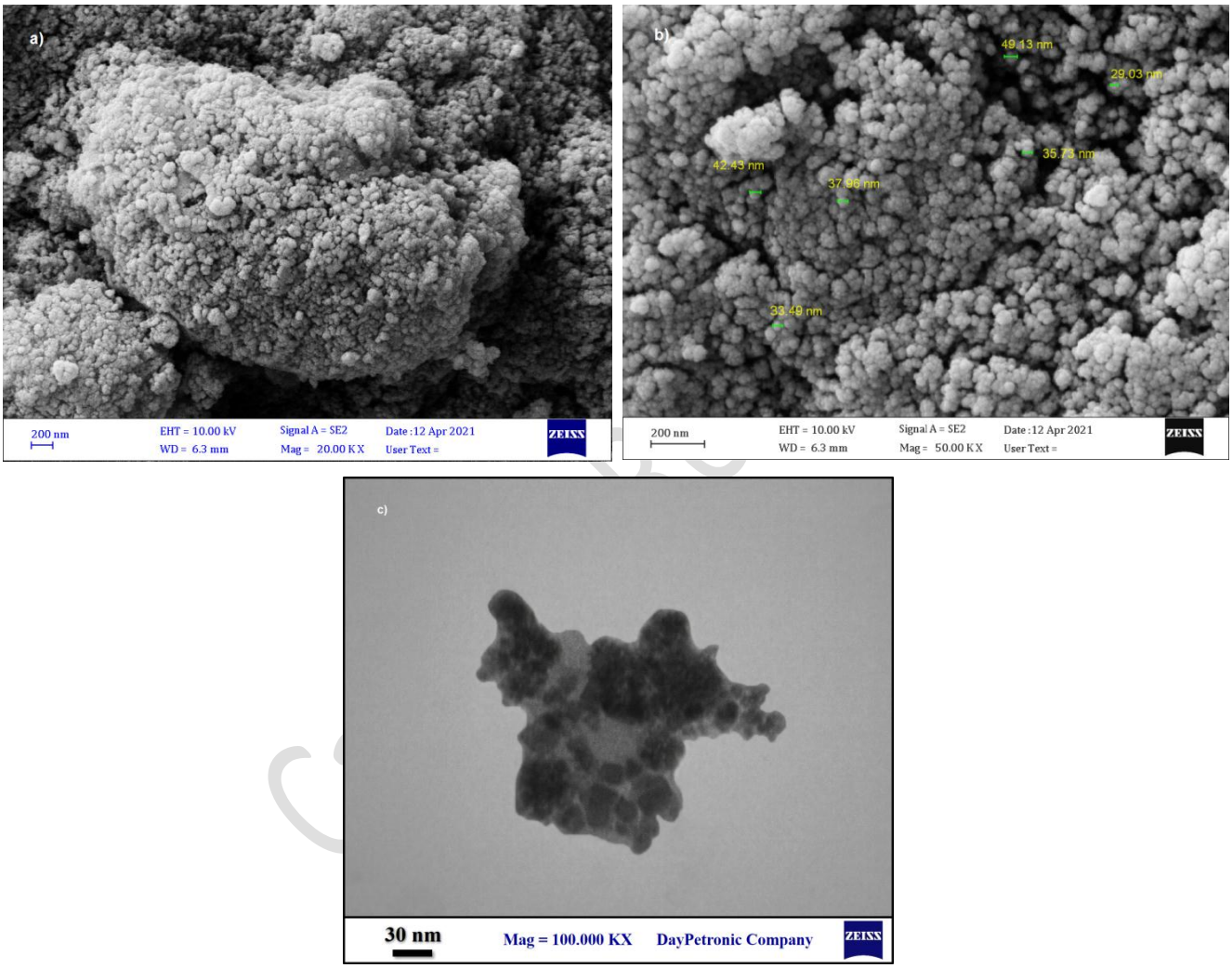


Fig. 3. SEM (A and B), and TEM (C) images of CoFe2O4@Ag nanocomposite



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Fig. 4. DLS analysis of $CoFe_2O_4$@Ag nanocomposite

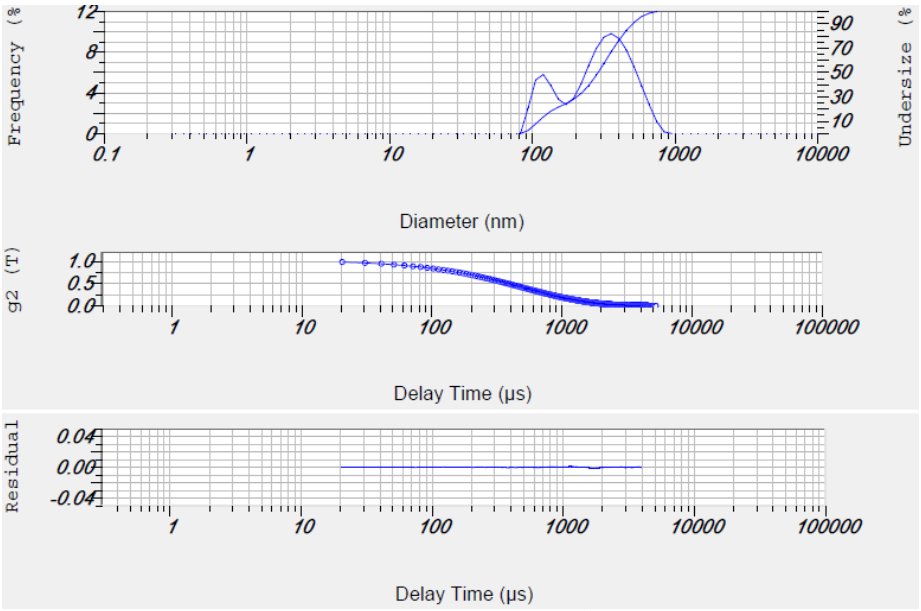


Fig. 5. Zeta potential of $CoFe_2O_4$@Ag nanocomposite

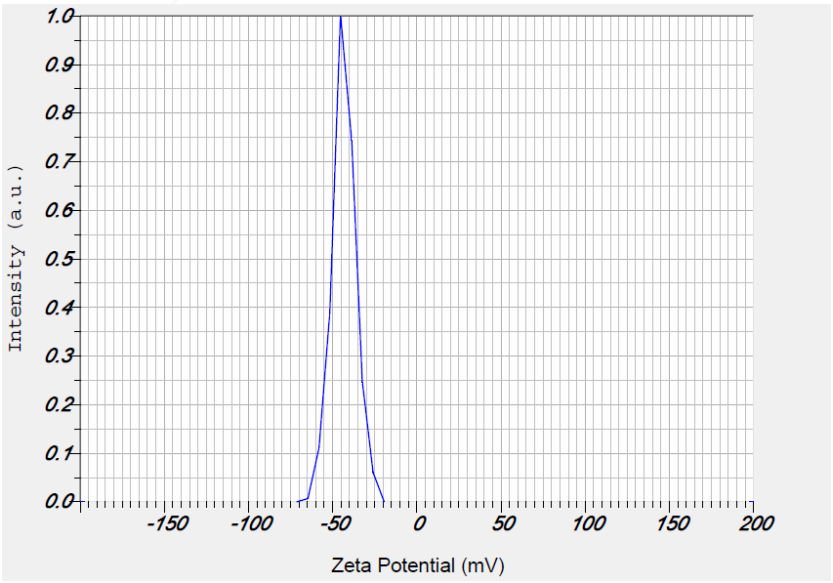


Fig. 6. EDS-mapping of $CoFe_2O_4$@Ag nanocomposite

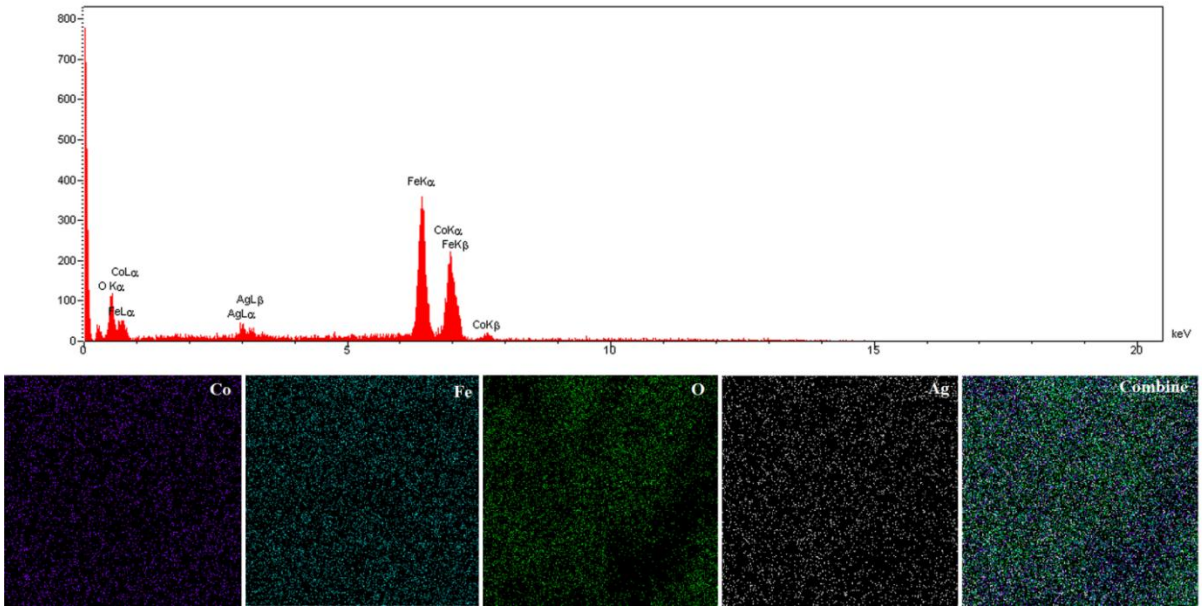
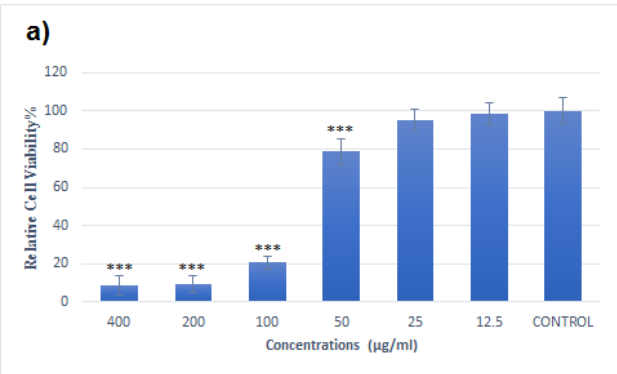


Fig. 7. Viability of SW480 (A) and HEK293 (B) cells after treating with $CoFe_2O_4$@Ag nanocomposite (the assay was performed in three replicates, $p<0.05$)



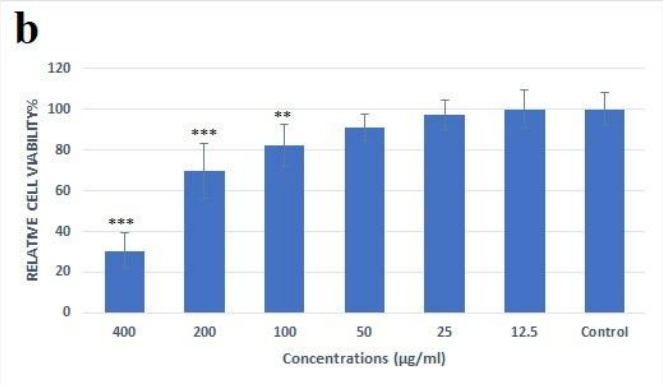


Fig. 8. Flow cytometry analysis of A) control, and B) $CoFe_2O_4$@Ag treated cells. (Q1: Necrotic cells, Q2: Late apoptosis, Q3: Early apoptosis, Q4: Live cells)

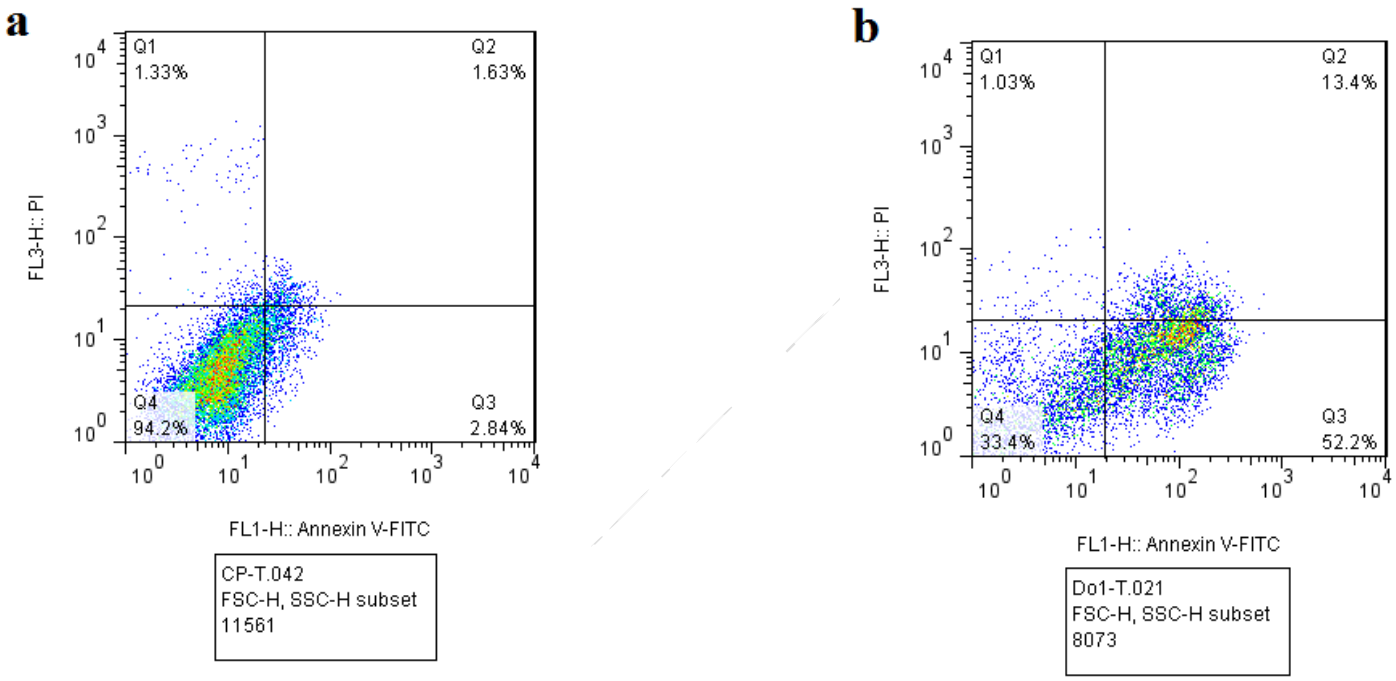


Fig. 9. The expression of the *BAX* and *Bcl-2* genes in control and $CoFe_2O_4$@Ag treated cells (the assay was performed in three replicates, $p<0.05$)

