Subacute Toxicity Analyses of Green Synthesized Magnetite Nanoparticles on Lemna minor L. (Duckweed) Plant

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Research Article

Keywords: Lemna minor, green synthesis, magnetite, nanoparticle, water ecosystem, phytotoxicity

Posted Date: July 20th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1794925/v1

Abstract

Magnetite nanoparticles (MNPs) are widely used in medicine, environmental technologies and biotechnology and green synthesis of MNPs could be an option to minimize potential environmental pollution by their usage. In this study, subacute toxicity of green synthesized magnetite (Fe_3O_4) nanoparticle was evaluated on *Lemna minor*, a main autotroph in lakes. Laurel (*Laurus nobilis*) leaf extract was used to synthesize the magnetite nanoparticles. Characterization of the nanoparticles were performed by UV/Vis spectrophotometer, Fourier transform infrared spectroscopy (FT-IR), Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Zeta size and potential and Scanning electron microscopy (SEM-EDS) analysis. Nanoparticles were around 108.5 nm, spherical in shape and capped with phyto-content. Subacute toxicity of magnetite nanoparticle was tested according to modified OECD 221 protocol, by treating *L. minor* plants with different MNPs concentrations (0.1, 1, 10, 100, 1000, 2000 mg L^{-1}) in petri dishes containing Steinberg medium for 15 days. The MNPs up to 1000 mg L^{-1} did not cause any toxic effect on *Lemna minor* even it promoted growth and development of the plant in the concentrations less than 100 mg L^{-1}. The number of fronds, colonies and photosynthetic pigment contents significantly decreased by magnetite nanoparticle application of 1000 and 2000 mg L^{-1} concentrations. Moreover, in these concentrations the nanoparticle caused oxidative stress indicated by the increased hydrogen peroxide and superoxide anion content and lipid peroxidation level. As a conclusion, this study showed that 1000 mg L^{-1} green synthesized MNPs concentration is the starting point of subacute toxicity for *L. minor.*

Introduction

Metal nanoparticles (NP) are extensively used in biomedical and engineering fields. Among them, magnetite (Fe_3O_4) nanoparticles as the strongest magnetic compound in the earth become prominent (Coricovac et al. 2017) with their powerful adsorbent, catalytic and antimicrobial properties (Mody et al., 2010). Magnetite NPs have been shown as an efficient inorganic and organic pollutant removal agent from water and soil (Giraldo et al., 2013). Additionally, as a low-cost adsorbent in drinking and wastewater treatment systems magnetite has been used. Another field for magnetite NP is biomedical applications in cancer therapy and drug delivery, magnetic resonance diagnoses due to their unique physicochemical nanoscale properties, such as magnetic-field generation, heat, and enhanced reactivity (Lu et al., 2010).

Iron is an essential nutrient for most life forms and magnetite nanoparticles are natural part of soils and water reservoirs. However, its intensifying usage in wastewater along with drinking water treatment and biomedical applications raises concern for accumulated magnetite contamination for aquatic life forms particularly primer producers. Environmental risks for surface waters may also be increased by flow of NPs into lakes and rivers during their manufacturing, transport, and discharge (Kumari et al. 2019).

Magnetite NPs known as less toxic metal nanoparticle to use many fields than other metal nanoparticles (Reviewed by Spanos et al. 2021). Researches about magnetite toxicity mostly focused on animals and

human over plants because of their usage in biomedical application such as carriers for cancer treatments cancer drug delivery or magnetite resonance (Tombuloglu et al. 2019). In fact, intensifying magnetite NP discharge to water ecosystem in different ways, should need to comprehensive evaluation for autotrophs which are the first cycle to reach to human in food chain.

Toxicity of iron oxide NPs which are chemically synthesized are reported in a broad spectrum of both terrestrial and aquatic plants. *Arabidopsis thaliana* growth inhibition and chlorophyll content decrease by iron oxide NPs exposure were reported de la Rosa et al. (2017). Decrease in total chlorophyll content in leaves of *Helianthus annuus* L. (Martínez- Fernández et al. 2016) by maghemite NPs and of corn (*Zea mays* L.) by γ-Fe_2O_3 NP exposure were reported (Li et al., 2016).

Recent report also revealed that chemically synthesized different iron oxide nanoparticles (akaganeite predominance + hematite) caused death of *Lemna minor* plants in 7 days in all treated concentrations between 30–50 mg L^{-1} (Souza et al. 2019).

Specific to magnetite NPs, there are very limited studies which used *Lemna gibba* (Barhouimi et al., 2015) and *Lemna minor* (Blinova et al. 2017) as test organisms to determine toxicity level. Barhouimi et al. (2015) used the performance index of PSII activity as a sensitive bioindicator to toxicity and found that PSII functions, growth rate, chlorophyll content dropped in *Lemna gibba* plants treated with chemically synthesized Fe_3O_4 (400 µg mL^{-1}) for 7 days. In contrast to above mentioned report, Blinova et al. (2017) observed that 27.2 ± 9.8 nm size chemically synthesized magnetite NPs led low toxicity level (> 100 ppm) to *L. minor* individuals in acute toxicity assay for 7 days.

Discrete reports on magnetite NP toxicity could be resulted by physiochemical features of engineered nanoparticles. In fact, synthesis method may change the nanoparticles' shape, size, structure, element components and dispersion which impact on the toxicity level of the magnetite NP on life forms (Liu et al., 2012). Physical and chemical synthesis methods have many disadvantages such as use of toxic chemicals and the formation of toxic by-products (Hussain et al., 2016). Over to physical and chemical methods, green synthesis is environmentally friendly and easy to apply for large-scale synthesis (Hoffmann et al. 2022). One of the objectives of green synthesis technology is to minimize potential risks to the environment and human (Allafchian et al. 2018) due to its wide usage in biomedical fields (Zhang et al. 2013).

Water ecosystem is another concern with accumulated magnetite NP discharge to water should be studied for autotrophs which are the basis of food chain and sustainable ecosystem. Thus, *Lemna minor* (duckweed) as an important model organism in ecotoxicological studies was used to monitoring magnetite NP toxicity for 15 days exposure by defining growth and biochemical parameters. To the best of our knowledge, this study is the first report revealing green synthesized magnetite nanoparticles toxicity on *Lemna minor* plant. Based on the obtained data, toxicity of green synthesized magnetite nanoparticle was found as relatively low, and its dose-dependent toxicity starts from 1000 mg L^{-1} concentration.

Materials And Methods

Synthesis and Characterization of Magnetite Nanoparticles

$FeCl_2.4H_2O$ and $FeCl_3.6H_2O$ (1:2) were dissolved in 100 mL of distilled water in a beaker and heated at 80°C for 10 minutes with constant stirring. The laurel extract was obtained from grinding of laurel leaves with liquid nitrogen into a fine powder and boiled in 100 ml distilled water for 10 min at 60°C. After filtration, 5 ml of the extract was added to the iron chloride mixture and stirred for 5 minutes. NaOH solution was added to the mixture to adjust the pH. The mixture was stirred for about 1 hour until the solution color turned to black. The synthesized nanoparticles were characterized by UV/Vis spectrophotometer, Fourier transform infrared spectrophotometer (FT-IR), inductively coupled plasma mass spectroscopy (ICP-MS), scanning electron microscope (SEM), and Zeta size analysis.

Cultivation and growth of L. minor

Lemna minor (duckweed) plant was obtained from Ege University Botanical Garden and their acclimation was performed according to OECD test protocol (OECD TG 221 − 34). The nutrient solution (Steinberg) used for *L. minor* growth was prepared as stated in the OECD 221 guidelines (2006). Acclimation *L. minor* was achieved in 250 ml flasks containing 100 ml Steinberg medium with 2–3 leaves in each flask for 8 weeks. The nutrient solution was renewed every 7 days. Plants were grown at 24 ± 1°C with 16: 8 photoperiod and 6000 lux light intensity.

Determination Of Magnetite Nanoparticle Toxicity

The toxicity tests were carried as described in the OECD guidelines (OECD 2006). Plants were treated with different concentrations of biologically synthesized magnetite nanoparticles (0.1, 1, 10, 100, 1000, 2000 mg L^{-1}) in petri dishes with a diameter of 60 mm containing 10 ml (Magnetite NP solution + Steinberg medium) for 15 days. Cultural media was renewed every 2 days. Morphological parameters, photosynthetic pigment content, hydrogen peroxide content, lipid peroxidation and superoxide anion accumulation assays were used to determine the toxicity of green synthesized magnetite nanoparticles.

Morphological Parameters

At the end of 15 days of testing, classical ecotoxicity parameters; frond fresh and dry weight, colony and frond number, growth inhibition were measured.

Frond numbers were counted with Dino Capture camera microscope (Dino-Lite Microscope USB, Taiwan). Fresh weight of the plants was measured after colonies washed and dry on filter papers, later they were left to dry for 24 hours in the oven set at 65°C for dry weight measurements. To determine plant growth,

leaves of each group were counted at the end of the test period. *L. minor* Average Growth Rate (μ) was calculated according to the formula given below:

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t_j - t_i}$$

Nj and Ni are the numbers of leaves at the end and beginning of the trial, respectively (OECD 221, 2006).

The potential toxic effects of the magnetite NPs, the Growth Inhibition Rate (IGR) was calculated based on leaf count measurements, as stated in the formula given below:

$$\mathrm{IGR} = \frac{(\mu_c - \mu_r)}{\mu_c} \times 100,$$

μc and μr are the average number of leaves for control and magnetite NP groups, respectively (OECD 221, 2006).

Determination Of Photosynthetic Pigment Content

hlorophyll a, b, and total chlorophyll contents were determined according to Arnon (1949) method in treatment and control plants. 0.1g of *L. minor* leaves were homogenized with an 80% acetone solution. The obtained homogenate was filtered with filter paper and absorbance measurements were made in the spectrophotometer at 663, 645, and 470 nm wavelengths.

Determination Of Hydrogen Peroxide (Ho) Content

The hydrogen peroxide content of the treated plants was determined by Sergiev et al. (2000) method. 0.1 grams of plant leaves were taken in a mortar and homogenized by adding 3 ml of cold 0.1% trichloroacetic acid (TCA) with liquid nitrogen. The homogenate obtained was centrifuged at 4500 rpm at + 4°C for 25 minutes. Some of the supernatants obtained were set aside for the lipid peroxidation experiment. 0.05 ml of the remaining supernatant was transferred to the tube. Tubes with 0.5 ml of 10 mM KH_2PO_4 buffer (pH: 7.0) and 1 mL of 1M KI were vortexed. The absorbance value was measured and recorded at 390 nm. The results were calculated as the amount of H_2O_2 per tissue ($\mu molg^{-1}$ tissue) by proportioning the standard graph.

Determination Of Lipid Peroxidation Content

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) using the thiobarbituric acid method. To correct the differences that may arise from pigment materials, 2 separate systems with and without tribarbituric acid (TBA) were prepared.

In the first tube, 0.5 ml supernatant was mixed with 1.5 ml 20% TCA (w / v). In the other tube 0.5 ml supernatant was mixed with 20% TCA (w / v) containing 1.5 ml 0.5% TBA. The reaction mixture was incubated in boiling water for 30 minutes and the reaction was stopped by placing the tubes in an ice bath. For the calculation of lipid peroxidation, absorbances at 532 nm and 600 nm wavelengths were measured and the results were calculated over the E = E'532-E'600 equation.

Microscopic Determination Of Superoxide Anion Accumulation

The accumulation of superoxide anion, one of the reactive oxygen species, in *Lemna minor* plants exposed to magnetite NPs for 15 days was determined by a light microscope. Samples were prepared in a solution containing 0.1% (w/v) nitroblue tetrazolium 15 mM, sodium azide, and 50 mM potassium phosphate buffer were incubated for 15 minutes. To terminate the reaction, duckweed plants passed through ethanol series from 70–95% were taken into the preparations and microscopic images were recorded.

Statistics

The "Statical Package for Social Sciences (SPSS for Windows 24.0, IBM, Armonk, New York)" program was used to determine and evaluate the standard errors of the experiments carried out on the *Lemna minor* plant. The differences between the averages were made using LSD test with a significance level of $p < 0.05$.

Results And Discussion

Nanoparticle characterization

Magnetite nanoparticles have been synthesized biologically using the laurel plant (*Laurus nobilis*). In an aqueous colloidal solution, the stability and formation of magnetite nanoparticles were confirmed by using UV-Vis spectral analysis. As a result of UV-Vis measurements of the obtained nanoparticle, the highest peak value was found in the wavelength range of 235–250 nm which represents the surface plasmon resonance of the magnetite NPs (Fig. 1). The higher absorbance between 200–250 confirms the formation of magnetite nanoparticles (Yew et al. 2016).

Identification of the possible biomolecules responsible for stabilization and capping of nanoparticles were carried out by FT-IR spectra of synthesized magnetite nanoparticles. The peaks correlated with

magnetite nanoparticles are shown in Fig. 2. FT-IR was used to examine the presence of biomolecules responsible for magnetite nanoparticle synthesis. According to the FT-IR spectrometer results, the peaks around 590–610 cm^{-1} could be assigned to the Fe–O stretching vibrational mode of Fe_3O_4. The peak occurring at 3308 cm^{-1} can be referred as -OH band groups and to the traces of molecular water and the molecules come from the laurel extract. In the study of Souza et al. (2019), it was mentioned that the Fe–O stretching vibration parallel to the c-axis modes corresponds to the bands at 445–690 cm^{-1}. In the same study, it was also mentioned that the band near 690 cm^{-1} might refer to iron hydroxide structures such as amorphous goethite, defective hematite (proto/hydrohematite), and errihydrite and is recurrent in hematite spectra. Chernyshova et al. (2007) referred to same bands for Fe-O stretching also added that the band around 3500 cm^{-1} could be associated to the surface hydration layer.

According to the Zeta size analysis results, the size of the magnetite nanoparticles' average was determined as 108.5 nm (Fig. 3a). The presence of the extra hydrated layers attached to the surface cause the difference in hydrodynamic size which usually occurs greater than the actual size measurements of the Fe_3O_4 NPs. Approximate size, surface morphology, shape and elemental composition were defined using scanning electron microscopy (SEM) (Fig. 3b) and EDS (Fig. 3c). SEM analysis showed that nanoparticles were spherical in shape and have 247 nm mean size. Dispersion/aggregation behavior of nanoparticles is difficult to determine through dried solutions from microscopic data from like SEM. This situation usually causes a difference between SEM and Zeta size results for nanoparticle size. EDS results showed that the green synthesized nanoparticle contained 51% Fe.

ICP-MS results showed there was 590 mg Fe in 1 kg magnetite NP sample. According to ICP-MS results the Fe content in 2000 mg mL^{-1} (highest applied concentration) was 1.18 mg while this content was 0,059 mg for 10 mL of growth medium.

Toxicological Effects Of Magnetite Nanoparticles Growth Parameters

L. minor plants treated with 0.1, 1, 10, 100, 1000 and 2000 mg L^{-1} magnetite nanoparticles for 15 days. The frond number, colony number, dry weight test results were examined for the physiological effects of the biological synthesis product magnetite nanoparticle on duckweed.

At the end of treatment, a significant decrease was observed in the number of leaves (frond number) at 100 mg L^{-1} and higher concentrations (Fig. 4a). In case of the number of colonies, the lowest treatment concentration (0.1 mg L^{-1}) stimulated the colony development (16%) while the highest two concentrations 1000 and 2000 mg L^{-1} caused a significant decrement in the number of colonies around 19 and 39%, respectively, compared to the control group (Fig. 4b).

Iron oxide magnetite nanoparticles can function as a plant growth stimulator (Shao et al. 2022). Blinova et al. (2017) revealed that at concentrations ≤ 100 mg L^{-1} magnetite nanoparticles with numerous hydrochemical compositions were not toxic to duckweed (*L. minor*) in waters. Similar results were obtained in the studies with *Lemna gibba* (Barhoumi et al. 2015) and *Chlorella vulgaris* (Chen et al. 2012; Barhoumi and Dewez, 2013) which showed that low toxic effect at concentrations higher than 100 mg L^{-1}. According to previous studies, chemically synthesized magnetite nanoparticles in concentrations less than 100 mg L^{-1} did not pose a threat to aquatic vegetation. In another study, the internalization and distribution of magnetite nanoparticles in the plants and their effects on plant growth were studied. The results demonstrated that magnetite nanoparticles stimulated alfalfa and soybean growth in 50 and 100 mg L^{-1} concentrations. The stimulations were found related to citric acid coating of the nanoparticles (Iannone et al. 2021).

In the current study, the results illustrated that green synthesized Fe_3O_4 NPs are 10 times less toxic (threshold concentration 1000 mg L^{-1}) when compared to chemically synthesized Fe_3O_4 NPs which are toxic in the concentrations ≤ 100 mg L^{-1} in waters (Barhoumi et al. 2015, Chen et al. 2012; Barhoumi and Dewez, 2013))

Photosynthesis And Mass Accumulation

Photosynthesis counts as a good method to assess the overall performance of a plant since it is the only energy entry point in plants (Kalaji et al. 2014). Also, it functions as a sensor for interpreting plant physiology and metabolism. Thus, to exhibit the effect of stress factors in the plant, photosynthetic pigments and activity are commonly used (Rastogi et al., 2017).

There were no negative impact of magnetite NP treatment on photosynthetic pigment content. Chlorophyll a/b ratio (Fig. 5a) which is an important parameter for toxicity on pigments, maintained similar to control level. This result indicated that magnetite NP has no hazardous effect on pigment system. Magnetite NP treatment did not cause any change in total carotenoids content (Fig. 5b) which are also important parameters to measure plant response to stress treatments (Rmiki et al., 1999). As photosynthetic accessory pigments and the components of antioxidant metabolism, their level and integrity were not damaged nor induced by magnetite NP treatment. These results, also are consistent with data from oxidative stress parameters (Fig. 7a,b).

In the study with *Hordeum vulgare*, the moderately increased pigment content up to 250 mg L^{-1} was observed, then gradual a reduction with the enhanced concentrations of NPs was recorded (Tombuloglu et al., 2019). Furthermore, in *Citrus maxima* plants, as compared with control chlorophyll content was considerably enhanced by 50 mg L^{-1} g-Fe_2O_3 NPs treatment (23.2%) whereas 100 mg L^{-1} g-Fe_2O_3 NPs remarkably reduced chlorophyll content (Hu et al. 2017). Likewise, iron oxide NPs increased the chlorophyll level in soybean (Ghafariyan et al. 2013).

The photosynthetic pigment content maintenance as control group level was not reflected by dry weight of plants. The lower concentrations of magnetite NPs (0.1 and 1 mg L^{-1}) induced mass accumulation in *L. minor* plants, indicating that an induction in biosynthesis reactions, however, dry mass of treated plants significantly decreased after 100 mg L^{-1} and higher concentrations in comparison to control. As parallel with the decrement of the number of the leaves from 100 mg L^{-1}, dry mass accumulation was blocked by the treatments higher than 100 mg L^{-1}. As a border of the inhibition of biosynthetic reactions it seems 100 mg L^{-1} is a prominent concentration for water plants. Overall, it seems, photosynthetic pigments were not affected other biosynthesis reactions are suppressed by high magnetite concentrations. The increased dry weight of plants treated with the 0.1 and 1 mg L^{-1} concentrations could be a result of *Lemna*'s ability to accumulate metals. In resemblance to the these results, in the study of Horvat et al. (2007), dry weight of the *L. minor* plants that were treated with was increased after the treatments with Pb, Mn, Ni, Zn and Fe due to *Lemna*'s ability to accumulate metals.

Hydrogen Peroxide Content And Lipid Peroxidation

The magnetite NP toxicity is prominent for 1000 and 2000 mg L^{-1} concentrations showed by hydrogen peroxide content (Fig. 7a). As an oxidative stress indicator of cellular metabolism, enhanced H_2O_2 content in the highest two concentrations point out that increase in oxidative stress level in cells of *L. minor* fronds. This result was confirmed by data of Superoxide anion accumulation experiments which displayed cell injury in 1000 and 2000 mg L^{-1} concentrations of magnetite NP (Fig. 8). Although oxidative stress and resulting injury were obvious for 1000 and 2000 mg L^{-1} concentrations of magnetite NPs, necrosis on fronds were more prominent for 2000 mg L^{-1} of magnetite NP application. These results are in accordance with malondialdehyde (MDA) production which is significantly increased in only for 2000 mg L (30%) (Fig. 7b). It may be speculated that the higher concentrations of treatments may cause oxidative stress in cells, but plant membranes could be protected up to 2000 mg L^{-1} by antioxidant mechanisms to defend cell integrity.

MDA is a universally acknowledged lipid peroxidation (oxidation stress) biomarker. Polyunsaturated fatty acids form MDA after ROS peroxidation. Formed MDA reacts with TBA and creates a red-coloured TBA-MDA adduct from lipid peroxidation. The more colorization indicates more production of TBARS which means higher lipid peroxidation (Tsikas et al., 2017). Souza et al. (2019) reported, different iron oxide nanoparticles (akaganeite predominance + hematite) caused a dose dependent lipid peroxidation in the plant increased with rising iron oxide NP concentration for all iron-based nanoparticles tested.

The treatment to *Brassica napus* with different concentrations of iron oxide NPs caused a significant increase at the accumulation of H_2O_2 in the study of Palmqvist et al. (2017). The obtained results in the current study, showed similarity for H_2O_2 and MDA contents which statistically significant difference was observed after 1000 mg L^{-1} treated plants.

Iron release from magnetite NPs might trigger Fenton reactions which forms hydroxyl radicals with H_2O_2. Through a Fenton's reaction, H_2O_2 reaction with Fe^{2+} produces hydroxyl radicals while with Fe^{3+} generates the superoxide anion (Jalali et al. 2017). Accordingly, to the present study's results, the high H_2O_2 content for the concentrations above 1000 mg L^{-1} might resulted in higher superoxide anion accumulation.

Conclusions

Granting all the previous studies, magnetite NPs are accepted as safe products, in this study it has been shown that greater concentrations than 100 mg L^{-1} inhibited the frond growth for individuals and it affected population by 1000 mg L^{-1} concentrations. The dose-dependent toxicity of magnetite NPs toxicity related with oxidative stress and membrane damage is obvious for 2000 mg L^{-1}. This study, demonstrated that green synthesized magnetite is safer than chemically synthesized equivalents which only showed toxicity symptoms in 1000 mg L^{-1} threshold.

Declarations

Author contribution: Buse Filizler was responsible for performing the experiments, writing original draft. Selin Haseki helped with performing the experiments. Melisa Ayisigi, was responsible for performing the experiments, writing-review & editing. Lale Yildiz Aktas was responsible for conceptualization, writing-review & editing.

Funding: This project was supported by Ege University Scientific Research Projects Coordination Unit with the project number of FYL-2018-20043.

Data availability: No supplementary information.

Declarations

Ethics approval: Not applicable.

Consent to participate: Not applicable.

Consent for publication: Not applicable.

Competing interests: The authors declare no competing interests.

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Figures

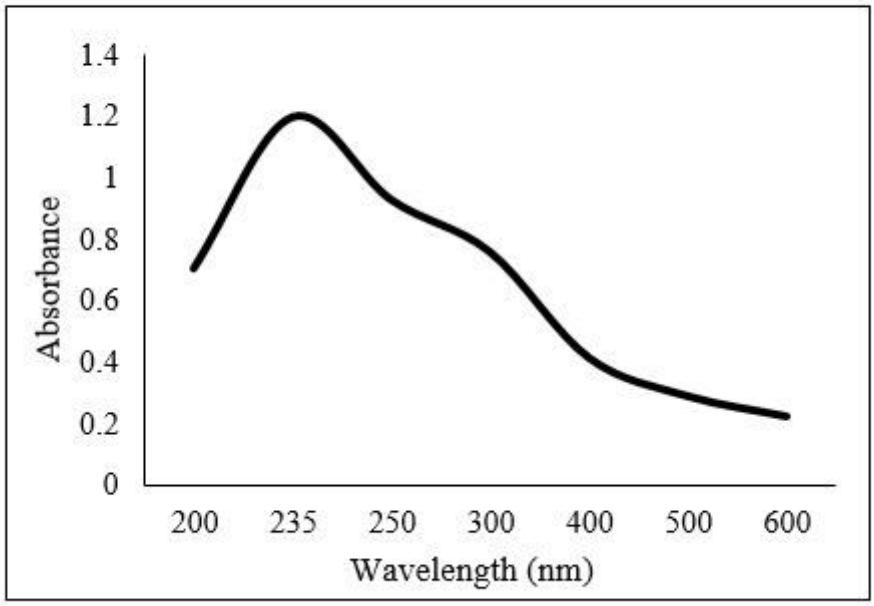

Figure 1

Characterization of green synthesized Magnetite NPs, UV- Vis spectra and specific SPR peak of NPs.

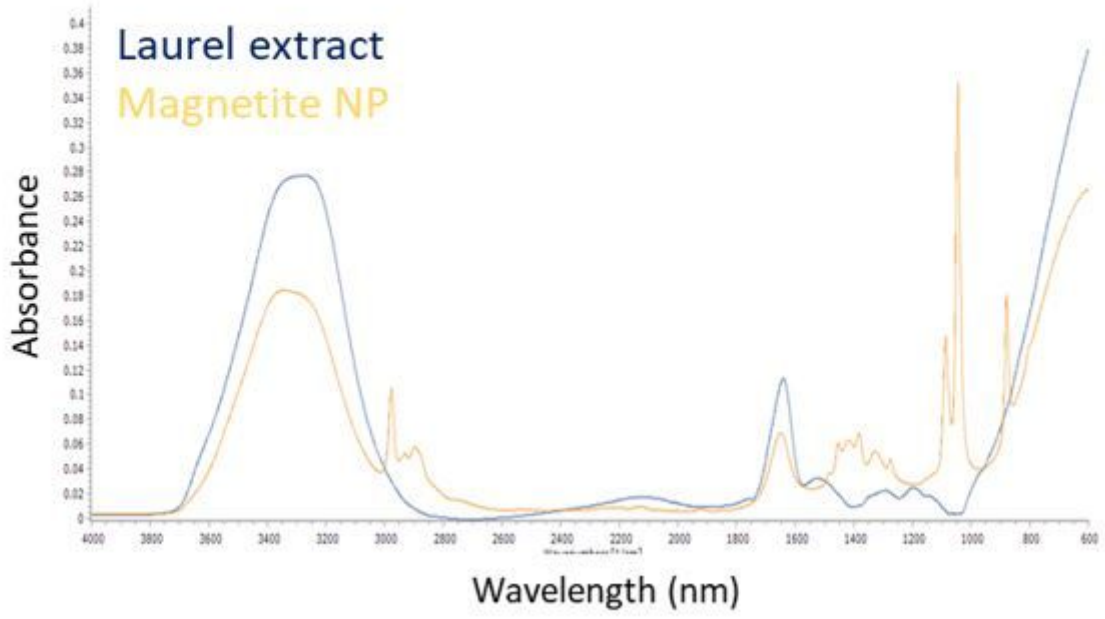

Figure 2

FT-IR spectra of green synthesized magnetite NPs.

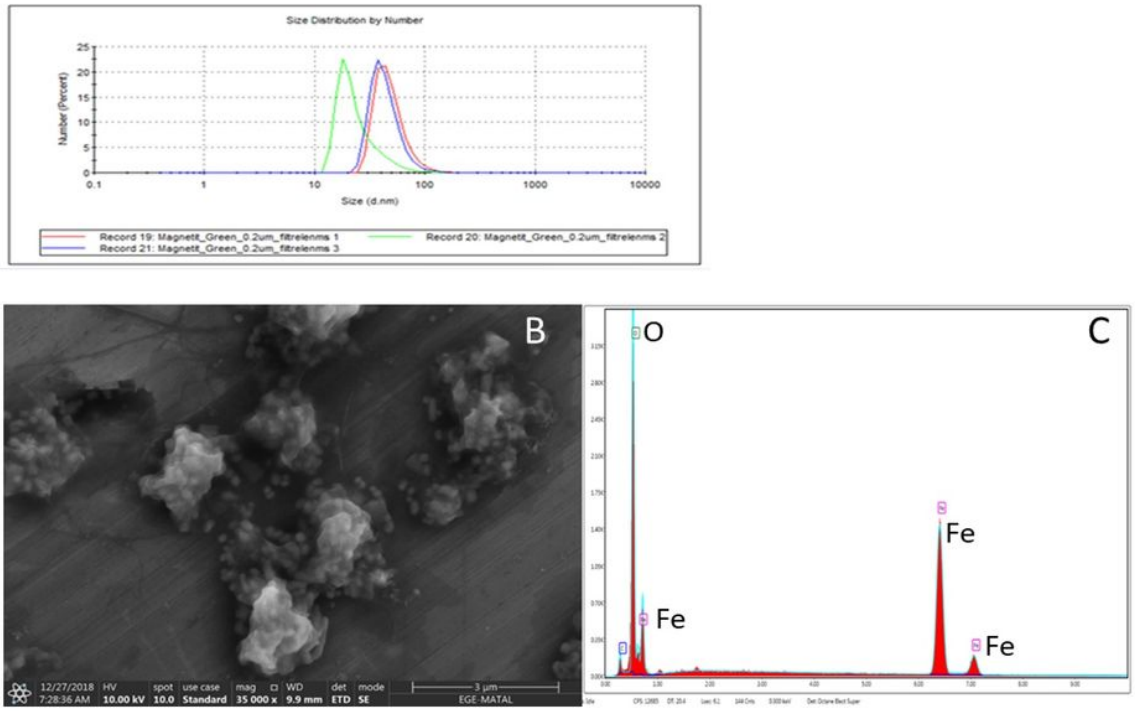

Figure 3

Zeta size **(a)** and, SEM images **(b)** presents the morphology and size of the nanoparticles, EDS results and Fe percent **(c)** in the sample of green synthesized Magnetite NPs.

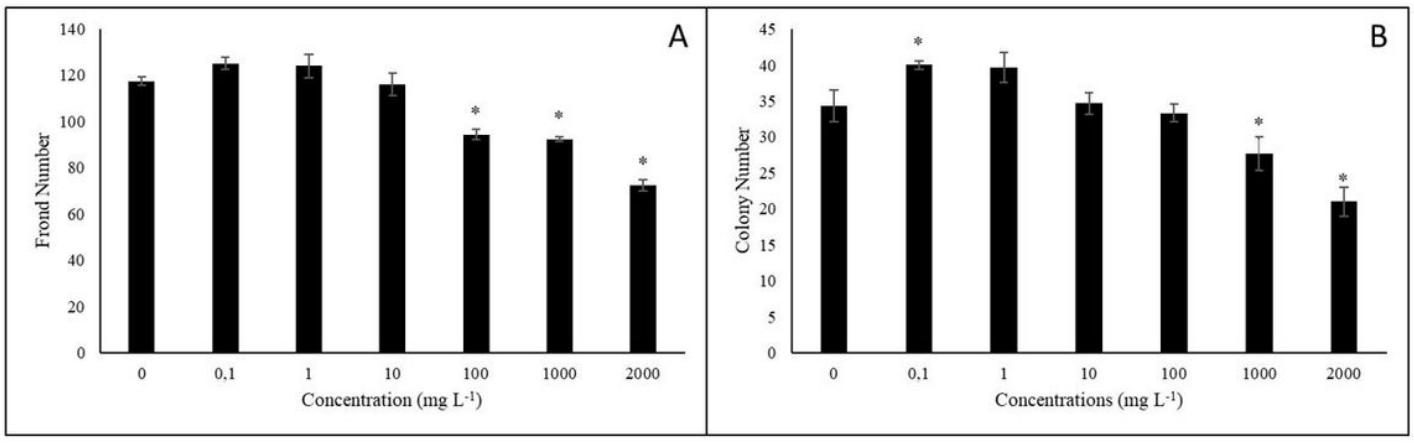

Figure 4

Frond number **(a)**, colony number **(b)** of Magnetite NP applied *L. minor* plants.

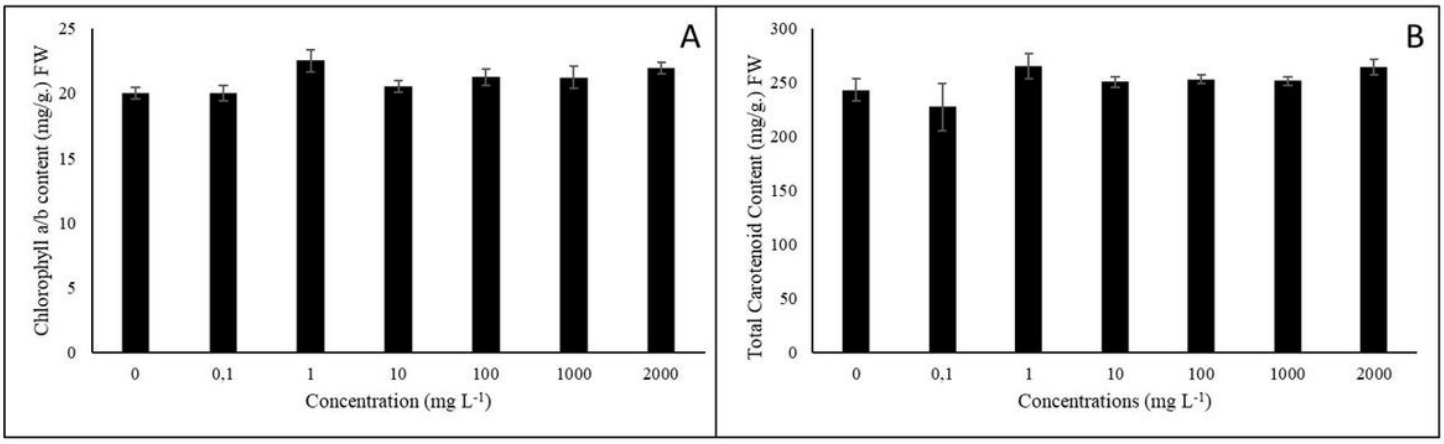

Figure 5

Chlorophyll a/b ratio **(a)** and total carotenoid content **(b)** of Magnetite NP applied *L. minor* plants.

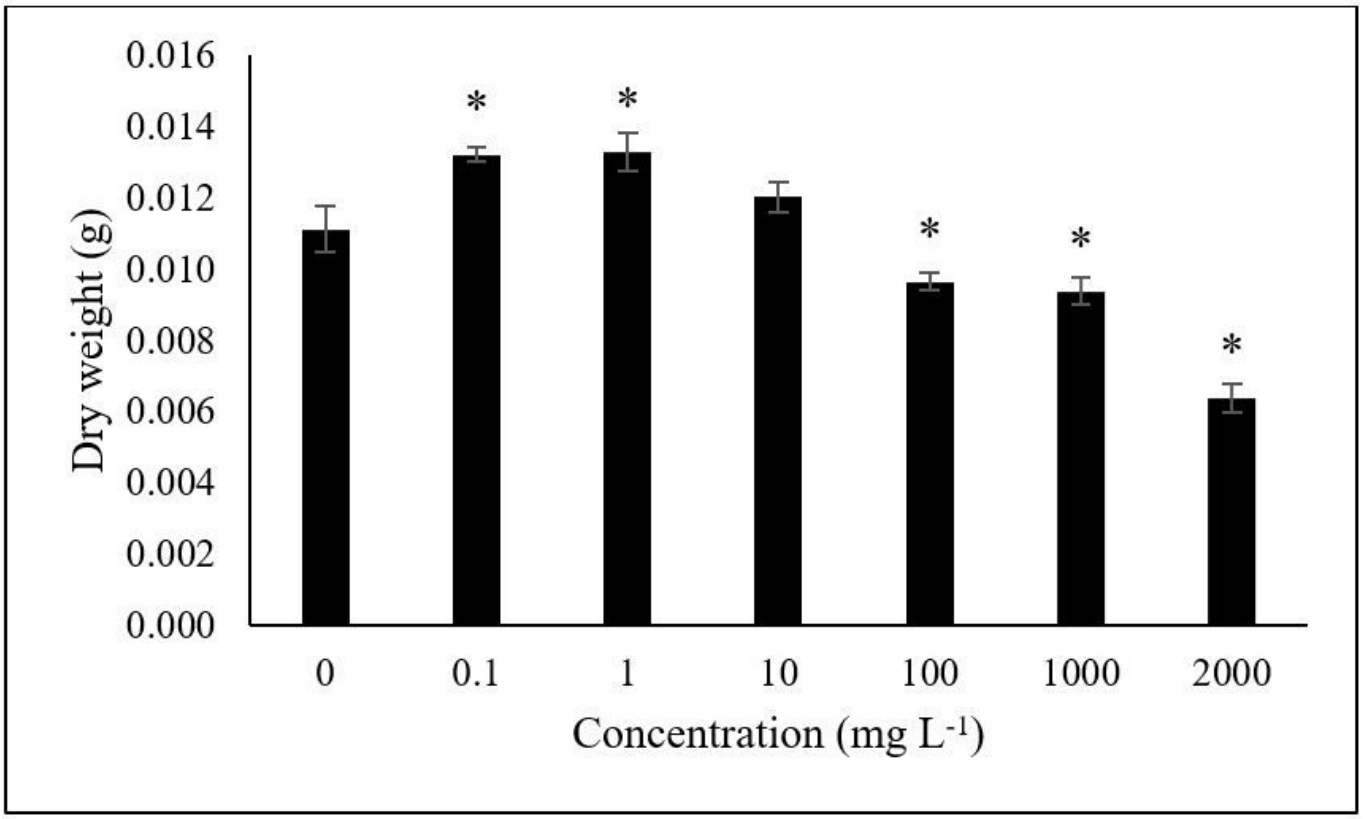

Figure 6

Dry mass of Magnetite NP applied *L. minor* plants.

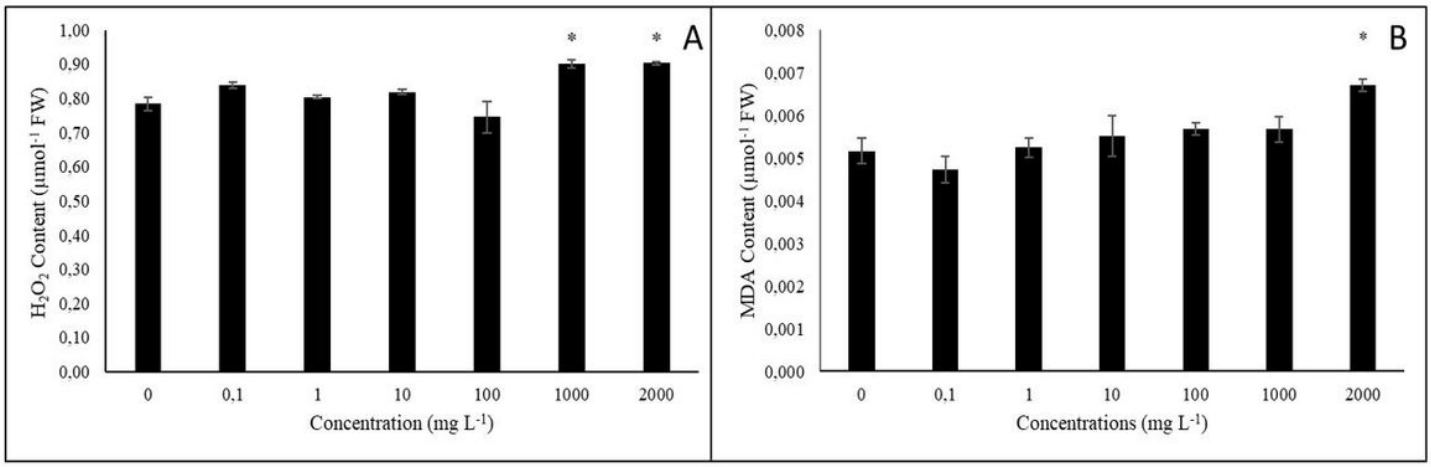

Figure 7

Hydrogen peroxide **(a)** and malondialdehyde content **(b)** of Magnetite NP applied *L. minor* plants.

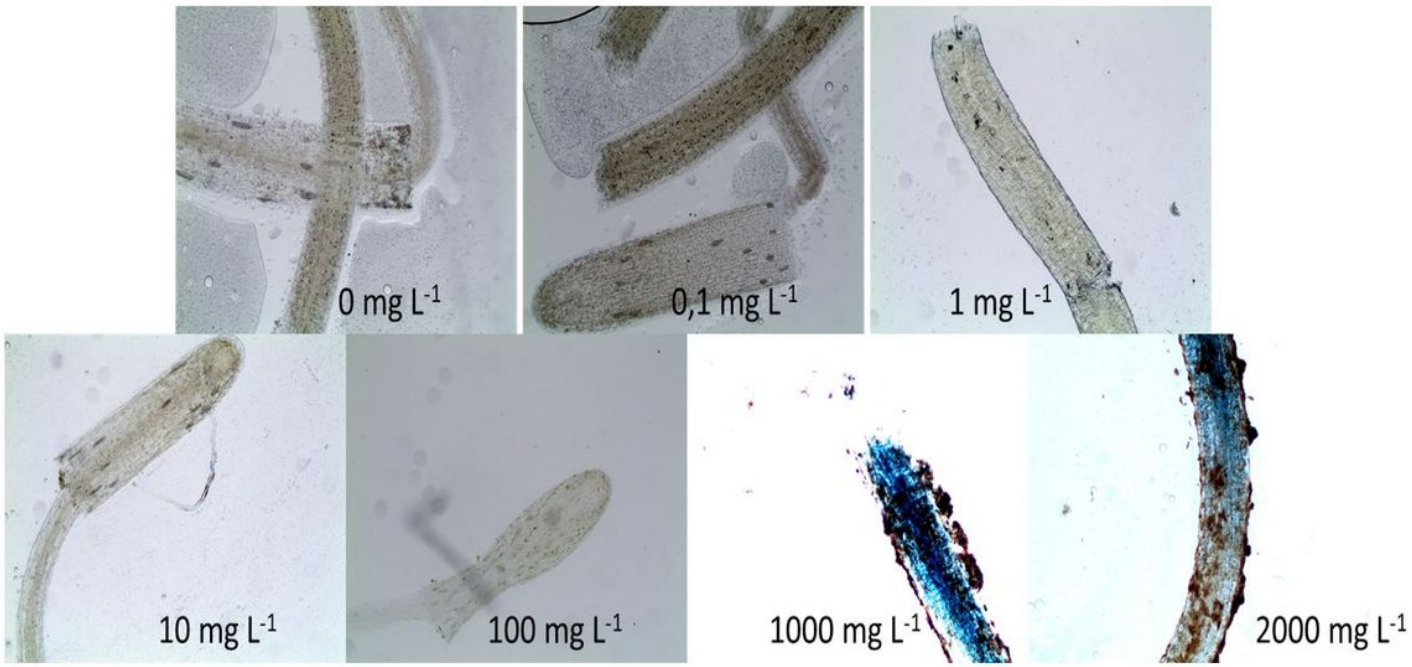

Figure 8

Superoxide anion accumulation in Magnetite NP applied *L. minor* plants.