RESEARCH ARTICLE

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Investigations of Cu_2ONPs - Orange Peels: Biosynthesis, Characterization, and Anticancer Activity on a Cytotoxic Breast Cancer Cell Line

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Abstract

Background: The study aimed to evaluate the efficacy of nanocomposites extracted from orange peels in targeting and combating MCF-7 cells. **Methods:** We characterized the nanoparticles using XRD, FTIR, SEM, and GC-MS, and evaluated their cytotoxicity and activity using the MTT colorimetric assay and double staining technique.Results: The obtained results showed that the nanoparticles ranged from 19 to 66 nanometers in size and contained active compounds in the range of 4,000-500 cm^{-1}. However, the XRD sizes of the crystalline nanoparticles ranged from 15 to 23 nm. Orange peel contains important compounds such as octanal, 1, 6-octadien-3-ol, 3, 7-dimethyl, and limonene. At 800 mg/ml, DPPH can act as a strong antioxidant and reduce free radicals by 78.29%. The addition of the molecules resulted in a trace amount of 3.99%, a viability of 74.671%, and a half-maximal inhibitory concentration of 43.75 g/mL for the cell line. The compatibility with the low concentration effectively repaired the damaged cells. Conclusions: We expect the present study to provide important insights into the influence of molecular structure on anti-cancer action.

Keywords: Cuprous oxide nanoparticles-orange peels- MCF-7 cell line- DAPI

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Introduction

Recently, there has been a growing interest in nanotechnology thanks to its advantages and potential in producing effective compounds for diagnosing and treating diseases. Nanotechnology offers the ability to target toxic cells, assist in cell diagnostics, and support applications such as biological testing and cell imaging. Researchers have discovered the negative side effects associated with chemotherapy drugs such as cisplatin, doxorubicin. Cancers such as breast, colon, and cervical cancer can lead to symptoms like nausea, vomiting, urinary tract and kidney dysfunction, among others. In the search for alternatives, scientists have found that utilizing natural resources could offer a viable solution to help manage the negative side effects of these harmful drugs [1].

It is trustworthy to note that there is a close relationship between nanoparticles and DNA as nanoparticles can reduce toxicity and neutralize free radicals that damage DNA. Therefore, researchers have resorted to plants as a primary source for developing medicines to leverage their effective properties and characteristics in the manufacturing of nanoparticle-based treatment. Plants contain effective chemical compounds such as flavonoids, polyphenols, hydroxyls, proteins, limonene, and other important compounds that play an important role and cause changes in cancer cells and DNA [2]. Maintaining a healthy diet and treating the cells' metabolism is crucial for controlling their metabolism, balance, and receptors. Due to their properties, active compounds, and organic products, phenols play a significant role in regulating the course of cell metabolism, reducing the harmful effects of toxic cells, and promoting the maintenance of healthy cells [3].

Reducing the size of cancer cells by controlling the DNA strands and metabolic processes using plant-synthesized nanoparticles has revolutionized biotechnology. This approach enables minimal toxicity, while efficiently tracking pathways to toxic cells due to nanoparticles' unique physical and chemical properties, which play an important role in the biological systems. The small size of copper oxide nanoparticles of copper oxide, in particular, is significant in the nanotechnology system [4]. There are many ways to detect toxic cells within free cells, structural units, and molecules in salivary glands, blood cells etc. Nanoparticles attach to the targeted cells by regulating cell growth (size) and proliferation (number).

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Additionally, the adhesion of nanoparticles weakens the outer wall, reducing their effectiveness and preventing their spread, while preserving healthy cells. This selective targeting is one of the main advantages of controlling inheritance nanoparticle-based treatments, offering precise control over cellular inheritance and activity [5].

Citrus fruits are a rich source of physiologically active substances that stimulate cell proliferation through enzymatic activity. Recent research has corroborated the use of citrus fruits, particularly the peel, as a natural reservoir of antioxidants. These analyses have revealed the presence of numerous hydroxyl groups, the primary flavonoid, and limonene, which acts as a protective antioxidant in several biological systems. Besides, numerous epidemiological studies have shown that citrus fruits, rich in hesperidin, offer benefits in combating degenerative diseases, including cardiovascular problems and some types of cancer. Their pharmacological characteristics include anti-inflammatory, antihistamine, and antiviral activities. Moreover, they work synergistically with vitamin C to reduce cholesterol levels [6]. The combination of plant extracts and nanoparticles, particularly metal oxides or elements with significant biological benefits, has proven to be environmentally friendly and non-toxic to healthy cells. The Capping Agent Factor plays a crucial role in stabilizing nanoparticles, preventing their agglomeration, and ensuring their proper distribution on the cell surface, thereby enhancing their activity in targeting harmful cells [7].

Using nanoparticles synthesized from plants have revolutionized, by enabling precise control over DNA strands and metabolic processes. Due to their unique physical and chemical properties, these nanoparticles are highly effective in locating and targeting toxic cells within biological systems. The Nano size of copper oxides plays an important role in the nanotechnology system [8].

The study's goal is to find out how well cuprous oxide nanoparticles from orange peel can fight cancer in the MCF-7 cell line by synthesizing them, characterizing them, and testing their effectiveness.

Experimental design of the material

This study was to determine the ability of cuprous oxide nanoparticles from orange peels to synthesize, characterize and evaluate their anticancer activity on MCF-7 breast cancer cell line (Flow Diagram.1)

Materials and Methods

Orange Peel Extraction: In December 2022, I collected oranges from the Sabaa Akbar area in central Baghdad, Iraq. I weighed and ground 25 grams of orange peels. Next, I added 120 ml of deionized distilled water and heated the mixture at 60°C for half an hour. We cooled the solution, filtered it using Whitman filter paper, and centrifuged it at 5,000 rpm for five minutes to obtain a clear solution,

Diagram 1. The Biosynthesis of Copper Oxide Nanoparticles from Orange Peels Demonstrated Their Potential as a Drug for the MCF-7 Cell Line

which we kept at 4°C until needed [9].

Synthesis of copper-orange peel nanoparticles: A 0.05 ml/L solution of copper acetate Cu (CH_3COO). 2H 2O was mixed with 100 mL of deionized water and 25 mL of orange peel extract. We stirred the mixture at 1,200 rpm while applying heat. We then filtered the liquid to eliminate any unreacted copper acetate. We rinsed the filtered solution with deionized water until the pH approached 7. This process ensures the removal of any residual copper acetate, allowing the solution to reach a neutral PH. We then dried the Cu_2 O nanoparticles at 60°C for a limited time. We can use the resulting orange peel Cu_2 O NPs for further characterization and various applications [10].

Characterization of Orange Peel Cu $_2$O *Nanoparticles*

I used a Philips PW1730 X-ray diffraction (XRD) instrument to determine the crystal structure of the nanoparticles. The XRD pattern revealed the characteristic peaks of orange peel Cu_2O nanoparticles [11]. To image and determine the shape and size of the nanoparticles, a MIRA III Tescan field emission scanning electron microscope (FESEM) was used. Subsequently, FTIR was used to evaluate the vibrational properties and chemical bonding and identify the functional groups present in the nanoparticles [12].

Evaluation of antioxidant Cu $_2$O-*NPs –orange peels*

DPPH free radical scavenging:We used the technique, preparing standard solutions or DMSO (control) and adding them to the DPPH methanolic solution at different concentrations. After vigorously shaking the mixture and letting it sit in the dark at room temperature for a while, we calculated the absorbance at 517 nm [13] using the following formula:

Effect DPPH % = [Abs control- Abs sample / Abs control] × 100

Reactive oxygen species (ROS):tested to mimic the increase in ROS found in toxic cancer cells. HDF cells in 96-well plates were seeded and cultured. Next, we added 1 ml of phosphate buffer with salt to the obtained cell mixture solution to separate the culture medium. We washed 2 ml of the collected cells at 1500 rpm for 5 min. The sample contained 1 mL of the cell mixture. We prepared the samples by adding the following to each tube: As a control, Tube 1 had no staining agent. Tube 2 had DCFH-DA. Tube 3 had phosphate buffer with salt. And Tube 4 had both 2',7'-Dichlorodihydrofluorescein diacetate and propidium iodide. After the incubation period, we added phosphate buffer with salt to Tube 4. We centrifuged the sample and measured its absorbance to analyze the effects of the staining agents and experimental conditions [14].

MTT assay

We prepared MCF-7 cells in vitro using 10% FBS and GIBCO, added them to the tubes, added a 1% penicillin/streptomycin antibiotic solution, and stored the cells at room temperature with 5% CO2. We treated the cells with different concentrations of copper oxide nanoparticles (orange peel) after they reached a certain density. Then we measured the absorbance at 570 nm to observe the different effects of concentration on cancer cells. We dissolved MCF-7 formazan crystals in DMSO with dimethyl sulfoxide. Then, we calculated the viability and cytotoxicity of MCF-7 cells [15]. The formula is as follows:

%Viability= (Control Absorbance−Medium AbsorbanceTreated Absorbance−Medium Absorbance) ×100%

IC_{50} =Concentration at lower viability+ (Difference in viability %50−Lower viability %× Concentration interval).

DAPI assay

Fluorescent microscopy staining analysis was performed to evaluate the activity of the synthesized nanoparticles on toxic DNA using 40,6-diamidinophenylindole (DAPI) stain, different concentrations were taken and added to MCF-7 cells, and the apoptosis of toxic cells was observed, according to Weber et al. [16].

Results

Study characterization of Cu $_2$O *NPs-orange peels*

Scanning electron microscopy confirms the presence of Cu $_2$ONPs orange peel particles. The sample was looked at (a) 1 µm and (b) 500 µm, as shown in Figure 1. The particles have a crystalline shape and are between 15 and 68 nm in size.

We investigated the size of Cu $_2$O nanoparticles in orange peel using XRD patterns. The pore size of the

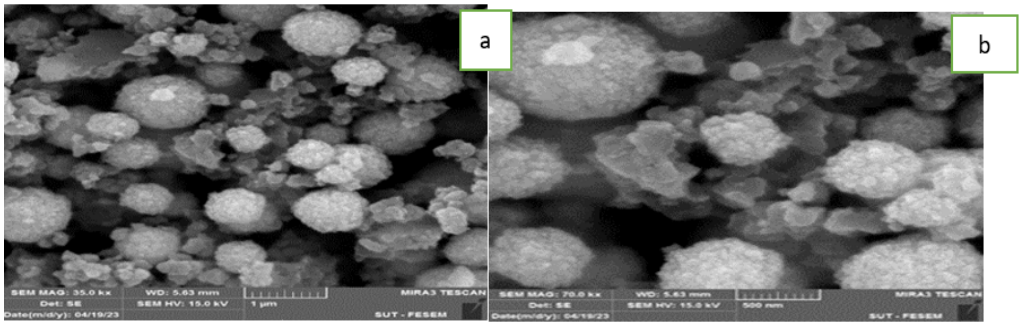

Figure 1. SEM of Cu 2ONPs Orange Peel

Table 1. XRD OF Nanopartial Cu_2O-Orange Peels

2-Theta-	d (nm)	BG	Height	I%	Area	I%	FWHM	XS(nm)
Cu_2O-Orange peels								
29.959	0.298	1204	671	6.3	6081	5.4	0.363	23
36.839	0.2437	2024	10685	100	113191	100	0.424	20
42.695	0.2116	1754	3416	32	39814	35.2	0.466	18
61.797	0.15	2024	2526	23.6	34082	30.1	0.54	17
74.093	0.1278	2182	1317	12.3	19323	17.1	0.587	17
77.857	0.1225	1841	456	4.3	7559	6.7	0.663	15

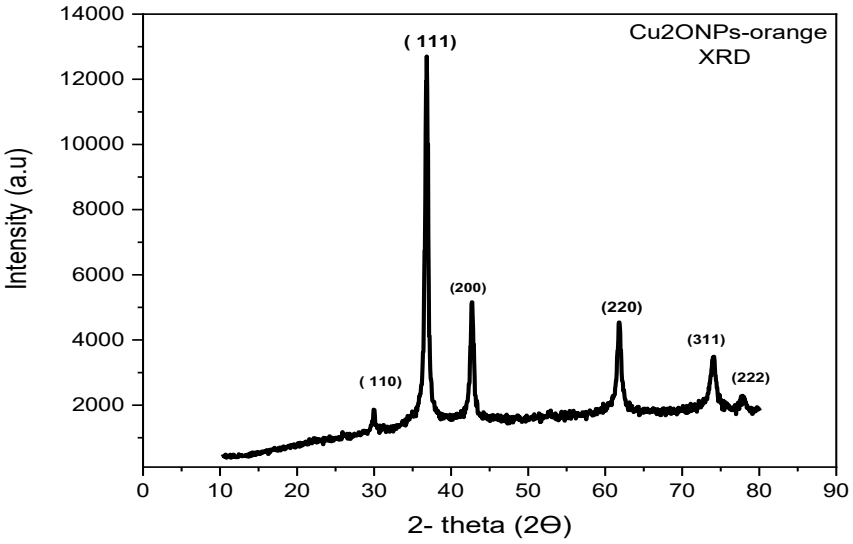

Figure 2. XRD of Cu 2O NPs-Orange Peel

nanoparticles was in the range of 15-23 nm as shown in Table 1, and a strong peak appeared at 2 theta = 36.839° in (111) direction. The sharpness and intensity of the diffraction peaks suggest that the Cu_2O nanoparticles in the orange peel are crystalline. The diffraction pattern shows the peaks of Cu_2O nanoparticles at 2 theta = 29.959, 36.839, 42.695, 61.797, 74.093, and 77.857, indicating their crystalline nature as shown in Figure 2.

Evaluation antioxidant of Cu_2O NPs-orange peels

To estimate the antioxidant activity and value of Cu_2O NPsderived from orange peels, we used a free

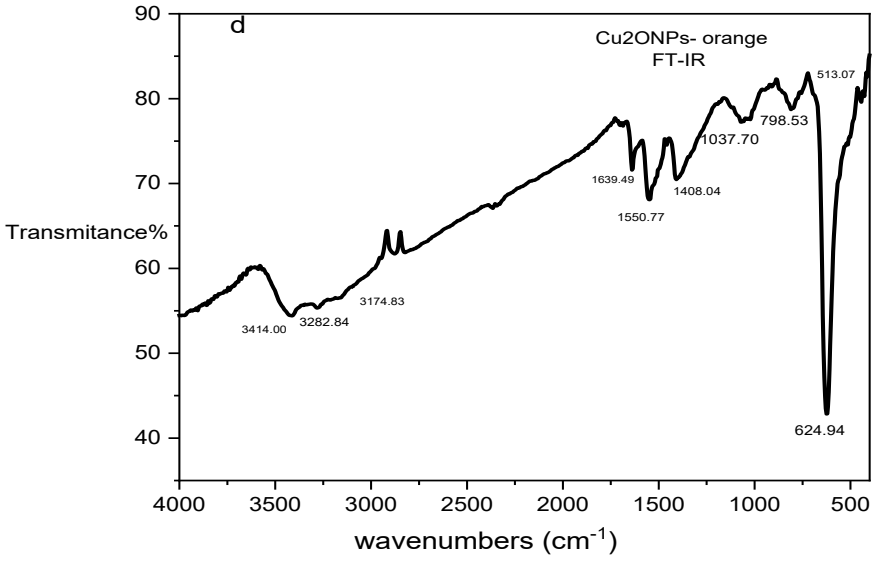

Figure 3. FT-IR of Cu_2O NPs-Orange Peel

Table 2.GC-MS of Orange Peels) Sweet Orange)

No.	name compounds	formula	Prob%
1	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	$C_{10}H_{18}O$	55.1
2	2,6-Octadienal, 3,7-dimethyl-, (Z)-	$C_{10}H_{16}O$	50.8
3	1-Cyclohexene-1-carboxaldehyde, 4-(1-methylethenyl)-	$C_{10}H_{14}O$	28.1
4	1,6-Octadien-3-ol, 3,7-dimethyl-	$C_{10}H_{18}O$	84.3
5	2,6-Octadienal, 3,7-dimethyl	$C_{10}H_{16}O$	40.5
6	2-Octen-1-ol, 3,7-dimethyl-	$C_{10}H_{20}O$	24
7	2-Cyclohexen-1-one, 3-methyl-6-(1-methylethenyl)-, (S)-	$C_{10}H_{14}O$	30.8
8	7-Octenal, 3,7-dimethyl-	$C_{10}H_{18}O$	27.7
9	Limonene oxide, trans-	$C_{10}H_{16}O$	44.3
10	Octanal	$C_{8}H_{16}O$	69.9
11	β-Myrcene	$C_{10}H_{16}$	42.3
12	3-Cyclohexene-1-methanol, α,α4-trimethyl-	$C_{10}H_{18}O$	46.5
13	1(2H)-Naphthalenone, octahydro-4a,8a-dimethyl-7-(1-methylethyl)-, [4aR-(4aα,7β,8aα)]-	$C_{15}H_{26}O$	61
14	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aα,4aα,7β,7aβ,7bα)]-	$C_{15}H_{24}O$	46.5
15	Caryophyllene oxide	$C_{15}H_{24}O$	50.1

Table 3. Ant Oxidation of DPPH Cuprous Oxide Cu_2ONPs- Orange Peels A

Concentration (mic/mL)	Scavenging %
10	18.18
50	35.84
100	37.86
200	45.27
400	58.35
600	72.91
800	78.29

radical scavenging test with the DPPH (2,2-diphenyl-1-picrylhydrazyl) dye. The procedure involved distributing the DPPH solution and adding varying concentrations of Cu_2O NPs. This method helps evaluate the ability of the nanoparticles to scavenge free radicals, which is an indicator of their antioxidant activity, as shown in Figure 4. The colour changes from purple to yellow, which indicates the activity and antioxidant effect of Cu_2ONPs. Table 3 shows that the nanoparticles were 78.29% active and efficient, and capable of scavenging free radicals at 800 g/mL.

Figure 5 A shows the high incidence of free radicals in cancer cells (MCF-7 cell line). However, when we added Cu 2O NP-orange peels to the cell line, we observed a 3.26% reduction in reactive oxygen species (ROS), as shown in Figure 5B. This demonstrated the effectiveness of orange peel copper oxide nanoparticles in reducing cell toxicity and oxidative stress. The addition of the nanoparticles resulted in a significant reduction in toxicity in the MCF-7 cell line treated compared to untreated. This suggests that the nanoparticles have a protective or beneficial effect on the cells, potentially reducing harmful effects or enhancing cell viability [18].

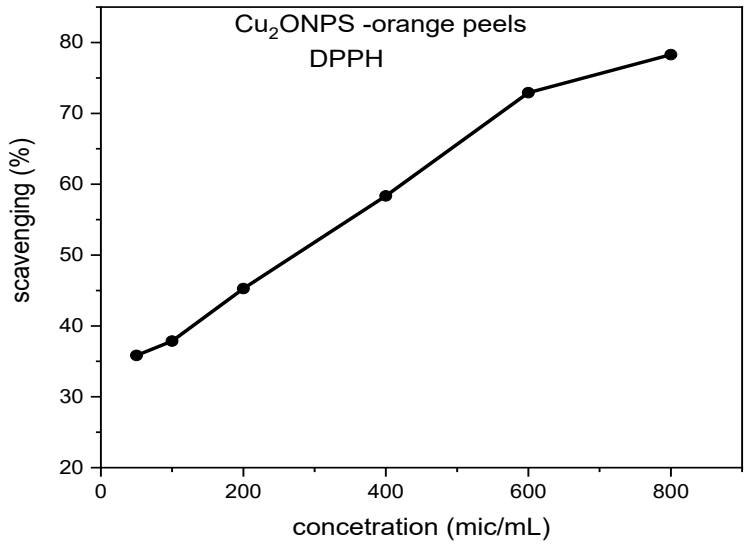

Figure 4. Standard Curve of Antioxidant of Cuprous Oxide Nanoparticle of Orange Peels

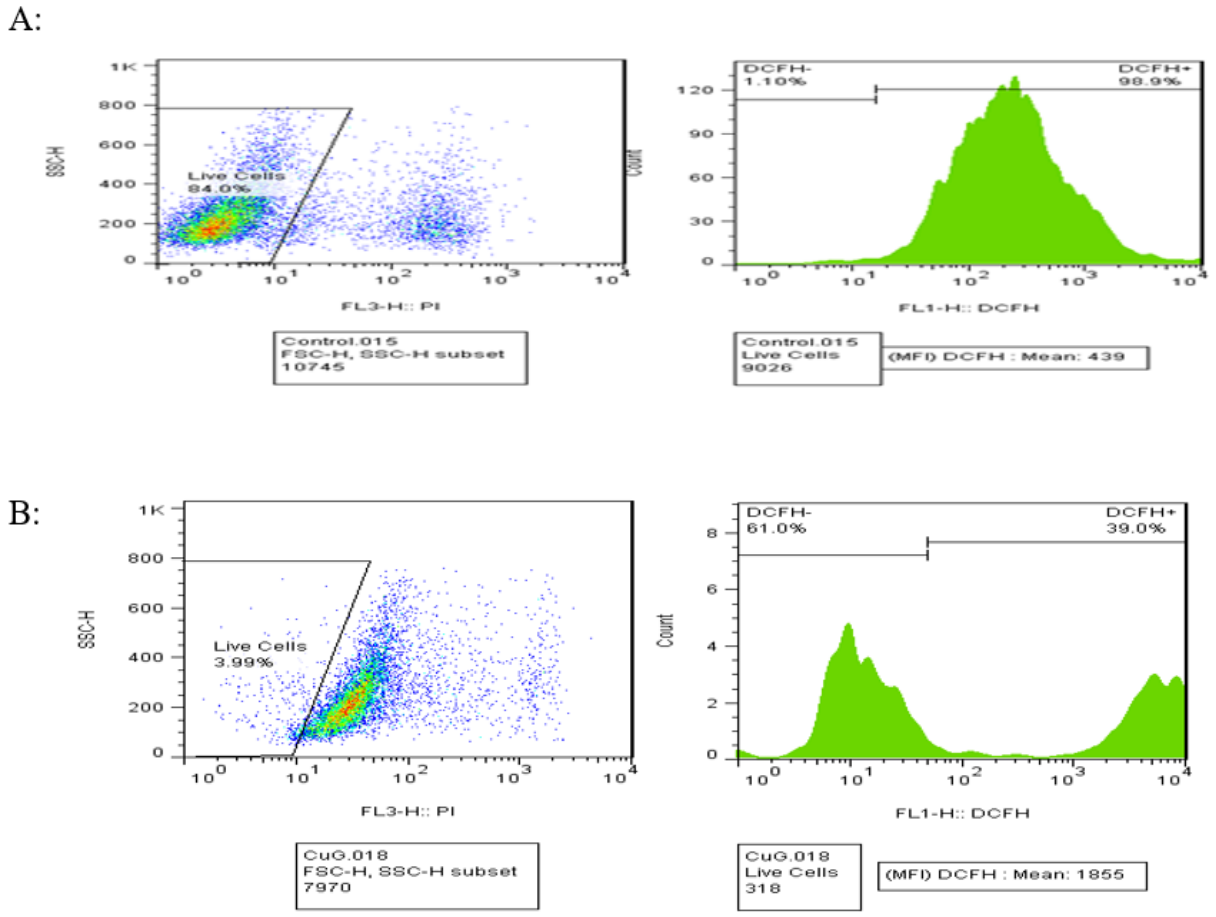

Figure 5. A: cytotoxic cell line before adding the treatment ofCu_2O nanoparticles of orange peels; B: after injecting the treatment Copper oxide nanoparticles- orange peels

Table 4. Evaluation of Cell Viability by MTT of Cu_2 ONPs-Orange Peels

Con.(µg/ml)/	OD	OD2	OD3	Average	Cell viability (%)
500	0.083	0.061	0.073	0.072333	15.01730104
250	0.134	0.129	0.147	0.136667	28.37370242
125	0.153	0.168	0.14	0.153667	31.90311419
62.5	0.296	0.322	0.3	0.306	63.52941176
31.25	0.356	0.345	0.378	0.359667	74.67128028
0	0.456	0.493	0.496	0.481667	100

Evaluation Biology activity of cooper oxide nanocomposites- orange peels

We evaluated the cytotoxicity of biosynthesized Cu_2O-Nano derived from orange peels as a potential treatment for breast cancer. Specifically targeting the MCF-7 cell line. Due to their cytotoxic targeting ability and antioxidant activity, these nanoparticles show promise as an alternative therapeutic option for breast cancer treatment [19] The MTT assay evaluated the cytotoxicity of orange peel Cu_2O nanoparticles by examining their interaction with the MCF-7 cell line. As shown in Table 4, at different concentrations, With an IC50 value of 43.75 g/mL, we found that the cell viability was higher at 62.5 and 31.25 g/mL, and the survival rate was 63.52 and 74.671%, respectively. Figure 6 shows that the viability of MCF-7 cells increased significantly as the concentration decreased.

Discussion

The researchers evaluated and used cuprous oxide nanoparticles synthesized from orange peels as a primary source and studied their effect on the MCF-7 cell line [21]. The conducted characterization processes revealed that these methods are crucial for understanding the physical and chemical properties of the synthesized nanocomposite, as shown in Figure 1. Characterization provides insights into some parameters, including dimensions, shape and chemical composition. As can be seen in Table 1, the sizes are 15-23 nm, which enhances the ability to enhance

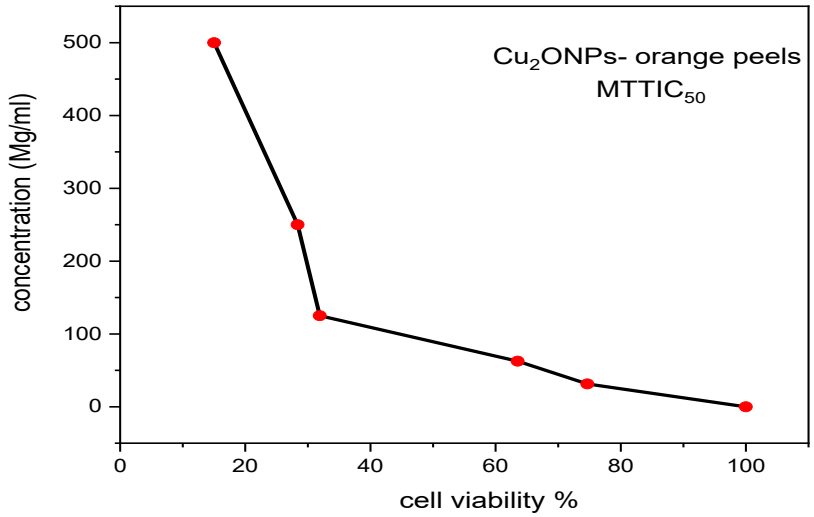

Figure 6. Standard Curve MTT Test Cu_2 O NPs - Orange Peels

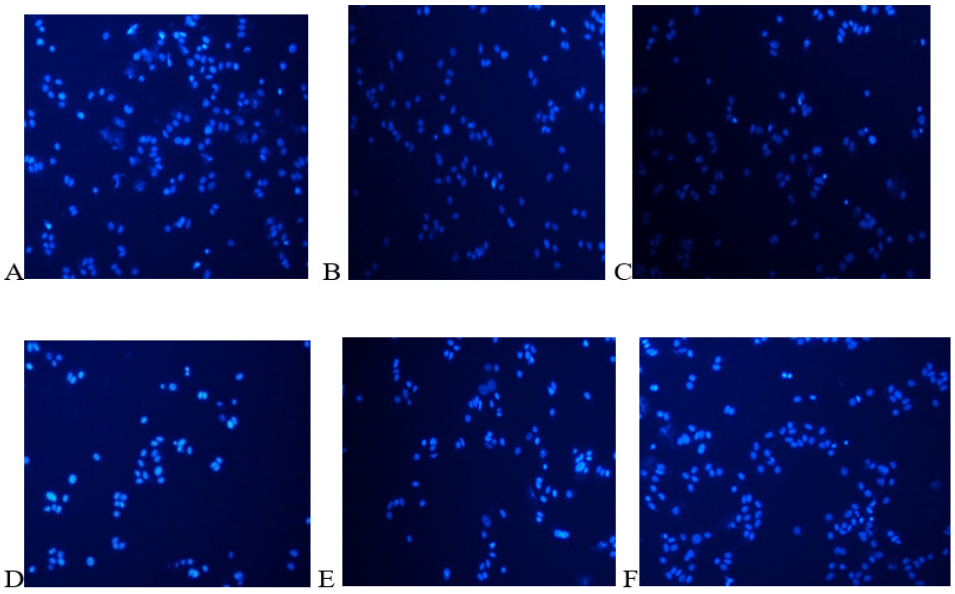

Figure 7. DAPI Stain of Cell Line Cu_2ONPs-Orange a) control b) 500, c) 250, d) 125, e) 62.5 f) 31.2

their effectiveness in selectively inducing cell death. This detailed analysis allows for the study of the fine details and surface morphology of the nanocomposite with high accuracy [22]. The characterization determined the types of chemical bonds present, including O-H bonds, carboxyl groups, C-O bonds indicating ester groups, chlorine-containing organic compounds, and silica groups (as shown in Figure 3). The presence of these functional groups enhances the chemical effectiveness of the nanocomposite in scavenging free radicals [23]. It was found that the nanocomposite contains chemical compounds such as 1,6-Octadien-3-ol, 3,7-dimethyl-, Caryophyllene oxide, Octanal, Limonene oxide and trans in high concentrations, and other active compounds that have a direct effect on toxic cells in the MCF-7 cell line selected in our work as shown in Table 2.

Using 2, 2-diphenyl-1-picrylhydrazyl, we evaluated the synthesized nanocomposite by measuring the absorbance at 570 nm. The results showed that higher concentrations of the nanocomposite led to increased free radical scavenging activity [24], with the highest scavenging activity recorded at a concentration of 800µg/mL, as shown in Table 3 and Figure 4. In addition, Table 4 shows that lower concentrations of the nanocomposite were associated with increased cell viability.Injection of Cu_2O NPs-orange peel nanoparticles into MCF-7 cancer cells resulted in a 3.99% reduction in free radicals. It indicates that the nanoparticles show promising therapeutic effects and May harm normal cells at higher concentrations.. Figure 7 shows the necrotic stage of MCF-7 cells at concentrations of 62.5 and 31.25 µg/mL, as observed using the fluorescent DAPI stain. The cell viability was 63.52% and 74.671%, respectively. In addition, the orange peel complex with Cu_2ONPs had the highest IC_{50} of the tested sample, at 43.75 g/mL. This indicates its potential application in biopharmaceutical [25].

The Cu_2ONPs-orange complex is moderately cytotoxic to cancer cells. The complex's bioactivity demonstrates its properties in the MCF-7 cell line, where researchers found about 50% of the synthesized complex's metal ions to be effective anticancer agents [26]. In addition, careful and appropriate selection of the ligand can improve the active properties of the compounds. Researchers have investigated nanoparticles based on zinc that can serve as chemotherapeutic drugs for copper and gold [27]. Citrus fruits containing active chemical compounds are compatible with most biological systems. Limonene compounds have the ability to affect proteins that play a role in various biological processes, including oxidative stress. This effect may contribute to improved protection against oxidative stress and alleviation of inflammation. However, further research is needed to confirm these effects and evaluate their impact on clinical applications, including transcription, degradation, and apoptosis. The presence of various chemical compounds in orange peels, which may act as proteasome inhibitors, suggests that these nanoparticles may be useful in cancer therapy.Further research and clinical trials are required to confirm the efficacy and safety of this approach as a cancer treatment, despite the promising therapeutic potential highlighted by these studies [28].

In conclusion,the present study aimed to manufacture nanoparticles from natural sources that are non-toxic and effective in killing cancer cells. We manufactured copper oxide nanoparticles from orange peels.. The results of the tests have shown that the plant is an effective user containing active compounds that play a significant role in increasing immunity and fighting free radicals. The immune and metabolic indicators were in their optimal ranges. This is clearly demonstrated by the FT-IR characterization results, which show absorption within the range of 500 to 4000 cm^{-1}, indicating the presence of various functional groups. The analysis revealed O-H bonds, carboxyl groups, and C-O bonds, suggesting the presence of ester groups and chlorine-containing organic compounds, along with silica groups. The nanoparticle sizes ranged from 15 to 23 nm, and XRD analysis confirmed the crystalline structure of the synthesized compound.

The characteristics using scanning electron microscopy showed that it has distinctive surface patterns. In addition, it enhanced the highest antioxidant activity. As the nanoparticles adhered to harmful cells, they became more powerful and showed exceptional antioxidant capabilities against DPPH. The MCF-7 cell line confirmed high viability when exposed to several doses and concentrations, resulting in a significant reduction in free radicals.Reducing the dose revealed the concentration toxic to cells, indicating its maximum potential for biopharmaceutical use in the tested sample. It substantiates potential uses in the treatment of cervical and colon cancer and acts as an antiviral, thus helping to eliminate harmful cells. Furthermore, it has the potential to deliver drugs to infected cells with greater precision, as well as help in disease detection and visualization.

Author Contribution Statement

All authors contributed equally in this study.

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None.

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