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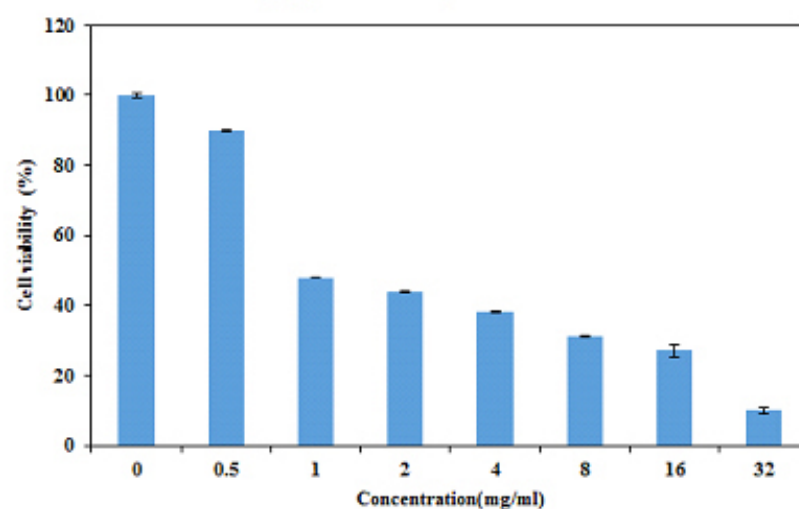
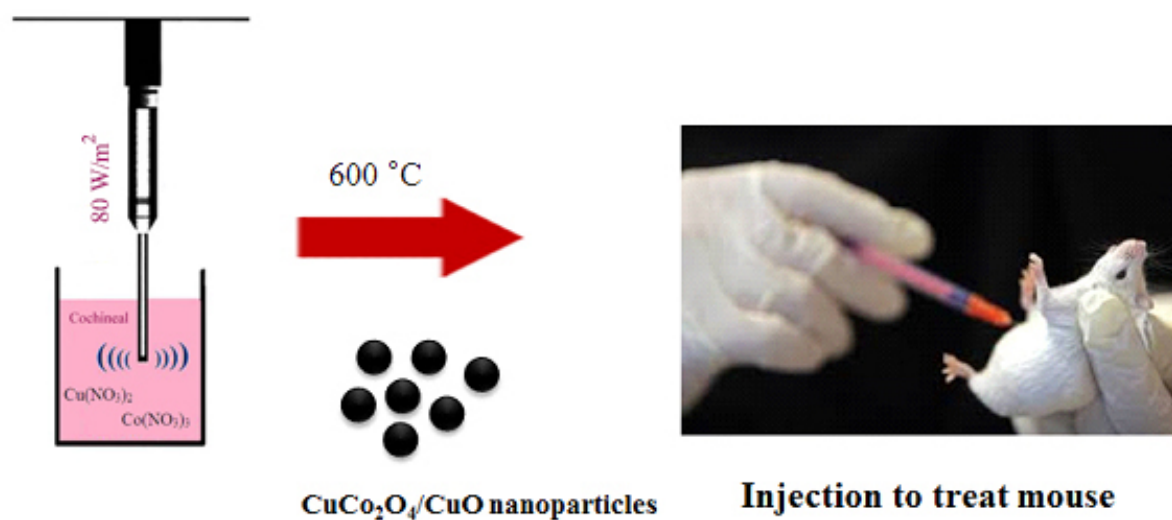
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Anti cancer results

Sonochemical assisted thermal decomposition method for green synthesis of $\text{CuCo}_2\text{O}_4/\text{CuO}$ ceramic nanocomposite using *Dactylopius Coccus* for anti-tumor investigations

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Abstract

Despite of great attention concerned on ceramic nanostructures in bioapplications, little is known about the toxicity of these NPs at the cellular and molecular levels. In this work, mesoporous nanocomposite of $\text{CuCo}_2\text{O}_4/\text{CuO}$ synthesized by sonochemical assistant thermal decomposition and the anti-cancer effect was studied as well. The environmental friendly method by surfactant free as well as low temperature was applied. A natural complex of copper-cobalt-coccus prepared by sonochemical method and finally decomposed thermally at 600 °C. $\text{CuCo}_2\text{O}_4/\text{CuO}$ mesoporous nanocomposites were successfully obtained through one-way reaction and characterized by; x-ray diffraction pattern (XRD), scanning electron microscope (SEM), transmission electron microscopy (TEM), energy dispersive spectrometry (EDS). Furthermore, vibrating sample magnetometer (VSM) was used to study the magnetic properties of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles. MTT assay was performed in order to determine the anti-proliferative effect of $\text{CuCo}_2\text{O}_4/\text{CuO}$ samples against 4T1 breast cancer cell lines. $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles induced dose-dependent cytotoxic effects against 4T1 breast cancer cells with IC_{50} of 0.82 mg/ml. Nevertheless, it is essential to perform more investigations to find the exact related mechanisms.

Keywords: Nanocomposite; Nanostructures; Anticancer; 4T1 cells; $\text{CuCo}_2\text{O}_4/\text{CuO}$; Sonochemical.

1. Introduction

The properties of ceramic materials dictated by some factors like types of presented atoms as well as bonding between them and the way the atoms are packed together. For determination of synthesized materials properties, bonding and structure type of precursors play an important role. One important kind of crystalline and non-metallic solids materials that can be produced by heating and cooling are ceramics [1]. Ceramic nanocomposites properties attributed to the mechanical and functional characteristics that have significant application in various fields. The newest application areas on ceramic nanocomposites are as follows: solar hydrogen production [2], photoreduction of heavy ions [3], near infrared bioimaging [4], catalysis [5], photocatalysis [6], gas sensing [7], environmental remediation [8], electrocatalytic oxidation [9] and Nitrogen oxide reduction [10].

The most significant application of ceramic nanoparticles is the biological one, besides, the utilizing of inorganic nanoparticles for biomedical applications has advanced [11]. In particular, magnetic iron oxide nanoparticles, including magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$), have received enormous consideration for their various biomedical applications counting biosensors, magnetic resonance imaging (MRI) contrast agents, and magnetic fluid hyperthermia [12-14] because of their tunable size-dependent magnetic properties in addition to their biodegradable and nontoxic nature [15, 16].

Notwithstanding of the significant effort of researches, cancer is still responsible for 25% of all deaths in developed countries [17]. There is a crucial need for more sensitive, cost-effective and precise methods for detecting and treating cancer. Inorganic nanoparticles and nanoparticles-biomaterials composites with their exceptional optical, thermal and electromagnetic properties have indicated excellent potential in novel methods for characterization, detection and cancer therapy [18]. Up to now, there are few reports about inorganic toxicity of nanoparticles against the cancer cells and in vivo investigations. In the past decades, the most used nanoparticles were Au and Ag [19-21]. Since the 1950s, Au has been used as a radioactive label in biomedical applications [22]. The use of biocompatible and nontoxic nanoparticles is vital for medical applications. Lately variety of

nanoparticles such as ZnO [23], Fe₂O₃ [24], Fe₃O₄ [25], Au-Fe₃O₄ [26], SiO₂ [27] has been used for cancer therapy. For instance, Ahmad M. et al. [28] reported the synthesis of ferromagnetic Ni doped CeO₂ nanoparticles and examined their cytotoxic effect on HEK-293 healthy cell line and SH-SY5Y Neuroblastoma cancer cell line. Also Asadpour et al. [29] demonstrated GPX activity in PC12 and N2a cell lines was reduced in the presence of ZrO₂ nanoparticles owing to cytotoxic effect. Moreover, M. Yazdi et al. [30] investigated that Se nanoparticles have appropriate potential against 4T1 breast cancer.

To the best of our knowledge, no reports has been published on anti-cancer activity of CuCo₂O₄/CuO nanoparticles till now. In the present work, we introduced a novel CuCo₂O₄/CuO nanocomposite for cancer therapy that were synthesized by novel, simple and eco-friendly ultrasonic method, besides, the cytotoxic effect of the nanoparticles was experienced against 4T1 breast cancer for the first time.

2. Experimental

2.1. Materials and physical measurement

All chemical and reagents used for the synthesis of CuCo₂O₄/CuO nanocomposites such as; Cu (NO₃)₂.5H₂O, Co(NO₃)₂.6H₂O were purchased from Merck Company. X-ray powder diffraction (XRD) patterns were recorded by a Philips diffractometer using X'PertPro and the monochromatized Cu K α radiation (λ = 1.54 Å). Fourier transform infrared (FTIR) spectra of the products were obtained using 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). A Philips EM208 transmission electron microscope with an accelerating voltage of 200 kV was applied for Transmission electron microscopy (TEM) images. The magnetic properties of the sample was detected at room temperature using a vibrating sample magnetometer (VSM, Meghnatis Kavir Kashan Co., Kashan, Iran). From Sigma (Steinheim, Germany), BSA (fraction V, minimum 98%) was purchased. 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 [31] pigment was obtained from the dried bodies of female scale insect species, *Dactylopius Coccus*, which were feed on wild cacti. The insects were dried at 70 °C in an oven until reaching a constant weight; then, grounded and stored before use.

2.3. *Synthesis CuCo₂O₄/CuO nanocomposite*

To synthesis the nanocomposite, 1mmol Cu(NO₃)₂ and 2mmol Co(NO₃)₂ dissolved in distilled water to a clear solution. Then, certain amount of coccus added to the solution under strong magnetic stirring at room temperature. The solution was stirred under ultrasonic waves (power = 80 W/cm²) for 30 min. After that, aqueous solution was heated by stirring at 80 °C for 30 min. The product was collected by centrifugation at 4000 rpm for 5 min, and washed repeatedly with distilled water and ethanol several times respectively. The resulted products were dried at 80 °C under vacuum for 2h, then calcined at 600 °C for 3h. The preparation conditions of CuCo₂O₄/CuO nanocomposite have been illustrated in Table 1.

2.4. *Cell culture*

Highly invasive, tumorigenic and transplantable tumor cell line (murine mammary carcinoma 4T1 cell line) was generously supplied from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). The 4T1 tumor cell was designated; because of its great similarity to human mammary cancer and transplantable to BALB/c mice.

2.5. Cytotoxicity assay

MTT-based assay [32] was used to evaluate the nanostructures cytotoxicity effect. To do this test, 1×10^4 cells of 4T1 cell lines was seeded in a 100 μ l growth medium in 96-well plate, and treated by increasing concentrations of sample no#5 (0, 0.5, 1, 2, 4, 8, 16, 32 μ M). Incubation of treated cells was performed at 37 °C in 5% CO₂ for 24 h. Then the absorbance was measured at 420 nm by ELISA reader (BioTeks Elx 800). The amount of the nanocomposites causing 50% inhibition of the proliferation (IC₅₀) was also calculated based on viability difference between untreated and treated cells by using 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 study the morphological variations of the cell lines, current Protocols of immunology was used by acridine orange/ethidium bromide double staining. Briefly, 5×10^5 cells/well were cultured in six-well plates and incubated overnight, afterwards treated with CuCo₂O₄/CuO nanoparticles at IC₅₀ concentration, harvested, and washed twice with PBS. Finally, ethidium bromide/acridin orange solution was added to the cell suspension and the nuclear morphology was evaluated by fluorescence microscopy (Zeiss, Germany).

2.7. Determination of nuclear morphology by DAPI staining

5×10^5 4T1 cells/well in 12-well plates were exposed to CuCo₂O₄/CuO nanoparticles at IC₅₀ for 1 day to see the presence of apoptotic bodies and nuclei morphology using DAPI nuclear staining. Afterwards, the cells were fixed in 4% paraformaldehyde-PBS solution for 15 min and stained with DAPI (0.3 μ M) for 30 min at room temperature. Fluorescence microscopy was used to understand the cells for nuclear morphology. In order to recognize the apoptotic cells, the presence of condensed, fragmented, and degraded nuclei used.

2.8. Cell apoptosis assay

To distinguish apoptosis from necrotic cell death induced, Annexin-V staining was used. Annexin-V protein that is located from the inner to the outer leaflet of the plasma membrane has a high affinity to phosphatidyl serine. The conjugation of Annexin-V with the fluorescent probe PE facilitates measurements by flow cytometric analysis. The use of 7AAD staining helps differentiate between apoptosis and necrosis owing to modifications in permeability of 7AAD through the cell membranes of damaged and living cells. To simulate the experiments conditions the same number of cells, culture situations, and concentrations used in the cell cycle analysis. 4T1 cells treated by CuCo₂O₄/CuO nanocomposites harvested and washed twice in PBS. The staining was carried out based on the manufacturer's instruction (eBioscience Annex in V Apoptosis Detection Kit PE, Catalog Number: 88-8102) as follows; 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). Then, 300 μ l binding buffer added to each sample, in an ice. The 488-nm laser was 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 analyses were achieved using the Flowjo version 7.6.1.

2.9. Cell cycle assay

Cell cycle analysis was implemented by staining the DNA with propidium iodide (PI), followed by flow cytometric measurement of the fluorescence. Approximately 3.5×10^5 4T1 cells/well were seeded in a six-well plate and incubated for 24 h. The medium was separated and stored after treatment with CuCo₂O₄/CuO nanoparticles (IC₅₀ concentration) for 24 h. Cells were washed with PBS and trypsinized, harvested in the stored medium, and finally centrifuged. The pellet were washed in PBS, fixed in ice-cold ethanol (70%), and stored at 4 °C for at least 12 h. Before flow cytometry analysis, cells were washed in PBS and stained with PI in RNase (40 μ g ml⁻¹ PI and 100 μ g ml⁻¹ RNase A) and incubated at 37 °C for 30 min, followed by incubation at 4 °C until

analysis. Flow cytometry analysis was done using FACS Calibur at 488 and 610 nm of excitation and emission wavelength respectively. Data were collected and analyzed using Flowjo software.

2.10. In vitro cellular uptake of CuCo₂O₄/CuO nanoparticles

To estimate the in vitro cellular uptake of CuCo₂O₄/CuO nanoparticles, 1×10^6 4T1 cells/ml) cells were seeded in 6-well microplates and treated with CuCo₂O₄/CuO nanoparticles. After that, the cells were 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. Finally, Philips transmission electron microscope CM100 (Philips, Eindhoven, Netherlands) was used for imaging.

2.11. Intracellular ROS measurement

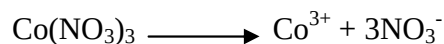
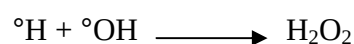
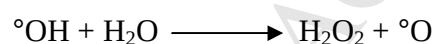
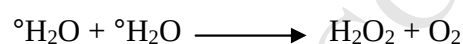
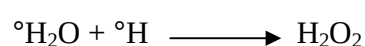
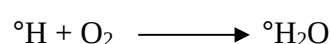
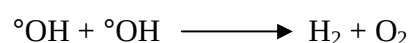
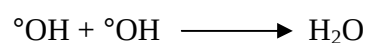
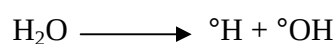
The net intracellular levels of ROS generated by synthesized compounds were measured by a non-fluorescence dye, DCFH-DA, which relates to intracellular ROS, to generate fluorescent 2,7- dichlorofluorescein (DCF). Briefly, 3.5×10^5 cells/ well were seeded into 24-well plates treated with IC₅₀ of CuCo₂O₄/CuO nanoparticles for 1 day. The positive control was freshly prepared hydrogen peroxide (H₂O₂, 0.1 mM in PBS). After treatment, the cells were incubated with 10 μ M DCFH-DA for 1 h, washed twice with PBS, and then suspended in 500 μ l PBS. The fluorescent intensity was detected, and then the amount of ROS was subsequently estimated from DCF production.

3. Result and discussion

3.1. Characterization of CuCo₂O₄/CuO nanoparticles

XRD pattern of CuCo₂O₄/CuO nanoparticles is indicated in Fig.1. The different peaks in this pattern can be indexed to monoclinic phase of CuO (JCPDS card no. 80-1917) along with cubic phase (JCPDS card no. 01-1155). The narrow sharp peaks demonstrates that the CuCo₂O₄/CuO nanoparticles were well crystallized. The mean crystallite diameter of the CuCo₂O₄/CuO nanocomposites (sample no. 2) calculated utilizing Debye Scherrer

formula [31] has been found to be 19 nm. To study the effect of the ultrasonic irradiation on the $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanocomposites morphology, sample no#1 was synthesized in the absence of the ultrasonic irradiation. Fig. 2a displays SEM images of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanocomposites prepared without ultrasonic irradiation. As shown in the Fig. 2a particles are agglomerated, but by using the ultrasonic irradiation, the smaller particles were synthesized (Fig. 2b) with an average size of 100-200 nm. To obtain intended nanoparticles, the calcination temperature should be reduced more. Therefore, samples prepared with ultrasonic irradiation and then calcined in 600°C (Fig. 3). As shown in Fig. 3 uniform nanoparticles of $\text{CuCo}_2\text{O}_4/\text{CuO}$ with average size of 20 nm and porous structures were obtained, that 50-200 nm size hollows were seen. The role of ultrasonic wave was investigated according to a reported method [33]. Throughout the solvent sonolysis, radicals of H and OH were produced. Both radicals were active and played important role in sonochemical reaction. The related ultrasonic mechanisms are illustrated as follows [33]:



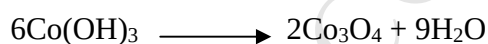
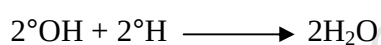
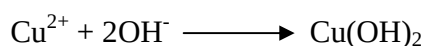
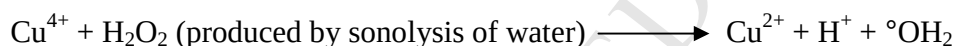
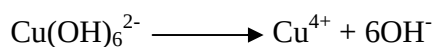
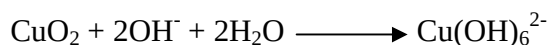
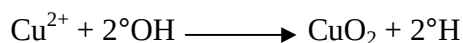
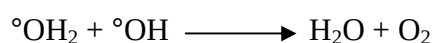
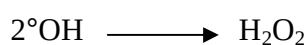
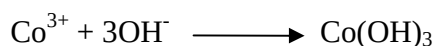
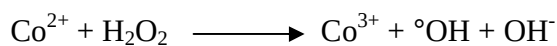
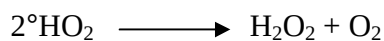
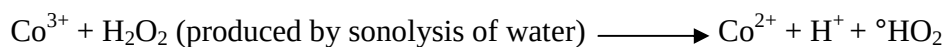
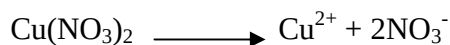


Fig. 4 represents EDS pattern of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanocomposites (sample no# 2). The presence of Cu, Co and O are clearly observed in this pattern. The EDS mapping analysis was done as well (Fig.5). Furthermore, CuCo_2O_4 and

CuO atoms are homogeneously dispersed on the surface of composite, as it is shown in the SEM-EDX mapping images.

TEM images of $\text{CuCo}_2\text{O}_4/\text{CuO}$ displayed in Fig. 6. TEM image clearly presented the formation of a uniform nanoparticles with average size 20 nm. In addition, the porous structure of sample is obvious as well.

The magnetization evaluations were performed by VSM in a magnetic field of upto 10 kOe in an air atmosphere and at room temperature. Fig. 7 demonstrates the magnetic hysteresis loops for nanoparticles. Usually, the loops of all samples shift along positive or negative H axes whereas the shift is denoted by an exchange bias field defined as $H_E = (H_{C1} + H_{C2})/2$, where H_{C1} (< 0) is the left coercive field and H_{C2} (> 0) is the right one. Correspondingly, the coercivity is defined as $H_c = |H_{C1} - H_{C2}|/2$. The magnetic hysteresis curves illustrate the weak magnetic response to a varying magnetic field.

33.2. Anticancer activity evaluation of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles

The anti-proliferative results showed that, $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles significantly inhibited the proliferation of breast cancer cell lines of 4T1 cells as depicted in Fig. 8. Sample no. 2 induced dose-dependent cytotoxic effects against different cell types with IC_{50} of 0.82 mg/ml.

3.3. Apoptosis induction

The 4T1 cells were treated with IC_{50} values of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles to understand whether the mechanism of cell death is through apoptosis or not, and the results were estimated by acridine orange/ethidium bromide staining methods (used to visualize nuclear changes and apoptotic body formation that are characteristics of apoptosis). Fluorescence microscope images demonstrated the stained apoptotic cells in comparison with the control cells in Fig. 9(a,b). The apoptotic cells are the cells whose nuclei are observed as orange spots, whereas the green ones are the viable cells. The cells treated with $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles presented an extended apoptosis relative to the control cells [23].

After treated with $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles, cells were stained by DAPI to realize nuclear morphology alterations associated with apoptosis more precisely (Fig. 10(a,b)). The results indicated that, the untreated cells have homogeneous nuclei, whereas $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles treated cells indicated fragmented nuclei.

Apoptosis analysis using flow cytometry was applied to further analyze the feature of cell death induced by $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles. The loss of cell membrane phospholipid asymmetry that resulted in the externalization of phosphatidylserine (PS) bonds on the cells surface is an important marker of apoptosis. PE Annexin-V as a fluorescent-labeled Annexin-V dye binds very precisely to externalize PS ligands on the surface of cells leading to the discover of early apoptotic cells. Since, externalization of PS happens in the earlier stages of apoptosis, PE Annexin-V staining can identify apoptosis at an earlier than assays based on nuclear changes like DNA fragmentation. The loss of membrane integrity, which is preceded by PE Annexin-V staining, accompanies the latest stages of cell death, resulting from either apoptotic or necrotic processes. 7-amino-actinomycin (7-AAD) is a vital dye to allow recognition of early apoptotic cells (7-AAD negative). The membranes of dead and damaged cells are permeable toward 7-AAD, while Viable cells intact with membranes exclude 7-AAD. Therefore, cells that are reflected viable ones are PE Annexin-V and 7-AAD negative; whereas, cells that are in early apoptosis are PE Annexin V positive and 7-AAD negative; however, late apoptotic or already dead cells are both PE Annexin V and 7-AAD positive. For distinguishing the degree of apoptosis in cells exposed to $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles PE Annexin V and 7-AAD staining using flow cytometry was applies. Fig. 11(a,b) confirmed that apoptosis was the mechanism of cell death caused by $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles. In fact, 24 h treatment by $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles (IC_{50}) brought a 2% early apoptosis, 25% late apoptosis and 3% necrosis. This results presented that, the maximum mortality was by apoptosis pathway (27%), but only small portion of mortality was necrosis (3%). Hence, $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles could be used as a potent chemotherapeutic agent to inhibit cancer cell growth in invasive breast cancer. According to past reports, these results are reasonable and appropriate [34, 35].

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

Fig. 12(a,b) exhibits the effects of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles on cell-cycle progression. The cell cycle analysis study indicated the drastic change of the 4T1 cell cycle distributions after treatment by $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles. After 24 h sample no#5 treatment (0.82 mg/ml), 40% of the 4T1 cells were in G1 phase, 33% were in S phase, and 27% were in G2 phase in contrast the untreated control cells consisting of 58% of the cells in G1 phase, 32% in S phase and 10% in G2 phase. For treated cells, a noteworthy decrease in the percentage of cells in the G1 phase and a significant increase in the percentage of cells in the G2 phase was determined. Moreover, 4.51% population was in sub_G1, which is related to apoptotic cell. This cell arrest in G2 phase by $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanocomposites treatment is due to arrest of cell division because of either DNA cleavage or damage to the cell division machinery.

4. Conclusions

Increasing application of nanoparticle in different fields such as clinical nanotechnologies urges the development of environmentally friendly synthesis methodology. $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles have been synthesized by green precursor via ultrasonic assisted precipitation method using cochineal powder as a natural precursor. By using thermal treatment at 600°C , uniform $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles were obtained. The effect of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles was investigated against the 4T1 Breast Cancer. $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles prepared in the present work induced dose-dependent cytotoxic effects against different cell types with IC_{50} of 0.82 mg/ml. A significant decrease in the percentage of cells in the G1 phase and a significant increase in the percentage of cells in the G2 phase was demonstrated. Furthermore, a population was in sub_G1 (4.51%), which is related to the apoptotic cell. This cell arrest in G2 phase after $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles treatment was due to arrest of cell division as a result of either DNA cleavage or damage to the cell division machinery. Therefore, these green $\text{CuCo}_2\text{O}_4/\text{CuO}$ NPs may have promising application as a novel anticancer agent for the treatment of breast cancer.

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Figure captions

Fig. 1. XRD pattern of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles.

Fig. 2. SEM images of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles: (a) sample no. 1, (b) sample no. 3.

Fig. 3. SEM images of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles: (c) sample no. 2.

Fig. 4. EDS spectrum of representative $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles (sample no. 2).

Fig. 5. EDS mapping (A) and FESEM-EDS image of $\text{CuCo}_2\text{O}_4/\text{CuO}$ (B). The scale bar is 1 μm .

Fig. 6. TEM images of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles (sample no. 2).

Fig. 7. VSM analysis of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles (sample no. 2).

Fig. 8. Cell viability of 4T1 treated with $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles (IC_{50} of 0.82 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. 9. Fluorescent microscopy of 4T1 cells stained with acridine orange/ethidium bromide, a) Un-treated cells, b) Treated cell by sample no. 2.

Fig. 10. Fluorescent microscopy of 4T1 cells with DAPI staining, a) Un-treated cells, b) Treated cell by sample no. 2.

Fig. 11. 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 sample no. 2.

Fig. 12. Flow cytometry based assay of cell cycle of control. a) Un-treated cells. b) Treated cell by sample no. 2.

Table 1. The reaction conditions of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles synthesized in this work.

Table 1

Sample no.	Ultrasonic irradiation (W/cm ²)	Temperature (°C)	Time (h)
1	-	600	3
2	80	600	3
3	80	700	3

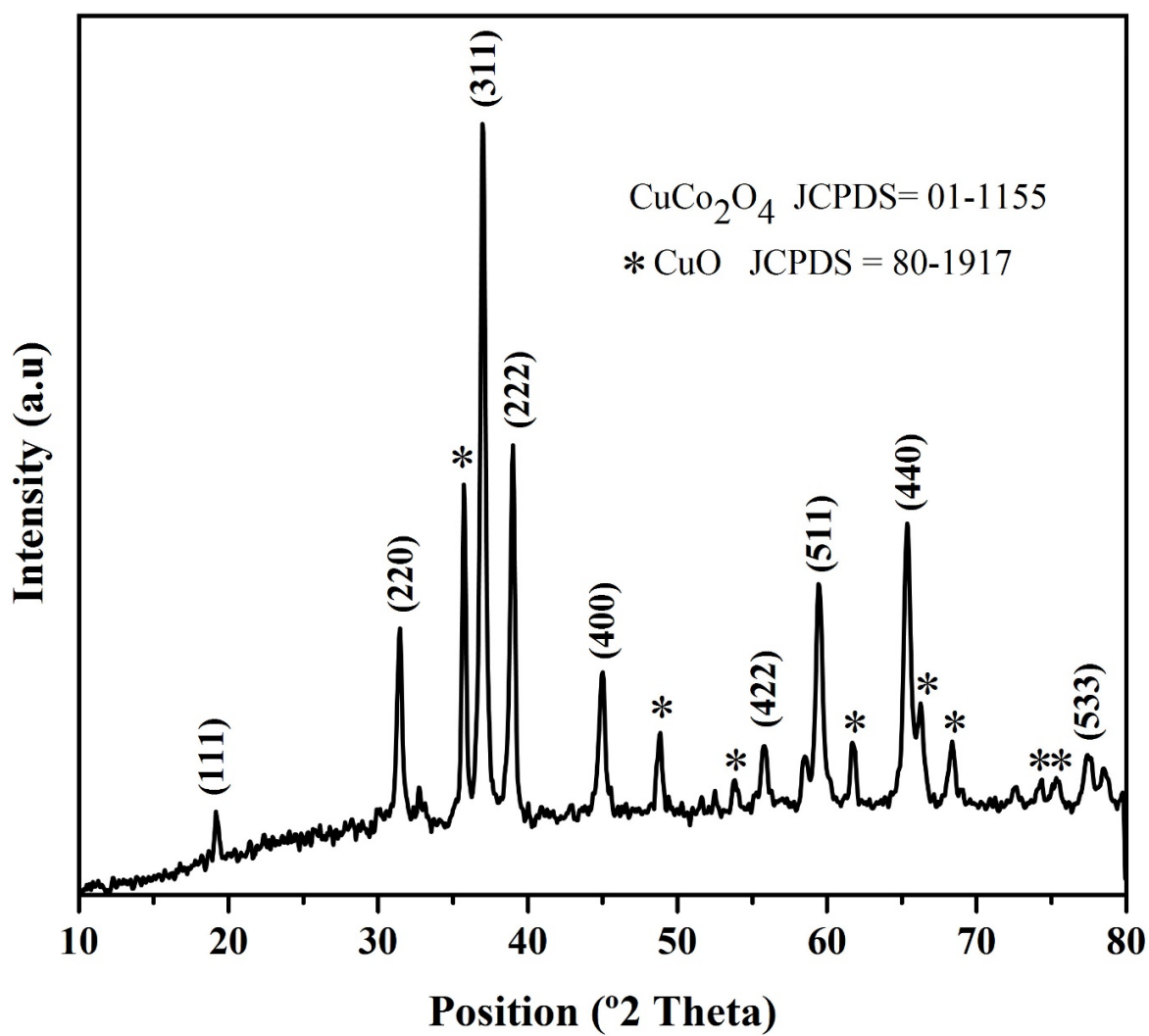


Fig. 1

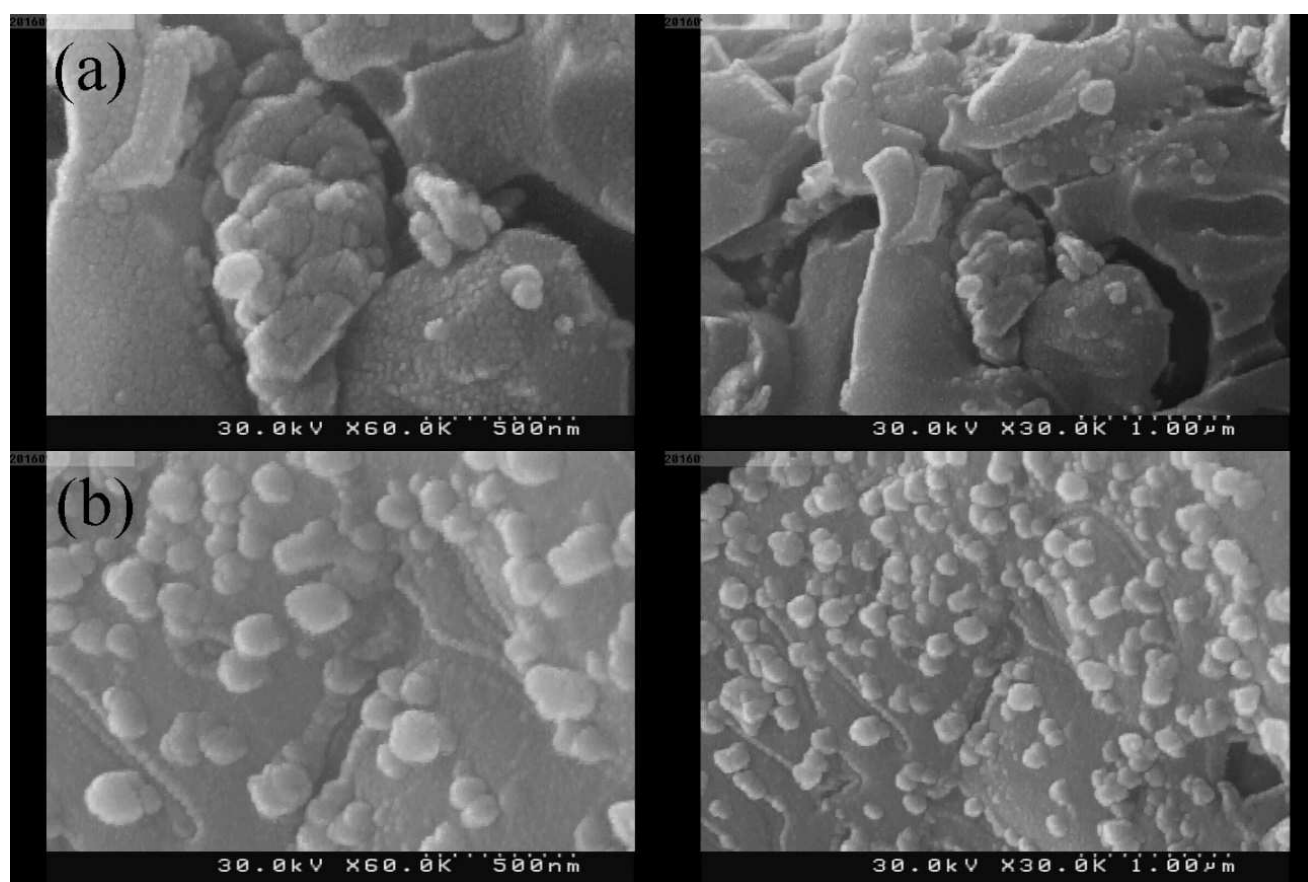


Fig. 2

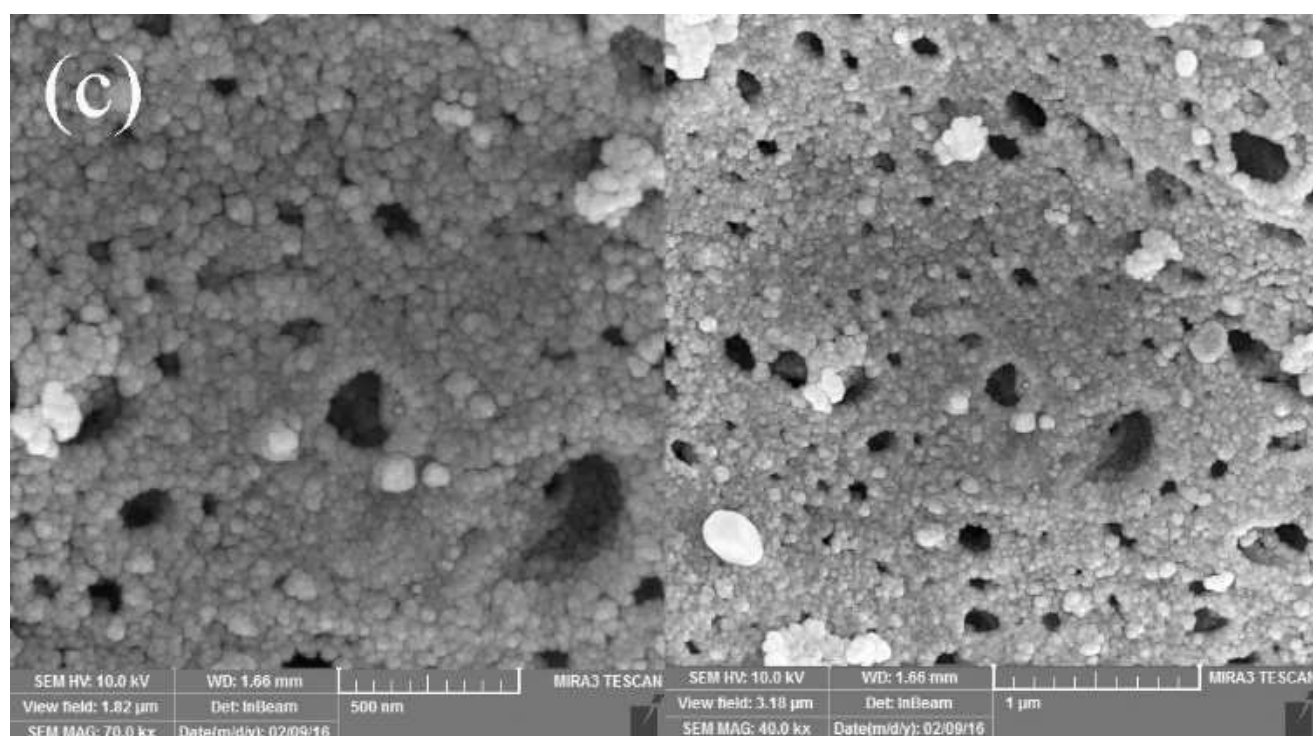


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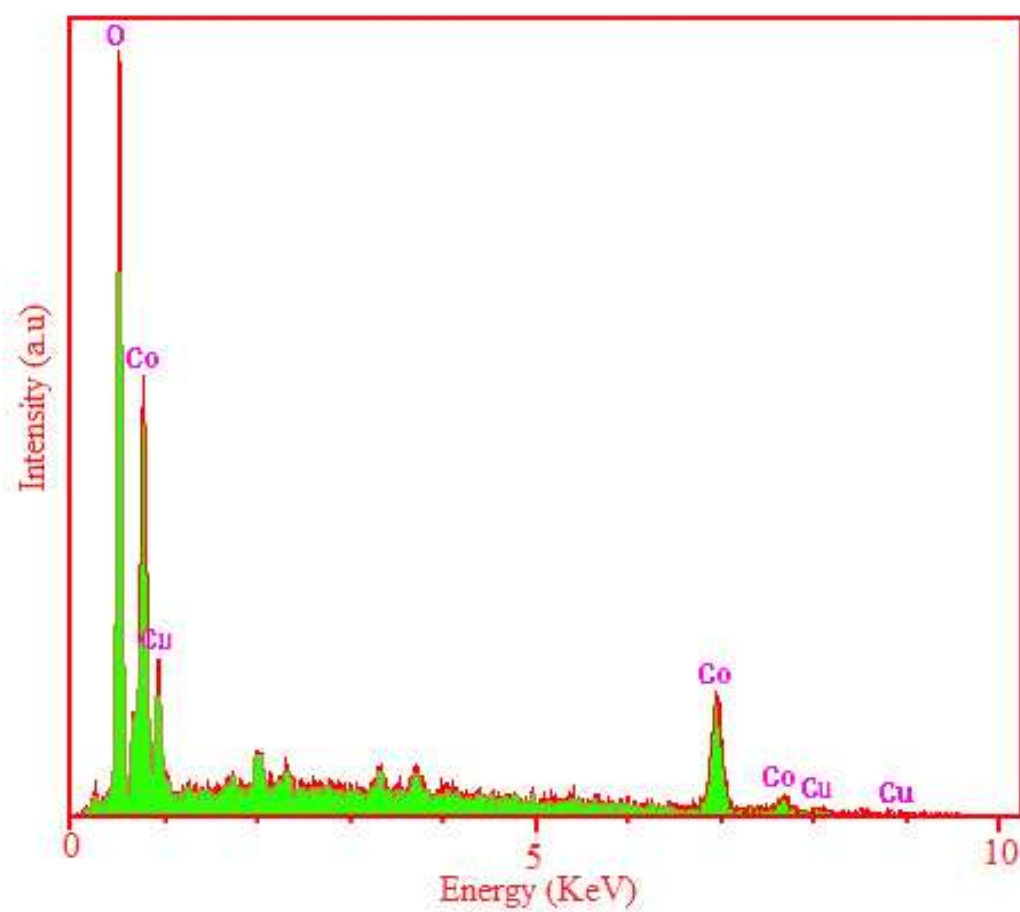


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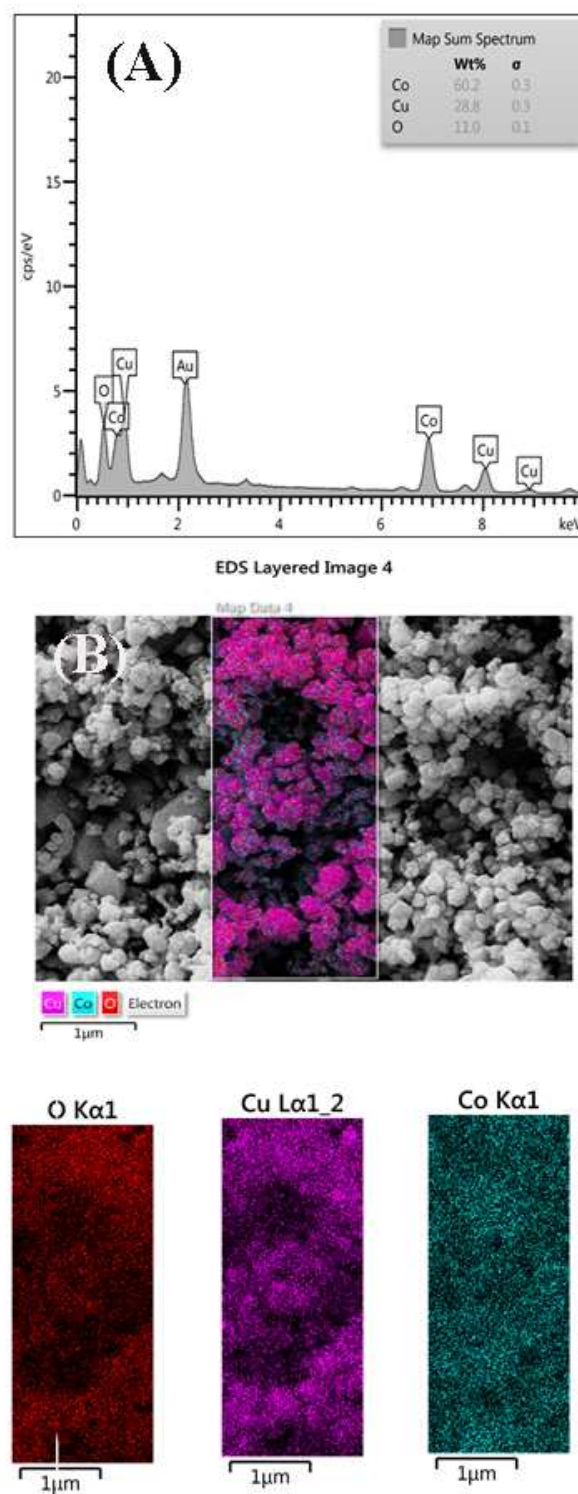


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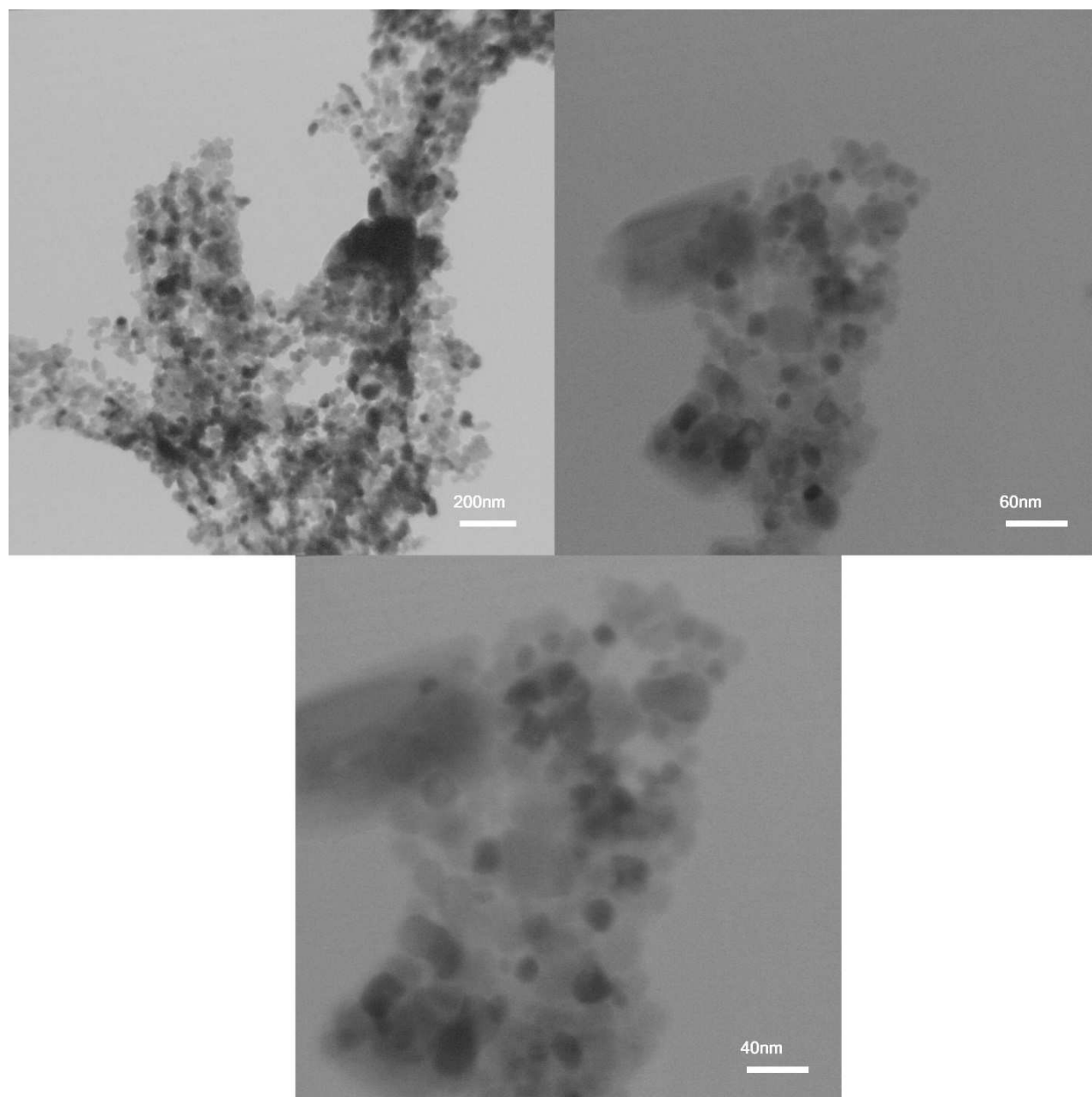


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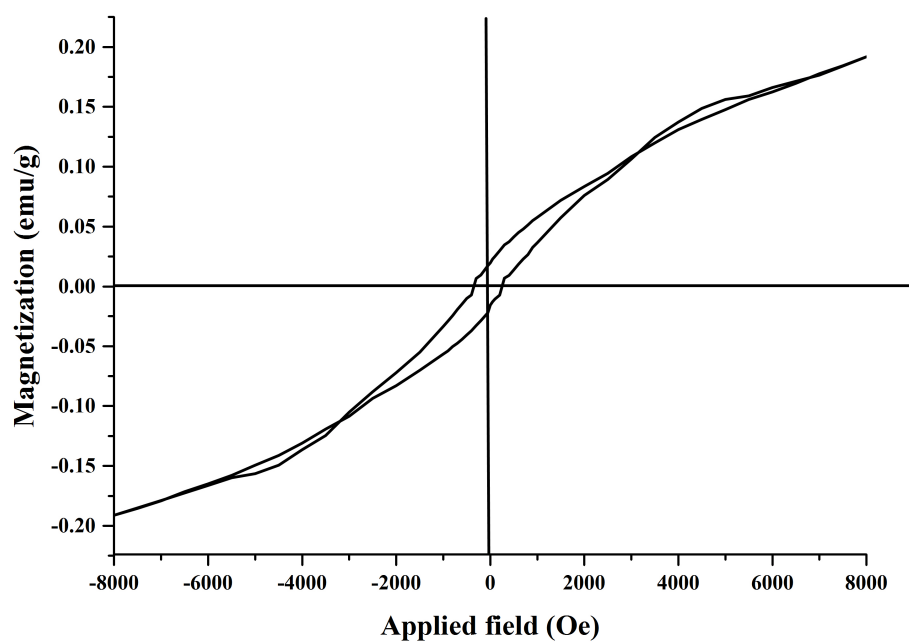


Fig.7.

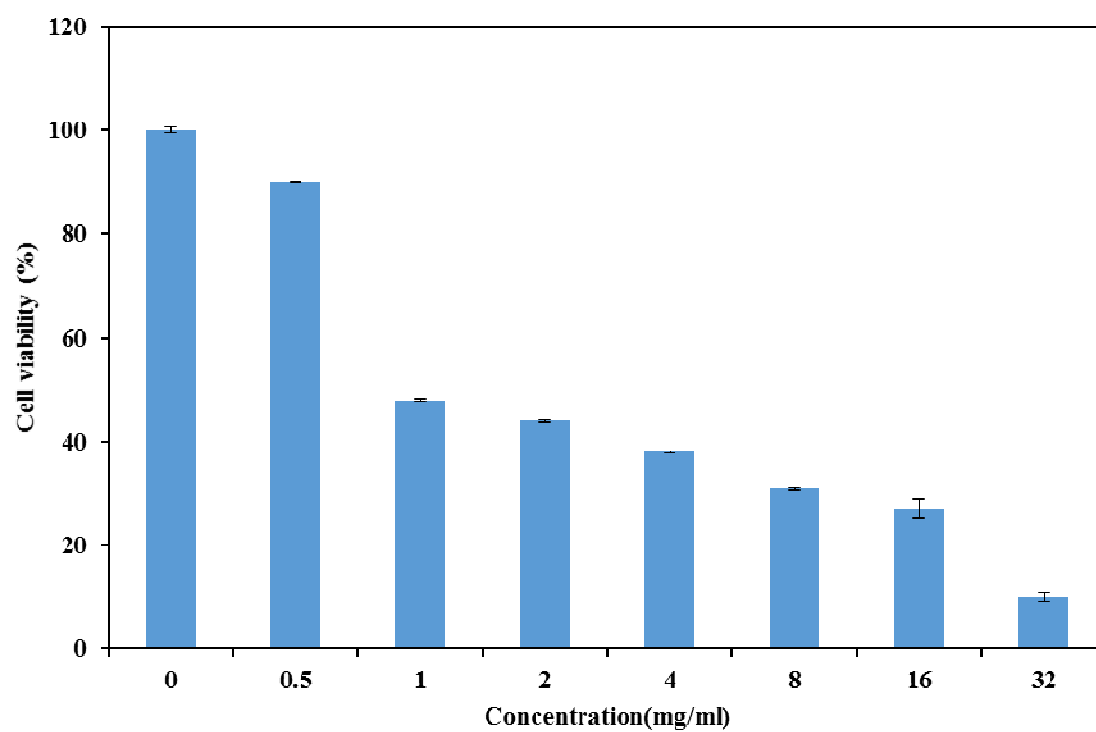


Fig. 8

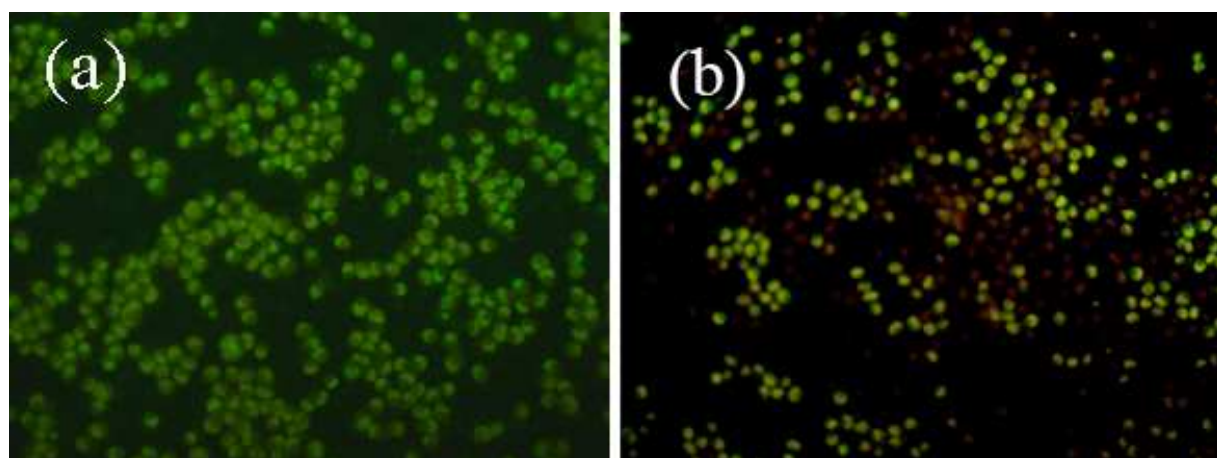


Fig. 9

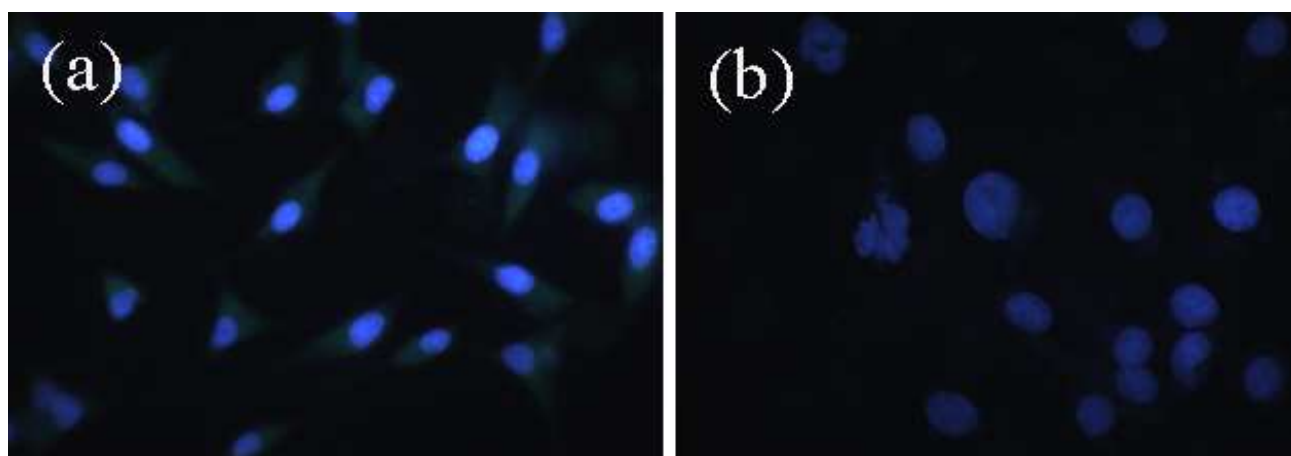


Fig. 10

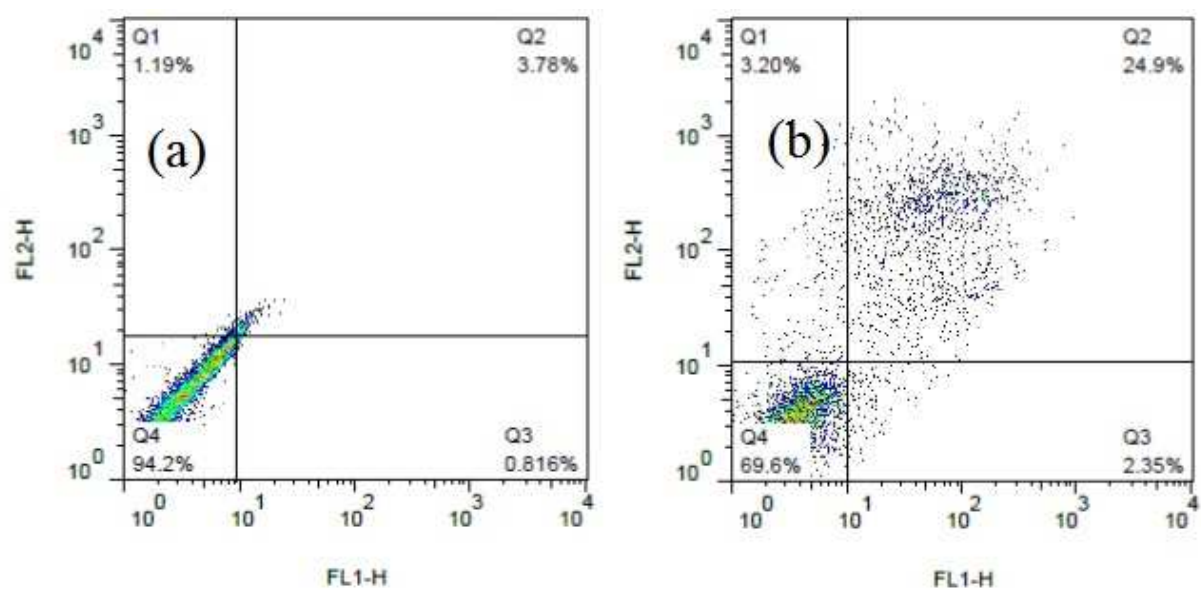


Fig. 11

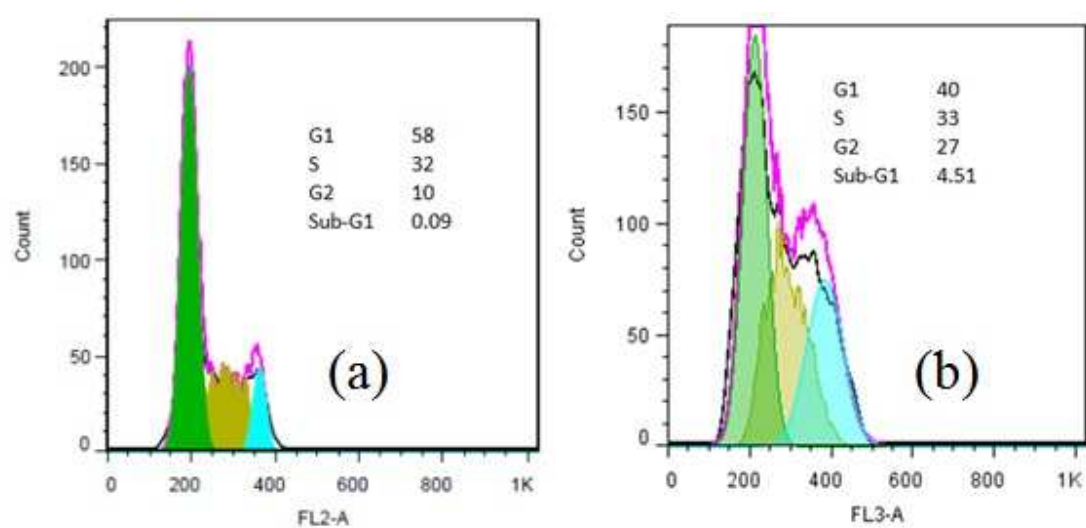


Fig. 12

- $\text{CuCo}_2\text{O}_4/\text{CuO}$ 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.
- $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles were characterized by FESEM, TEM, EDS, XRD and VSM.
- For the first time the anticancer of $\text{CuCo}_2\text{O}_4/\text{CuO}$ nanoparticles against 4T1 cells lines.