Research Article

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Green synthesis, characterization, cytotoxicity, and antimicrobial activity of iron oxide nanoparticles using Nigella sativa seed extract

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Abstract: Green synthesis of nanoparticles (NPs) is a more sustainable, safe, and environmentally friendly method. This study aimed to investigate the synthesis procedure of iron oxide nanoparticles (IONPs) using the seed extract of Nigella sativa (N. sativa) as a strong reducing agent and to estimate their cytotoxic and antibacterial properties. The obtained IONPs were characterized by field-emission scanning electron microscopy, X-ray diffraction, dynamic light scattering (DLS), zeta potential (ZP), and Fourier-transform infrared spectroscopy analyses. The cytotoxicity of the biosynthesized IONPs was demonstrated by the MTT assay. Furthermore, the antibacterial activity of the fabricated biosynthesis metal oxide NPs was tested on Escherichia coli (E. coli) (ATCC 35218) and Staphylococcus aureus (S. aureus) (ATCC 29213) bacterial strains using the Kirby–Bauer disk diffusion method. This study showed the formation of a well-dispersed, highly stable (ZP $\zeta = -51.8$ mV) 10 NPs with an average diameter of about 31.45 nm. Moreover, the biosynthetic NPs (IONPs) exhibited a significantly nontoxic effect when analyzed by the MTT assay. The biosynthetic NPs (NS-IONPs) exhibited excellent antibacterial activity against E. coli and S. aureus, where the inhibition zones were 12.34 ± 0.58 and 11.52 ± 0.58, respectively.

Keywords: antibacterial activity, cytotoxicity, iron oxide nanoparticles, Nigella sativa, green synthesis

1 Introduction

Magnetic iron oxide nanoparticles (IONPs) are one of the most commonly used inorganic nanoparticles (NPs) in various industries, including chemical, medicinal, pharmaceutical, electronic, and agricultural industries, due to their unique properties such as high saturation magnetization, which allows for easy magnetic separation in the presence of an external magnetic field [1,2]. IONPs can be synthesized using various methods, such as sol–gel, chemical reduction, co-precipitation [3], hydrothermal synthesis, and pulsed laser ablation in dimethylformamide (DMF) [3–6]; however, the chemicals used in these processes are considered hazardous to the environment [7]. Green NP synthesis using bioresources has sparked considerable interest as a promising method not only for reducing the toxicity of NPs that is often linked with traditional chemical synthesis methods but for its low cost, convenience of use, and environmentally friendly nature [8].

Plant extracts are used as a reducing and capping agent in the biosynthesis of NPs, decreasing the need for toxic-reducing chemicals [9]. Recently, numerous
investigations have been performed on the environmentally friendly production of iron-based NPs using diverse plant parts such as *Cynometra ramiflora* fruit extract, *Persea americana* rind and *Punica granatum* seed extract, and *Avicennia marina* flower extract; *Nigella sativa*; *Glycyrrhiza glabra*; *Cynodon dactylon*; and fungi such as *Aspergillus flavus* and *Phoma exigua* [10–15]. Various reducing agents such as DMF, hydrazine carbon monoxide (CO), sodium borohydride (NaBH₄), and others have been utilized in the synthesis of IONPs. These reducing agents are highly reactive compounds that negatively affect the environment and impair IONP biocompatibility, resulting in restricted biomedical applications of IONPs [16,17].

So, IONPs must be entirely biocompatible for biomedical applications [18]. As a result, biogenic reduction/green synthesis methods that are novel and environmentally friendly are in high demand [19].

Biogenic reduction techniques, which involve bacteria, fungi, algae, and higher plant extracts, are among the best options for producing metal and metal oxide NPs [20]. These more environmentally friendly methods are cost-effective, produce a good yield, and are fairly reproducible [21].

There are a couple of successful studies in synthesizing IONPs by using plant extracts, for instance, fruit extract of *Ficus carica* (common fig) [22], leaf extract of *F. carica* [23] and *Carica papaya* [24], and also seed extract of fenugreek seed [25]. However, there are only finite studies on the synthesis of IONPs from the seed extract of *N. sativa*.

*N. sativa* is a plant belonging to the Ranunculaceae family. It is a traditional medicinal plant that grows in various locations worldwide, particularly in Iraq and Saudi Arabia [26]. *N. sativa* has a rich source of nutritionally important elements, and its oil contains polyunsaturated fatty acids and other phytochemicals with high antioxidant activities and a glucose-lowering impact [27].

In this study, we used *N. sativa* as a reducing agent in the green synthesis technique to synthesize IONPs, combining the therapeutic properties of *N. sativa*’s seed extract with the critical properties of IONPs and evaluating their antibacterial effectiveness against *E. coli* and *S. aureus*.

## 2 Materials and methods

### 2.1 Chemicals and plant collection

All chemicals used in this study, including tetrahydrates of iron–iron oxide (FeCl₂·4H₂O, 99%), hexagonal-ferrous chloride (FeCl₂·6H₂O, 99%), ammonia (NH₃, 25%), tetramethylammonium hydroxide (25%), NaOH (99.99%), absolute ethanol (≥99.8%) (GC), and nutritional broth, were obtained from Sigma-Aldrich Pty Ltd (Darmstadt, Germany) and used without further purification. In June 2021, *N. sativa* seeds were obtained from the faculty of the University of Al-Qadisiyah, Al-Diwaniyah, Iraq. The seeds were collected and stored at 4°C until further study.

### 2.2 Bacterial strains

The tested bacteria were Gram-positive and Gram-negative, including *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 35218).

### 2.3 Synthesis of IONPs

#### 2.3.1 Preparation of the plant extract

Following the Jalali and Tehranipour [28] method, with some modifications, about 100 g of *N. sativa* seeds were mixed with 70% ethanol in a Soxhlet extractor in the central laboratory of the College of Veterinary Medicine, University of Al-Qadisiyah. Under low pressure, 32% of the obtained extract was concentrated and stored at 4°C until required.

#### 2.3.2 Gas chromatography-mass spectrometry (GC-MS) analysis

The seed extract of *N. sativa* was analyzed using GC-MS (Shimadzu GC 17A, Japan). For the analysis, 1 μL of the extract was injected at a 20:1 split ratio. Helium gas (99.9%) was used at 1 μL per minute in the gaseous form. The experiment was carried out in the electron impact (EI) mode with a 70 eV ionization energy. The temperature of the injector was held constant at 250°C. The temperature of the column oven was set at 50°C (held for 3 min), increased to 280°C (held for 3 min) at 10°C per min, and finally at 300°C held for held for 10 min. After comparing the spectral configurations acquired with the available mass spectral databases (NIST and WILEY libraries [multiplier voltage of 1 kV]), the compounds were characterized [29–31].

#### 2.3.3 Preparation of green-synthesized (*N. sativa*-iron oxide) NPs

The biosynthesis of IONPs was carried out using a method described earlier by Bibi et al. [32]. To prepare IONPs,
distilled deionized water was used as the solvent. In all, 4.0 mL of FeCl₃ (1 M) and 1.0 mL of FeCl₂ (2 M) were mixed in a 100 mL container under vigorous shaking. The coprecipitation method, the most common method, was used for preparing magnetic IONPs for biomedical applications [33]; an aqueous solution of NH₃ (50 mL and 1.0 M) over 5 min was slowly added to this mixture. The residue was then treated with 25% tetramethylammonium hydroxide and the reaction mixture was gently stirred for 2–3 min. The powder was accurately weighed, transferred to a flask, and dissolved in ultrapure water by ultra-sonification for 5 min using the dispersion method.

2.3.4 Characterization of green-synthesized IONPs

2.3.4.1 Field-emission scanning electron microscope (FESEM)
The morphological features and size of green-synthesized IONPs were studied using an FESEM (MIRA3 TESCAN-XMU, Brno, Czech Republic) at a 20 kV electron acceleration.

2.3.4.2 X-ray diffraction (XRD) test
Applications of Cu–Kα-wavelength (\(T = 1.5405 \text{ Å}\)) were produced using a Phillips diffractometer in samples of green-synthesized IONPs. The voltage used was 40 kV, and the current intensity was 25 mA.

2.3.4.3 DLS measurement
DLS measurements of the prepared green-synthesized IONPs were carried out using Horiba SZ-100 nanoparticle analyzer. An appropriate powder concentration of 0.01 g per 100 mL is distributed in DMSO. The same medium has been used to estimate the cytotoxicity effect on the distribution of DMSO particles.

2.3.4.4 Determination of zeta potential (ZP)
ZP measurements were performed using Horiba SZ-100 nanoparticle analyzer to investigate particles’ surface charge of prepared green-synthesized IONPs. The particle’s electrostatic potential was calculated in ultrasonic dispersion of 0.01 g per 100 mL in DMSO at room temperature.

2.3.4.5 FTIR analysis
To study the chemical bonds formed between the \(N. sativa\) (NS) seed extract and IONPs, IR spectra analysis of the NS-IONP layers was performed. The spectra were acquired in the 400–3,500 cm⁻¹ wavenumber range in an attenuated total reflection (ATR) mode using an FTIR instrument (Shimadzu Corporation, Japan) with 4 cm⁻¹ resolution. The spectra have been acquired in transmittance mode by placing the samples on the diamond–ZnSe crystal plate of the ATR unit inserted into the spectrometer and analyzed using Version 1.25 Sp5, supplied by Shimadzu. The spectrum of the NS-IONP layer was recorded after 32 scans.

2.3.4.6 MTT assay
To measure the cytotoxic effect and biocompatibility of prepared green-synthesized IONPs, the MTT assay was used by following the instructions from the manufacturer. At a density of \(1 \times 10^5\) cells per well, Vero cultures were incubated at 37°C in the presence of 5% \(CO_2\). Green-synthesized IONPs were added in five concentrations 12.5, 25, 50, 100, and 200 μg·mL⁻¹ for 48 h, followed by washing twice before introducing 100 μL of cultivated medium and 0.5 mg·mL⁻¹ of MTT reagent each to a fresh source. Green-synthesized IONPs were washed twice with phosphate-buffered saline (PBS). The unlabeled cells were used as the control group, while Triton-X was used as the positive control. Later, the labeled cells were incubated at 37°C in 5% \(CO_2\) for 4 h. The medium was tactfully aspirated and replaced by 100 μL of fresh DMSO. The absorbance of the dissolved formazan product was examined at a wavelength of 570 nm.

2.3.4.7 Antibacterial activity
Kirby–Bauer disk diffusion method was used to test the antibacterial activity, which was considered a suitable method for evaluating the antibacterial activity of a newly synthesized material [34]. Briefly, four or five bacterial colonies were collected using a sterile inoculating loop and suspended in 2 mL of sterilized PBS; the studied bacteria include \(S. aureus\) (ATCC 29213) and \(E. coli\) (ATCC35218). The turbidity of bacterial suspension has been modified to a 0.5 McFarland level by diluting it with sterile PBS. Swabs that are free from bacteria were put into the inoculum channels. Bacteria were streaked onto Muller–Hinton agar plates by using swabs. Green-synthesized IONP suspensions were
prepared by dissolving 0.1 mg of green-synthesized IONPs in 1 mL of distilled water. Sonication was carried out on the suspension for 10 min before usage. In all, 35 μL of green-synthesized IONP suspensions and distilled water (as a negative control) were used to impregnate the standard antibiotic disc (as a positive control). The final step was to conduct a disc diffusion assay on Mueller–Hinton agar plates against two different bacterial strains (Merck, Germany). Sterile forceps were used to put disks impregnated with culture medium on the surface of the agar. The bacterial activity was evaluated using plates incubated at 37°C for 24 h. The zone of inhibition (this activity) was measured in millimeters. The assays were repeated three times.

### 2.3.4.8 Statistical analysis

The mean ± standard deviation (SD) was used to analyze our results. For assessing statistical significance, Excel was also used. A p-value less than 0.05 or 0.01 was considered statistically significant. All required statistical analyses were performed using the statistical program SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

### 3 Results and discussion

#### 3.1 Gas chromatography-mass spectrometry analysis

The ethanolic extract of *N. sativa* seeds was subjected to gas chromatography–mass spectrometry analysis, revealing various peaks. The database of the spectrum of known components from the GC-MS library was used to interpret the chromatogram peaks. The extract contained 15 main elements, according to GC-MS profiling. The peak regions for several chemicals are shown in Table 1, and Figure 1 lists the discovered compounds and their peak area (%) and retention duration (RT).

GC-MS analysis confirmed the presence of many *N. sativa* seed extract compounds, including amino acids, proteins, carbohydrates, fixed oil, steroids, flavonoids, alkaloids, phenols, and quinones. The difference between the current study and others is the number of compounds detected in the extract; the present study discovered 15 compounds, another study discovered 23 [30], and 31 was found in ref. [35].

Many plant bioactive compounds are found and used to enhance good health and combat infection in humans and animals, and many of these compounds are sold as food or herbal medications [36,37]. Herbal medicine’s popularity among the general public has soared in recent years, in part due to the ease with which it can be obtained without a prescription, the low cost, and the ease of scheduling an appointment with a healthcare provider, but also because of the widespread belief that natural remedies are less dangerous than synthetic ones [38]. Many compounds in the *N. sativa* plant include volatile oils, cardiac glycosides, terpenoids, flavonoids, anthraquinones, sterols, alkaloids, saponins, and volatile bases [39].

### 3.2 Characterization

#### 3.2.1 FESEM

The fabricated green-synthesized IONPs (Figure 2), with an average size of 31.45 nm, were visualized using the FESEM technique for displaying the size and shape of relatively spherical NPs.

#### 3.2.2 XRD

The diffraction peak appeared at 2θ with 30.3°, 35.7°, 43.1°, 53.6°, 57.4°, and 62.6°, respectively, corresponding to the following diffraction planes: (111), (200), (220), (311), (222), and (331), respectively.
to the crystal structure of magnetite (220), (311), (400), (440), (531), and (533), indicating the formation of the anatase phase of green-synthesized IONPs (Figure 3). The peaks in Figure 3 show a broadening of the half maximum of the peak in green-synthesized IONPs and increased signal concerning the crystallographic plane 311. The XRD pattern of green-synthesized IONPs matched well with that of the standard reference for Fe₃O₄ (magnetite) from the database (JCPDS file no. 00-003-0863), and our results are also in good agreement with the previously reported studies [40].

### 3.2.3 Dynamic light scattering (DLS) and zeta analysis

The DLS and ZP analysis of prepared green-synthesized IONPs are shown in Figures 4 and 5. The nature of hydrodynamic size (diameter) in DMSO of prepared green-synthesized IONPs was studied. DLS showed the hydrodynamic size distribution of small particles, and it refers to the size of the core and the materials used to cover it. For the green-synthesized IONP suspension in DMSO, a clear indicator for stability without particle settlement was found by the ZP analysis (Figure 5) (ζ = −51.8 mV), with an electrophoretic movement (mean) of −0.000402 cm²V⁻¹s⁻¹. The study on the prepared suspension also confirms the general ZP criteria negatively for improved stability [41,42].

### 3.2.4 FTIR analysis

FTIR analysis was used to determine the functional groups of green-synthesized IONPs (Figure 6). Based on the peak value in the infrared radiation region, an FTIR spectroscope was used to determine the functional groups of active substances. The FTIR spectra of produced NPs ranged from 3,500 to 400 cm⁻¹. The FTIR spectra of the produced IONPs were used to determine the groups responsible for NP capping and stabilization. Thirteen peaks were recorded including 3384.04, 2921.79, 2851.83, 2349.42, 1722.80, 1619.13, 1528.23, 1461.96, 1392.79, 1376.84, 1054.53, 582.29, and 443.39 cm⁻¹. From these 13 peaks, about 4 peaks were extreme bands which are 3384.04 cm⁻¹ (br), 1722.80 cm⁻¹ (m),

![Chromatogram of the ethanolic extract of N. sativa.](image)
1054.53 cm$^{-1}$ (m), and 582.29 cm$^{-1}$ (m), as shown in Figure 5 (m). These four bands have been linked to those found in Fe$_2$O$_3$ [43,44]. Peaks at 582.29 cm$^{-1}$ (Fe–O) and 1722.80 cm$^{-1}$ (H$_2$O bending vibration), and a large peak at 3384.04 cm$^{-1}$ are the vibration bands (H$_2$O str). Instead of three distinct peaks, a broad peak of about 582.29 cm$^{-1}$ (Fe–O str) was noticed, which could be due to an organic molecule from $N$. sativa extract on the surface of IONPs [40].

3.3 MTT results

The MTT assay was used to evaluate the viability/proliferation of green-synthesized IONPs at various concentrations, 12.5, 25, 50, 100, and 200 μg·mL$^{-1}$ for 48 h (Figure 7). Compared to the control group, the negative control and green-synthesized IONPs did not show any mentioned cytotoxicity at concentrations of 12.5, 25, 50, 100, and 200 μg·mL$^{-1}$ for 48 h.

The current study agreed with that of Izadiyan et al., which approved no significant toxicity of green-synthesized IONPs ($J$. regia-IONPs) on normal cell lines at varying doses [42]. These findings show that these $J$. regia-IONPs can be used in various biomedical applications. Another study found the bio-functional starch/IONPs to have non-toxic effects on normal and malignant cervical cell lines, making them ideal candidates for various biological applications [45]. Khatami et al. fabricated super-paramagnetic IONPs (SPIONPs), utilizing a zero-calorie stevia extract as a reducing and stabilizing agent, and showed that the antioxidant activity of the generated NPs was found to be within acceptable limits [46–48]. These can be attributed to the presence of phytochemicals and bioactive substances such as flavonoids, quinones,
tannins, and other compounds that play an essential role in the creation, capping, and stabilization of iron (II) oxide NPs. These polyphenols and antioxidants protect the NPs from oxidation and aggregation and eventually inhibit the toxic effect of synthesized IONPs [49–51].
3.4 Antibacterial activity

Figure 8 shows that green-synthesized IONPs have more efficient antibacterial activity on *S. aureus* and *E. coli*. Green-synthesized IONPs exhibited attractive antimicrobial decrease by enhancing the specific surface area [52]. The disc diffusion test was used to determine the antibacterial impact of green-synthesized IONPs against *E. coli* and *S. aureus* (Figure 9), where an evident zone of growth inhibition of green-synthesized IONPs at a concentration of 0.1 mg·mL⁻¹ was 12.34 ± 0.58 for *E. coli* and 11.52 ± 0.58 for *S. aureus*, respectively.
Antibacterial properties of green-synthesized IONPs were investigated against different pathogenic bacterial strains such as *E. coli*, *S. aureus*, *B. subtilis*, *S. epidermidis*, *K. pneumoniae*, and *P. aeruginosa* at various concentrations using the disc diffusion assay [53]. In general, all bacterial strains were susceptible to IONPs, except *S. epidermidis* and *P. aeruginosa* [54]. Similarly, antibacterial activity was observed for IONPs produced through the co-precipitation approach from *Balanites aegyptiaca* oil [55]. Khashan et al. used pulsed laser ablation in liquid for investigating the synthesis of IONPs at various concentrations doped with carbon nanotubes for antibacterial activity and wound dressing repair [56]. Medicinal plants with antibacterial activity have been employed infrequently in the production of IONPs. Our findings indicate that the green synthesis of the *N. sativa*-IONP compound possesses vigorous antibacterial activity against pathogenic bacterial strains (*E. coli*, *S. aureus*) [57].

Green-synthesized IONPs stuck in a suspension on bacterial surfaces, resulting in green-synthesized IONPs being adsorbed on the bacteria’s surface, which could be combined with a photocatalytic oxidation reaction to

**Figure 8:** Inhibitory effects of the green-synthesized IONPs, IONPs, *N. sativa* extract, and erythromycin (C++) against pathogenic bacteria (*E. coli* and *S. aureus*) using disc diffusion assay. The results were compared with the DW as a negative control (C−). ** Highly significant differences * p < 0.05; * significant differences p < 0.05.

**Figure 9:** Disc diffusion assay exhibiting the impact of green-synthesized NPs as antibacterial agents against *S. aureus* and *E. coli*. (1) Green synthesized (*N. sativa*-iron oxide) NPs, (2) IONPs, (3) *N. sativa* extract, (4) erythromycin (C +ve), and (5) DW (C –ve).
inactivate the bacteria [58]. Several possible mechanisms explain bacteria’s impact on green synthesis IONPs [59]. Scientific evidence shows that the formation of hydroxyl radicals, superoxide radicals, singlet oxygen, and hydrogen peroxide, collectively known as reactive oxygen species, can damage bacteria’s proteins and DNA, resulting in bactericidal action on IONPs [60].

4 Conclusion

In this work, we used the seed extract of N. sativa to synthesize IONPs in a green and environmentally friendly method. Chemically, the N. sativa seeds are highly diverse, and the GC-MS analysis revealed 15 substances in the plant extract. The synthesized NPs are 31.45 nm, stable and well distributed, and noncytotoxic to Vero cell lines. They have good antimicrobial activity against pathogenic E. coli and S. aureus bacterial strains.

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