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Green biosynthesis, structural characterization and anticancer activity of copper oxide nanoparticles from the brown alga *Cystoseira myrica*

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ABSTRACT

Copper oxide nanoparticles (CuONPs) were synthesized by a green method using aqueous extract of *Cystoseira myrica* as capping and reducing agent. A UV-visible spectrophotometer was used to characterize the produced nanoparticles. The aqueous extract of *C. myrica* at pH 5 and 100°C after 150 minutes was shown to be the optimal condition for the synthesis of copper oxide nanoparticles. The Structure and characters of synthesized nanoparticles were confirmed using TEM, DLS, XRD, and FTIR. The efficiency of different concentrations of copper oxide nanoparticles (25, 50, 75, 100) µg/ml synthesized from aqueous extract of *C. myrica* exhibit high antitumor-activity against some cancer lines such as HepG2 and MCF-7. The percent of cell viability decreases as the concentration of nanoparticles rises. Copper oxide nanoparticles at a concentration of 100 µg/ml were found to have the lowest percentage of cell viability in HepG2 (18.7%), while MCF-7 was (19.27%).

INTRODUCTION

Scientists have recently focused on the biosynthesis of Metal-Based NPs as it is one of the most promising technology, biologically safe, cost-effective as compared with chemical and physical methods (**Zhang *et al.*, 2020)**. Metal oxide nanoparticles are getting popular in a wide range of applications. The recent convergence of biology and nanotechnology has resulted in the development of a new discipline called nanobiotechnology, which reveals the use of biological products for example plants and algae in a variety of biophysical and biochemical processes (**Shah *et al.*, 2015**). It concerned the development and synthesis of different types of nanoparticles with diameters ranging from 1 to 100 nm **(Zhou *et al.*, 2006)**.

Metallic nanoparticles (NPs) are being acclaimed as the material of the future, then they have gotten a lot of attention and used in a variety range of applications (**Senapati *et al.*, 2005)**. They also have distinct catalytic, electrical, magnetic, and optical

properties that distinguish them from bulk metals (**Ghorbani *et al.*, 2015**). They also use in nanodevices, optoelectronics, information storage, nanoelectronics, nanosensors, and catalysis (**El-Trass *et al.*, 2012**). Also they are widely used in environmental remediation, industry, medical, and even home applications, however, there are few publications on metal oxide NPs biosynthesis, particularly employing algae (**Fawcett *et al.*, 2017**).

Copper oxide nanoparticles (CuONPs) are considered the simplest forms of copper compounds. These nanoparticles have a wide range of industrial uses due to their low production costs and potential physical features (**Amiri *et al.*, 2017)**. CuONPs have gotten a lot of interest for their bioactive applications because of their varied functionalities like catalysis, photothermal, and photoconductivity (**Schmid, 1992**), anticancer, antimicrobial (**Devasenan *et al.*, 2016),** biocidal properties (**Gudej and Tomczyk, 2004**). Also, CuONPs are high chemical, less toxic, long self-life, and physically stabile as compared to the organic antimicrobial agents (**Mahapatra *et al.*, 2008**).

Biological systems can serve as a "bio-laboratory" to synthesize metal oxide and pure metal particles at the nanometer scale using a biomimetic approach, avoiding the drawbacks of chemical and physical methods, and suggesting environmentally friendly alternatives to produce energy-efficient, low-cost and non-toxic CuONPs (**Mohanpuria *et al.*, 2008**). Copper oxide nanoparticles which is biosynthesized through green routes are eco-friendly, cost-effective, safe, stable, and longer shelf life. Most studies on the green synthesis of CuONP used plant extracts **(Chung *et al.*, 2017; Batoool and Masood, 2017; Rehana *et al.*, 2017; Yugandhar *et al.*, 2017; Buazar *et al.*, 2019; Awwad and Amer, 2020; Murthy *et al.*, 2020; Wu *et al.*, 2020; Zhao *et al.*, 2020**).

Algae is a diverse group in the plant kingdom that is not only accumulates metals by chelation and chemical transformation but also reported to synthesize metal nanoparticles and bio-mineral structures (**Azizi *et al.*, 2014**). Recently, the biosynthesis of nanoparticles using algae got a great attention due to its high efficacy and easy access (**Shah *et al.*, 2015).** Compared to other biological sources, algal extracts are rich source of bioactive metabolites that act as reducing and stabilizing agents for the production of nanoparticles (**Waris *et al.*, 2021).**They have acted as eco-friendly precursors for NPs synthesis with various applications. Few researches have been reported on the biosynthesis of copper oxide nanoparticles from algae and its anticancer activities (**Abboud *et al.*, 2014; Bhattacharya *et al.*, 2019; Ramaswamy *et al.*, 2016)**.

The present study aimed to confirm the ability to synthesis copper oxide nanoparticles by *Cystoseira myrica* extract. Different characterization approaches used to investigate their properties and cytotoxic effect against two tumor cells.

MATERIALS AND METHODS

Collection of algal samples:

Cystoseira myrica, a brown seaweed, was obtained off the coast of Hurghada, Egypt, on the Red Sea. The algal samples were collected and washed under running fresh

water to eliminate sand and other extraneous materials. The seaweed was collected and placed in a polyethylene bag before being transported to the laboratory. The seaweed was washed twice with distilled water to eliminate any metallic compounds, and then three times with deionized water until the pH of the wash solution was equivalent to deionized water. According to **Soliman *et al.* (2018)** the seaweed was harvested, completely dried in the shade at room temperature, then ground in a mechanical grinder before being passed through a 0.2 mm sieve. It was then placed in a dry place for further use.

Preparation of algal extract:

5 g powdered alga and 50 mL deionized water were mixed in a round flask, stirred thoroughly on a rotary shaker for 1 hour, allowed to boil at 70°C, and then cooled to room temperature (**Hashemi *et al.*, 2015**). Filter paper Whatman No. 1 was used to filter the extracted solution. For further studies, the filtrate was kept at 4°C (**Thamer *et al.*, 2018**).

Preparation of copper oxide nanoparticles:

The extract of *Cystoseira myrica* was mixed with 1mM $CuSO_4.5H_2O$ (Sigma Aldrich Company, USA) solution at a mixing ratio (1:9) to determine the optimum conditions according to **Kashyap *et al.* (2019)**. The reaction solution was incubated for 30, 60, 90,120 and 150 minutes. The pH of the reaction was adjusted to (5, 7 and 9) **(Kredy, 2018)** using 0.1 N HCl and 0. 1 N NaOH (**Chen *et al.*, 1997**). After setting the pH to 5. The reaction temperature was maintained at 25°C and by water bath heating at 50, 75, and 100°C **(Hashemi, *et al.*, 2015)**. The effect of these parameters on the production CuONPs was investigated using a UV-visible spectrophotometer.

Characterization of synthesized copper oxide nanoparticles

1. **UV spectral analysis**

The presence of the synthesized CuONPs was confirmed using a UV-visible spectrophotometer. Ultraviolet spectral measurements were taken in the wavelength range of 200-500 nm. In the laboratory of the chemistry of the faculty of education, Ain Shams University, the UV-visual spectra recorded using a spectrophotometer (Nicolet evolution 100, Cambridge) with digital data capture.

2. **Transmission Electron Microscope**

Transmission Electron Microscopic (TEM) Analysis (JEOL JEM-2100) at the Egyptian Petroleum Research Institute, Egypt. Characterization of scale, morphology was performed. Drops of an aqueous suspension of CuONPs were transferred onto carbon-coated TEM grids to prepare samples for TEM research. Before the analysis, the film on the TEM grid dried.

3. DLS and Zeta-potential

A Zeta sizer Nano-ZS, MALVERN, (United Kingdom), Nanotechnology Centre, Egyptian Petroleum Research Institute (EPRI), Egypt, and a JEOL JEM-2100 electron microscope, Japan used to assess the zeta potential of CuONPs biosynthesized from *Cystoseira myrica* extract.

4. X-ray diffraction

The measurements X-ray diffraction of CuONPs was performed using XRD diffractometer (JED-2300T) Cu-Kα X-rays of wavelength 1.54060 Å and data were taken for the range of 5° to 80° with a step of 0.026°. Drops of copper oxide nanoparticle solution were placed on a slide and air dry at 35°C for 2 hours. The X-ray generator was turned on with Cu kal radiation at a 2 angle.

5. Fourier-transform infrared (FTIR)

An FTIR spectrometer (FT/IR-6100 type A) was used to measure the spectra in the wavelength range of 4000 to 400 nm^{-1}. FTIR to identify the bioactive compounds in *Cystoseira myrica* extract that are responsible for the bio-reduced CuONPs to create nanoparticles are identified using FTIR.

Evaluation of anticancer of copper oxide nanoparticles

1. Cell culture

Cell lines of human hepatocellular carcinoma (HepG2) and breast carcinoma cells (MCF-7) donated by the tissue culture section of the holding firm for Biological Products and Vaccines (VACSERA), Giza, Egypt. According to **Saintigny *et al.* (200٢)** the cytotoxicity of biosynthesized copper oxide nanoparticles was tested against human hepatocellular carcinoma cells (HepG2) and breast carcinoma cells (MCF-7).

2. Dimethyl thiazolyl tetrazolium bromide assay

The cell viability assay of human hepatocellular carcinoma (HepG2) and breast carcinoma cells (MCF-7) cell lines was determined using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described by **Mosmann (1983)**. Cell cultures were seeded in 96-well plates either with a series of 0.1 to 100 μg/ml concentrations of CuONPs or alone (negative control) (**El-Menshawi *et al*., 2010)** and incubated for 24 h at 37°C in a humidified environment of 95% air and 5% CO_2. After adding 100 ul/well MTT to the cultures and incubating them for 4 hours, 1 ml dimethyl sulfoxide (DMSO) was applied to each well. With DMSO as a blank, the absorbance at 570–620 nm was determined using a UV spectrophotometer. Data were collected from three independent experiments **(Berridge *et al*., 2005).** The percentage of viable cells was calculated as follows:

Cell viability percentage = (OD of treated cells / OD of untreated cells) X 100 **(Chen *et al*., 2009)**.

Statistical analysis

The experimental data was analyzed using Minitab 19. Inferential statistics were employed to compare the findings of different groups. Different comparisons were explored using One-way analysis of variance under the fit General linear model (ANOVA). All group interactions were subjected to post hoc analyses using the Tukey test for pairwise comparisons. At 0.05, P values were determined to be significant.

RESULTS

Metal oxide nanoparticles are distinguished from other nanoparticles such as nanoparticles, semiconductor dots, magnetic and polymeric because of their unique surface plasmon resonance (SPR). In this work, the extract of *Cystoseira myrica* was used to examine the potentiality for the biosynthesis of CuONPs.

1. Characterization and dispersion of CuONPs

a. Ultraviolet-Visual Spectroscopy of CuONPs

i. pH

One of the important factors influencing the production of copper oxide nanoparticles was the pH of the reaction mixture. (Figure 1). CuONPs synthesis reported at pH 5, 7, and 9 during the different incubation durations per minute was treated to 1mM of $CuSO_4.5H_2O$. CuONPs formation was measured using UV spectroscopy (Fig. 1).

The SPR band was recorded by extract of *Cystoseira myrica* around 243.5nm at various incubation reaction times at low pH 5 (Fig. 1A). The peaks of CuONPs around 247nm were also reported to be supported by neutral pH 7, as seen in (Fig. 1B), while in an alkaline medium with a pH 9, *C. myrica* extract has SPR peaks around 248nm (Fig. 1C). The current investigation found that changing pH levels did not affect the synthesis and maximal absorption of NPs. At the optimized CuONPS concentration of 1 mM $CuSO_4.5H_2O$, the synthesis was observed to form in the pH 5 when using algal extract of *C. myrica*.

ii. Temperature

One of the most important influencing factors in reactions is temperature. The solution temperature was heated from 25 to 50, 75, and 100°C, respectively, to describe the effects of the heating procedure in copper oxide nanoparticles synthesis. It was discovered that as the temperature increases, the absorption peaks for copper oxide nanoparticle biosynthesis by *C. myrica* extract increased (Fig. 2). The effect of different temperatures on the rate of synthesis of copper oxide increased the intensity of SPR and was confirmed by UV- Vis Spectra. The UV-Visible spectrum revealed that at 25°C and 50°C, the UV–vis records an absorption peak of approximately 243.5nm as shown in (Figs. 2A and B). UV–vis results, on the other hand, reveal an absorption peak of about 248nm at 75°C, as shown in (Fig. 2C).

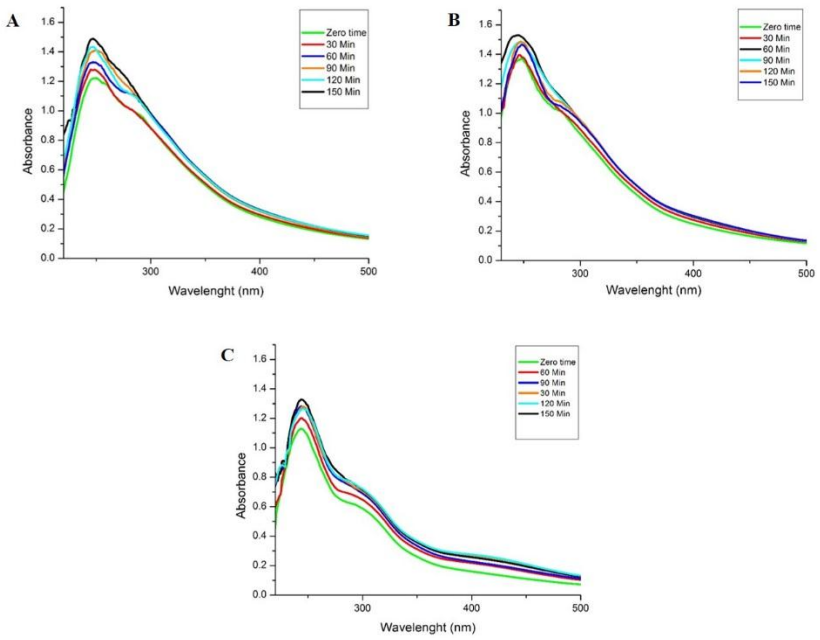

Fig. 1. UV-Vis spectra of copper oxide nanoparticles synthesized by extract of *Cystoseira myrica* at different pH ; (A) pH=5, (B) pH=7, and (C) pH=9 at different incubation periods per minutes (zero time, 30,60,90,120 and150).

The results showed that as the temperature increases, the distinctive absorbance peaks became stronger, peaking at 249.5nm at 100°C employing *C. myrica* extract, as shown in (Fig. 2D).

When using *C. myrica* extract with $CuSO_4.5H_2O$ concentration of 1 mM, the synthesis rate increased with an increase in reaction temperature up to 100°C, which demonstrated maximum synthesis and stayed stable for a longer time.

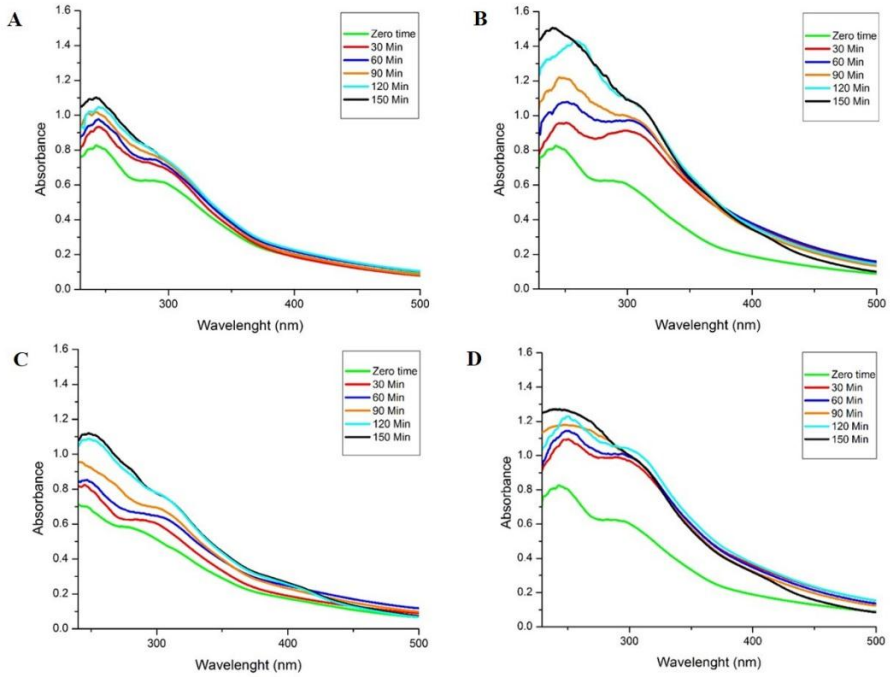

Fig. 2. UV-Vis spectra of copper oxide nanoparticles synthesized by extract of *Cystoseira myrica* at different Temperature; (A) T=25°C, (B) T=50°C, (C) T=75°C and (D) T=100°C different incubation periods per minutes (zero time, 30,60,90,120 and150).

b. Morphological and particle size distribution analyses

The morphology of CuONPs produced by *Cystoseira myrica* extract using TEM has been demonstrated to be spherical throughout the size range of 11-80nm with a mean size of 21.78nm, as illustrated in (Fig. 3). The curve of particle size estimation and distribution of CuONPs was recorded by dynamic light scattering (DLS). The results demonstrate that the average size was less than 100nm (Fig. 4). The biosynthesized CuONPs were found to keep their characteristics for at least four months of storage at 4**°C**. In this work, zeta potential measurement shows a value around -18.8 mV (Fig. 4).

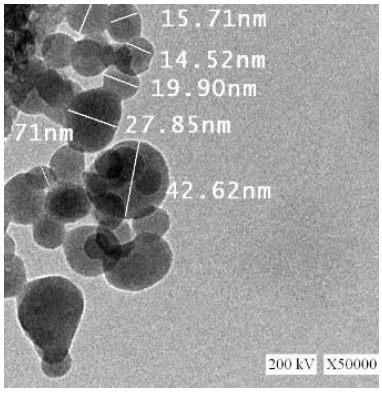

Fig. 3. TEM image of the copper oxide nanoparticles synthesized by extract of *Cystoseira myrica*.

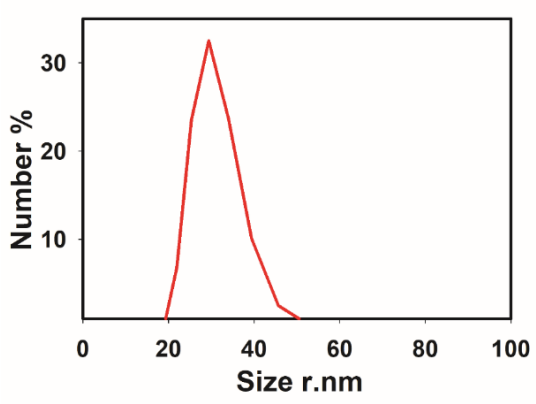

Fig. 4. DLS Curve of copper oxide nanoparticles synthesized by extract of *Cystoseira myrica*.

c. X-Ray diffraction analysis

The CuONPs synthesized by the extract of *Cystoseira myrica* have an XRD diffraction pattern. CuONPs are crystalline in nature and the 2 values of the XRD pattern ranged from 5 to 80°, with six Bragg diffraction peaks at degrees degree = 4.56 º, 28.27 º,31.67 º,41.2º,45.37 º and 46.6 º (Fig. 5).

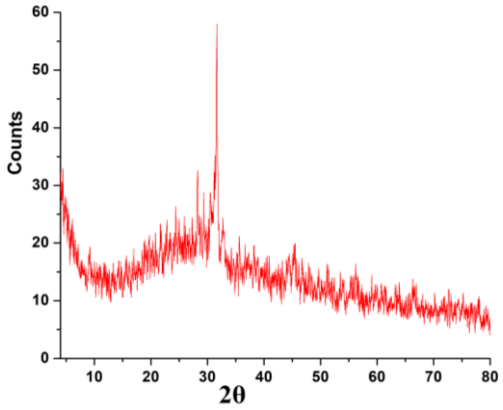

Fig. 5. X-ray diffraction analysis of copper oxide nanoparticles synthesized by extract of *Cystoseira myrica*.

d. Surface functionalities: FTIR analysis

FTIR was used to identify the functional groups of biomolecules in copper oxide nanoparticles biosynthesized by *Cystoseira myrica* extract. The FT-IR spectrum of CuONPs synthesized by the extract of *C. myrica* showed different absorption peaks (cm^{-1}) (Fig. 6).

It indicates the existence of the phenol or alcohol compounds' –OH strong stretching band at 3334cm^{-1}. The peak at 2060cm^{-1} represents N=C=S stretching bond (isothiocyanate). The absorption bands 1636cm^{-1} are characteristic of -C=O- stretching vibration of Amide I indicate the presence of protein, due to the N–H bending vibration of secondary amines and the carbonyl β unsaturated ketone amide (lipid, protein). The absorption peaks at 1103cm^{-1} showed the presence of secondary alcohol (-C-O- stretching bond). C-Br stretching peaks represented at 540cm^{-1} represented alkyl halides compound (lipids) and it exhibits the vibrational modes of CuO.

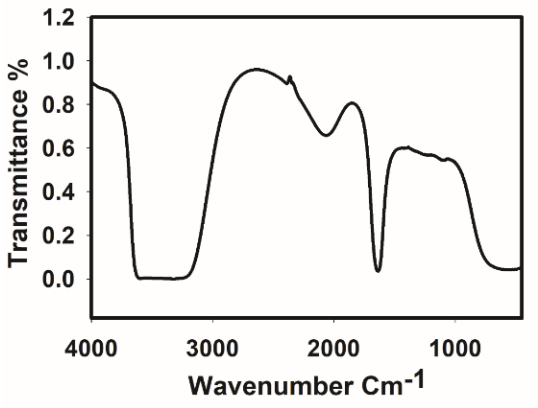

Fig. 6. FT-IR spectrum data of CuONPs synthesized by extract of *Cystoseira myrica*.

2. Cytotoxicity of copper oxide nanoparticles

The toxicity of different concentrations of copper oxide nanoparticles (from 0.1 µg/ml to 100 µg/ml) against human hepatocellular carcinoma (HepG2) and breast carcinoma cells (MCF-7) cell lines investigated in (Figs. 7Aand B). After 48 hours, the percentage of cell viability of human hepatocellular carcinoma (HepG2) increased from 18.7±2.03 to 67.21±3.75 when the concentration of CuoNPs was decreased from 100 to 0.1 µg/ml, respectively. While the percentage of cell viability of human breast carcinoma cells (MCF-7) increased from 19.27±1.33 to 86.31±1.65 when the concentration of CuONPs was decreased from 100 to 0.1 µg/ml, respectively.

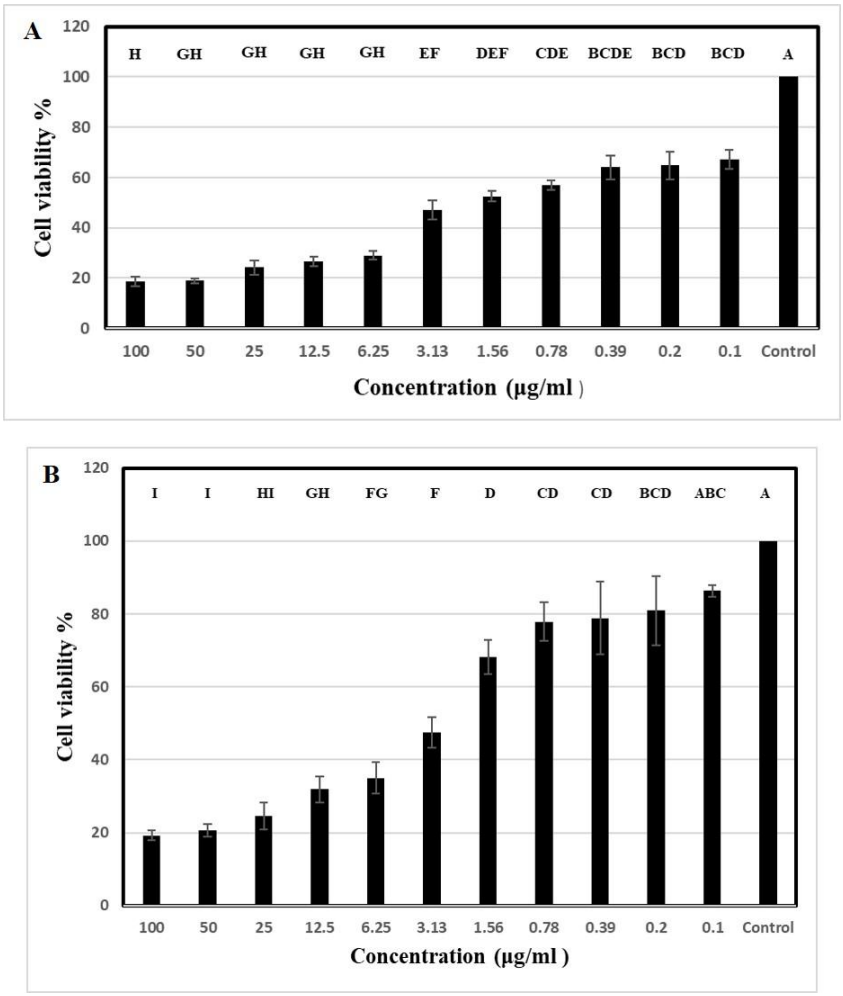

Fig. 7. Cytotoxic effect on human hepatocellular carcinoma cell line HepG2 (A) and breast carcinoma cell line MCF-7 (B) treated with different concentration of copper oxide nanoparticles (from 0.1 to 100 µg/ml) synthesized by extract of *Cystoseira myrica* compared to HepG2 human hepatocellular carcinoma cell line and MCF-7 breast carcinoma cell line and free from any treatment (Control) after 48 hours of time exposure (different letters shows the significance between different groups and same letters represent the non-significance between different groups and Error bars represent standard deviation (SD)).

DISCUSSION

Concentration of the extract, pH, and temperature are the main factors that affect the synthesis of CuONPs (**Akintelu *et al.*, 2020).**

The pH of a nanoparticle was one of the parameters that influenced its shape, composition, and size (**Hulkoti and Taranath, 2014**).The absorption peak positions are dependent on particle shape and size, so ultraviolet-visible spectroscopy is used (**Din and Rehan, 2017**). The present study revealed that all CuO nanoparticles created from the extract of *C. myrica* have a band at 243.5, 247, and 248nm at pH 5, 7, and 9 respectively, these findings were consistent with those of **Rehana *et al.*, (2017).**

In comparison to other pH values, pH 5 was found to be better to produce copper oxide nanoparticles because it speeds the reaction and lowers the size of nanoparticles. This pH may be the most suitable for the synthesis of essential biomolecules, particularly proteins involved in the bio-reduction of copper salts to CuONPs. The maximum absorption of the synthesized NPs was unaffected by the pH change (**Ghareib *et al.*, 2018**).

Another most important influencing parameter in reactions is temperature (**Patricia *et al.*, 2019).** The results showed that when the temperature increased, the typical absorbance peaks increased. The increase in the temperature is related to a change in the kinetics of the reaction, atoms movement on the solution (Brownian movement), and the aggregative mechanisms of nanoparticles (**Piñero *et al.*, 2017).**

At the optimal $CuSO_4.5H_2O$ concentration of 1 mM, the synthesis rate increased with an increase in reaction temperature up to 100°C, indicating maximal synthesis and staying stable for longer periods, indicating stable synthesis. Despite the great number of studies on CuONPs synthesis, only a few papers on optimization have been published. The increased rate of CuONPs synthesis at optimum conditions, according to the current study, could be a direct result of the effect of pH and temperature on the principal biomolecule responsible for the reduction in *C. myrica* extract.

In addition, the particles biosynthesized by *C. myrica* extract were 11-80nm in size and spherical. Our findings are consistent with previous research (**Dastjerdi and Montazer, 2010; Abboud *et al.*, 2014; Thamer *et al.*, 2018**).

The average size of the copper oxide nanoparticles biosynthesized by extract of *C. myrica* was with a mean size of 21.78nm as proven by HRTEM and zeta potential measurement records a value around -18.8 mV, these zeta potential values imply that these copper oxide NPs have higher stability in the solution, These results were in agreed with (**Tomaszewska *et al.*, 2013; El-Naggar *et al.*, 2018**).

The XRD diffraction pattern of the biosynthesized CuONPs produced by extract of *C. myrica* was concerned with the reflection lines of CuO nanoparticles. Similar findings were reported by **Das *et al.* (2013)**. The spectrum is characterized to pure CuO, indicating single-phase CuO formation with indexed to monoclinic structure according to with JCPDS (Joint Committee on Powder Diffraction Standards). The experimental results were recorded to conform with **Vaseem *et al.* (2008)**.

Generally, the broad absorption band at $3334cm^{-1}$ is due to the –OH groups of the phenol or alcohol compounds and $2060cm^{-1}$ are present in N=C=S stretching bond (isothiocyanate). The absorption bands $1636cm^{-1}$ are characteristic of -C=O- stretching vibration of Amide I indicate the presence of protein (**Prakash *et al.*, 2013**). The absorption peak at $1103cm^{-1}$ represented the presence of secondary alcohol (-C-O- stretching bond). A strong absorption peak C-Br represented at $540cm^{-1}$ represented alkyl halides compound (lipids) and it reveals the vibrations modes of the CuO functional

group. The presence of nano-sized CuO particles was confirmed. A similar pattern was also reported by **El-Trass *et al.* (2012)** and **Jeronsia *et al.* (2016)**.

Our experiment indicates that when the concentration of CuONPs rises, the percentage of cell viability decreases. After 48 h of exposure, the percentage of cell viability decreases due to dysfunction of mitochondrial and the results showed that CuONPs were found to decrease HepG2 and MCF-7 cell viability percentage in a dose-dependent manner (**Siddiqui *et al.*, 2013; Jeronsia *et al.*, 2016; Ramaswamy *et al.*, 2016).** The HepG2 cell line showed less effect than the MCF-7 cell line when both were treated with CuONPs at different concentrations and at the same time, demonstrating that cancer cell lines are more sensitive to nanoparticles than normal cells. Copper oxide nanoparticles have a cell-selective effect as a result.

CONCLUSION

This study presents green, environmentally friendly synthesized CuONPs utilizing *Cytoseira myrica*. The technique of nanoparticles synthesis using marine algae extract has the potential advantage of being stable in solution, which is a significant advantage now in use. This synthetic technology has the potential to be used to synthesize various metal oxide nanoparticles and can be valuable in biotechnological, environmental, and medical applications. The results suggested that CuONPs showed promising anticancer activity against human hepatocellular carcinoma (HepG2) and human breast cancer cell line (MCF-7).

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