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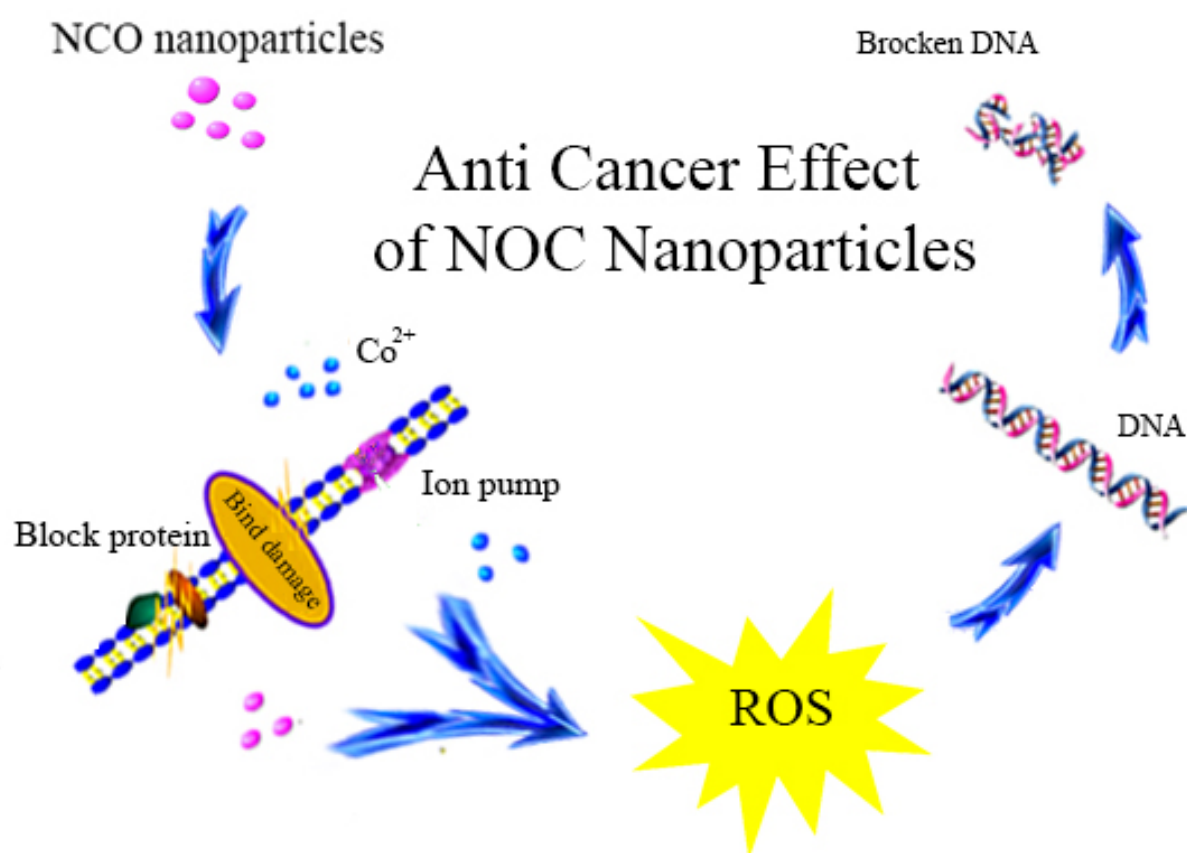
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Effective induction of death in breast cancer cells with magnetite NiCo₂O₄/NiO nanocomposite

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Abstract

With the objective of finding an avenue for development of magnetic nanomaterials which were incorporated into cells for induction of cell death in human cancerous cell lines, an environmental friendly synthesis method was investigated. The present work aimed to synthesis magnetic NiCo₂O₄/NiO nanoparticles by a novel, simple, environmental friendly and cost effective method in the presence of *Dactylopius Coccus*. The ultra-fine NiCo₂O₄/NiO ceramic nanocomposites (NNC) were characterized by X-ray diffraction pattern (XRD), scanning electron microscope (SEM), transmission electron microscopy (TEM), energy dispersive spectrometry (EDS) as well as vibrating sample magnetometer (VSM) used to study the magnetic properties of NNC nanoparticles. MTT cytotoxicity assay performed to determine the anti-proliferative effect of NNC nanoparticles against 4T1 breast cancer cell lines. The results demonstrated that the NNC nanoparticles meaningfully reduced the survival percentage of the 4T1 cells of the murine invasive breast cancer with an IC₅₀ was of 4 mg/ml. The dominant mechanism of cell death was apoptosis, along with an increase in cellular oxidative stress. Consequently, based on the obtained results, it can be concluded that NiCo₂O₄/NiO nanoparticles can potentially be used as a chemotherapeutic agent against invasive breast cancer cells.

Keywords: NiCo₂O₄/NiO; Nanoparticles; Green synthesis; Nanocomposite; MTT cytotoxicity.

1. Introduction

Cancer is considered as one of the most extremely life-menacing diseases with systematic widespread global incidence which results in a high rate of death or lasting disabilities in patients [1]. Several toxicological studies indicate that the nanoparticles could also be the reason of some opposing effects. Ex vivo toxicity of these particles commonly are investigated in numerous cell lines by MTT assay to provide some hints about the hazards effect of these carriers on cellular [2]. Nanotechnology is the industrial revolution fourth wave, the huge phenomenon that has spread throughout the scientific community, that is developing quickly as possible [3,4]. Nanoparticles with different structure, size, shape and chemical surface properties can indicate diverse biological and biomedical applications [5,6]. By reducing the nanoparticles size many changes in electrically, magnetic, structural, morphological and chemical properties will happen [7,8]. Since nanomaterials are presently used in paints, varnishes, insulation, electronic diodes, cosmetics, and sunscreens, therefore evaluation of products is important [9-12]. One of the most important factor for nanoscale designed materials is targeted cancer cells. Some inorganic nanoparticles due to their high level of surface-to-volume ratio have antibacterial properties, small size and good penetration into microorganisms [13]. Also, having photocatalytic, ionic and catalytic properties caused them to be widely used in the combat against pathogen microbe's human, bacteria, viruses and fungi [14-16]. magnetite nanoparticles (MNPs), are promising targeted materials within the biomedical field and have applications in drug delivery systems, magnetic resonance imaging (MRI), and magnetic hyperthermia [17, 18]. Researchers indicated that, ZnO nanoparticles which release Zn^{2+} causes the Schwann cells death [19]. Zn^{2+} is a dynamic component of proteins and enzymes, and is an ionic signal between numerous intracellular organelles [20]. However, excess free Zn^{2+} is cytotoxic and can induce serious neuronal injury [21-23]. ZnO nanoparticles have been revealed to exhibit special toxicity toward cancerous cells (HL60) in comparison with normal cells [24]. Numerous studies established that toxicity of nano-sized ZnO was encouraged by the generation of reactive oxygen species (ROS). Rendering to the close relationship between the physicochemical

properties of ZnO nanoparticles and their toxicity[25], in the present work the ability of Ni ions on cell death was studied. Investigations on the protective mechanism of metal oxide nanoparticles is increasing nowadays. Metal oxide nanoparticles have the ability of producing toxic elements in human tissues and cell cultures that rise oxidative stress that leads to cell death [26]. Large nanoparticles are absorbed by mitochondria and nuclei, producing a DNA mutation and finally cell death. For determining the toxicity of nanoparticles, some factors like size and dimensions, chemical composition, shape, surface structure, density and solubility play key role [27, 28]. Since cancer cells have negative zeta potential due to a high percentage of anionic phospholipids as well as specific groups on their surface, Positive charge nanoparticles via electrostatic interaction absorb into target cells [15, 29]. Though chemotherapy is frequently used nowadays, but in many cases owing to its resistance to the body, it may cause the patient death [30]. Therefore, many attempts were done to discover anticancer drugs having more toxic effects [31, 32]. In this study, NiCo₂O₄/NiO nanoparticles were synthesized for the first time, and their effects on the 4T1 cell lines were investigated.

2. Experimental

2.1. Materials and physical measurement

All the chemical and reagents for the synthesis of NiCo₂O₄/NiO nanocomposites such as Ni(NO₃)₂·5H₂O, Co(NO₃)₂·6H₂O were purchased from Merck Company and used without any purification. X-ray powder diffraction (XRD) patterns were recorded by a Philips diffractometer using X'PertPro and the monochromatized Cu K α radiation ($\lambda = 1.54 \text{ \AA}$). Fourier transform infrared (FTIR) spectra of the products were obtained utilizing KBr pellets on an FT-IR spectrometer (Magna-IR, 550 Nicolet) in the 400–4000 cm⁻¹ range. The energy dispersive spectrometry (EDS) analysis was carried out by Tescan mira3 microscope. FESEM images were investigated using a Tescan mira3 and Hitachi S-4160 field emission scanning electron microscopes (FESEM). Transmission electron microscopy (TEM) image was obtained on a Philips EM208 transmission electron

microscope with an accelerating voltage of 200 kV. The magnetic properties of the sample was detected at room temperature using a vibrating sample magnetometer (VSM, Meghnatis Kavir Kashan Co., Kashan, Iran). BSA (fraction V, minimum 98%) was purchased from Sigma (Steinheim, Germany). RPMI cell culture and fetal bovine serum (FBS) were purchased from Gibco (United States). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCFH-DA), penicillin, and streptomycin were all obtained from Sigma (United Kingdom), and dimethyl sulfoxide (DMSO) was purchased from Merck (Germany). 7-AAD, Annexin-V dyes, and annexin-V binding buffer were all purchased from BD Biosciences (San Jose, CA). All organic solvents used for TEM were received from VWR, Norway. 4,6-Diamidino-2-phenylindole (DAPI) was purchased from Invitrogen Life Technologies (Eugene, OR, USA).

2.2. *Coccus powder*

Coccus pigment was obtained from the dried bodies of female scale insect species, *Dactylopius Coccus*, which feed on wild cacti. The insects were dried at 70 °C in an oven until attainment to a constant weight, finally grounded and stored before use.

2.3. *Synthesis NiCo₂O₄/NiO nanocomposite*

Magnetite NNC nanoparticles (MNPs) with a diameter of ~10 nm were synthesized using the following method. To synthesis the nanocomposite 1 mmol Ni(NO₃)₂.5H₂O and 2 mmol Co(NO₃)₂.6H₂O dissolved in distilled water to form a clear solution. Definite amount of coccus powder added to this solution under strong magnetic stirring at room temperature. The solution was put under ultrasonic waves (power = 80 W/cm²) for 30 min. The aqueous solution heated at 80 °C for 30 min under stirring. Finally, the product was collected by centrifugation at 4000 rpm for 5 min, washed repeatedly with distilled water and ethanol several times, dried at 80 °C under vacuum for 2 h and formerly calcined at 600 °C for 3 h. The conditions for the synthesis of NiCo₂O₄/NiO nanocomposite have been illustrated in Table 1.

2.4. Cell culture

The National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran) were kindly supplied the murine mammary carcinoma 4T1. The 4T1 tumor designated due to its appropriateness for experimental animal model for human mammary cancer and transplantability to BALB/c mice. These cells are highly tumorigenic, invasive and transplantable tumor cell line.

2.5. Cytotoxicity assay

MTT-based assay for the investigation of cytotoxicity effect of the NNC nanoparticles was applied as follows:- 1×10^4 4T1 cells were seeded in a 100 μ l growth medium in 96-well plate and treated by increasing concentrations of NiCo₂O₄/NiO nanoparticles (0, 0.5, 1, 2, 4, 8, 16, 32 μ M). Following incubation of treated cells was achieved at 37 °C in 5% CO₂ for 24 h. The amount of the nanostructures causing 50% inhibition of proliferation (IC₅₀) was also calculated based on viability difference between untreated and treated cells by the following formula:

Cell viability was expressed as 100% for untreated cells (control). All samples were performed in triplicates and the survival rate (%) was calculated as the following equation:

$$\text{Survival rate (\%)} = (\text{OD in treatment group} / \text{OD in control group}) \times 100$$

2.6. Nuclear characteristic of 4T1

In order to investigate the morphological variations of the breast cancer cells, an acridine orange/ethidium bromide double staining following Current Protocols of Immunology was applied. Briefly, 5×10^5 cells/well were cultured in six-well plates, incubated overnight, treated with NNC nanoparticles at IC₅₀ concentration, harvested, and finally washed twice with PBS. Ethidium bromide/acridin orange solution added to the cell suspension for evaluating the nuclear morphology by fluorescence microscopy (Zeiss, Germany).

2.7. Determination of nuclear morphology by DAPI staining

For examination of the presence of apoptotic bodies besides nuclei morphology, DAPI nuclear staining were used. Briefly, 5×10^5 4T1 cells/well in 12-well plates exposed to NNC nanoparticles at IC_{50} concentration for 24 h. after that, cells were fixed in 4% paraformaldehyde-PBS solution for 15 min, then stained with DAPI (0.3 μ M) for 30 min at room temperature. In order to visualize nuclear morphology of the cells, fluorescence microscopy was used to understand the presence of condensed, fragmented, and finally degraded nuclei and apoptotic cells were recognized as well.

2.8. Cell apoptosis assay

For better understanding the apoptosis mechanism, Annexin-V staining was done to differentiate apoptosis from necrotic cell death caused by synthesized NNC nanoparticles. Annexin-V protein was used, attributable to high affinity of it toward phosphatidyl serine, which is translocated from the inner to the outer leaflet of the plasma membrane at early-stage of apoptosis. The key role is the conjugation of Annexin-V with the fluorescent probe PE, which facilitates the measurements by flow cytometric analysis. 7AAD staining can help to distinguish apoptosis and necrosis based on variances in permeability of 7AAD through the living and damaged cell membrane. Cell number, concentrations, and culture conditions hold similar to those used in the cell cycle analysis. NNC nanocomposites treated 4T1 CELLS harvested and washed twice in PBS. The manufacturer's instruction (eBioscience Annexin V Apoptosis Detection Kit PE, Catalog Number: 88- 8102) followed for staining: Firstly, cells were incubated in the dark for 15 min at room temperature in 200 μ l binding buffer containing Annexin V-PE (10 μ l) and 7AAD (5 μ l), 300 μ l binding buffer was added to each sample, and retained in an ice box. The 488-nm laser used for excitation with BD FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometry system. PE was detected in FL-2, while 7AAD was detected in FL-3. 1×10^4 cells were acquired and data analyzed using Flowjo version 7.6.1.

2.9. Cell cycle assay

For analyzing the cell cycle, Propidium iodide (PI) staining of DNA followed by flow cytometric measurement of the fluorescence applied. Nearly, 3.5×10^5 4T1 cells/ well were seeded in a six-well plate, incubated for 24 h, the NiCo₂O₄/NiO nanoparticles treatment (IC₅₀ concentration) for 24 was done, the medium was separated and stored. Cells were washed with PBS and trypsinized, harvested in the stored medium, centrifuged and finally, the pellet washed in PBS, fixed in ice-cold ethanol (70%), and stored at 4 °C for at least 12 h. Before doing the flow cytometry analysis, cells washed in PBS and stained with PI in RNase (40 µg ml⁻¹ PI and 100 µg ml⁻¹ RNase A) and incubated at 37 °C for 30 min, followed by incubation at 4 °C until analysis. Flow cytometry analysis performed using FACS Calibur at an excitation and emission wavelength of 488 nm and 610 nm respectively. Flowjo software used to analyze obtained data were using.

2.10. In vitro cellular uptake of NiCo₂O₄/NiO nanoparticles

1X10⁶ 4T1cells/ml were seeded in 6-well microplates and treated with NNC nanoparticles. After that, the cells washed with PBS, fixed by 2% glutaraldehyde, post-fixed in 1% osmium tetroxide and dehydrated in alcohol before embedding in Epon. Thin sections (80 nm) of resin embedded 4T1 cells were cut on a Leica Ultracut UCT and imaging was done by Philips transmission electron microscope CM100 (Philips, Eindhoven, Netherlands).

2.11. Intracellular ROS measurement

In order to measure the net intracellular levels of ROS generated by synthesized NNC nanocomposites, DCFH-DA that is a non-fluorescence dye used, which interact with intracellular ROS for generating fluorescent 2,7-dichlorofluorescein (DCF). Briefly, 3.5×10^5 cells/well seeded into 24-well plates and treated with IC₅₀ of NiCo₂O₄/NiO nanoparticles for 24 h. freshly prepared hydrogen peroxide (H₂O₂, 0.1 mM in PBS) was used as the positive control in the assay. The cells incubated with 10 µM DCFH-DA for 1 h after treatment, washed

twice with PBS, and finally suspended in 500 μ l PBS. The fluorescent intensity detected, the amount of ROS subsequently estimated from DCF production as well.

2.12. Statistical analysis

Statistical analysis of the ROS generation and apoptosis assay were performed using ANOVA and a post hoc two sample Student's t-test comparing fluorescent ratios to the control. To indicate significant differences defined by the following ranges: $p < 0.05$, was included in the figures.

3. Result and discussion

3.1. Characterization of $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles

XRD pattern of $\text{NiCo}_2\text{O}_4/\text{NiO}$ ceramic (NNC) nanoparticles is revealed in Fig.1 A (a, b). The different peaks observed in this pattern display cubic phase of NiO (JCPDS card no. 22-1189) and cubic phase of NiCo_2O_4 (JCPDS card no. 73-1702). Fig.1 a illustrates the XRD pattern of synthesized NNC nanoparticles in the presence of ultrasonic waves and Fig. 1 b demonstrates synthesized nanoparticles in the in absent of ultrasonic waves. It is obvious that, ultrasonic waves caused the reduction in the nanoparticles size and the peaks became wider. The narrow sharp peaks specify that the $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles were well crystallized and are in nano range size. The mean crystallite diameter of the NNC nanocomposites (sample no. 2) calculated using Debye Scherrer formula [8] was 10 and 9 nm for sample 1 and sample 2 to be respectively (Table 1).

To control the chemical purity and stoichiometry of NNC nanocomposites (sample 2), an EDS analysis was applied. Fig. 1 B displays the EDS pattern that confirmed the presence of Ni, Co and O elements, but there were no impurity peaks. For investigating the power ultrasonic effect and the calcination temperature on the morphology and size of nanoparticles, 4 samples in several dissimilar condition were synthesized. Various nanoparticles were gained and data summarized in Table 1. Fig. 1C represents the plot of magnetization versus applied field (M-H) at 300 K for NNC nanocomposites and the hysteric curve with nearly saturated nature at

high fields. VSM analysis indicates that, NNC nanocomposite has ferromagnetic behaviour. The saturation magnetization and coercivity are found 4.48 emu/g and 200 Orsted (Oe) respectively.

In Fig. 2 (a,b), the SEM images of NNC nanocomposites prepared in the present and absence of ultrasonic irradiation power, proved the key factor for the formation of nanoparticles. As shown in Fig. 2a, nanostructures were shaped in several shapes (nanoparticles, nanocubics and nanorods), Nonetheless, in the absent of ultrasonic irradiation there was not any separating factor to form small nanoparticles; consequently, the nanoparticles was agglomerated with the size of about 150-200 nm (Fig. 2b). To investigate the effect of temperature on the morphology and size of $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles, sample 3 and sample 4 prepared at 500 °C and 700 °C respectively as shown in Fig. 3 (a,b). Fig. 3a demonstrates the SEM images of $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles synthesized in presence of ultrasonic irradiation and calcined in 500 °C. An ultrafine porous NNC nanostructures were found. The nanoparticles size of is about 20 nm. This nanostructures can be an excellent candidate for desired application. The exact morphology and size of $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanostructures was illustrated by TEM images. Fig. 3c which contains homogenous ellipsoidal and spherical nanoparticles with about 30-40 nm.

3.2. Anticancer evaluation of $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles

The MTT cytotoxicity assay results presented that $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles were significantly inhibited the proliferation of breast cancer cell lines of 4T1 cells (Fig. 4). The cytotoxic effect of $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles against different cell types was dose-dependent with IC_{50} of 4 mg/ml.

3.3. Apoptosis induction

Acridine orange/ethidium bromide staining after cell treatment with IC_{50} values of NNC nanoparticles to evaluate the cell death mechanism. In order to characterize the apoptosis, nuclear changes and apoptotic body formation can be easily visualized using the stain. Fig. 5 (a,b), indicates the fluorescence microscopic images of apoptotic and control cells. In these images, orange spots nuclei are apoptotic cells, while green spots

demonstrates viable cells. In addition, treated cells with NNC nanoparticles presented a prolonged apoptosis comparative to the control cells. For observing nuclear morphology cells were stained by DAPI after treated with NNC nanoparticles (Fig. 5 (c,d)). The obtained results specified that the untreated cells showed homogeneous nuclei, whereas treated cells displayed fragmented nuclei. For further analyze of the cell death, flowcytometry apoptosis analysis was achieved (Fig. 6 (a,b)). The damage in cell phospholipid asymmetry, results in the externalization of phosphatidylserine (PS) bonds with the cells surface that is an initial marker of apoptosis. PE Annexin-V as a fluorescent-labeled Annexin-V dye binds very specifically to externalize PS ligands on the cells surface that leading to the finding of early apoptotic cells. Meanwhile, the externalization of PS happens in the earlier stages of apoptosis, PE Annexin-V staining can identify apoptosis based on nuclear variations, such as DNA fragmentation. 7-amino-actinomycin (7-AAD) is a valid dye to permit recognition of early apoptotic cells (7-AAD negative). Viable cells with complete membranes eliminate 7-AAD, while the membrane of dead and damaged cells are permeable to 7-AAD. Consequently, cells that are measured viable are PE Annexin-V and 7-AAD negative one; cells that are in early apoptosis are PE Annexin V positive and 7-AAD negative; in addition late apoptotic or already dead cells are both PE Annexin V and 7-AAD positive. Apoptosis degree of cells exposed to $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles was measured by PE Annexin V and 7-AAD staining using flow cytometry. Fig. 6 (a,b) emphasize that the cell death mechanism induced by NNC nanoparticles is apoptosis. Really, NNC nanoparticles induced 5% early apoptosis, 30% late apoptosis and 3% necrosis, after 24 h treatment by IC_{50} values. Base on the obtained result, more mortality was formed by apoptosis pathway (35%) and less portion of mortality was created by necrosis (3%). Therefore, synthesized $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles can be used as a powerful chemotherapeutic agent to prevent cancer cell growth in invasive breast cancer cell lines.

3.4. Effect of $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles on cell cycle

The effects of NNC nanoparticles on cell-cycle progression in 4T1 cells are presented in Fig. 6 (c,d). The cell cycle analysis, after treatment of cancer cell lines by $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles designated the drastic

modification of 4T1 cells. Based on cell cycle analysis, 32% of the 4T1 cells were in G1 phase, 56% in S phase, and 12% in G2 phase compared to the untreated control cells that indicated 58% of the cells in G1 phase, 32% in S phase, and 10% in G2 phase. An important decrease in the cells percentage stayed in the G1 phase and a significant increase in the cells percentage in the S phase was determined for treated cells. Additionally, a large population was in sub_G1 (4.13%), which is associated to apoptotic cell. This cell arrest in G2 phase by NiCo₂O₄/NiO nanoparticles treatment can be due to DNA damage after treatment.

3.5. Effect of NiCo₂O₄/NiO nanoparticles in cellular ROS

In order to know the effect of NiCo₂O₄/NiO nanoparticles on oxidative stress, H₂DCF-DA assay of ROS generation was applied. The creation of ROS leads to homeostasis distribution in the enzyme system of ROS scavenging antioxidants. As shown in Fig. 7, the ROS levels of treated cell, generated in response to NNC nanoparticles, were significantly higher than those of normal cells (control) and even higher than control positive. Moreover, ROS level indicated in dose-dependent manner. These results designated that cell death was mediated by ROS production, which may change the cellular redox status.

4. Conclusion

In the present research, for the first time, a novel biosynthetic source was employed for preparing the NiCo₂O₄/NiO nanoparticles. Uniform sub 10 nm magnetic nanostructures were prepared via sonochemical process followed thermal treatment method at 500 °C in the presence of cochineal dye, an environmental friendly precursor, as a green reducing and capping agent. The NiCo₂O₄/NiO nanoparticles against cancer cells, induced cell death in dose-dependent manner. There are some symptoms like increased permeability of treated cell toward acridine orange/ethidium bromide, nuclear variations toward fragmented nucleus production and quantitative data of flow cytometry that confirmed apoptosis is the cell death path. Synthesized NiCo₂O₄/NiO nanoparticles were also found to induce oxidative stress via ROS generation. Based on the obtained results, it

can be concluded that $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles are good candidate as chemotherapeutic agent against invasive breast cancer cells; however, additional pharmacological investigations are needed definitely.

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Figure captions:

Fig. 1. (A): XRD patterns of NiCo₂O₄/NiO nanoparticles: (a) sample no. 1, (b) sample no. 2, (B): EDS spectrum of representative NiCo₂O₄/NiO nanoparticles synthesized at 600 °C, for 3 h (sample no. 2) and (C): M–H hysteresis at room temperature for NiCo₂O₄/NiO nanoparticles.

Fig. 2. SEM images of NiCo₂O₄/NiO nanoparticles: (a) sample no. 1, (b) sample no. 2.

Fig. 3. SEM images of NiCo₂O₄/NiO nanoparticles: (a) sample no. 3, (b) sample no. 4 and TEM image of NiCo₂O₄/NiO nanoparticles (sample no.2) (c).

Fig. 4. Cell viability of 4T1 treated with of NiCo₂O₄/NiO nanoparticles (IC₅₀ of 4 mg/ml). The concentration of nanoparticles is depicted on the x-axis. First column is the untreated cells as negative control. Each column shows the mean value of three experiments. The error bars were calculated by dividing the standard deviation by the square root of the number of measurements.

Fig. 5. Fluorescent microscopy of 4T1 cells stained with acridine orange/ethidium bromide, (a) Un-treated cells, (b) Treated cell by NiCo₂O₄/NiO nanoparticles; Fluorescent microscopy of 4T1 cells with DAPI staining, (c) Un-treated cells, (d) Treated cell by NiCo₂O₄/NiO nanoparticles.

Fig. 6. Two-dimensional contour density plots for determination of fractions of early apoptotic, late apoptotic, and necrotic cell death of live non-treated 4T1 cells treated, respectively. (a) Un-treated cells. (b) Treated cell by NiCo₂O₄/NiO nanoparticles; Flow cytometry based assay of cell cycle of control. (c) Un-treated cells. (d) Treated cell by NiCo₂O₄/NiO nanoparticles.

Fig. 7. ROS generation in NiCo₂O₄/NiO nanoparticles.

Table 1. The reaction conditions of NiCo₂O₄/NiO nanoparticles.

Table 1

Sample number	Ultrasonic waves	Temperature (°C)	Grain size (nm)	Particle size (nm)	Particle shape
1	Yes	600	9	40-80	Nanoparticles, nanocubic and nanorod
2	No	600	10	20-200	agglomerated
3	Yes	500	-	20-25	Porous nanostructures
4	Yes	700	-	100-800	agglomerated

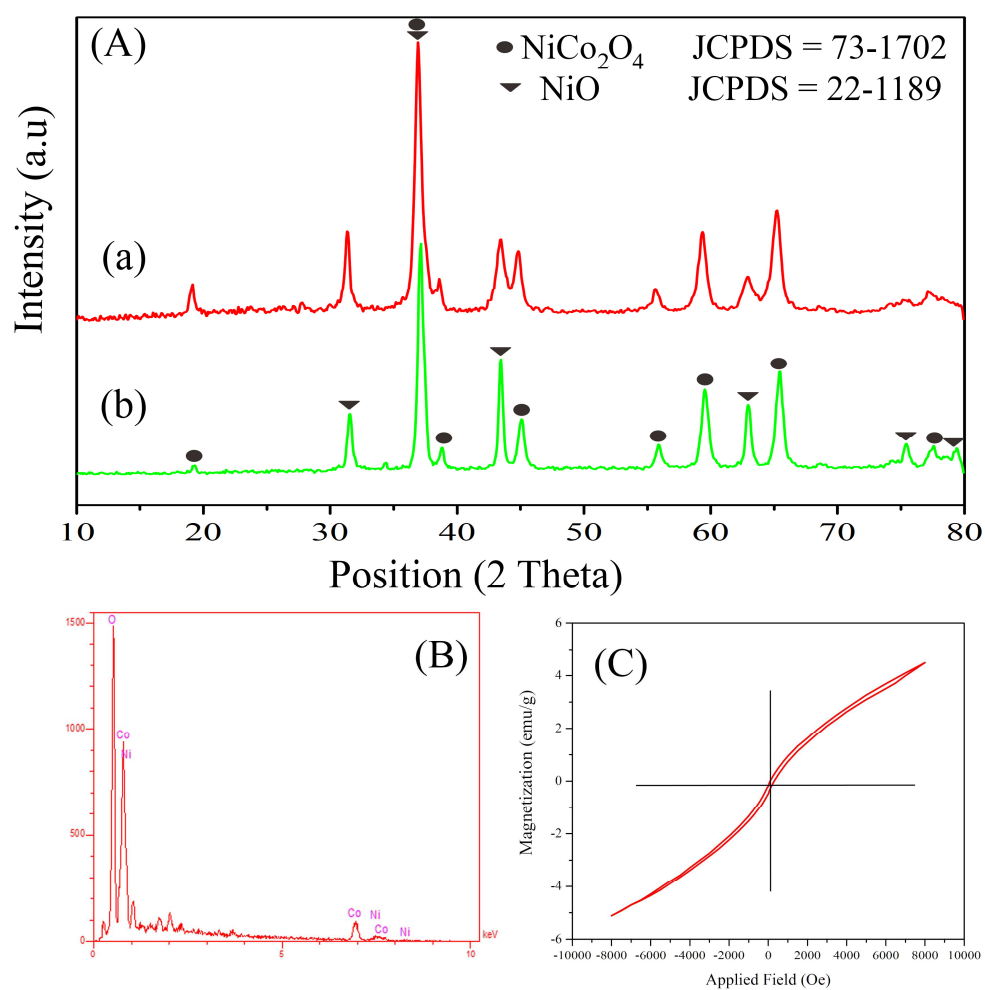


Fig. 1

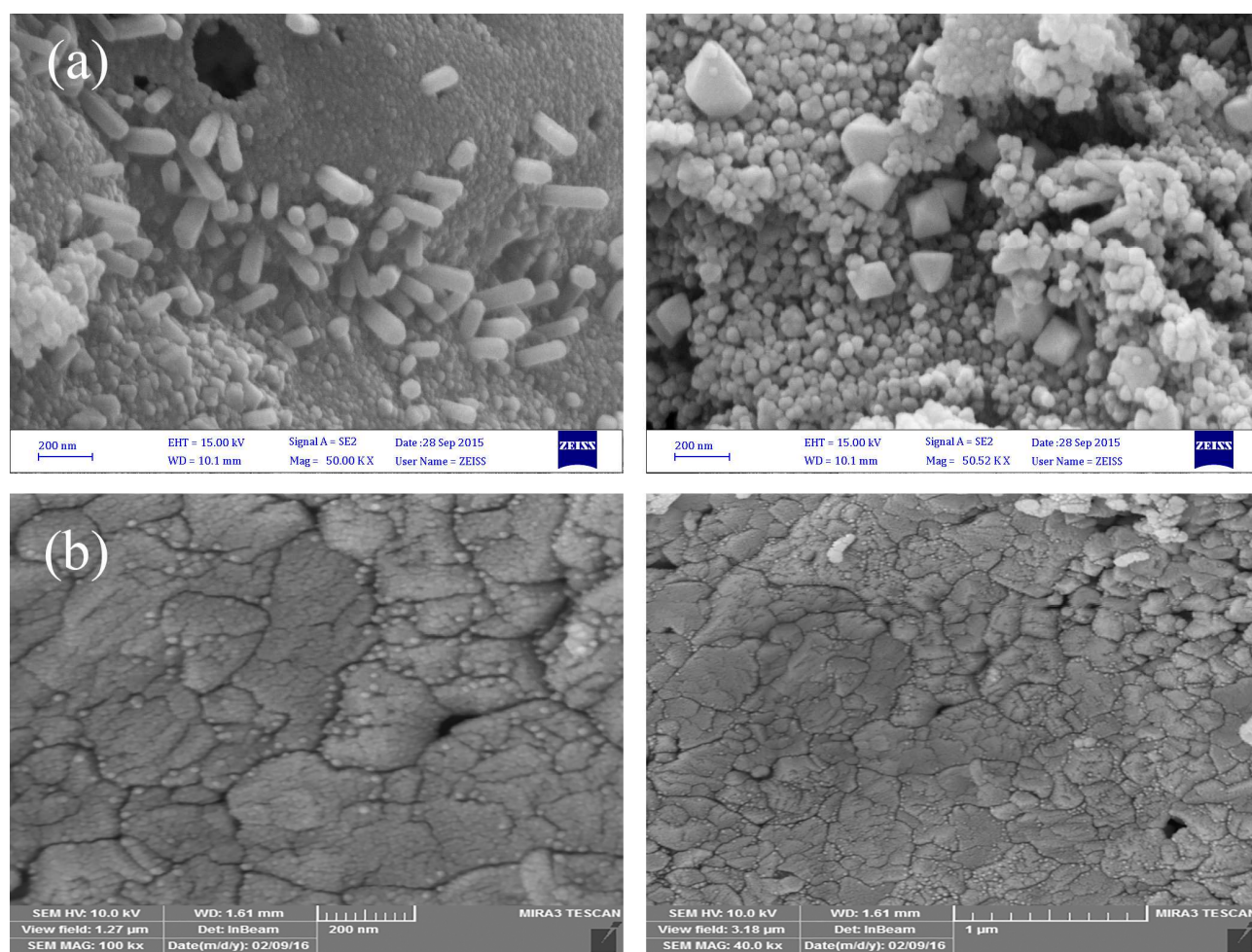


Fig. 2

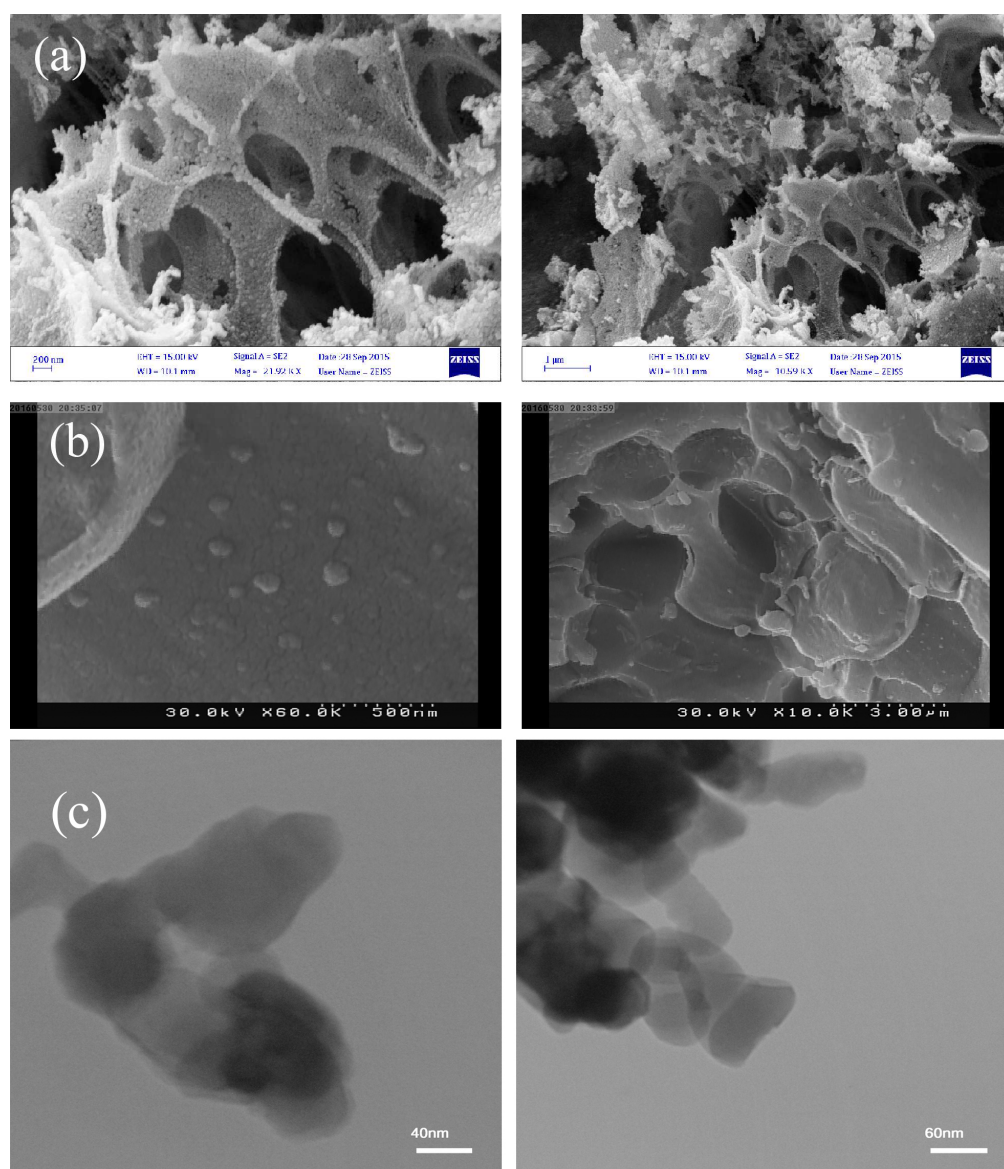


Fig. 3

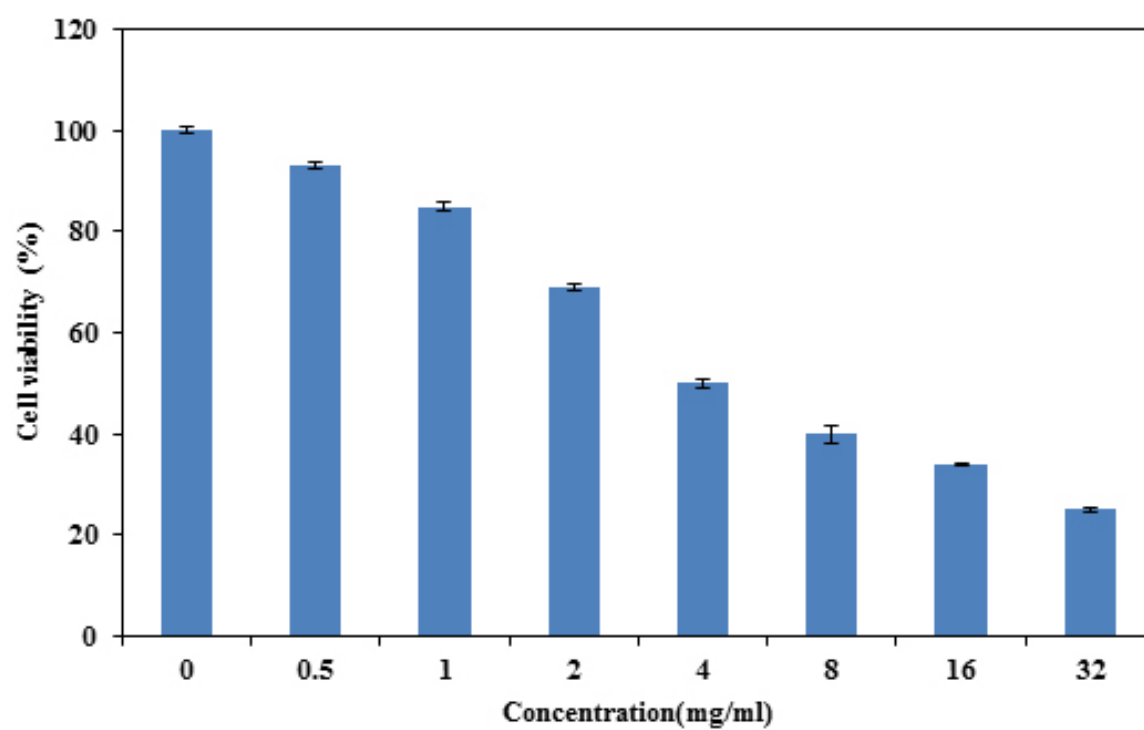


Fig. 4

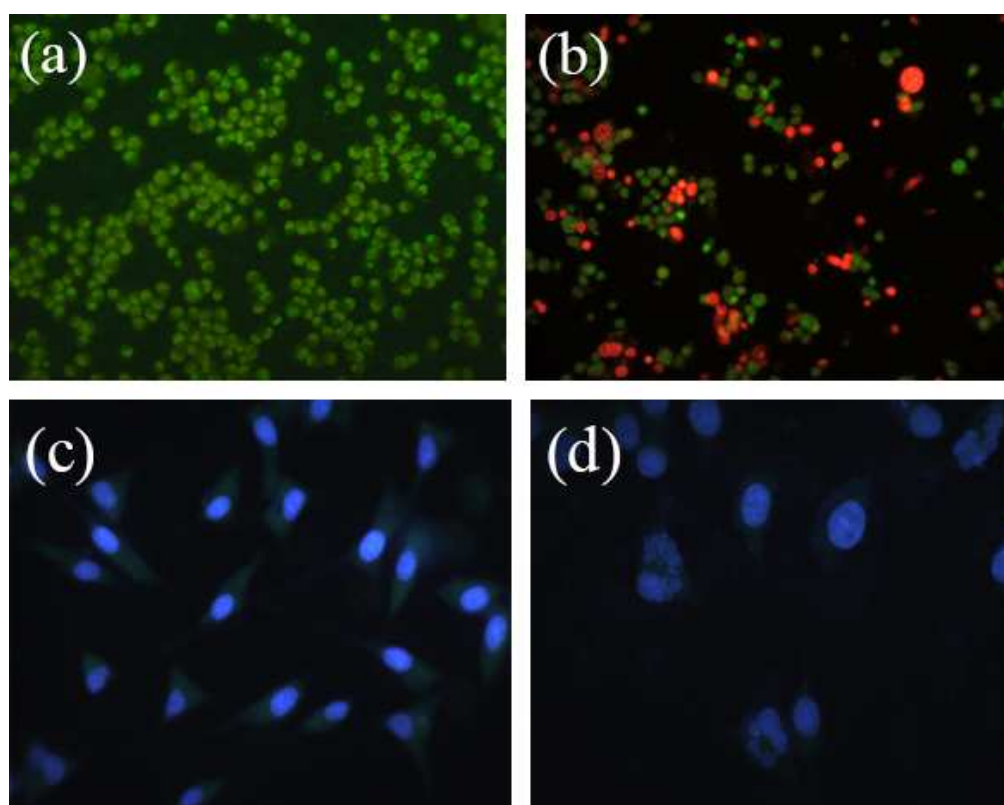


Fig. 5

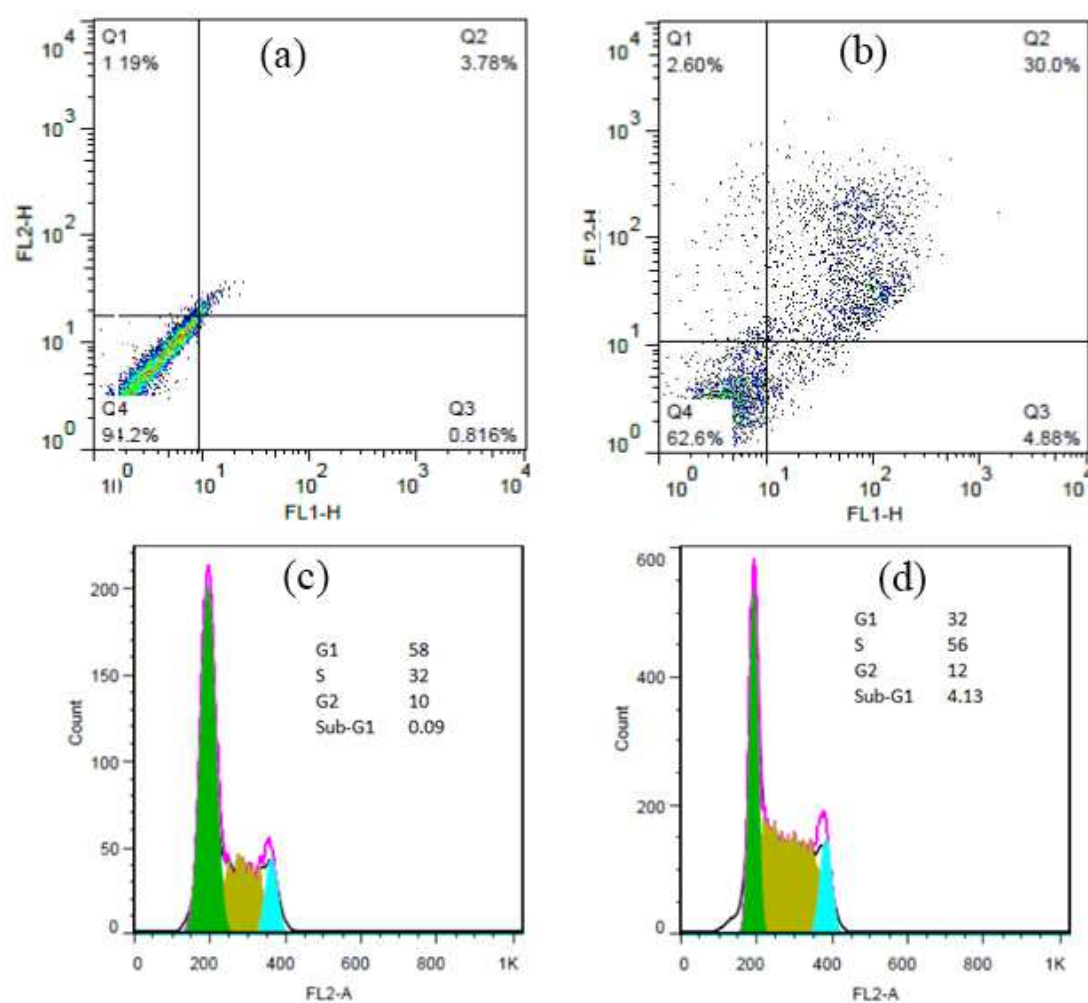


Fig. 6

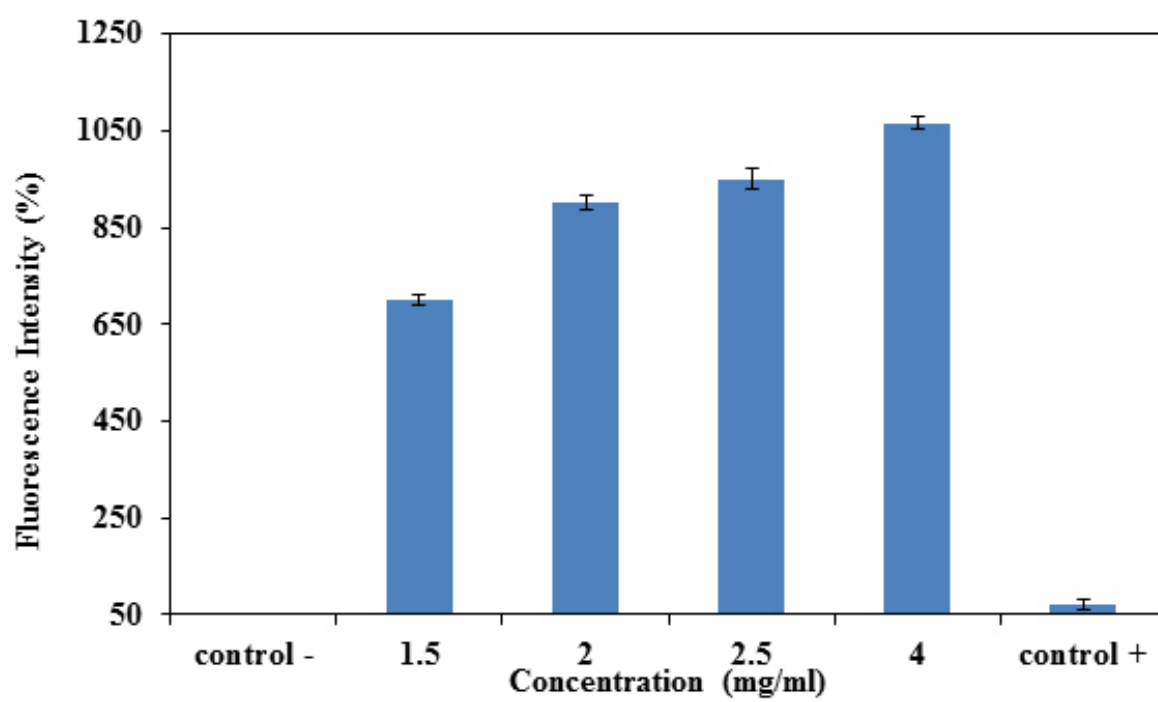


Fig. 7

- $\text{NiCo}_2\text{O}_4/\text{NiO}$ porous nanocomposites has been synthesized by assistance green cochineal dye for the first time.
- These uniform nanoparticles has been prepared using ultrasonic assisted thermal treatment method.
- The role of ultrasonic waves and mechanism was investigated.
- For the first time the anticancer of $\text{NiCo}_2\text{O}_4/\text{NiO}$ nanoparticles against 4T1 cells lines.
- MTT cytotoxicity assay was performed to determine the anti-proliferative effect of nanoparticles against 4T1.