Int. J. Pure Appl. Sci. 9(2);344-351 (2023)

IJPAS
ijpas@munzur.edu.tr
ISSN: 2149-0910

Research article/Araştırma makalesi
DOI:10.29132/ijpas.1304265

Effect of Copper Oxide Nanoparticles on Prostate Cancer PC3 Cells

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Received date:31.05.2023, Accepted date:28.06.2023

Abstract

Copper oxide (CuO NPs) nanoparticles were synthesized by green synthesis and characterized by UV-Vis spectroscopy, FT-IR spectroscopy, SEM and EDX analysis. The shape of the CuO nanoparticles obtained is approximately spherical and their particle sizes range from 40 to 85 nm. According to the experimental results, two peaks at 305 and 318 nm in the 270-400 nm range in the UV-Vis absorption spectrum and one peak at 536 cm^{-1} in the FT-IR spectrum confirm the presence of copper oxide nanoparticles. In addition, the presence of biomolecules in aesculus hippocastanum(horse chestnut) extract on the surface of the copper oxide nanoparticles synthesized by green synthesis was determined by the peaks seen in the range of 3300 cm^{-1} - 1000 cm^{-1} in the FTIR spectrum. The images, shape and size of the copper oxide nanoparticles were determined by SEM, and the weight percent of the elements were determined by EDX. PC3 prostate cell lines were used in the study. After measuring the cytotoxic effect of the agents used in the study on prostate cancer cells, the apoptotic effect of this effect was determined by Hoechst/propidium iodide (HOPI) staining. Graphpad prism program was used to compare all parameters between groups, and "one-way analysis of variance" (one-way ANOVA) method and dunnet's test were applied to test whether there was any difference between at least two groups. It has been determined that the drug obtained from copper oxide nanoparticles has a cytotoxic effect on PC3 prostate cancer cells and this effect occurs through the apoptosis pathway.

Keywords: Green synthesis, copper oxide nanoparticle, prostate cancer, PC3 prostate cell

Bakır Oksit Nanopartiküllerinin Prostat Kanseri PC3 Hücreleri Üzerindeki Etkisi

Öz

Bakır oksit(CuO NP'ler) nanoparçacıkları, yeşil sentez yolu ile sentezlenmiştir ve UV-Vis spektroskopisi, FT-IR spektroskopisi, SEM ve EDX analizi ile karakterize edilmiştir. Elde edilen CuO nanoparçacıklarının şekli yaklaşık olarak küreseldir ve parçacık boyutları 40 ile 85 nm arasında değişmektedir. Deneysel sonuçlara göre, UV-Vis absorpsiyon spektrumunda 270-400 nm aralığında 305 ve 318 nm'de görülen iki pik ve FT-IR spektrumunda 536 cm^{-1}'de bir pik, bakır oksit nanopartiküllerin varlığını doğrulamaktadır. Ayrıca, yeşil sentez yolu ile sentezlenen bakır oksit nanoparçacıklarının yüzeyindeki aesculus hippocastanum(at kestanesi) ekstraktındaki biyomoleküllerin varlığı FTIR spektrumunda 3300 cm^{-1} - 1000 cm^{-1} aralığında görülen pikler ile belirlenmiştir. Bakır oksit nanoparçacıklarının görüntüleri, şekli ve boyutu SEM ile, elementlerinin yüzde ağırlık oranları ise EDX ile tespit edilmiştir. Çalışmada PC3 prostat hücre hatları kullanılmıştır. Çalışmada kullanılan ajanların prostat kanseri hücreleri üzerindeki sitotoksik etkisi ölçüldükten sonra bu etkinin apoptotik etkisi hoechst/propidium iodide(HOPI) boyaması ile belirlenmiştir. Gruplar arasında tüm parametrelerin karşılaştırılmasında graphpad prism programı kullanılmıştır ve en az iki grup arasında herhangi bir farklılığın olup olmadığını test etmek için "tek yönlü varyans analizi" (one-way ANOVA) yöntemi ve dunnet's testi uygulanmıştır. Bakır oksit nanopartiküllerinden elde edilen ilacın PC3 prostat kanseri hücreleri üzerinde sitotoksik etkiye sahip olduğu ve bu etkinin apoptoz yolu ile gerçekleştiği belirlenmiştir.

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Anahtar Kelimeler: Yeşil sentez, bakır oksit nanoparçacık, prostat kanseri, PC3 prostat hücresi

INTRODUCTION

Nanotechnology examines the properties and applications of structures in nanoscale. The structures with sizes between 1 nm and 100 nm are generally described as nanostructures (Willems, 2005). The nanostructures are used in various fields such as disease diagnosis, disease control, and microelectronics (Iravani, 2011; Thakkar et al., 2010). Metal nanoparticles, which are among the nanostructures, can be produced by various methods. These methods are physical, chemical and biological methods (Iravani, 2011; Mittal et al., 2013). Physical and chemical methods used in nanoparticle synthesis are disadvantageous due to their high cost, toxic effects and high energy needs (Danhalilu et al., 2019). Recently, environmentally biological methods such as microorganism, enzyme and plant extract are recommended as an alternative to chemical and physical methods to synthesize nanoparticles. In biological methods, fungi, algae, bacteria and plants are used to synthesize of the nanoparticles (Iravani, 2011; Erdoğan et al., 2022; Thakkar et al., 2010). The use of plant extract for the synthesis of nanoparticles is more advantageous than other biological methods. It can also be scaled for large-scale syntheses and produced quickly (Kharissova et al., 2013). Nanoparticles synthesized using plant extract are defined as "green synthesis". In green synthesis, plant extracts obtained from the leaves, roots, fruits, shells and seeds of plants are used (Iravani, 2011; Omer et al., 2021; Kharissova et al., 2013). The factors such as the structure of the plant extract, metal concentration, pH, temperature can affect the production and other properties of metal nanoparticles (Danhalilu et al., 2019). The reason for using plants in the synthesis of nanoparticles is the presence of reducing and stabilizing molecular structures such as amino acids, proteins, polyphenols, aldehydes and ketones in plant extracts (Mittal et al., 2013). The copper oxide, which is among the metal nanoparticles, is a p-type semiconductor with 1.2 ev band gap (Nethravathi et al., 2015). In particular, CuO nanoparticles have been characterized due to their antimicrobial effects, and their antimicrobial effects have been investigated (Das et al., 2013; Nethravathi et al., 2015; Sivaraj et al., 2014). Furthermore, it is very sensitive to prokaryotes and eukaryotes compared to other metal nanoparticles (Tri et al., 2019). The copper oxide nanostructures have a wide range of applications including optical and electrical devices (Aruna et al., 2015), sensors (Wang et al., 2016), anode material for lithium-ion batteries (Wang et al., 2011), catalysis (Zaman et al., 2012), nanocomposite films (Shankar et al., 2017), solar cells (Sharma et al., 2015) and antibacterial materials (Nethravathi et al., 2015).

Copper oxide nanoparticles have gained importance due to their versatile use in industry and medicine. These nanoparticles have been shown to have an inhibitory effect against many bacteria and microorganisms (Das et al., 2013). Also, the CuO nanoparticles can act as anticancer agents, as they reduce the development of cancer cells. The current chemotherapy drugs used in the treatment of cancers have serious side effects and are disadvantageous as they significantly affect the quality of life of patients (Gnanavel et al., 2017). For this reason, it is necessary to search new ways of treatment and to develop alternative therapies in the treatment of cancers. One of the alternative ways at this stage is to use nanoparticles in this field and try to get results. For this purpose, the effects of copper oxide nanoparticles synthesized by green synthesis on cancer cells were studied. In the literature, it has been determined that the copper oxide nanoparticles synthesized by this method affect and destroy cancer cells such as human breast cancer cell (MCF-7) (Jeronsia et al., 2016; Rehana et al., 2017; Sivaraj et al., 2014), human lung cancer cell (A549) (Kalaiarasi et al., 2018; Rehana et al., 2017; Sankar et al., 2014), human colon cancer cell (HCT-116) (Gnanavel et al., 2017), cervical (HeLa) (Nagajyothi et al., 2017; Rehana et al., 2017), epithelioma (Hep-2) (Rehana et al., 2017), normal human dermal fibroblast (NHDF) cell (Rehana et al., 2017), epithelioma (Hep-2) (Rehana et al., 2017), MDA-MB-231 human breast cancer lines (Yugandhar et al., 2017), HL-60 cell line and PC3 (prostate cancer) (Naz et al., 2020).

In this study, we synthesized copper oxide nanoparticles for the first time using the extract of aesculus hippocastanum(horse chestnut) leaves and determined that these nanoparticles have a cytotoxic effect on PC3 prostate cancer cells. We characterized the structure and properties of copper

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ISSN: 2149-0910

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oxide nanoparticles synthesized by UV-Vis (Ultraviolet and visible region spectroscopy), FTIR (Fourier transform infrared spectroscopy), EDX (Energy diffusion X-ray diffraction), SEM (Scanning electron microscopy). The cytotoxic effect of copper oxide nanoparticles on prostate cancer cells was investigated by MTT test. Apoptosis measurement was determined by HOPI staining.

MATERIALS AND METHODS

Materials

Aesculus hippocastanum (horse chestnut) leaves were collected from the garden of Ulubatlı Hasan Anatolian High Schools, Bursa, Turkey. Copper(II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$, 99.5%) was purchased from Sigma-Aldrich.

The preparation of Aesculus Hippocastanum (Horse Hhestnut) Leaf Extract

5 g of leaves collected from the aesculus hippocastanum tree in the garden of Ulubatlı Hasan Andolu High School were firstly washed with deionized water and left to dry for 24 hours. To get a better extract, we cut the leaves into small pieces and put them in the erlenmeyer flask with 165 g of deionized water. We then extracted the leaves by heating this erlenmeyer at 80 °C for 20 minutes. Finally, the leaf and extract mixture in the erlenmeyer was filtered with the help of filter paper and the extract was obtained.

The synthesis of Copper Oxide Nanoparticles

20 mL of copper(II) sulfate pentahydrate (100 mM) solution was added to a 200 mL beaker. While this solution was also mixing in the magnetic stirrer, 20 mL of liquid aesculus hippocastanum extract was added dropwise and stirred at room temperature for 20 minutes. The resulting chemical solution was centrifuged at 10000 rpm and then the pellet was washed with distilled water and ethanol to remove organic residue. Nanoparticles obtained in powder form were kept at room temperature for further study.

Characterization of Copper Oxide Nanoparticles

The absorption spectrum of copper oxide nanoparticles and aesculus hippocastanum extract was recorded at room temperature in the range of 190-1100 nm by Ultraviolet-visible(UV-Vis) spectrophotometer(SCINCIO - NEOSYS200). The presence of functional groups of plant biomolecules on copper oxide nanoparticles was characterized by fourier transform infrared(FT-IR) spectroscopy (Nicolet - iS50) in the range of 400-4000 cm^{-1}. The properties and structure of the surface of copper oxide nanoparticles were determined by Scanning Electron Microscopy(Zeiss / Gemini 300-SEM). The elemental compositions of the obtained copper oxide nanoparticles were analyzed by EDX (Zeiss / Gemini 300).

Cell Culture and MTT Assay

In this study, the toxic effect of copper oxide nanoparticles on prostate cancer cells was investigated. Androgen-independent PC3 prostate cell lines were used in the study. After the cells were removed from nitrogen, they were dissolved in a water bath. Then, medium containing 10% FBS (Fetal bovine serum), 1% L-glutamine and penicillin-streptomycin was added and centrifuged at 800 rpm for 5 minutes. After centrifugation, the supernatant was discarded and the growth and proliferation of cells was ensured in 75 flasks by adding fresh medium. After the cells reached 80-90% occupancy, they were removed with trypsin-EDTA and transferred to 15 falcon tubes. It was centrifuged at 800 rpm for 5 minutes and then the supernatant was discarded. After the addition of fresh medium, the cells were counted with a thoma slide and 105 cells per ml were planted in 48 well plates and kept in an incubator with 5% CO_2 at 37 °C for 24 hours. After 24 hours, copper oxide nanoparticles at different concentrations (5, 10, 20, 40 µM) were added to the cells and left to stand in the same conditions in the incubator for 24 hours. Then, 40 µl of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye was added to each well and left in the incubator for 4 hours. Then 100 µl of DMSO was added to each well and the spectrophotometer at 560-620 nm' viability was measured.

Determination of Apoptosis

After measuring the cytotoxic effect of the agents used in the project on prostate cancer cells, the effect of this effect on the apoptotic pathway was determined by Hoechst/propidium iodide(HOPI) staining. In this context, cells were seeded into 24-well plates and incubated for 24 hours. After

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ISSN: 2149-0910

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incubation, 10 µM concentration of drug was added to the cells and allowed to incubate again for 24 hours. After 24 hours, cells were lifted with trypsin-EDTA and transferred to Eppendorf tubes. Then, after centrifugation at 800 rpm for 5 minutes, the supernatant was discarded and HOPI dye mixed with 50 µl of fresh medium was added to the tube at a ratio of 1:1. The cells were then incubated for 30-60 minutes and then examined under a fluorescence microscope.

Statistical Analysis

In the comparison of all parameters between the groups, using the Graphpad Prism program, "one-way analysis of variance" (one-way ANOVA) method and Dunnet's test were applied to test whether there was any difference between at least two groups, and all data were compared with the control group. $P<0.05$ was considered statistically significant in the evaluations. All numerical values are expressed as arithmetic mean ± standard error.

RESULTS AND DISCUSSION

UV-Vis Spectra Analysis

The presence of CuO nanoparticles in the liquid solution was investigated by taking UV-Vis absorption spectra in the wavelength range of 200-1000 nm. The UV-Vis spectrum the solution containing CuO nanoparticiples and the extract of aesculus hippocastanum leaf is given in Figure 1. The broad maximum peak around 365 nm in UV-Vis spectrum originates from biomolecules belonging to extract of Aesculus hippocastanum leaf (Figure 1). The shift in the peak towards high frequency in this spectrum occurred with the effect of CuO nanoparticles. This shift supports the formation of the copper oxide nanoparticles. It also explains CuO nanoparticle formation the color change (light green) observed in the mixture of extract (brown) and copper sulfate (blue). The peaks appearing at 305 and 318 nm in the UV-Vis spectra are characteristic absorption peaks of copper oxide nanoparticles. The maximum two peaks seen at 305 and 318 nm indicate that synthesized copper oxide nanoparticles are formed. The UV-Vis absorption peaks of CuO NPs overlap with previous studies. In previous studies, similar peak values such as 285 nm (Sankar et al., 2014), 275 and 372 nm (Nethravathi et al., 2015), 380 nm (Das et al., 2013), 220 and 235 nm (Rehana et al., 2017), 291 and 355 nm (Reddy, 2017) were found.

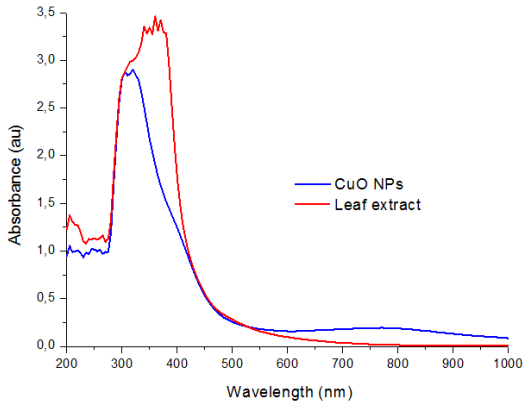

Figure 1. UV–Vis spectrum of synthesized colloidal CuO NPs and leaf extract of aesculus hippocastanum

FT-IR spectra analysis

FTIR spectroscopy measurements were performed to identify the biomolecules of the extract of aesculus hippocastanum leaf attached to the surface of the copper oxide nanoparticles. In addition, FT-IR spectroscopy is used to determine the Cu-O stretching vibrations of the CuO nanoparticles. Biomolecules ensure the stability of copper oxide nanoparticles and also change the properties of the surface. In this case, the extract of the leaf produces a significant effect on the CuO nanoparticles and causes it to acquire different properties. As seen in Figure 2, the peaks at 3284, 2926, 2849, 2638, 2287, 1710, 1591, 1436, 1351, 1267, 1056 and 536 cm^{-1} have supported the presence of biomolecules that bind to the surface of the CuO nanoparticle. It can be thought that these peaks are caused by functional groups in the extract of the leaf such as –O–H, –C–H, –C=O, –C=C, –C–N. The broad absorption band, which gives a peak at approximately 3284 cm^{-1} between 3600 and 3000 cm^{-1} in the FT-IR spectrum of the copper oxide nanoparticle, belongs to the –O–H group, which has a hydrogen bond. This absorption band can be caused by water molecules, alcohol or phenol. The absorption band seen in the range of 3000-2840 cm^{-1} is attributed to the aliphatic –C – H groups. The peak observed at 2287 cm^{-1} belongs to the –C≡N stretching in the triple bond region. It is attributed to carbonyl group (–C=O stretching) the peak appeared at 1710 cm^{-1}, but it is possible that this peak belongs

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to carboxylic acid, an amide or a ketone. The multiple bands around 1591 cm^{-1} are due to stretching vibration of the –C=C group or bending vibration of the –O–H group. The bands in the range of 1460-1360 cm^{-1} correspond to –C-H bending peaks. The absorption peaks observed at 1351 and 1267 cm^{-1} can be attributed to –C–N and –

C–O stretching vibrations. The peak at 1056 cm^{-1} can be due to aliphatic fluoro (C–F stretching) or –C–O–C stretching vibration. The results of FT-IR

analysis show that the surface of copper oxide nanoparticles is covered by biomolecules found in the leaf extract of the aesculus hippocastanum and remains stable for a long time. In the FT-IR spectra, the peaks found between 400 and 600 cm^{-1} are caused by Cu-O stretching vibrations (Reddy, 2017; Rehana et al., 2017). The Cu-O stretching peak at 536 cm^{-1} belonging to the synthesized copper oxide nanoparticles appeared as multiple absorption band. This peak confirmed the presence of CuO in the CuO nanoparticle. Similar peak values were also found in previous studies (Jeronsia et al., 2016; Padil and Černík, 2013; Rehana et al., 2017; Sivaraj et al., 2014).

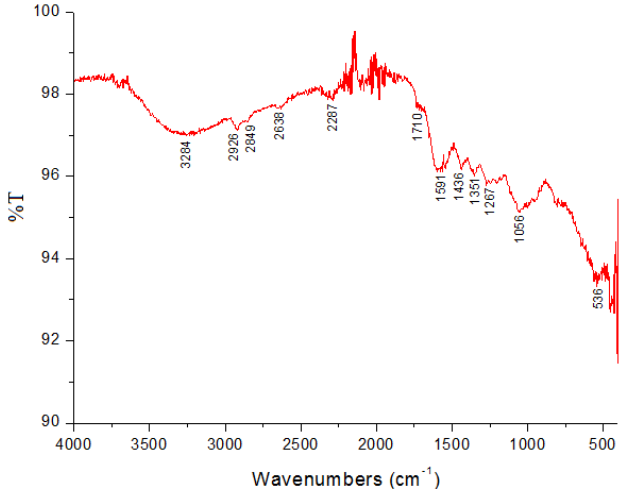

Figure 2. FTIR spectrum of CuO NPs synthesized using extract of aesculus hippocastanum leaf

SEM analysis

Structural and morphological properties of copper oxide nanoparticles were determined by SEM analysis. SEM images of copper oxide nanoparticles obtained at different magnification rates are given in Figure 3. The copper oxide nanoparticles from the SEM images given in Figure 3 are quite small in size, gray, bright, and approximately spherical regions. The sizes of these nanoparticles range from 40 nm to 85 nm.

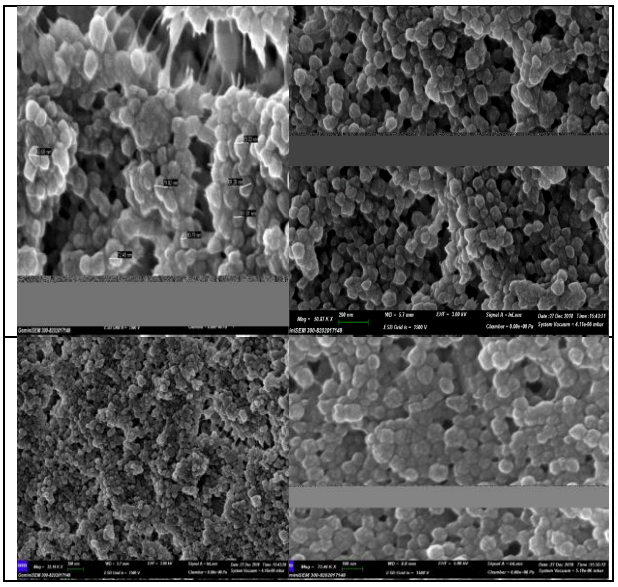

Figure 3. SEM images of CuO nanoparticles at different magnification rates

EDX analysis

EDX analysis was performed to determine the presence of copper oxide nanoparticles. The EDX spectrum is given in Figure 4. As seen in Figure 4, there are other elements besides the presence of copper in the spectrum. These elements that appear in EDX analysis are C, O, S, Au, Pd and Si (Table 1). It is estimated that the source of these elements is due to the biomolecules of the extract of the aesculus hippocastanum leaf on the surface of the copper nanoparticles. In addition, the plastic (centrifuge tube surface) substrate used for the EDX data is thought to cause an increase in the amount of carbon and oxygen elements. The elements found in the EDX analysis are given in Table 1.

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ISSN: 2149-0910

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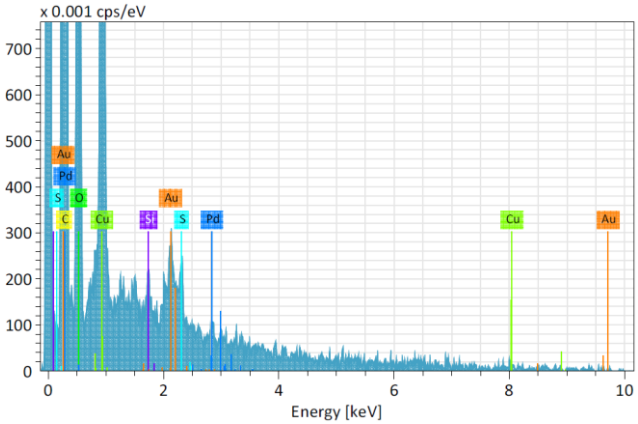

Figure 4. EDX analysis of CuO nanoparticles obtained through green synthesis. Additionally, the plastic substrate used for obtaining EDX data has increased the amount of carbon and oxygen elements

Table 1. *Percent weight ratios of the elements obtained from CuO nanoparticles as a result of EDX analysis. In addition, the plastic (centrifuge tube surface) substrate used for the EDX data is thought to cause an increase in the amount of carbon and oxygen elements*

Element	Weight (%)
Carbon (C)	48.77
Oxygen (O)	23.33
Copper (Cu)	19.10
Gold (Au)	5.41
Sulfur (S)	1.61
Palladium (Pd)	1.02
Silicon (Si)	0.77

Investigation of the Activity of Copper Oxide Nanoparticles in Prostate Cancer PC3 Cells

Cytotoxicity Measurement

The cytotoxic effect of copper oxide nanoparticles on prostate cancer cells was investigated by the MTT test and the results are shown in Figure 5. Accordingly, after 10 µM concentration, the cytotoxic effect of the drug was found to be around 50%. However, it was observed that the toxic effect of the drug increased with increasing drug concentration. The cytotoxic activities of CuO NPs have been determined in previous studies (Jeronsia et al., 2016; Rehana et al., 2017; Sivaraj et al., 2014).

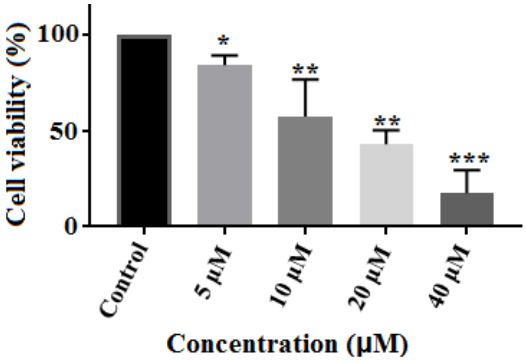

Figure 5. Cytotoxic effect of the drug obtained from copper oxide nanoparticles applied at different concentrations to prostate cancer cell line PC3 cells (*p<0.05, **p<0.01, ***p<0.001).

Apoptosis Measurements

Apoptosis measurement was determined by HOPI staining. After the MTT test, a concentration of 10 µM was determined to be appropriate and apoptosis measurements were made at this concentration. Figures and graphics obtained as a result of HOPI staining are shown in Figure 6. Accordingly, it was determined that the number of apoptotic cells in the drug administered group increased significantly when compared to the control group.

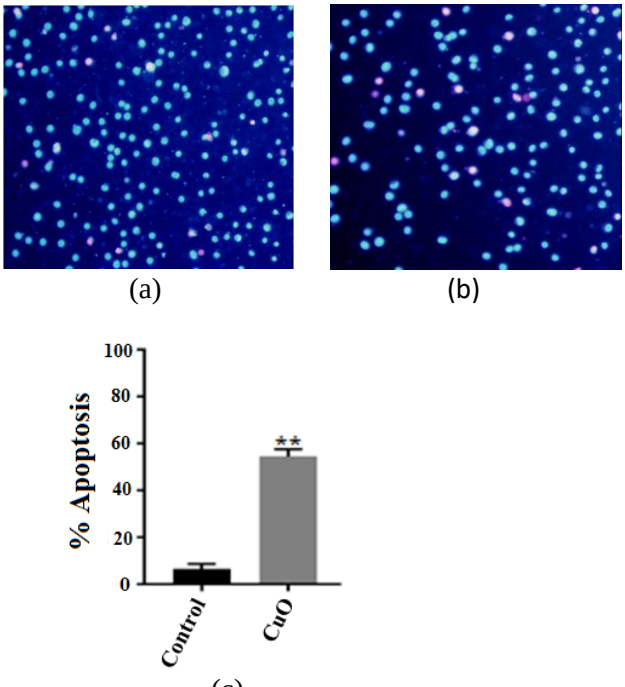

Figure 6. a) HOPI images of control and b) drug groups and c) apoptosis rates of these groups (**p<0.01).

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CONCLUSIONS

In this study, the structure, size, shape and morphological properties of copper oxide nanoparticles obtained through green synthesis were characterized by UV-Vis, FT-IR, SEM and EDX techniques. It supported the presence of copper oxide nanoparticles two peaks that appeared at 305 and 318 nm in the UV-Vis spectrum. FT-IR spectroscopy confirmed the presence of biomolecules on the surface of copper oxide nanoparticles and Cu-O bond. The CuO NPs synthesized from SEM images were determined to have a spherical shape and size in the range of 40-80 nm. Elements of biomolecules (C, O, Au, S, Pd and Si) of leaf extract found on the surface of CuO nanoparticles and copper element have been identified with EDX analysis.

Considering the data obtained in this study, in which the toxic effect of copper oxide nanoparticles and the possible mechanism of apoptosis in PC3 cells, which are the prostate cancer cell line, were examined, it was determined that the drug obtained from the aforementioned copper oxide nanoparticles had a cytotoxic effect on prostate cancer cells and this effect was realized through the apoptosis pathway.

CONFLICT OF INTEREST

The Authors report no conflict of interest relevant to this article.

RESEARCH AND PUBLICATION ETHICS STATEMENT

The authors declare that this study complies with research and publication ethics.

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