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ORIGINAL ARTICLE

Green preparation of copper nanoparticle-loaded chitosan/alginate bio-composite: Investigation of its cytotoxicity, antioxidant and anti-human breast cancer properties

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Abstract In this study, an eco-friendly and low-cost procedure for the in *situ* fabrication of Cu nanoparticles by using chitosan/alginate hydrogel. The prepared Cu NPs@CS/Alg nanocomposite were characterized by advanced physicochemical techniques like Fourier Transformed Infrared spectroscopy (FT-IR), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Energy Dispersive X-ray spectroscopy (EDX) and X-ray Diffraction (XRD) study. It has been established that chitosan/alginate-capped gold nanoparticles have a spherical shape with a mean diameter from 10 to 20 nm. In the cellular and molecular part of the recent study, the treated cells with Cu NPs@CS/Alg nanocomposite were assessed by MTT assay for 48 h about the

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cytotoxicity and anti-human breast cancer properties on normal (HUVEC) and breast cancer cell lines i.e. infiltrating lobular carcinoma of breast (UACC-3133), inflammatory carcinoma of the breast (UACC-732), and metastatic carcinoma (MDA-MB-453). In the antioxidant test, the IC50 of Cu NPs@CS/Alg nanocomposite and BHT against DPPH free radicals were 344 and 193 μg/mL, respectively. The IC50 of Cu NPs@CS/Alg nanocomposite were 297, 386, and 359 μg/mL against KYSE-270, OE33, and ESO26 cell lines, respectively. The viability of malignant breast cell line reduced dose-dependently in the presence of Cu NPs@CS/Alg nanocomposite.

1. Introduction

Nanotechnology is the field to yield modern systems, tools, and materials by taking control at the atomic and molecular levels using the features that appear on those surfaces. Applications for nanotechnology in medical diagnostics, food, medicine, biotechnology, environment, energy, chemistry, physics, etc., introduce this technology as an interdisciplinary and cross-sectoral context (Arunachalam, 2003; You et al., 2012). The interdisciplinary nature of nanoscience and nanotechnology as the field to yield modern systems, tools, and materials with precision atoms and molecules, will eventually affect the health and medical sector (You et al., 2012; Mao, 2016; Veisi et al., 2018). Drug use is currently volumetric, so most cells in the body need medication. In the new method, the drug is directed directly to specific cells with new injection devices and delivered to the required location. By this mechanism, small and large diseases can be diagnosed and treated at the beginning of their formation (Liao et al., 2015; Fazaeli et al., 2010). The National Nanotechnology Project is being implemented in European countries, the United States and Japan with high priority in various fields. Nanotechnology and nanoscience emerging fields can move materials very accurately, to understand and control unprecedented fundamental components of physical objects (Arunachalam, 2003; You et al., 2012; Mao, 2016; Veisi et al., 2018; Liao et al., 2015). It seems that these developments will change the way we design and build everything from vaccines to computers. The plan would increase investment in nanotechnology about twice as much each year as last year. A branch of nanotechnology is the formulation of new drugs with metal nanoparticles (Fazaeli et al., 2010; Konda et al., 2014; Mazaahir et al., 2012).

Nanoparticles have become very popular due to their wide applications in biology, medicine and medicine. Structurally, their size is in the range of 100 nm. Several drugs such as small hydrophobic and hydrophilic drugs, molecules, and vaccines of biological nanoparticles can be administered by these nanoparticles (Arunachalam, 2003; You et al., 2012; Mao, 2016). They are widely used in improving the treatment and diagnosis of diseases. Nanoparticles in nanoliposomes, carbon nanotubes, nanofibers, nanospheres have been widely used for drug carriers and in the manufacture of cell scaffolds (Veisi et al., 2018; Liao et al., 2015). Applications of nanoparticles in drug delivery include drug carriers in diseases such as cancer, cardiovascular disease, and Alzheimer's. The use of these nanocarriers is very effective for neurological diseases such as Alzheimer's (Veisi et al., 2018; Liao et al., 2015; Fazaeli et al., 2010; Konda et al., 2014; Mazaahir et al., 2012). Due to their size, these nanoparticles can cross the blood-brain barrier, which has always been a barrier to the passage of drugs to the affected area in this type of destructive brain disease. Due to their small size, nanoparticles can also be used in brain cancers (Mazaahir et al., 2012; Kiasat et al., 2013; Celardo et al., 2011). The goal in making nanoparticles is to control the surface properties, particle size, and release of a specific and efficient drug in a specific place and time for the drug to be as effective as possible (Konda et al., 2014; Mazaahir et al., 2012; Kiasat et al., 2013; Celardo et al., 2011). Nanoparticles are widely used in tissue engineering scaffolds, targeted drug delivery and disease diagnosis. At present, many drug delivery systems are made of nanoparticles and different materials have been used as drug stimulants or enhancers to ameliorate the effectiveness of treatment and the durability and stability as well as the safety of anticancer drugs (De Jong and Borm, 2008; Borm et al., 2006; Stapleton and Nurkiewicz, 2014). The substances used to release cancer drugs are divided into different polymers, magnetic, and biomolecules. These materials can also provide surface modifications such as binding to target antibodies and ligands to make the nanoparticles act purposefully to increase the effectiveness of the treatment (Borm et al., 2006; Stapleton and Nurkiewicz, 2014; Patra et al., 2018).

Recently, various nanoparticles have been specifically designed to deliver anticancer drugs and nucleic acids such as DNA and RNA to cancer cells and as a result, they open up new avenues in cancer treatment strategies. These nanoparticles can be classified as organic or inorganic (De Jong and Borm, 2008; Borm et al., 2006; Stapleton and Nurkiewicz, 2014; Patra et al., 2018; Itani and Al Faraj, 2019). The last decade has seen the promising emergence of nanoparticles in cancer treatment systems such as drug delivery and recombinant proteins with anti-tumor properties. Special features of the microenvironment around the tumor allow nanoscale systems to accumulate at the tumor site (De Jong and Borm, 2008; Borm et al., 2006; Stapleton and Nurkiewicz, 2014; Patra et al., 2018). Therefore, some nanoparticles with adjuvant properties, when they carry peptides or proteins, can increase the activity of cells in the reticuloendocardial system and activate macrophages and dendritic cells (Borm et al., 2006; Stapleton and Nurkiewicz, 2014; Patra et al., 2018; Itani and Al Faraj, 2019). Activated macrophages and dendritic cells swallow and process the complex, and immune responses are formed more efficiently. These nanoparticles can increase the response of the immune system to the target antigen, as well as to direct and direct this system to create a specific type of response (Trojer et al., 2013; Liu et al., 2014). By using these nanoparticles as antigen carriers, the amount of recombinant protein used as well as antigen toxicity is reduced and the destructive effects of proteases on protein antigen are reduced (Celardo et al., 2011; De Jong and Borm, 2008; Borm et al., 2006; Stapleton and Nurkiewicz, 2014). This strategy enhances the efficiency of the target protein in inducing immune responses against the tumor, which is important in advancing functional goals such as protein and effective drug delivery (Itani and Al Faraj, 2019; Trojer et al., 2013; Liu et al., 2014).

Chitosan is one of the distinctive and most versatile biopolymers encompassing linear polysaccharides with reactive amino groups (*N*-deacetylated chitin), with relatively biocompatible, and inexpensive abundance compared to other biopolymers (Veisi et al., 2018). Alginate is another homopolymer consisting of guluronic (G) and mannuronic (M) acid blocks (Liao et al., 2015). The duo is compatible to each other forming covalent or co-ordinates attachments. The fusion of biopolymers and metal NPs can afford fascinating nano biocomposite for biological application. So, we have been prompted to use naturally occurring polysaccharide biomolecules, chitosan/alginate composite for the in *situ* synthesis and stabilizing of Cu nanoparticles. This kind of double shell-core structure affords additional stability to the copper nanoparticles from self-aggregation and unwanted oxidation. Also, the properties of Fe_3O_4/PEG_{2000}/Cu NPs against common

breast cancer cell lines i.e. infiltrating lobular carcinoma of breast (UACC-3133), inflammatory carcinoma of the breast (UACC-732), and metastatic carcinoma (MDA-MB-453) were evaluated.

2. Experimental

2.1. Preparation of the Cu NPs@CS/Alg nanocomposite

First, 0.5 g of chitosan (98% NH_2) and 0.5 g sodium alginate were dissolved in100 mL of 2% (v/v) acetic acid solution and sonicated for 30 min. Next it stirring at room temperature overnight to obtain chitosan/sodium alginate composite (CS/Alg). Then pH of the chitosan/alginate solution was adjusted to pH 10 (NaOH, 3 wt%) and an aqueous solution of $CuSO_4{\cdot}5H_2O$ (50 mg in 10 ml) is added drop-wise over 10 min, at room temperature. The mixture is mechanically stirred for 6 h at 100 °C. The progress of the reaction could be monitored by the change in color from watery blue to the dark-brown color of the aqueous solution was due to the excitation of the surface plasmon resonance and SPR band which both play an important role in the confirmation of copper nanoparticles formation. The prepared Cu NPs@CS/Alg nanocomposite were collected by centrifugation and washed several times with DI-water and dried.

2.2. Antioxidant activities of Cu NPs@CS/Alg nanocomposite

The free radical scavenging test was first performed by Blois in 1958, and after some modification by numerous studies in its current form. DPPH method is one of the most widely used methods for estimating antioxidant content. DPPH is a stable radical that reacts with hydrogen atom compounds. This test is based on the inhibition of DPPH, which causes the decolorization of DPPH solution by adding radical species or antioxidants. DPPH changes color from purple to yellow by taking an electron from the antioxidant compound. The free radicals in DPPH are adsorbed at 517 nm, which follows Beer Lambert's law, and decreased absorption is linearly related to the amount of antioxidants; the higher the amount of antioxidants, the more DPPH is consumed and the more purple turns yellow (Lu et al., 2021).

This experiment was performed with few changes in the method of Lu et al (Lu et al., 2021). 0.5 ml of 0.1 mM DPPH solution prepared in 95% ethanol was mixed with 100 µl of Cu NPs@CS/Alg nanocomposite. The resulting solution was kept in the dark at 38 °C for 31 min. The absorbance of the samples was then read at 518 nm (Lu et al., 2021).

To compare the activity of Cu NPs@CS/Alg nanocomposite, standard BHT compound was used as a standard antioxidant (Lu et al., 2021).

To determine the amount of IC50 (IC50 is defined as the concentration required to inhibit 50% of the antioxidant activity) for Cu NPs@CS/Alg nanocomposite, experiments were performed at eleven different concentrations of the desired nanoparticle solution and BHT. Each experiment was performed in three shifts and the mean values were calculated (Lu et al., 2021).

Percentage of radicalization activity was calculated through the following equation (Lu et al., 2021):

$$\text{Inhibition } (\%) = \frac{\text{Sample A.}}{\text{Control A.}} \times 100$$

In this regard, the blank adsorption indicates the adsorption of the control solution, which contains 0.5 ml of DMPH solution and 100 µl of 95% ethanol instead of Cu NPs@CS/Alg nanocomposite solution and adsorption of the reaction indicates the adsorption of the solution content of the Cu NPs@CS/Alg nanocomposite sample (Lu et al., 2021).

2.3. Anti-human breast cancer properties of Cu NPs@CS/Alg nanocomposite

One of the cytotoxicity test methods to measure the rate of cell death is the MTT method, which is based on the formation of formazan dye by reducing the substance MTT (dimethyl thiazole 2 and 5 diphenyltetrazolium bromide) or other tetrazolium salts (Lu et al., 2021). By breaking the MTT tetrazolium ring by mitochondrial enzymes in living cells, insoluble purple formazan crystals are formed. The formation of these crystals indicates the activity of respiratory chain enzymes and is a measure of cell viability. By measuring the amount of absorption by spectrophotometer at specific wavelengths, the number of living cells can be determined. This test is performed according to ISO 10993-5 and its purpose is in vitro evaluation of cytotoxicity. Cytotoxicity test is performed according to ISO10993-5 standard and in three ways: NRU test, CFU test, MTT test and XTT test. The most common method for assessing cytotoxicity is to measure cell survival by MTT (Lu et al., 2021). The basis of MTT method is based on the intensity of dye produced by the mitochondrial activity of cells, that measured at a wavelength of 540–630 nm and directly proportional to the number of living cells, the increase or decrease in the number of living cells is linearly related to the activity of cell mitochondria. MTT tetrazolium dye is revived in active (metabolically) cells. Mitochondrial dehydrogenases in living cells produce NADH and NADPH, leading to an insoluble purple precipitate called formazan. This precipitate can be dissolved by isopropanol or dimethyl sulfoxide (Lu et al., 2021). Dead cells, on the other hand, are unable to perform this conversion due to the inactivity of their mitochondria and therefore do not show a signal. In this method, dye formation is used as a marker for the presence of living cells. In recent years, MTT testing has been the most important measurement method to evaluate the toxicity and anti-cancer effects of metal nanoparticles (Lu et al., 2021).

In this study, infiltrating lobular carcinoma of breast (UACC-3133), inflammatory carcinoma of the breast (UACC-732), and metastatic carcinoma (MDA-MB-453) cells were used to evaluate the anticancer effect of Cu NPs@CS/Alg nanocomposite on cell culture. For this purpose, each cell line was placed separately in T25 flasks with a complete culture medium (including DMEM (Dulbecco's Modified Eagle Medium, 10% complementary bovine fetal serum, and 1% penicillin–streptomycin solution) and at 37 °C in the incubator, cell culture was incubated with 5% CO_2.

After obtaining 80% cell density, the sample was exposed to 1% trypsin-EDTA solution and after 3 min of incubation at 37 °C in a cell culture incubator with 5% CO_2 and observation of cells removed from the bottom of the plate, the sample was centrifuged at 5000 rpm for 5 min and then the cell precipitate was decrypted by adding trypsin culture medium. Then, the cell suspensions after adding trypan blue dye were counted by neobar slide and cytotoxicity test was performed by MTT

method. For this purpose, in each well of 98 cell culture plate, 10,000 KYSE-270, OE33, and ESO26 cells were introduced with 200 µl from the complete cell culture medium and to achieve the cell monolayer density, the plate was re-exposed to 5% CO_2 at 37 °C. After reaching 80% cell growth, the culture medium was removed and the cell surface was first washed with PBS buffer, again, in all wells, a complete two-concentration culture medium of 100 µl was introduced and 100 µl of a solution of Cu NPs@CS/Alg nanocomposite dissolved in PBS (mg/mL^2) was introduced into well No. 1. After mixing the nanoparticles in the culture medium, 100 µl of it was removed and added to the second well. In the next step, 100 µl of the second well was removed after stirring the medium and added to well 3. This operation was performed up to well 11 and thus the amount of nanoparticles in each well was halved, respectively. Well No. 12 contained only one cell and complete culture medium of one concentration and remained as a control. The plate was again exposed to 5% CO_2 at 37 °C for 24 h and after 24 h the cytotoxicity was determined using tetrazolium dye. 10 µl of tetrazolium dye (5 mg/ml) was added to all wells, including the control, and the plate was exposed to 5% CO_2 at 37 °C for 2 h. The dye was then removed from the wells and 100 µl of DMSO (Dimethyl sulfoxide) was added to the wells, the plate was wrapped in aluminum foil and shaken thoroughly in a shaker for 20 min. Finally, cell survival was recorded in ELISA reader at 540 nm (Lu et al., 2021):

$$\text{Cellviability } (\%) = \frac{\text{Sample A.}}{\textit{Control A.}} \times 100$$

Then, based on the absorption rate of each well and its comparison with the control, the inhibitory concentration of 50% (IC50) was obtained (Lu et al., 2021).

After collecting data, Minitab statistical software was used for statistical analysis. Evaluation of antioxidant results in a completely randomized design and comparison of means was Duncan post-hoc test with a maximum error of 5%. To measure the percentage of cell survival in factorial experiments with the original design of completely randomized blocks and compare the means, Duncan post-hoc test with a maximum error of 5% was used. The 50% cytotoxicity (IC50) and 50% free radical scavenging (IC50)) was estimated with ED50 plus software (INER, V: 1.0). Measurements were reported as mean ± standard deviation.

3. Results and discussion

Cancer is now one of the leading causes of death worldwide. Existing treatments have not been able to meet the treatment needs for various types of cancer. Therefore, the use of new technologies in the prevention and treatment of cancer can be helpful. Extensive research on nanoparticles has been conducted in recent years (Gao et al., 2015; Mohammed et al., 2016; Li and G f., 2014; Yang et al., 2011; Xinli, 2012). The advent of nanotechnology has had a profound effect on many areas of healthcare and scientific research. Common cancer treatments, including chemotherapy, radiation and surgery, may reduce the size of the tumor, but the effect of these methods is transient and has no positive effect on patient survival. Therefore, replacing more effective, more specific therapies with fewer side effects with higher anti-cancer activity is a dominant issue in clinical oncology (Yang et al., 2011; Xinli, 2012; Allen, 2002; Byrne et al., 2008; Torchilin, 2007).

The gradual maturation of nanotechnology has been considered not only for treating cancer but also for a wide variety of applications, especially for drug delivery and diagnostic and imaging cases. There are many types of nanoparticles available and choosing the right carriers according to demand is a key issue (Torchilin, 2007; Pranali, 2013; Zhang et al., 2014; Matsumura et al., Cancer 2004). Nanoparticles are very close in size to biological molecules in terms of size and can easily penetrate into the cell, for this reason, one of the goals of nanotechnology is to mount molecules and drugs on nanoparticles and transfer them to the target cell (Torchilin, 2007; Pranali, 2013; Zhang et al., 2014; Matsumura et al., Cancer 2004). It is also possible to create different surface properties for nanoparticles by attaching protective ligands to increase the nanoparticles' resistance to the immune system and increase their presence in the bloodstream, and even binding ligands to specifically bind the nanoparticles to the target tissue (Zhang et al., 2014; Matsumura et al., Cancer 2004,; Nie et al., 2007; Gao et al., 2002; Davis et al., 2008).

3.1. Structural characterization of synthesized Cu NPs@CS/Alg nanocomposite

A post-synthetic modification approach was followed in the strategically developed Cu NPs@CS/Alg nanocomposite. It was based on preparing a hydrogel by two biopolymers, chitosan and alginate, that then further applied for in *situ* synthesized Cu NPs following the green process. The final Cu NPs@CS/Alg nanocomposite was subsequently analyzed through several techniques, such as, FT-IR, SEM, TEM, EDX, XRD and ICP-OES.

In order to rationalize the stepwise synthesis of Cu NPs@CS/Alg nanocomposite, FT-IR spectra of CS, Alig, CS/Alg composite and Cu NPs@CS/Alg nanocomposite have been depicted in Fig. 1. The characteristic absorption bands of chitosan are represented in Fig. 1a as, 1028 cm^{-1} (C—N stretching), 1382 cm^{-1} (C—O stretching of CH_2-OH linkage), 1591 cm^{-1} (N—H bending), 1648 cm^{-1} (C═O stretching of amide linkage) and 3400–3500 cm^{-1} (O—H and N—H stretching) (Fazaeli et al., 2010). In the IR spectrum of sodium alginate (Fig. 1b), the asymmetric and symmetric stretching frequencies of C═O from COONa appeared at 1618 and 1418 cm^{-1}. The aliphatic C—H stretching and O—H stretching vibrations appear at 2852 and 3512 cm^{-1} respectively (Konda et al., 2014; Mazaahir et al., 2012; Kiasat et al., 2013). Fig. 1c, representing the FT-IR spectrum of CS/Alg composite is literally an amalgamation of both individual component peaks with the slight shifting of characteristic peaks, which is a clear sign of successful hydrogelation of the biomolecules. And finally in the spectrum of Cu NPs@CS/Alg nanocomposite, as seen in Fig. 1d, due to strong complexation of in situ synthesized Cu atoms with the surface functions like NH_2, OH, CHOH, CH_2OH, C═O etc, the corresponding IR absorption bands are observed to be shifted to higher or lower regions respectively.

The innate microstructural features, morphology, size and shape of the as-synthesized Cu NPs@CS/Alg nanocomposite was resolved by SEM and TEM images. Fig. 2 shown the SEM image of the quasi-spherical Cu NPs@CS/Alg nanocom-

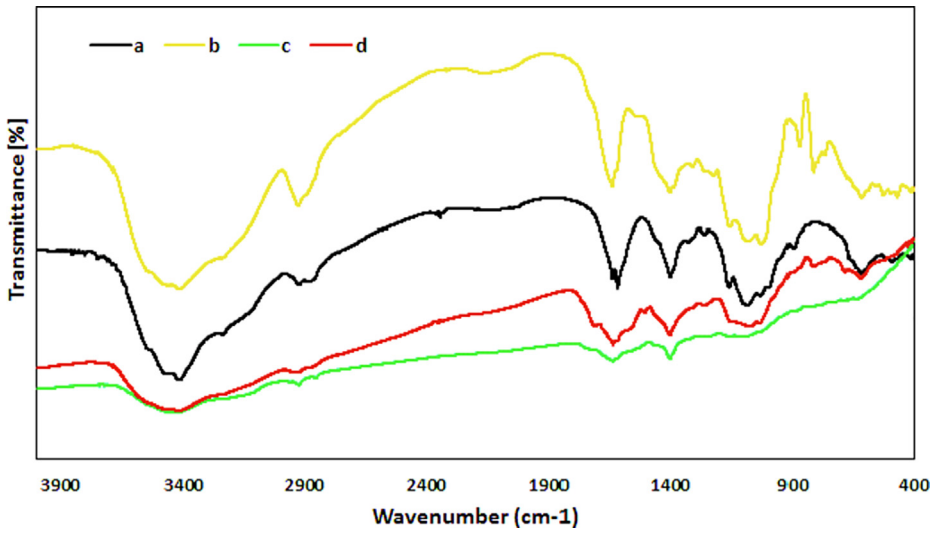

Fig. 1 FT-IR spectra of a) CS, b) Alig, c) CS/Alg and d) Cu NPs@CS/Alg.

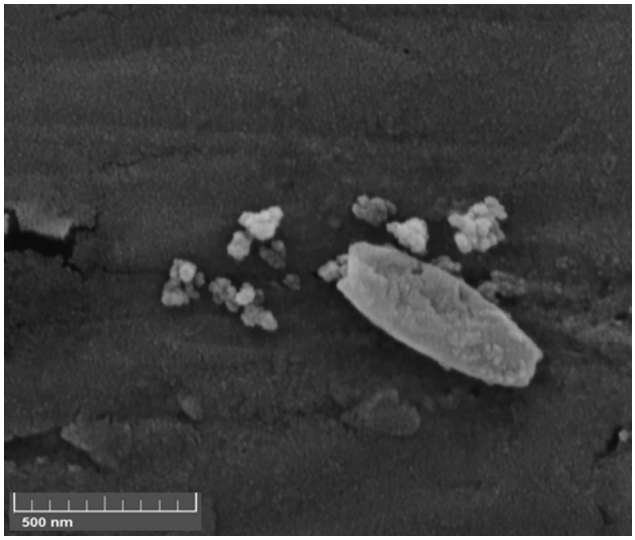

Fig. 2 FE-SEM image of the Cu NPs@CS/Alg nanocomposite.

posite. Functional modifications can be imagined from the surface coating over the particles. To have an idea of the more detailed structure of the nanocomposite, TEM analysis was carried out (Fig. 3). The related outcomes also justify the SEM results. It reveals the perfectly spherical nanoparticles. The average diameter of the Cu NPs was around 10–20 nm. The particles are observed to be well dispersed without any sign of aggregation.

In order to have an idea of the chemical composition of the Cu NPs@CS/Alg nanocomposite, EDX analysis was carried out and the profile is shown in Fig. 4. The elements C, N, O and Na are attributed to CS/Alg composite. The presence of Cu element confirmed the successful fabrication of Cu NPs over CS/Alg composite. The results were further justified by SEM elemental mapping analysis (Fig. 5). The compositional map reveals the C, N and Cu species to exist with excellent dispersion throughout the matrix surface.

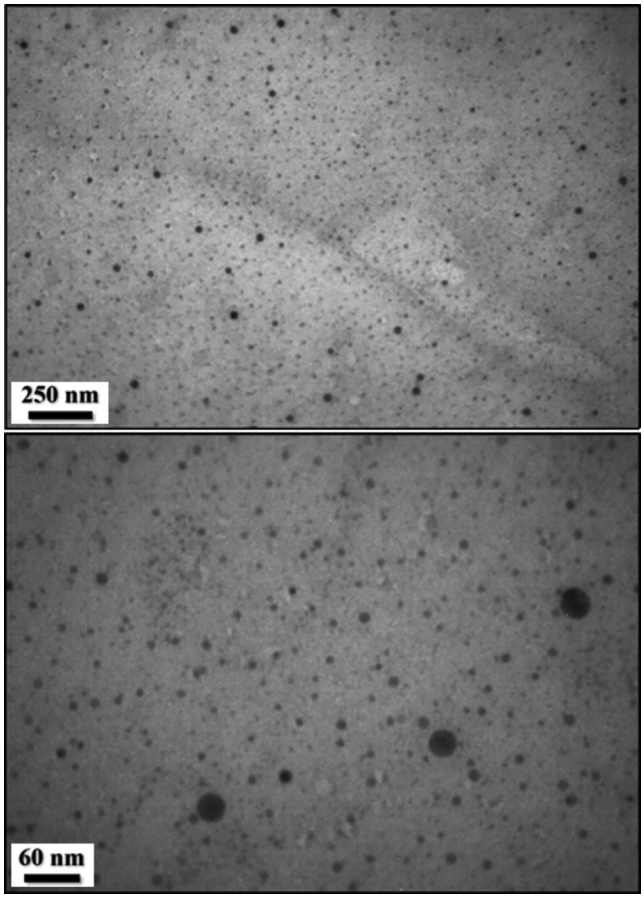

Fig. 3 TEM images of the Cu NPs@CS/Alg nanocomposite.

Finally, the phase structure and crystallinity of the prepared Cu NPs@CS/Alg nanocomposite was ascertained from XRD analysis (see Fig. 6). While the initial phase in the 2θ region up to 20° was attributed to CS/Alg composite and non-crystalline. Additionally, the several diffraction peaks appeared at the 2 Theta degree of 43.2°, 50.3° and 74.1° corre-

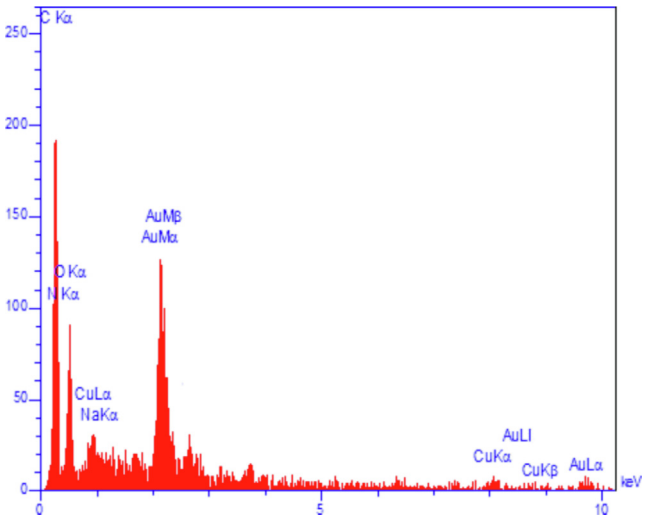

Fig. 4 EDX spectrum of the Cu NPs@CS/Alg nanocomposite.

sponds to the crystal planes (1 1 1), (2 0 0), and (2 2 0), respectively. All the peaks are authenticated standard data which that satisfies the highly pure crystalline Cu nanoparticles (JCPDS No. 04-0836).

3.2. Analysis of the antioxidant potentials of Cu NPs@CS/Alg nanocomposite

Oxidative stress is caused by an imbalance between the production of free radicals and metabolic reactions, which leads to damage to lipids, proteins and nucleic acids. These damages may be due to low levels of antioxidants or an excessive increase in the production of free radicals in the body (Namvar et al., 2014; Sankar et al., 2014; Katata-Seru et al., 2018). In humans, oxidative stress is associated with chronic diseases such as diabetes and cancer. Therefore, the production of synthetic and natural antioxidants is necessary to prevent oxidative stress and its destructive effects.

Antioxidants effectively and in various ways reduce the harmful effects of free radicals in the biological and food systems and cause detoxification (Sankar et al., 2014; Katata-Seru et al., 2018; Sangami and Manu, 2017). In this regard, green nanoparticles can be used (using plant substrates to prepare nanomaterials that are environmentally friendly and do not contain any harmful chemicals) that show antioxidant properties. At present, the use of non-toxic substances in synthesizing nanoparticles to prevent biological hazards, especially in medical and pharmaceutical applications is considered (Namvar et al., 2014; Sankar et al., 2014; Katata-Seru et al., 2018; Sangami and Manu, 2017; Beheshtkhoo et al., 2018; Radini et al., 2018). Many researchers have focused on bioactive substances derived from plants or other sources such as bacteria,

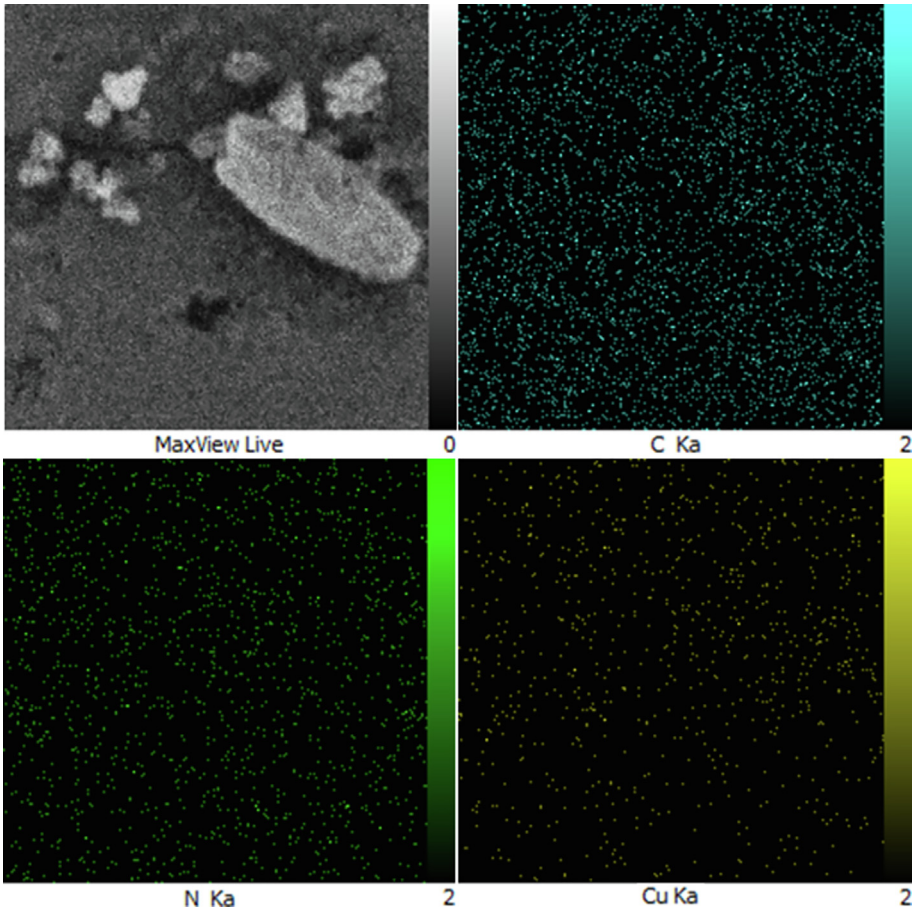

Fig. 5 Elemental mapping of Cu NPs@CS/Alg nanocomposite.

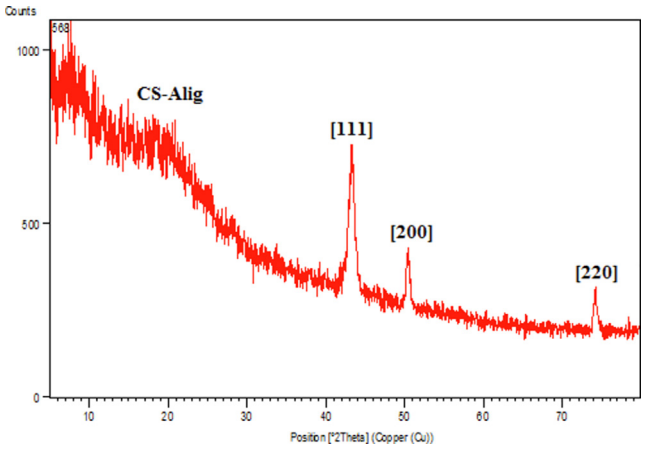

Fig. 6 XRD pattern of the Cu NPs@CS/Alg nanocomposite.

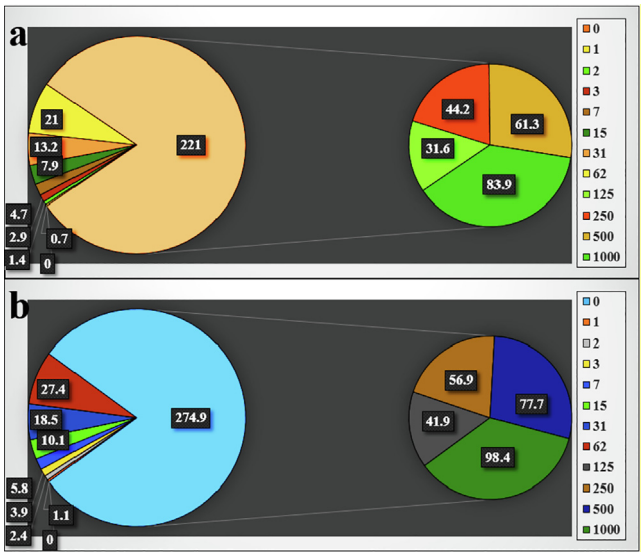

Fig. 7 The antioxidant properties of Cu NPs@CS/Alg nanocomposite (a) and BHT (b) against DPPH.

fungi and yeast for synthesizing nanoparticles. The green synthesis method is thought to increase the biocompatibility and performance of metal nanoparticles for biological applications due to removing harmful chemicals (Davis et al., 2008; Namvar et al., 2014; Sankar et al., 2014; Katata-Seru et al., 2018). During the bioproduction stages of nanoparticles, their extracellular production using plants or their extracts is more beneficial and their production can be adjusted in a controlled way based on size, distribution and shape for different purposes (Davis et al., 2008; Namvar et al., 2014; Sankar et al., 2014; Katata-Seru et al., 2018; Sangami and Manu, 2017; Beheshtkhoo et al., 2018).

In the recent study, the scavenging capacity of Cu NPs@CS/Alg nanocomposite and BHT at different concentrations expressed as percentage inhibition has been indicated in Table 1 and Fig. 7. In the antioxidant test, the IC50 of Cu NPs@CS/Alg nanocomposite and BHT against DPPH free radicals were 334 and 193 µg/mL, respectively (Table 1).

3.3. Analysis of the anti-human breast cancer potentials of Cu NPs@CS/Alg nanocomposite

The unique physical and chemical properties of copper nanoparticles have attracted the attention of the scientific community due to their high plasmonic properties, heat transfer, chemical stability and antibacterial effects. Using copper is nothing new; it dates back to Hippocrates, who used it as an antibacterial to control wounds (Radini et al., 2018; Beyene et al., 2017; Chen and Schluesener, 2008). Today, copper nanoparticles are used in many commercial products, including soap, food, plastics, catheters, textiles, and bandages. However, their mechanism of action is still unknown. Many factors (shape, size, surface chemistry, morphology, density, charge, and purity) affect the biological activity of copper nanoparticles (Alexander, 2009; Bhattacharya, 2011; Jo et al., 2015). Another important feature of copper nanoparticles is their role in treating cancer. Copper nanoparticles are a promising tool as an anti-cancer agent in diagnosis and evaluation. They have many benefits with strong effects against different cancer cell lines. Their better penetration and ability to detect copper nanoparticles in the body make them a more effective tool in treating low-risk cancers compared to standard treatments (Bhattacharya, 2011; Jo et al., 2015; Rai et al., 2014; Riehemann et al., 2009). The unique properties of copper nanoparticles, such as their optical properties, easy synthesis and high surface-to-volume ratio, make them suitable for treating cancer. Copper nanoparticles can also be conjugated to various molecules, including DNA and RNA, to target different cells and antibodies or polymers. These important factors are important for increasing the half-life for circulating in vivo, which is very important in drug and gene delivery applications. In addition, copper nanoparticles are used as a cancer cell erosion tool because of their ability to convert radio frequency into heat (Jo et al., 2015; Rai et al., 2014; Riehemann et al., 2009; Huang et al., 2017; Conde et al., 2012).

Copper nanoparticles induce changes in cell morphology, decrease cell metabolic activity, increase oxidative stress leading to mitochondrial damage, and ultimately damage DNA by producing reactive oxygen species (ROS). Adsorption of copper nanoparticles occurs through endocytosis (Riehemann et al., 2009; Huang et al., 2017; Conde et al., 2012; Bhattacharyya et al., 2011). Examination of cancer morphology shows that synthesizing copper nanoparticles can significantly increase cell death. Now, if chitosan is combined with copper nanoparticles, it increases the rate of cell death. The mechanism of anti-cancer activity of copper nanoparticles is that copper nanoparticles induce apoptosis in cancer cells (Rai et al., 2014; Riehemann et al., 2009; Huang et al., 2017). Copper nanoparticles are capable of altering the regula-

Table 1 The IC50 of Cu NPs@CS/Alg nanocomposite and BHT in the antioxidant test.

	Cu NPs@CS/Alg nanocomposite	BHT
IC50 (µg/mL)	334 ± 0[b]	193 ± 0[a]

The numbers indicate the percent of free radical (DPPH) inhibition at the concentrations of 0–1000 µg/mL of Cu NPs@CS/Alg nanocomposite (a) and BHT (b).

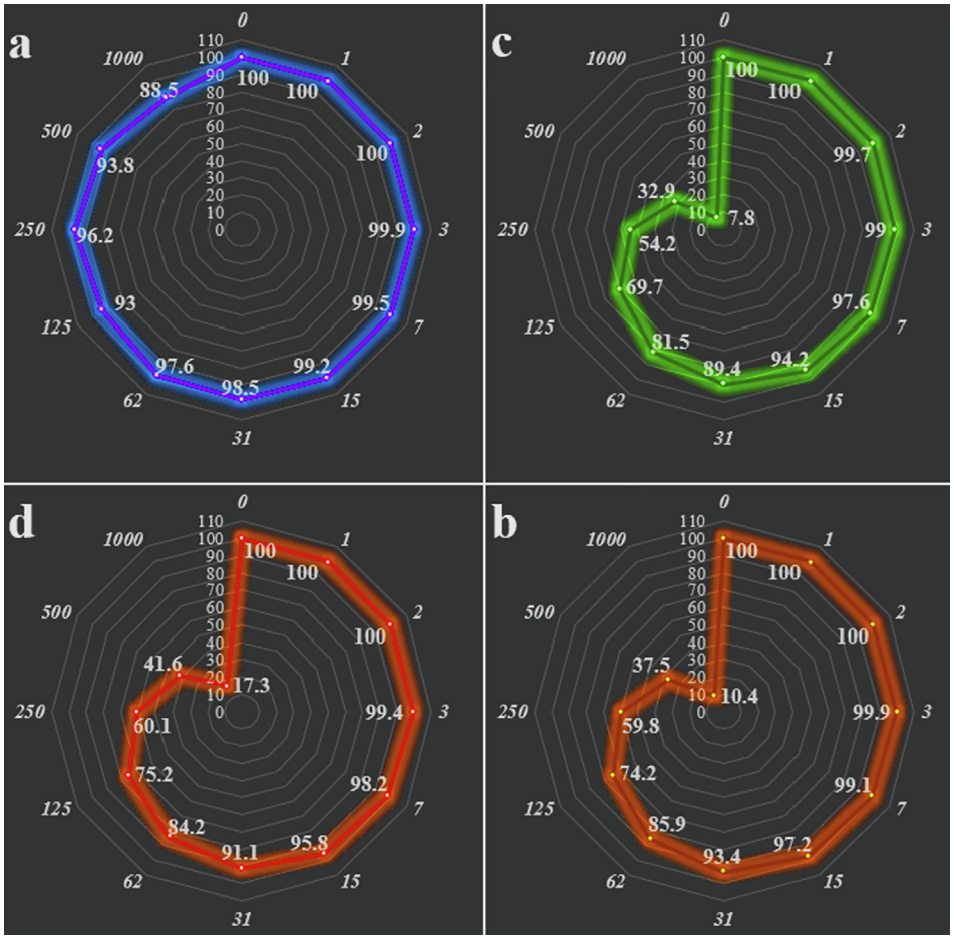

Fig. 8 The anti-human breast cancer properties (Cell viability (%)) of Cu NPs@CS/Alg nanocomposite (Concentrations of 0–1000 μg/mL) against normal (HUVEC: a) and human breast cancer (infiltrating lobular carcinoma of breast (UACC-3133 (b)), inflammatory carcinoma of the breast (UACC-732 (c)), and metastatic carcinoma (MDA-MB-453 (d))) cell lines.

tion of more than 1,000 genes. Among these genes are metallothianonine, chaperones, and histones. Autophagy is one of the anti-cancer mechanisms of copper nanoparticles, which induces cell death (Huang et al., 2017; Conde et al., 2012; Bhattacharyya et al., 2011). Nanoparticle-induced autophagy is an important cell degradation process, and increased autophagy can also increase cell death. Recent studies show that copper nanoparticles can induce autophagy through the accumulation of autophagolysosomes in ovarian cancer cells. Therefore, autophagy can affect performance; At low levels it can increase cell survival and at high levels it can cause cell death. Another mechanism of copper nanoparticles is the increase in the level of internal ion concentration, which increases the production of ROS. On the other hand, uncontrolled production of ROS can lead to serious cellular damage, including DNA and mitochondrial damage, leading to programmed cell death (apoptosis) (Bhattacharyya et al., 2011; Sau et al., 2010; Sperling and Parak, 2010; Pelaz et al., 2015; Day et al., 2009). The cytotoxic activity of copper nanoparticles in breast cancer cells is through activation of caspase 3, p53, pErk1/2 and lack of Bcl2 expression. In particular, copper nanoparticles induce cell death through various processes including ROS production and increased lactate dehydrogenase secretion, induction of apoptosis, induction of autophagic genes, mitochondrial dysfunction and activation of caspase and DNA damage (Beyene et al., 2017; Chen and Schluesener, 2008; Alexander, 2009; Bhattacharya, 2011; Jo et al., 2015; Rai et al., 2014). Copper nanoparticles, like other biomaterials, can cause toxic effects in living organisms. The toxicity created by these particles depends on their properties and route of entry. The toxicity induced by copper nanoparticles is due to oxidative stress, which accumulates in the cytoplasm and cell nucleus, leading to the production of free radicals (Huang et al., 2017; Conde et al., 2012). Copper nanoparticles are initially separate particles that have the highest toxicity due to their high contact surface, but their toxicity decreases over time and joins together (Riehemann et al., 2009; Huang et al., 2017; Conde et al., 2012; Bhattacharyya et al., 2011; Sau et al., 2010).

In this investigation, the treated cells with different concentrations of the present Cu NPs@CS/Alg nanocomposite were assessed by MTT assay for 48 h about the cytotoxicity properties on normal (HUVEC) and breast malignancy cell lines i.e. infiltrating lobular carcinoma of breast (UACC-3133), inflammatory carcinoma of the breast (UACC-732), and metastatic carcinoma (MDA-MB-453) (Fig. 8).

The absorbance rate was evaluated at 570 nm, which represented viability on normal cell line (HUVEC) even up to

Table 2 The IC50 of Cu NPs@CS/Alg nanocomposite in the anti-human breast cancer test.

	HUVEC	KYSE-270	OE33	ESO26
IC50 (μg/mL)	–	297 ± 0[a]	386 ± 0[b]	359 ± 0[b]

The numbers indicate the percent of cell viability at the concentrations of 0–1000 μg/mL of Cu NPs@CS/Alg nanocomposite against several human breast cancer cell lines.

1000 μg/mL for Cu NPs@CS/Alg nanocomposite (Table 2 and Fig. 8).

The viability of malignant breast cell lines reduced dose-dependently in the presence of Cu NPs@CS/Alg nanocomposite. The IC50 of Cu NPs@CS/Alg nanocomposite were 297, 386, and 359 μg/mL against KYSE-270, OE33, and ESO26 cell lines, respectively (Table 2).

It seems that the anti-human breast cancer effect of recent nanoparticles is due to their antioxidant effects. Because tumor progression is so closely linked to inflammation and oxidative stress, a compound with anti-inflammatory or antioxidant properties can be an anticarcinogenic agent (Namvar et al., 2014; Sankar et al., 2014).

Many nanoparticles have pharmacological and biochemical properties, including antioxidant and anti-inflammatory properties, which appear to be involved in anticarcinogenic and antimutagenic activities (Sankar et al., 2014; Katata-Seru et al., 2018). Today, nanoparticles synthesized by biological methods play a vital role in treating many diseases, including cancer (Katata-Seru et al., 2018; Sangami and Manu, 2017; Beheshtkhoo et al., 2018). Nanoparticles synthesized by biological methods are no longer the only ones in traditional medicine, in addition, they have been able to adopt an industrial line of natural products for treating various cancers. Various cell lines from cancers of the prostate, ovary, lung, liver, and pancreas have been treated with metallic nanoparticles (Namvar et al., 2014; Sankar et al., 2014; Katata-Seru et al., 2018).

4. Conclusion

Due to the advantages of nanoparticles such as the ability to carry drugs, reduce toxicity, controlled drug release and specific drug delivery to the target tissue, these structures have been able to attract the attention of many researchers. As a result of these features, nanotechnology has great potential for cancer treatment that can move from a research laboratory to a patient's bedside. One possible concern that limits the administration of some nanoparticles in treating cancer is their toxicity, which needs further investigation. However, nanotechnology-based cancer therapies will continue to be developed to improve treatment outcomes.

This study demonstrates an effective, simple, green and cost-effective method for preparing Cu NPs@CS/Alg nanocomposite applying CS/Alg bio-composite as a green reductant and stabilizer agent of the copper NPs. The particles were characterized by FTIR, SEM, EDS, TEM, XRD and elemental mapping techniques.

The viability of malignant breast cell lines reduced dose-dependently in the presence of Cu NPs@CS/Alg nanocomposite. The IC50 of Cu NPs@CS/Alg nanocomposite were 297, 386, and 359 μg/mL against infiltrating lobular carcinoma of breast (UACC-3133), inflammatory carcinoma of the breast (UACC-732), and metastatic carcinoma (MDA-MB-453) cell lines, respectively. The Cu NPs@CS/Alg nanocomposite showed the best antioxidant activities against DPPH. The IC50 of Cu NPs@CS/Alg nanocomposite and BHT against DPPH free radicals were 334 and 193 μg/mL, respectively. After clinical study, Cu NPs@CS/Alg nanocomposite can be utilized as an efficient drug in the treatment of breast cancer in humans.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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