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Green synthesis of Iron oxide and Iron dioxide nanoparticles using *Euphorbia tirucalli*: characterization and antiproliferative evaluation against three breast cancer cell lines

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ABSTRACT
Researchers have become increasingly interested in nanoparticles made from plants because of their stability and large surface area. In the current study, iron oxide and iron dioxide nanoparticles were synthesized using aerial parts of the *E. tirucalli* as a reducing agent. The nanoparticles were analyzed using various techniques, including Ultraviolet-visible spectroscopy, Fourier Transform Infrared spectroscopy, X-ray diffractometer, X-ray photoelectron spectroscopy, X-ray energy dispersive spectroscopy, Scanning electron Microscopy, and Transmission Electron Microscopy. The nanoparticles were then investigated for their antiproliferative effect against MCF-7, SK-BR-3, MDA-MB231, and Vero cell lines. The results confirmed the formation of FeO and FeO2 nanoparticles by color change and a UV absorbance peak between 220–390 nm. EDS analysis showed traces of Fe and O, while TEM confirmed the nanoparticle size of 100 nm. FTIR showed a peak at 514 nm. The FeO-RT NPs demonstrated over 80% antiproliferative activity against the MCF-7 cell line at a concentration of 10 μg/mL. While doxorubicin, FeO-RT NPs, and DCM extract showed similar activity against the MDA-MB231 cell line at 10 and 1 g/mL concentrations. However, Vero and SK-BR-3 cell lines showed decreased antiproliferative activity. This study highlights the environmentally friendly use and safe application of iron oxide NPs in cancer therapy.

1. Introduction
Cancer is a significant public health concern worldwide, ranking second only to cardiovascular disease as a leading cause of death [1]. Breast cancer is now the most commonly...
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diagnosed cancer worldwide, with 2.3 million new cases estimated in 2020, accounting for 11.7% of all cancer cases [2]. Unfortunately, 684,996 people died from the disease [2].

The COVID-19 pandemic regulations resulted in a rise in cancer mortality and morbidity rates due to delayed diagnosis and treatment, ultimately impacting global health and the economy negatively [3].

Breast cancer is not a single disease, but rather a group of diseases with different characteristics and clinical features. Because of this, there is no single treatment approach that can be effective for all types of breast cancer [3,4]. For example, positive receptor cancer cell lines such as MCF-7, BT474, KLP-4, SK-BR-3, and others can be treated with surgery, hormonal therapy, and chemotherapy, respectively [5]. However, triple-negative breast cancers such as MDA-MB231, BT-459, SUM185PE, and others are difficult to treat because they lack all three receptors [6]. Moreover, it is important to note that the current treatments have adverse side effects, are not selective, and have poor pharmacokinetics [7–9]. Therefore, to overcome these limitations, a new therapeutic approach concerning cancer treatment is urgently needed.

Nanoparticles (NPs) have caught the attention of many researchers in the field of diagnosis and treatment due to their versatile and beneficial characteristics. They have already been utilized for various purposes such as tumor imaging in vivo, bio-molecular profiling of cancer biomarkers, and targeted drug delivery [10]. The use of green chemistry to synthesize NPs is an eco-friendly, economical, and sustainable solution that has gained attention for its potential to reduce the use of hazardous chemicals and solvents, resulting in lower toxicity and fewer harmful by-products [11–14]. This approach also utilizes readily available natural resources, such as plant extracts or microorganisms, which reduces the need for expensive reagents and energy-intensive processes [15]. The versatility of this method enables the production of a wide range of NPs with varying properties, including size, shape, and surface chemistry, making it customizable for specific applications [16]. Furthermore, it generates lower waste byproducts, leading to reduced pollution [15]. It is also easily scalable, making it ideal for industrial applications that require large quantities of NPs [17].

Metal oxide NPs are known for their unique physio-chemical and optoelectronic properties, making them highly important [30–32]. Among the various metal oxides, iron oxide NPs have garnered significant attention due to their low toxicity, superparamagnetic properties, surface area and volume ratio, protein immobilization, and potential use in...
diagnostic magnetic resonance imaging (MRI), thermal therapy, and drug delivery [33]. In addition, they have demonstrated effectiveness in inhibiting MCF-7 cell lines in prior research [34]. However, no comparative study has been conducted to assess the antiproliferative effects of iron oxide and iron dioxide NPs against MCF-7, SK-BR-3, and MDA-MB231 cell lines.

Therefore, this study aimed to synthesize metal iron oxide and iron dioxide NPs using the aerial plant extract of *E. tirucalli* as a reducing agent, characterize the NPs, and compare their antiproliferative effect against three different subtypes of breast cancer cell lines. Our research focuses on investigating the potential anticancer properties of *E. tirucalli* against breast carcinoma.

2. Materials and Methods

2.1. Materials

In March 2017, the *E. tirucalli* plant species was commercially acquired from Gariep Nursery in Pretoria, South Africa (GPS coordinates 25°47′3″S, 28°18′58″E). Dr. Nolubabalo Matinise of iTHEMBA LABS, South Africa generously donated ferric nitrate nonahydrate salts. The extraction solvents, namely Hex, DCM, and methanol (MeOH), were purchased from Merck in South Africa. MCF-7, SK-BR-3, and MDA-MB231 cells were purchased from Cellonex in South Africa. Reagents including DMEM, trypan blue dye, and MTT salt were purchased from Sigma Aldrich in South Africa.

2.2. Methods

2.2.1. Plant extraction and preparation

Upon arrival at the laboratory, the aerial parts of the plant were washed with tap water and chopped into smaller pieces. The pieces were then air-dried and ground into a fine powder.

2.2.2. Preparation of FeO and FeO$_2$ nanoparticles

In order to synthesize NPs, the *E. tirucalli* finely ground powder weighing 30 g was boiled in distilled water at 80°C for a duration of 2 h. Following this, the extract was divided into two beakers and mixed with 5 g of Ferric nitrate nonahydrate. These solutions were then placed on a magnetic stirrer, maintained at 60°C for a period of 12 h, and closely observed for any changes in color which would indicate the formation of NPs. The resulting NP pellets were obtained by subjecting the solutions to centrifugation at 5000 rpm for 20 min, after which they were washed with double distilled water to remove any excess plant extract. These pellets were then dried in an oven at 80°C for 2 h. Once dried, the NPs were annealed at 500°C for a duration of 2 h to eliminate impurities and were subsequently stored at room temperature for characterization.

2.2.3. Preparation of *Euphorbia tirucalli* aerial extracts for cell culture

To extract compounds from the plant, a 10 g fine powder was sequentially extracted using various organic solvents (hexane, dichloromethane, methanol, and ethyl acetate) in 150 mL portions. The extraction process was carried out for 48 h on a shaker at room temperature. The resulting extracts were filtered and concentrated under reduced pressure at 45°C using a rotary vacuum evaporator from Buchi labotech Switzerland. Finally, the extracts were dried under a fume hood and stored at 4°C for future use.
2.2.4. Characterization of synthesized nanoparticles
Characterization of FeO and FeO$_2$ NPs was conducted using the established protocol by Shunmugadevi and Palanisamyb [35], with slight modifications. UV spectroscopy was utilized to investigate the absorption patterns, employing the Multiskan Go 1.01.10 spectrophotometer. Fourier Transform Infrared Spectroscopy (FTIR) was used to identify the functional groups involved in the capping process, with the Thermo Nicolet Nexus 670 spectrometer measuring the spectrum. The structural properties of the NPs were examined using the Bruker D8 X-ray diffractometer. The NPs’ chemical state was determined by using XPS with the Versa Probe XPS V1.4. The morphology and size of the particles were determined using SEM and TEM, respectively. The JEOL JEM 2010 UHR and Philips 100 transmission electron microscope were used for this purpose. Lastly, EDS was used to detect the nano constituents present in FeO and FeO$_2$ NPs. The Philips 100 transmission electron microscope was used for this process.

2.2.5. Phytochemical screening
A phytochemical analysis was conducted on the aerial part of *E. tirucalli* using Yadav and Agarwala’s [36] protocol, with some slight modification. Various tests were carried out to determine the presence or absence of alkaloids, glycosides, flavonoids, phenols, saponins, terpenoids, and tannins.

2.2.6. Antiproliferation screening of Euphorbia extracts
2.2.6.1. Cell culture. The MCF-7, SK-BR-3, and MDA-MB231 cell lines were cultured using Dulbecco’s modified eagle's medium (DMEM), DMEM: HAMS F12 (1:1), and Mc-Coy media, respectively. Each medium was supplemented with 10% fetal bovine serum (FBS) and the cells were kept in an environment with 37°C temperature and 5% CO2 humidity in an incubator. When the cells reached 80% confluency, they were sub-cultured.

2.2.6.2. Antiproliferative assay. To facilitate easy harvesting, the cells were treated with Trypsin. An automated cell counter (Countess Fl, life technology) was used to count the viable cells with trypan blue dye. 1 x 10$^5$ cells/ml were then seeded into each well of a 96-well microtiter plate and incubated under the same conditions for 24h. The cells were treated with unannealed and annealed NPs along with crude *E. tirucalli* at concentrations of 100, 10, and 1μg/mL in triplicate for 48h. Wells containing untreated cells were used as a negative control, while Doxorubicin drug was used as a positive control. Cell growth activity was measured using the tetrazolium dye (MTT assay) [37]. Cell viability and growth inhibition percentage were calculated, and graphs were plotted using Microsoft Excel 2008.

3. Results and discussion
3.1. Phytochemical analysis
In our previous research on *E. tirucalli*, we examined its phytochemicals and discovered the presence of tannins, glycosides, triterpenoids, and saponins [38]. These components are recognized for possessing anticancer and antioxidant properties, and also have the potential to reduce metal ions [39,40]. Additionally, plants that contain antioxidants typically have reducing abilities.
3.2. Synthesis of iron oxide and iron dioxide NPs

The aerial part of *E. tirucalli* was utilized as a reducing agent, leading to a noticeable shift in color from light brown to black upon the introduction of Ferric nitrate nonahydrate salt to the *E. tirucalli* extract, Figure 1. This indicates the stimulation of electrons and the formation of FeO and FeO$_2$ NPs. The formation of these NPs was caused by plant secondary metabolites specifically polyphenols that effectively reduced metal ions and stabilized the FeO NPs and FeO$_2$ NPs. These nanoparticles have phytochemicals attached to their surface that possess hydrophilic hydroxyl groups. These groups enable the nanoparticles to be evenly dispersed in aqueous solutions [41]. These results are consistent with those of katata-Seru et al. [42], who found a comparable color alteration from reddish brown to black, suggesting the presence of Fe NPs.

3.3. Characterization of synthesized NPs

3.3.1. UV-vis spectrum of iron oxide and iron dioxide NPs

To analyze the formation of iron nanoparticles, a UV-vis spectrophotometer was utilized to measure the absorbance in the range of 200 – 1000 nm. The results, depicted in Figure 2a,b, exhibit two absorption peaks between 220 – 390 nm. Figure 1a displays peaks at 220 and 380 nm, whereas Figure 1b shows peaks at 230 and 390 nm. These absorption peaks are attributed to the formation of iron nanoparticles. The absorption peak at 220 nm are attributed to the presence of tannins and triterpenoids in *E. tirucalli* extract [41]. The 220 nm and 230 nm peaks are consistent with Pattanayak and Nayak’s study [43], which suggests the presence of surface plasmon vibrations in metallic iron nanoparticle solutions. Furthermore, the peak observed at 390 nm is in line with the findings of Kumar and Prem’s study [44], which identified the formation of iron oxide nanoparticles.

3.3.2. FTIR spectrum analysis of iron oxide and iron dioxide NPs

FTIR spectroscopy was employed to investigate the functional groups within *E. tirucalli* and the synthesized iron NPs. The FTIR spectra of the iron NPs are shown on Figure 3a,b. The figures displayed peaks in the 500 – 4000 cm$^{-1}$ range, specifically at 3350 cm$^{-1}$, 1650 cm$^{-1}$, 1150–1050 cm$^{-1}$, 750 cm$^{-1}$, and 514 cm$^{-1}$. The 3350 cm$^{-1}$ peak signifies the presence of O-H groups, potentially originating from phenols or water in the *E. tirucalli* plant [45]. At 1650 cm$^{-1}$, the peak suggests hydrogen bonding between oxygen molecules in the iron oxide nanoparticles and hydrogen molecules in *E. tirucalli*, representing C=O.
The absorption peaks at 1150–1050 cm\(^{-1}\) are attributed to C-O groups, aligning with previous research confirming the formation of iron oxide nanoparticles [48]. The 750 cm\(^{-1}\) peak is due to CH groups present in aromatic compounds within \textit{E. tirucalli}. Furthermore, the presence of a peak at 514 cm\(^{-1}\) confirms the formation of both iron oxide and dioxide nanoparticles, corroborating findings from earlier studies [34,47,49].

**3.3.3. XRD analysis of iron oxide and iron dioxide NPs**

The XRD spectra of iron oxide and dioxide NPs synthesized using a green method are displayed in Figure 4a,b. The spectra showed various diffraction peaks at \(\theta = 30.91^\circ, 35.70^\circ, 43.14^\circ, 57.46^\circ,\) and 62.90\(^{\circ}\), which correspond to the planer reflections of (220), (311), (222), (400), (511), and (440) of a cubic phase for both types of NPs, respectively. These peaks are attributed to (00-graphitic) [50]. These reflection peaks can be indexed to the magnetite structure of iron oxide observed in previous studies by Kumar and Prem [44], Perumal et al. [50], and Taha et al. [51], and can further be compared with the data found in JCPDS card (77–1545). However, additional peaks were detected in both samples, indicating the presence of impurities.
3.3.4. XPS analysis of iron dioxide NPs

The XPS technique was employed to analyze the elemental composition and electron surface state of various components in FeO₂ NPs. Unfortunately, the analysis for FeO NP could not be completed as there was not enough material available. The resulting analysis, as shown in Figure 5a–c, revealed the presence of iron (Fe), magnesium (Mg), oxygen (O), carbon (C), and calcium (Ca) in FeO₂ NPs, thereby providing valuable insights into the chemical composition of the material. These findings were further affirmed through EDS analysis, which corroborated the presence of these elements in FeO₂ NPs. The peaks observed at 712.3, 710.3, and 708.5 eV were attributed to Fe 2p₁, Fe 2p, and Fe p₃, respectively, thereby providing evidence for the presence of Fe³⁺ octahedral species in the NP. These findings are consistent with previous studies [52] and highlight the importance of Fe³⁺ in the electronic properties of FeO₂ NPs. Moreover, the peaks observed at 530.5 eV and 532 eV were attributed to O 1s in the NPs and the presence of hydroxyl groups [53,54], respectively. Additionally, the peak observed at 529.2 eV indicated the presence of O on the surface of E. tirucalli FeO₂ NP, associated with O–H, O–C, and O=C compounds [55]. These findings suggest that the electronic properties of FeO₂ NPs are heavily influenced by the presence of O on the surface of the plant. Overall, the
valence states of Fe and O in FeO\textsubscript{2} NPs were found to be +3 and −2, respectively, as observed in the attributes of Fe 2p and O 1s core levels. These results provide valuable insights into the electronic properties of FeO\textsubscript{2} NPs and highlight the importance of understanding the elemental composition and electron surface state of materials in the field of nanotechnology.

### 3.3.5. SEM analysis of iron oxide and iron dioxide NPs

The morphology and size of iron oxide and dioxide NPs were examined using a SEM. The nanoparticles were found to measure 100 nm in size, as illustrated in Figure 6a,b. Figure 6a depicts the iron oxide nanoparticles in a mesoporous and matrix-like arrangement, with a flat plate shape that can bend into a rod shape. In contrast, Figure 6b shows a different morphology compared to the iron oxide nanoparticles. Iron dioxide nanoparticles are formed in a spherical shape with a few cubic shapes and a clustered pattern. They may have clumped together due to the consistency of the solution and the drying method used [56]. The variation in morphology between the two iron nanoparticles could be attributed to the reducing power of phytochemicals, pH levels, or other factors that can affect their formation [57].
3.3.6. TEM and EDS analysis
The TEM images in Figures 7a and 8a showcase the iron NPs that were synthesized, revealing hexagonal agglomerated NPs with a size of 100 nm. EDS profiling of all nanocomposites obtained after calcination indicated peaks, with a high characteristic peak of oxygen followed by carbon, iron, calcium, chloride, magnesium, potassium, sodium, sulphur and phosphorus present for both NPs in Figures 7b and 8b. Notably, sodium was not detected in the iron dioxide NP (Figure 8b). The presence of iron and oxygen confirmed the formation of iron oxide and dioxide NPs. The high oxygen and carbon peaks detected in both NPs suggest that polyphenols were involved in the synthesis of iron nanoparticles [58]. Furthermore, the oxygen and carbon peaks may represent the polyphenol present in the plant [59]. Additional weak composite peaks observed of calcium, sodium, and magnesium may arise from the glass that holds the sample during synthesis [52]. The phosphorus, potassium, sulphur, and chloride peaks observed were due to improper washing of the sample [60].

The composition of the iron oxide NPs was 53.4% of iron, 22.1% of carbon, 17.0% of oxygen, and all other composites were less than 5% (Figure 7b). On the other hand, the iron dioxide NPs were composed of 40.4% of iron, 28.0% of oxygen, 17.7% of carbon, 7.2% of calcium, and all other composites were less than 5% (Figure 8b). The difference in oxygen percentages between the iron oxide and iron dioxide NPs can be attributed to their formation process. They are both formed from Ferric nitrate nonahydrate precursor salt when iron reacts with oxygen from the plant extract’s metabolic constituents. Sometimes, when the oxygen from the water or air binds to iron, it increases the number of oxygen molecules within the compound, differentiating between the two oxides. The presence of oxygen and carbon is crucial as they stabilize and reduce the two metal oxides [61]. Although there were impurities present in the samples, they were in low concentrations, as shown in the results.

3.3.7. MTT assay
Breast cancer is characterized by the protein expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Because of these characteristics, it is necessary to compare the effects of the potential antiproliferation drugs or drug carriers in different breast cancer cells with clinically approved cancer drugs in order to assess the efficacy or potential benefits within new application techniques.
Antiproliferative effect of the crude extracts, annealed and unannealed Iron Oxide and Iron dioxide NPs against MCF-7, SKBR-3, MDA-MB231 and Vero cell lines was tested across concentrations of 1, 10, and 100 μg/mL in triplicates, and doxorubicin served as a standard drug. The level of toxicity exhibited by the treatment was observed to be concentration-dependent, with the most substantial toxicity observed at the highest concentration, Figure 9a–d.

The extracts from *E. tirucalli*, specifically Hex and DCM, displayed moderate antiproliferative effects against MCF-7 and MDA-MB231 cells. At concentrations of 100 and 10 μg/mL, these extracts inhibited cell growth by over 50% and 40%, respectively. However, the same extracts showed weak activity against SK-BR-3 and Vero cells, with cell growth inhibition rates of less than 40%. This suggests that SK-BR-3 is less susceptible to the extracts than MCF-7 and MDA-MB231. Previous research has also shown that stevioside and other compounds have higher cytotoxicity against MCF-231 and MCF-7 cell lines compared to SK-BR-3 cell line [62,63]. This anticancer activity could be due to estrogen and progesterone receptors as both cell lines are HER2 negative [62]. Additionally, Fe$_3$O$_4$NPs were reported to inhibit the growth of lung cancer cells Habibi et al. [64]. Moreover, Rajendran et al. [65] have demonstrated a considerable level of Fe$_3$O$_4$NPs cytotoxicity against various cancer cells, especially at lower concentrations, in comparison to normal cells. In a study conducted by Al-Shalabi et al. [66], it was found that IONPs were highly effective in inhibiting the growth of the MDA-MB231 cell line. The IC$_{50}$ value was measured at 0.3817 μg/mL, indicating a strong inhibitory effect. According to a research conducted by Pillai and colleagues [67], IONPs have been found to possess remarkable cytotoxicity against DLD-1 cancer cells. Interestingly, these nanoparticles have been
demonstrated to have minimal or no adverse effects on normal cells, thus making them a potentially efficient option for treating cancer. This finding suggests that IONPs may have potential as a therapeutic agent for breast cancer treatment.

Moreover, the synthesized unannealed FeO-RT NPs showed potent activity against MCF-7 cells with over 80% inhibitory activity at concentrations of 100 and 10μg/mL, which could be attributed to impurities and compounds present in the plant before calcination. After calcination, the antiproliferative activity decreased, indicating the degradation of phytochemicals due to increased temperature. Additionally, the annealed and unannealed FeO NPs showed higher inhibitory activity against MCF-7 than the Hex and DCM extracts, and about the same as the standard drug. Contrary, studies by Hernandes et al. [68] reported a 20% decrease in cell viability when MCF-7 and HeLa cells were treated with mesoporous silica NPs. Moreover, other studies revealed that MCF-7 cells treated with pure/uncoated iron oxide NPs had high cell viability [69].

The FeO NPs and doxorubicin standard drug showed almost the same activity at 10 and 1μg/mL concentrations against MDA-MB231, indicating moderate activity against the cell line. This activity shows the potential of FeO as a promising antiproliferative agent. The FeO$_2$ NPs showed decreased cell growth inhibition activity compared to the FeO NPs, which could be attributed to the chemicals and bonding abilities of FeO$_2$ and FeO.

4. Conclusion

Iron oxide and iron dioxide NPs were successfully synthesized using the aerial parts of Euphorbia tirucalli. The synthesized FeO-RT NPs together with the Hex and DCM extracts showed increased antiproliferative activity against MCF-7 and MDA-231 breast cancers compared to SK-BR-3 and Vero cells. The results show that the triple-negative breast cancer is not restricted to the receptor binding mode of action. The decreased antiproliferative activity against SK-BR-3 cells should be investigated further to determine the mechanism of action of the NPs and extracts. Further cytotoxic analysis should be conducted to determine the selectivity of the NPs and extracts. In conclusion, this study confirmed that the green-synthesized FeO NPs could be a potential antiproliferative drug because of the safety it displayed with the Vero cells.

Disclosure statement

The authors declare no conflict of interest.

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