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Phytosynthesis of magnetite (Fe_3O_4) nanoparticles using *Nepeta bornmuelleri* hairy roots

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ABSTRACT

Magnetic iron oxide nanoparticles (Fe_3O_4-NPs) offer numerous applications in agriculture, pharmaceutical, and food industries. In this study, Fe_3O_4-NPs were effectively phytosynthesized by environmentally friendly hairy root extracts. The A4 strain of *Agrobacterium rhizogenesis* was used to induce the hairy roots of *Nepeta bornmuelleri*. Leaves in *N. bornmuelleri* contain important ingredients such as 1, 8 cineol that is one of the most well-known secondary metabolites. The phytosynthesis of Fe_3O_4-NPs was verified and described by Ultraviolet-Visible spectroscopy, field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), high-resolution transmission electron microscopy (HRTEM), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), dynamic light scattering (DLS), and thermogravimetric analysis (TGA). The NPs were clustered with a size of less than 20 nm in FESEM analysis and 20-100 nm in TEM and HRTEM analysis. XRD pattern showed a peak of Fe_3O_4-NPs at 35.28° (220 nm). In the FTIR experiment, amines, alkanes, carbonyls, fluorides, and alcohols were detected as organic molecules involved in the synthesis of NPs. The average size of NPs in their liquid medium was about 53.39 nm, according to DLS analysis. According to TGA analysis, NPs maintained 82% of their weight at a temperature above 700 °C. According to the results of TGA study, *Nepeta*'s fast-growing hairy roots generated highly pure NPs. TGA analysis revealed that the weight loss of synthesized NPs in hairy roots was smaller than those identical samples produced in the extracts obtained from aerial parts of the plants. The 1, 8 cineol in the hairy root extracts measured by gas chromatography was found to be around 0.001%.

Key words: Fe_3O_4 nanoparticles, Hairy root extract, Nano-Phyto-synthesis, *Nepeta bornmuelleri*.

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INTRODUCTION

Metal nanoparticles (NPs) have the exceptional thermal, photosensitive, catalytic, super-paramagnetic, surface-to-volume ratio, electrical properties, and convenient separation under the magnetic field (Assa *et al.,* 2016). Metallic oxide has been used in cell therapy (Hosseini *et al.,* 2018; Jain *et al.,* 2018), drug delivery (Tietze *et al.,* 2015; Oroujeni *et al.,* 2018), cancer detection (Revia and Zhang, 2016), oil industry (Simonsen *et al.,* 2018), magnetic resonance imaging (Fatima and Kim, 2018), environmental pollution detection (Song *et al.,* 2019), and immunoassay (Hung *et al.,* 2014). Iron oxide contains both Fe^{2+} and Fe^{3+} ions with a hard-crystalline structure that is ferromagnetic or superparamagnetic when the sizes are less than 15 nm. Due to the presence of different polymorphs, Fe_3O_4 and γ-Fe_2O_3 are functional and suitable for medical and biological applications. Different physical and chemical methods such as sol-gel (Lemine *et al.,* 2012), thermal method (Liu *et al.,* 2015), sonochemical method (Zhu *et al.,* 2013), ultrasonic chemical coordination, chemical bonding, oxidation of $Fe(OH)_2$ by H_2O_2, microwave irradiation, irradiation to R radiation (Wu *et al.,* 2011) have been used for the synthesis of magnetic NPs. However, these common methods have limitations such as low stability, complexity and toxicity to the environment (Khatami *et al.,* 2015). The demands for metal oxide NPs have been increased rapidly, therefore, their synthesis is considered by many chemicals, physical and green approaches. The green techniques use live organisms and unlike chemical and physical methods, are environmentally friendly, sustainable, more stable and non-toxic (Mohammadi *et al.,* 2016; Zare *et al*., 2017; Keshavarzi *et al.,* 2018). Plants possess secondary metabolites such as alkaloids, flavonoids, essential oils, and phenols that can be used as green reducing agents to convert metal ions to metal NPs (Pourakbar *et al*., 2020). The synthesis of NPs by co-precipitation and the use of plant extract serve as both a precipitating agent and a capping agent for the NPs. Synthesis with plant extracts leads to better performance and reduced co-precipitation of other cations in solution (Awwad and Salem, 2012; Nnadozie and Ajibade, 2020). The use of plant extract and other parts of the plant for the green synthesis of magnetic NPs have been investigated extensively in mesenchymal stem cells (Elfick *et al*., 2017), wild oak leaves (Sari and Yulizar, 2017), *Stevia rebaudiana* (Alijani *et al*., 2019), hawthorn (Omidvari *et al*., 2014), seaweed (Yew *et al*., 2016), starch (Alishah *et al*., 2017), fruit extract of *Couroupita guianensis* (Sathishkumar *et al*., 2018). Recently, the cultivation of hairy roots has been gained more attention due to their genetic stability, high multiplication rate, and biosynthetic capacity. Herbal compounds often accumulated at a higher level in hairy roots compared to in cell culture. (Ono and Tian, 2011; Parizi *et al*., 2020).

The genus *Nepeta* belongs to the Lamiaceae family, which has more than 280 species, with 39 species endemic to Iran, commonly known as Pooneh (Valimehr *et al*., 2014). The genus *Nepeta* is widely used in traditional medicine due to its distinct chemical compositions. The main compounds of *Nepeta* are nepetalactone and 1,8 cineol (Salehi *et al*., 2018). In a study to investigate the different types of *Nepeta* compounds in Iran, it was shown that nepetalactone, caryophyllin, 1,8 cineol, linalool, β-pinene, α-terpinolene, di-germacrene, spatulenol are the most important compounds of *Nepeta* species (Kazemi *et al*., 2016). Secondary metabolites such as polyphenols, flavonoids, saponins, tri-terpenoids, and di-terpenoids have been reported in the root extracts of several species of *Nepeta* (Gautam *et al*., 2016; Sharma and Cannoo, 2016)., Natural compounds found in *Nepeta* can be employed as a stabilizing agent for the synthesis of NPs. To the best of our knowledge, neither the hairy root production nor a green synthesis of NPs has been reported in *Nepeta*. This study was conducted to produce the iron oxide NPs using eco-friendly, green, novel, and efficient methods in the hairy root extract of *N. bornmuelleri*.

MATERIALS AND METHODS

Plant material and growth conditions

Nepeta seeds were collected from the Baft region in Kerman province, Iran, and were verified at the Botanical Laboratory of Faculty of Agriculture, Shahid Bahonar University of Kerman. The pH of culture medium was adjusted to 5.7 before adding the agar and then was autoclaved for 20 min at 121 °C. Seeds were sterilized for 1 min in 70% ethanol, 15 min in 2.5% hypochlorite, and rinsed with sterilized sterile water 3 times before they were cultured in a complete MS medium (Murashige and Skoog, 1962). The media containing seeds were kept in a growth chamber at 25±2 °C under a 16 h light and 8 h dark photoperiod.

Bacterial strains and induction of hairy roots

Artificial inoculation by A4 strain of agropine (Chaudhuri *et al*., 2005) from *Agrobacterium rhizogenes* resulted hairy roots formation in 40-days-old leaf explants of *N. bornmuelleri.* An aliquot bacterial suspension containing MS: LB (3: 1) media

was poured on the wounded abaxial part of the explants. Next, the explants were placed in a medium containing half-strength MS and kept in dark at 25±2 °C for 48 h. Then, the explants were transferred to a new culture medium containing 500 mg/L of cefotaxime antibiotic to remove bacteria. This transfer was repeated every 48 h with a lower concentration of antibiotics until complete bacterial elimination. The explants were kept in a growth chamber in dark until the emergence of the root. To ensure complete removal of the bacteria, the roots were kept in an antibiotic-free medium for one week, then 2-3 cm of the tip of the fast-growing roots were separated and put into a 250 ml Erlenmeyer containing 50 ml of half-strength MS containing 3% (w/v) sucrose and 50 $mg.l^{-1}$ of cefotaxime and placed in the dark on a shaker at 120 rpm at 25±2 °C for 40 days. To obtain the required root fresh weight, they were sub-cultured every other week.

PCR analysis

Polymerase chain reaction (PCR) analysis was performed to validate the insertion of the *rolC* gene into the plant genome. The *rolC* primers were: 5′-TAACATGGCTGAAGACGACC-3′ and 5′-AAACTTGCACTCGCCATGCC-3′ (product size of 534 bp). The absence of virD was further investigated using the primers 5′-ATGTCGCAAGGCAGTAAGCCC-3′ and 5′-GGAGTCTTTCAGCATGGAGCAA-3′ (product size of 483 bp). The following conditions were used in the experiment: 2 min of pre-denaturation at 94 °C, 35 cycles of 50 s denaturation at 94 °C, 45 s annealing at 58 °C, and 45 s extension at 72 °C. As a positive control, the Ri plasmid from *A. rhizogenes* was employed (Ahmadian Chashmi *et al*., 2016; Parizi *et al*., 2020).

Preparation of hairy roots extract and determination of 1,8 cineol

About 30 g of hairy roots were weighed and washed three times with double sterilized water, then the roots were dried under a laminar airflow for 24 h. An aliquot of 20 ml of dichloromethane solvent was added per gram of root powder and every 8 h half of the previous amount of solvent was added again. The extract was filtered through 2.5 µm Whatman ® filter paper. Finally, to evaporate the solvent, it was placed in a Bain-marie bath at 40 °C. The extract was stored at -4 °C until freezing. To measure the amount of 1,8 cineole, the solid extract of hairy roots was dissolved in dichloromethane in a 1:50 ratio and injected into the GC-MS apparatus (Varian Saturn 2000, Agilent Technologies, USA).

Preparation of aqueous hairy roots extract and synthesis of Fe_3O_4-NPs

For the preparation of the aqueous hairy root extract, the previous protocol of the plant extract (Awwad and Salem, 2012) was used with some modifications. In brief, 30 g hairy root was harvested after 40 days, washed 5 times with sterile deionized water, and dried under laminar airflow for 24 h. One g of powder dried hairy roots was placed in a 80 ml beaker, then 20 ml sterilized deionized water was added and heated to 60 °C for 20 min. The extract was filtered through 2.5 µm Whatman filter paper and centrifuged for 20 min at 4500 rpm twice.

Preparation of Fe_3O_4-NPs

To synthesis the Fe_3O_4-NPs, we used $FeCl_2$. $4H_2O$, $FeCl_3$. $6H_2O$ and NaOH. To retrieve the iron, 100 ml of iron salts were mixed with a 2: 1 ratio of sterile deionized water and heated to 80 °C for 10 min using a stirrer magnet, then 5 ml of aqueous extract of hairy roots were added to the reaction mixture. After 5 min, 20 ml NaOH solution was added to the reaction mixture, 3ml per min. The reaction mixture was placed at room temperature until the NPs were gently precipitated. The solution of NPs with extract was washed with sterile deionized water 3 times at 7000 rpm for 10 min. The resulting NPs were divided into two parts: one part dried in an oven at 60 °C to get powder and another one stored in deionized water.

Characterization of Fe_3O_4-NPs

The synthesized NPs were scanned for the analysis of maximum absorption in range of 200-550 nm by Scan drop 250-211FO75 spectrophotometer (Analytic Gena, Germany). Field emission scanning electron microscopy (FESEM); MIRA3 model, Czech Republic) was used to reveal the crystalline structure of surface and the composition of synthesized NPs. To determine the functional groups and their possible role in the synthesis of Fe_3O_4-NPs, the FTIR (Fourier transform infrared spectroscopy) analysis was conducted (Specturam Two, Perkin Elmer, USA). Dynamic light scattering analysis (DLS); Zetasizer Nano, UK) was performed to investigate the average dispersion of synthesized NPs in liquid medium. To determine the exact shape and size of the synthesized NPs, the images of transmission electron microscopy (TEM) and high-resolution transmission electron microscopy (HRTEM) were taken with the electron beam microscope (Zeiss-EM10C-100K, Germany). To investigate the crystalline structure of Fe_3O_4-NPs, X-ray diffraction spectroscopy analysis was performed by XRD (X ‘Pert Pro, Panalytic). To study the behavior of Fe_3O_4-NPs against heat, thermal stability,

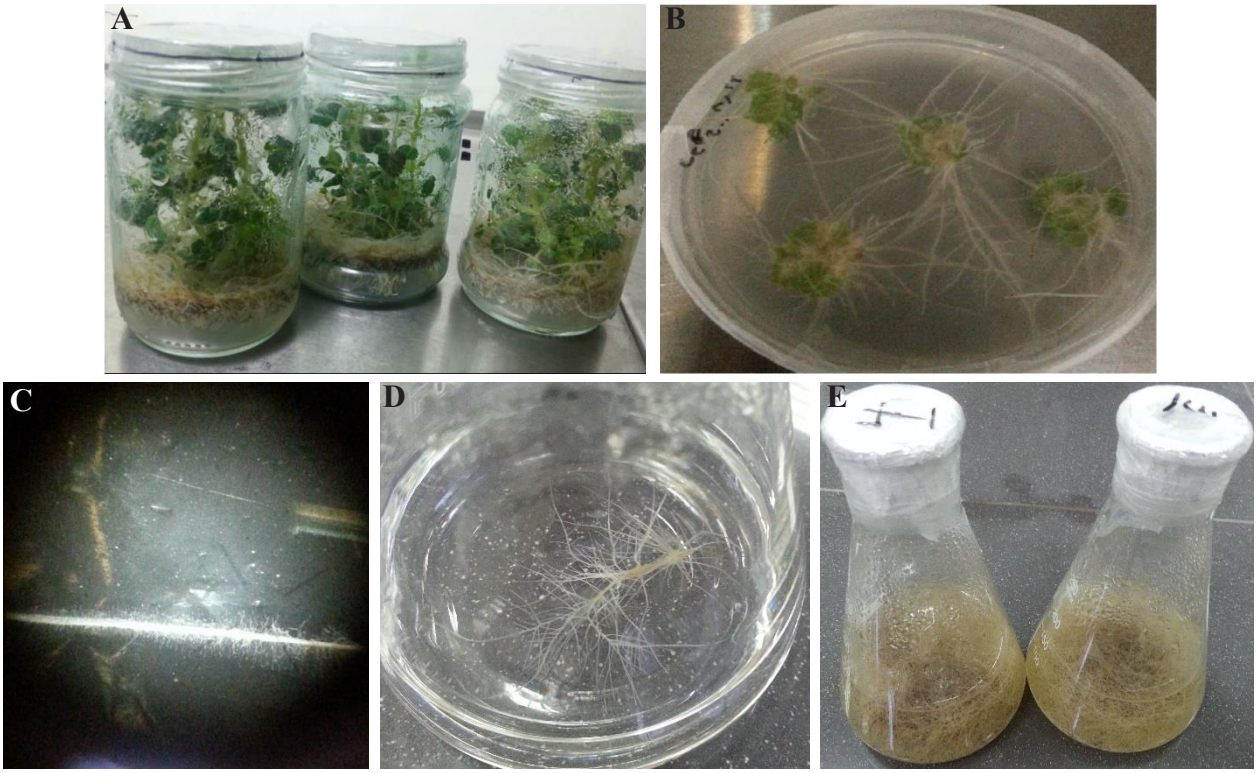


Figure 1. A: The 40-day-old seedlings of *N. bornmuelleri*. **B:** Transgenic roots emergence from leaf explants. **C:** Hairy roots under the binocular microscope. **D:** 7-day-old clones in the liquid medium. **E:** 40-day old clones in the liquid medium.

and degradation properties, the nanosheet weighting test was conducted at room temperature up to 700 °C (Rheometric Scientific STA 1500).

RESULTS AND DISCUSSION

The results of this study showed that after 13 days of inoculation, the first roots of explants emerged, and about 90% of the explants produced roots by the 20th day. The roots derived from leaf explants were branched, hairy and fast-growing which are characteristic of transgenic roots. The roots also grew well in the liquid medium, and their weight almost doubled every two weeks to reach 30 g on the 40th day (Figure 1). Various species and explants of *Nepeta* have been inoculated with *Agrobacterium rhizogenesis* to induce hairy roots. In *N. pogonosperma*, hairy roots emerged in the stem explants after one week (Valimehr *et al*., 2014). Hairy roots of stem explants were observed in *N. teydea* after 4 weeks (Fraga *et al*., 2017). This study described the complete protocol for induction of hairy roots in *N. bornmuelleri* leaf explant using *A. rhizogenesis* with high yield hairy roots induction (90 - 99%). Using this method, the hairy roots produced quite rapidly in less than a month.

Although the hairy roots can be easily distinguished from natural roots as they grow rapidly in hormone-free media, the transformation was confirmed using the PCR method by amplification of the *rol C* gene with the length of 534 bp and absence of *vir D* with the length of 483 bp in the transgenic roots (Figure 2). During gene transfer using *A. rhizogenesis*, the bacterium inserts a T-DNA region on its plasmid containing the rol genes with the cooperation of vir genes located outside of this region in the host genome, thus the transgenic plants contain the rol gene and this indicates a transformation.The results of gas chromatography analysis showed that the presence of secondary metabolite 1,8 cineol in a 50 fold diluted extract of the hairy roots of *Nepeta* was 0.001% (Figure 3). In nano-biotechnology, environmentally friendly methods of NP synthesis using plant secondary metabolites have been developed as inducers of reaction and conversion of metal ions to NPs as well as capping and stabilizing agents (Alishah *et al.,* 2017; Gopalakrishnan and Muniraj, 2021). Due to its adaptability to *in vitro* culture and recombinant metabolite synthesis, hairy root cultures are a promising source of plant secondary metabolites (Parizi *et al*., 2020). Synthesis of NPs in the hairy root extract of *L. maroccana* was reported (Borovaya *et al*., 2014). In our experiment, the hairy root extract of *N. bornmuelleri* was used to synthesise Fe_3O_4-NPs.

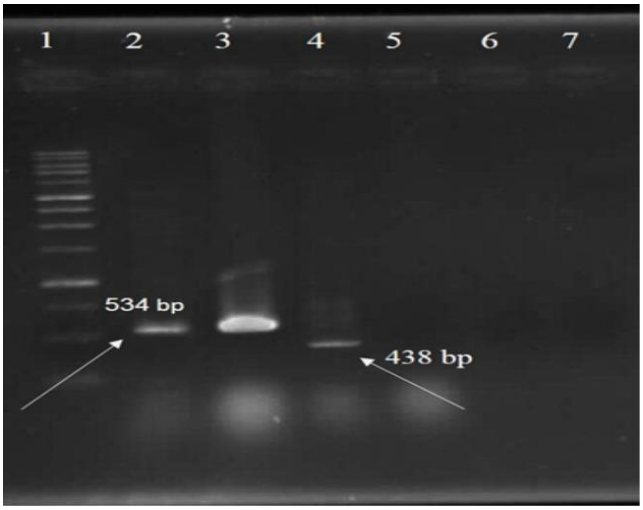


Figure 2. PCR amplification of *rol C* (534 bp) gene: lane (1), molecular size marker (100 bp ladder). Lane (2) hairy roots (genomic DNA extracted from transformed *N. bornmuelleri*. Lane (3 positive control (Ri plasmid DNA extracted from *Agrobacterium rhizogenes*. Lane (4) positive control (Ri plasmid DNA isolated from *Agrobacterium rhizogenes*). Lane (5) transformed hairy roots of *Nepeta bornmuelleri*. Lane (6) negative control (genomic DNA extracted from non-transformed *N. bornmuelleri*. Amplification of *vir D* (483 bp) gene using PCR: Lane (7) negative control (genomic DNA isolated from non-transformed *N. bornmuelleri*).

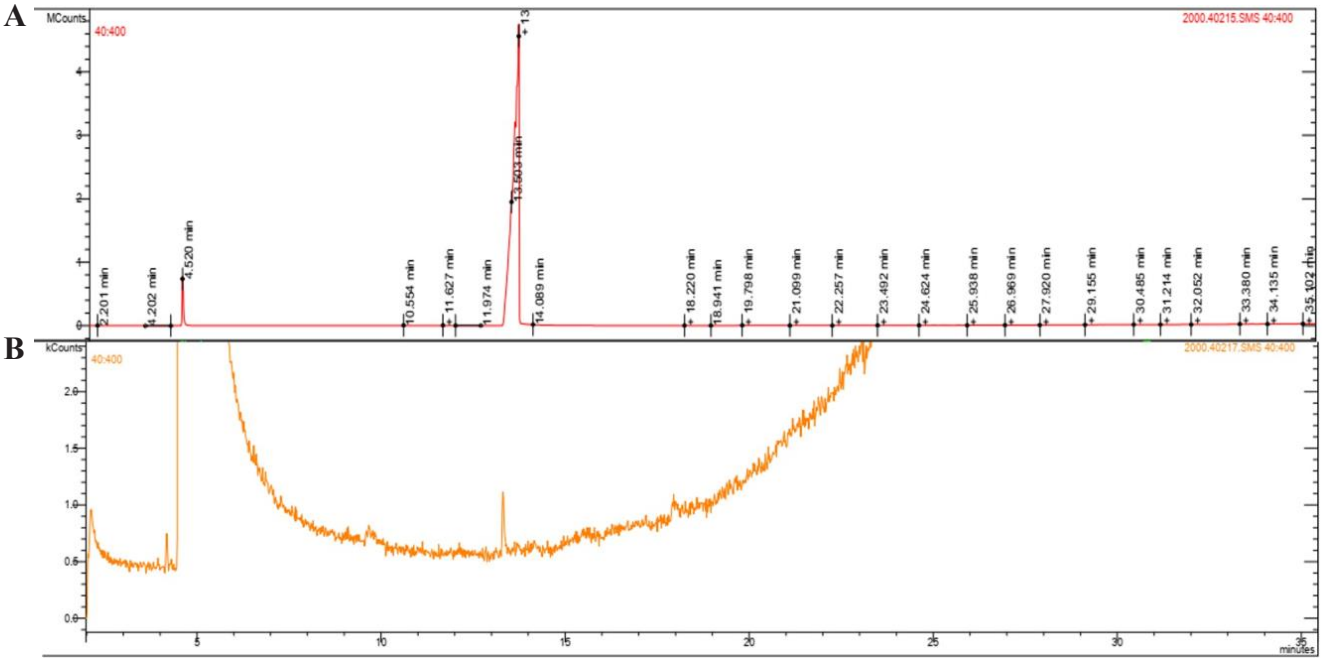


Figure 3. Chromatogram of gas chromatography. **A:** Peak related to the pure standard of metabolite 1, 8 cineole. **B:** Peak related to metabolite 1, 8 cineole in 50 fold diluted hairy roots extract.

Nepeta's hairy root extract has a transparent light brown color that, when added to an iron salt solution, changes the yellow color of the iron salt solution to a reddish brown color. Adding sodium hydroxide to the aforementioned solution temporarily suspended the black Fe_3O_4-NPs and eventually caused them to settle (Figure 4). This was similar to the results of previous studies on green synthesis of Fe_3O_4-NPs? (Assa *et al*., 2016; Nnadozie and Ajibade, 2020). UV–visible absorption was used to describe Fe_3O_4-NPs that is a valuable approach for characterizing NPs. Absorption wavelengths in 200–800 nm range are commonly employed to characterize metallic NPs (Mulvaney, 1996).

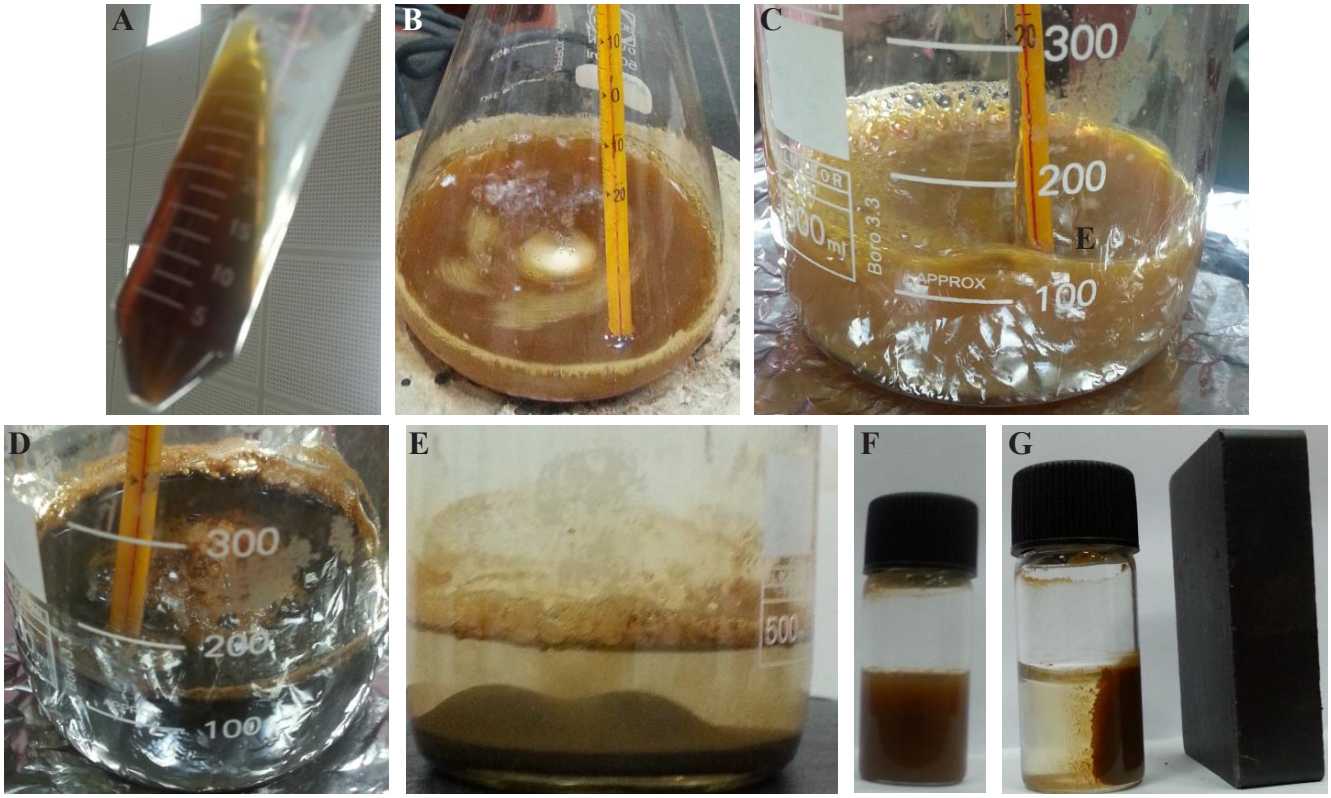


Figure 4. A: Hairy roots extract. **B:** Solution of Fe^{3+} and Fe^{2+} with 2:1 M ratio. **C:** The combination of iron solution and hairy roots extract. **D:** The mixture of the iron solution, hairy roots extract, and NaOH (1.0 M). **E:** Precipitated synthesized Fe_3O_4-NPs. **F:** Washed Fe_3O_4-NPs in deionized water. **G:** Separation of synthesized Fe_3O_4-NPs from water using an external magnet.

The stimulation of surface plasmon resonance (SPR) of the NPs caused the black hue of the Fe_3O_4-NPs solution. The UV–vis spectra of the reaction samples, on the other hand, were captured immediately after synthesis using a Scan Drop-type product (Analytik Jena, Germany). As shown in Table 1, Fe_3O_4-NPs exhibited optical absorbance around 220 nm which is in agreement with previous results (Awwad and Salem, 2012).

The FESEM image of synthesized Fe_3O_4-NPs, spherical morphology, crystalline particles agglomerated in organic compounds of *Nepeta* hairy root extract, and NPs with sizes of 14.57 and 16.43 nm can be clearly observed (Figure 5).

Strong signals demonstrated the presence of Fe, as shown in the EDS pattern of FESEM. Other signals from Cl, O, N, and Si atoms were detected, with ions such as Fe and Cl originating from the metal salts employed in the synthesis, and C, N, O, Al, and Si ascribed to elements found in the hairy root. The presence of carbon suggested that Fe_3O_4 NPs was capped by the phyto-constituents from aqueous hairy root extract of hairy roots of *Nepeta* (Figure 6).

Table 1. UV-visible absorption spectrum of synthesized Fe_3O_4-NPs in *Nepeta* hairy roots. Fe3O4 NPs showed absorbance near 220 nm.

Wavelength(nm)	Absorbance
175	12.5
200	12
220	37.5
250	16
300	12
350	10
400	7.5
450	5
500	5
550	5

The image of TEM and HRTEM (Figure 7) showed that NPs were spherical, polygonal in shape, and agglomerated with the size of 20-100 nm. Agglomeration might be due to the magnetic properties of Fe_3O_4-NPs, capping of NPs with the condensation properties of phytoextract, and its hydroxyl groups (Gawande *et al*., 2013; Venkateswarlu and Yoon, 2015; Nnadozie and Ajibade, 2020).

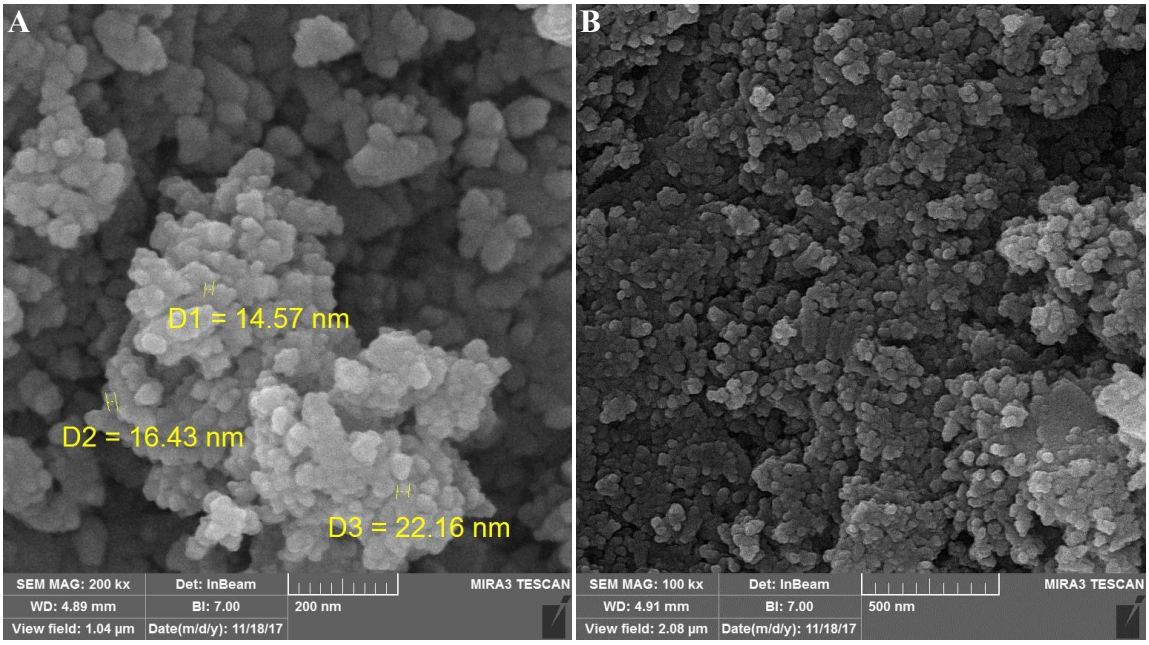


Figure 5. FESEM image of the synthesized Fe_3O_4-NPs in *Nepeta* hairy roots. (A) NPs in sizes of 14.57 and 16.43 nm, (B) Morphology of spherical crystalline cluster particles in organic compounds of *Nepeta* hairy root extract.

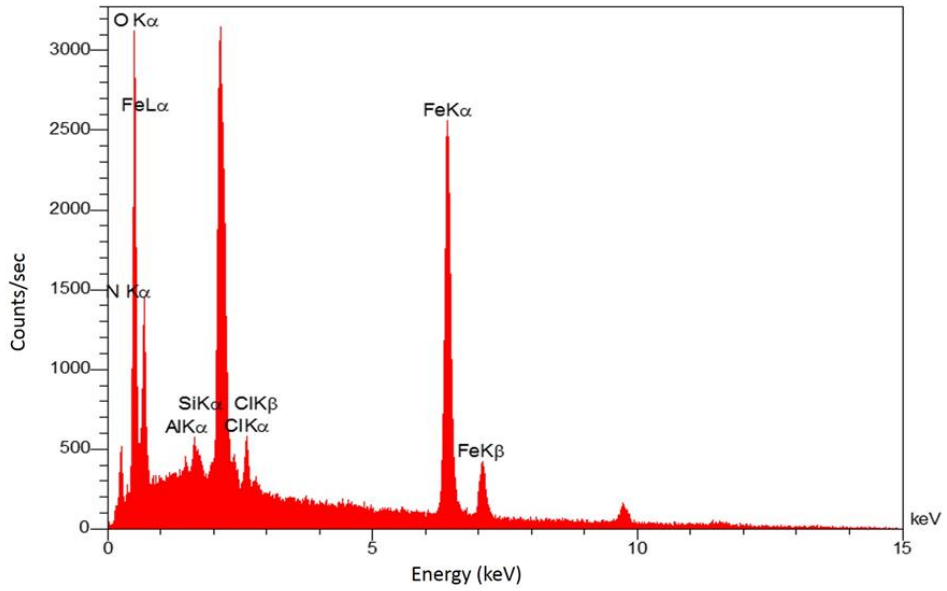


Figure 6. EDS graph of FESEM analysis.

X-ray diffraction (XRD) analysis revealed a peak at 35.28° (220), which is related to the NPs. The other peaks were very faint (Figure 8).

The results of dynamic light scattering (DLS) analysis revealed that the distribution of NPs in an aqueous solution was between 30-100 nm (Figure 9). Organic compounds involved in the green synthesis of Fe_3O_4-NPs were investigated by FTIR analysis. FTIR spectrum showed absorption bands at 168.97, 483.6, 623.91, 833.75, 1107.25, 1386.04, 2925.55, 3412.30 cm^{-1} (Figure 10). According to the communication table (Pavia *et al*., 2014), absorption bands are assigned to the N-H (amine), C-H (alkanes), C=O (carbonyl), C-X (fluoride), C-O (alcohol, ether, ester, carboxylic acid, anhydrase), respectively. Outside C-Hs representing crystalline structures that are used to identify two forms of α and β, chlorides and bromide iodide, stretching vibration.

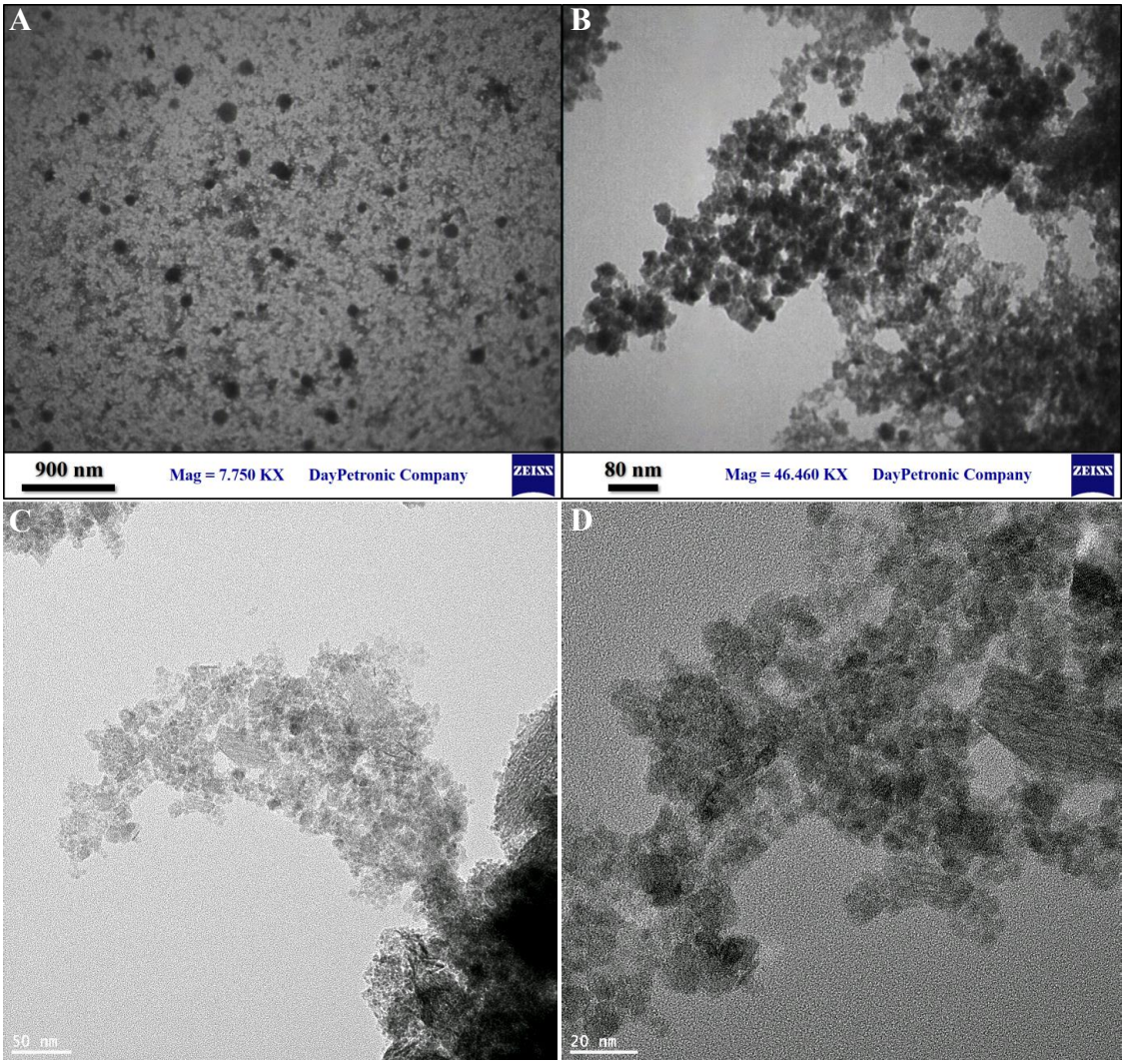


Figure 7. (A) TEM image of the synthesized Fe3O4-NPs at magnification of 7.7.50 KX showing NPs with the size under 900 nm, (B) TEM image of the synthesized Fe3O4-NPs at magnification of 46.460 KX showing NPs with the size of 80 nm, (C) HRTEM image of the synthesized Fe_3O_4-NPs with the size of 50 nm, (D) HRTEM image of the synthesized Fe_3O_4-NPs with the size of 20 nm.

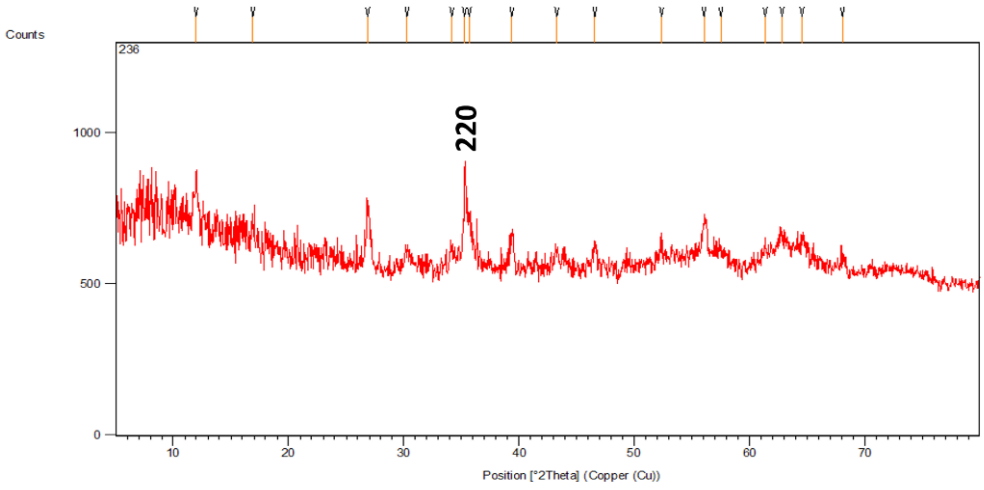
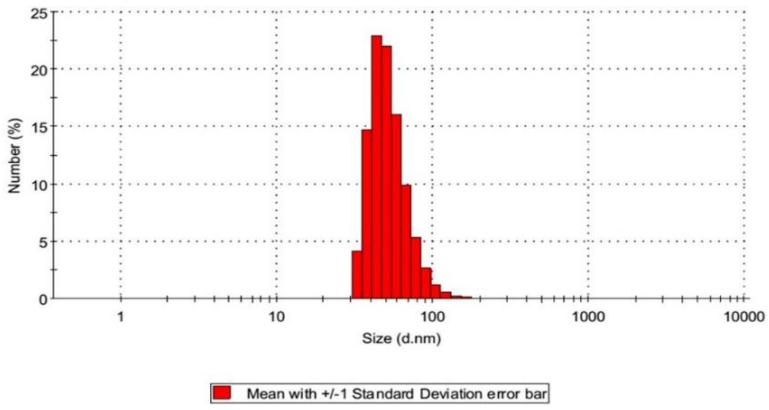


Figure 8. X-ray diffraction pattern of synthesized Fe_3O_4-NPs. The pattern shows a peak of NPs at 35.28° (220).



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size: d.nm	Number %
32.67	4.1
37.84	14.7
43.82	22.9
50.75	22
58.77	16.1
68.6	9.8
78.82	5.4
91.28	2.7
105.7	1.3
122.4	0.6
141.8	0.2

Figure 9. DLS studies of Fe_3O_4-NPs (size distribution of Fe_3O_4-NPs).

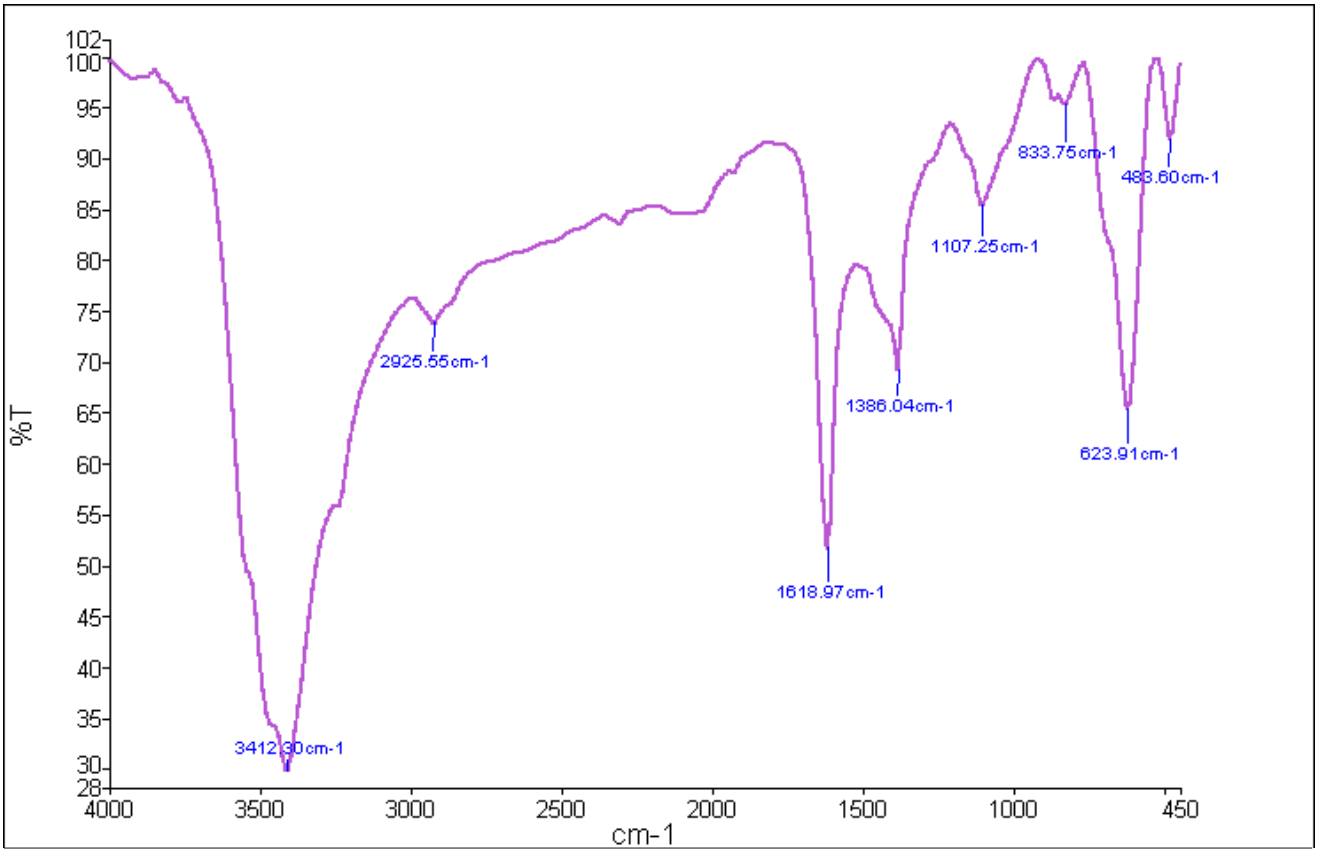

Figure 10. FTIR spectra of synthesized Fe_3O_4-NPs in aqueous hairy roots extract of *Nepeta*.

The results of thermogravimetric analysis (TGA) showed a steady weight loss of room temperature up to 750 °C (Figure 11). The weight loss of nanopowder up to 100 °C in Fe_3O_4-NPs was 17.28 due to the water evaporation and the desorption of bioorganic compounds (Khalil *et al.,* 2014). The remaining weight of the sample at 700 °C was 82.72%.

CONCLUSIONS

This study describes a complete protocol for the green synthesis of Fe_3O_4-NPs by hairy root extract of the *N. bornmuelleri* plant. Induction of hairy roots in *N. bornmuelleri* leaf explant using *Agrobacterium rhizogenesis* showed a practical, inexpensive, and

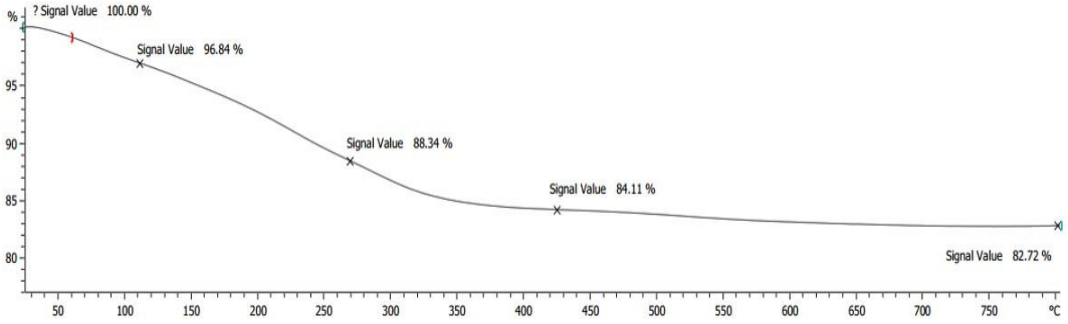

Figure 11. TGA curves of synthesized Fe_3O_4-NPs.

environmentally friendly approach for Fe_3O_4-NPs production. Synthesis of NPs in *Nepeta* hairy roots and its extract created the Fe_3O_4-NPs with a size of 20-100 nm that have many cutting edge applications. Examination of the FTIR spectrum of Fe_3O_4-NPs synthesized in hairy roots showed that the bases in hairy roots are not as many as those found in intact plants and plant leaf extracts resulted from optical activities such as photosynthesis. Based on the results of TGA analysis, fast-growing hairy roots of *Nepeta* can lead to the production of NPs with high purity. TGA analysis showed that the rate of weight loss of synthesized NPs in hairy roots was lower compared to similar samples synthesized in the extracts of aerial parts of the plant. This biological synthesis of NPs is non-toxic, eco-friendly, and low-cost technology for the large-scale production of well-characterized NPs.

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