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Research Article

Phyto-Synthesized Silver Nanoparticle Toxicity Effect on Aquatic Plant *Lemna minor* L.

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Abstract

Silver nanoparticles (AgNPs) are made up about 55% of all nanomaterials produced and are widely used in consumer products. Its is inevitable that these particles are released to the aquatic environment during production, use and disposal. In this study, subacute toxicity of AgNPs obtained by phyto-synthesis was investigated on *Lemna minor* L. (duckweed) plants. The formation of AgNPs obtained from laurel (*Laurus nobilis* L.) extract was determined by UV-VIS spectrophotometric measurements. The AgNPs synthesized by the phyto-synthesis method were characterized by Fourier transform infrared spectroscopy (FT-IR), Zeta size and potential, Inductively Coupled Plasma Mass Spectrometry and Scanning electron microscopy (SEM-EDS) analysis. The analysis results show that AgNPs are homogeneously distributed, spherical in shape with an average size of 34 nm and coated with phyto-content. For toxicity tests, plant stock cultures were grown in the climate room according to OECD 221 guidelines. After 8 weeks of acclimation, the plants were treated with AgNP concentrations ranging from 0.005 to 50 mg L^{-1} for 7- and 14-days. The increase in AgNP concentration caused a decrease in frond numbers. Growth inhibition data showed that the EC_{50} value of phyto-synthesized AgNP was 4.78 mg L^{-1} and the lowest observed effect concentration (LOEC) was 0.5 mg L^{-1} for 7-days. AgNP concentrations below LOEC level (0.05, to 0.5 mg L^{-1}) caused a significant decrease in growth rate by 20.07% after 7 days of exposure while it was found 4.03% for 14-days treatment at the highest AgNP concentration (0.5 mg L^{-1}). Similar trend was observed in fresh-and dry weight of plants indicating prolonged exposure time triggering tolerance mechanism which was corroborated by chlorophyll a/b and carotenoids content results. Based on higher NOEC, LOEC and EC_{50} values, phyto-synthesized AgNP usage may lead less environmental toxicity.

Keywords: Silver Nanoparticle, *Lemna minor*, Nanotoxicity, Phyto-synthesis.

Sucul Bitki *Lemna minor* L. Üzerinde Fito-Sentezlenmiş Gümüş Nanopartikül Toksisitesi Etkisi

Öz

Gümüş nanopartiküller (AgNP) üretilen tüm nanomalzemelerin yaklaşık %55'ini oluşturmakta ve tüketici ürünlerinde yaygın olarak kullanılmaktadır. Bu nanopartiküllerin üretim, kullanım ve bertaraf sırasında su ortamına salınması kaçınılmazdır. Bu çalışmada, fito-sentez yoluyla elde edilen AgNP'lerin *Lemna minor* L. (su mercimeği) bitkileri üzerinde subakut toksisitesi araştırılmıştır. Defne (*Laurus nobilis* L.) ekstraktı kullanılarak elde edilen gümüş nanopartiküllerin oluşumu UV-VIS spektrofotometrik ölçümüyle belirlenmiştir. Fito-sentez yöntemiyle sentezlenen AgNP'ler, Fourier transform infrared spectroscopy (FT-IR), Zeta boyut ve potansiyeli, Taramalı elektron mikroskobu Inductively Coupled Plasma Kütle Spektrometrisi (ICP) and Scanning electron microscopy (SEM-EDS) analizi ile karakterize edilmiştir. Analiz sonuçları, AgNP'lerin homojen olarak dağıldığını, ortalama 34 nm büyüklüğünde küresel şekilli olduğu ve bitkisel içerik ile kaplandığını göstermiştir. Toksiste testleri için bitki stok kültürleri, OECD 221 yönergesine göre iklim odasında yetiştirilmiştir. 8 haftalık alışma aşamasından sonra, bitkilere 7 ve 14 gün boyunca 0.005 ila 50 mg L^{-1} arasında değişen AgNP konsantrasyonları uygulanmıştır. Uygulanan AgNP konsantrasyonundaki artış yaprak sayılarında azalmaya neden olmuştur. Büyüme inhibisyonu verileri, fito-sentezlenen AgNP'nin EC_{50} değerinin 4.78 mg L^{-1} ve 7 gün boyunca gözlemlenen en düşük etki konsantrasyonunun (LOEC) 0.5 mg L^{-1} olduğunu göstermiştir. LOEC seviyesinin altındaki AgNP konsantrasyonlarında, 7 günlük uygulama sonrasında en yüksek AgNP konsantrasyonu (0.5 mg) büyüme oranınında %20.07'lik önemli bir düşüşe neden olurken, 14 günlük uygulama sonucu büyüme oranının %4.03 azaldığı belirlenmiştir. Benzer bir eğilim, bitkilerin taze ve kuru ağırlıklarında gözlenmiştir. Bu durum, uzun maruz kalma süresinin (14 gün) bitkide tolerans mekanizmasının tetikleyebileceğini, klorofil a/b ve karotenoid içeriği sonuçları ile de uyumlu olarak, işaret etmektedir. Yüksek NOEC, LOEC ve EC_{50} değerleriyle, fito-sentezlenmiş AgNP kullanımının daha düşük çevresel toksisiteye yol açabileceği sonucuna varılmıştır.
Anahtar Kelimeler: Gümüş Nanopartikül, *Lemna minor*, Nanotoksisite, Fito-sentez.

1. Introduction

The production and application of nanoparticles (NPs) has increased in consumer products. Although there is an increase in nanoparticle usage, the effects and behaviour of NPs in the environment have not been completely reviewed (Bundschuh et al., 2018). Among metal nanoparticles silver nanoparticle (AgNP) is the most diversely used one in many areas from textile to agriculture (Vance et al., 2015, Ayisigi et al., 2020).

Silver has been reported as a hazardous substance due to the toxicity of its dissolved or ionic forms in many organisms (Reinfelder and Chang, 1999). It has been pointed out; the same toxicity has been harnessed in the form of AgNPs. However, it also has been suggested that the toxicity of AgNPs could be the result of physical or chemical production methods which are costly and involve toxic materials (Khoshnamvand, et al., 2020). Hence, there is a need to develop eco-friendly and less costly synthesis methods for NPs. Biologically synthesized NPs were referred as green materials since they avoid the use of toxic chemicals compared to physically or chemically synthesized NPs (Huo et al., 2016). Moreover, it has been mentioned, the coating agents of biologically synthesized NPs are biological components that presented low toxicity (Newton et al., 2013).

In addition to the synthesis methods, parameters like nanoparticle concentration, temperature, composition in chemical salts, ionic strength, and pH affects agglomeration or stabilization of metallic nanoparticles in an aqueous solution is important to determine nanotoxicity (Jiang et al., 2009). Furthermore, the nanoparticle physicochemical properties such as the size, charge at particle surface, shape, the solubility of particles, finally chemical composition surface structure and area determine the bioavailability, uptake and toxicity potential within aquatic organisms for metallic nanoparticles (Oukarroum et al., 2013).

L. minor grows temperate regions and are often used for toxicity tests. The species has a floating or sunken discoid stem (leaf) and a stem emerges from under each leaf. Although flower formation is very rare in *Lemna spp.*, and the plants produce new leaves vegetatively. Compared to older plants, younger ones tend to be paler, and the roots are shorter. Lemna's small size, simple structure, asexual and short reproduction make the plants of this genus very suitable for laboratory tests (OECD 221, 2006). *L. minor*, aquatic plant, usually used to determine the impacts for a variety of substances released to the environment and recently they are being used to evaluate the toxic effects of NPs (Minogiannis et al., 2019).

The present study aimed to investigate toxic effects of AgNPs produced by environmentally friendly biological synthesis, which have become widespread in recent years, to aquatic ecosystems, on *L. minor*, a model aquatic plant.

2. Material and Method

2.1. Synthesis and Characterization of Silver Nanoparticles (AgNPs)

AgNP was synthesized by using laurel (*Laurus nobilis* L.) leaf extract with phyto-synthesis method. Laurel leaves were powdered in liquid nitrogen and homogenized by mixing 100 ml of pure water at 60°C for 10 minutes. The filtered laurel extract was mixed with 1 mM $AgNO_3$ solution at a ratio of 9:1 ($AgNO_3$/extract) at 90°C for 2 hours. The synthesis of AgNP was completed after the colour of solution turned to brown.

The synthesized AgNPs were characterized by different physicochemical techniques like UV-VIS, FT-IR, ICP-MS, Zeta-Sizer, Zeta-Potential, and SEM analysis. Synthesis of nanoparticles were determined by the detection of the absorbance between 400-435 nm, the specific localized surface plasmon resonance peak for AgNPs, with UV- VIS Spectroscopy (Thermo Scientific, UK). Fourier transform infrared spectroscopy (FT-IR) (FT-IR 8000 Series, Shimadzu, Japan) was used to identify the organic components of AgNPs come from laurel extract that coats the nanoparticles. The silver content of the nanoparticle was determined quantitatively and qualitatively by Inductively Coupled Plasma - Mass Spectrometer (ICP-MS) analysis (Agilent, 7500ce, USA). The surface potential and size of the nanoparticles were determined by zeta sizer-potential analysis (Malvern, UK). The shape and size of the nanoparticles were determined by imaging with a scanning electron microscope (SEM) and they were observed under high vacuum and 7.5 kV for Energy Dispersive X-Ray spectroscopy (EDS) study (Thermo Scientific, Apreo S-USA).

2.2. Cultures of *L. minor* plants

L. minor plant was obtained from Ege University Botanical Garden (Izmir-Turkey) and subjected to 8 weeks of acclimation under sterile conditions in 250 ml flasks containing 100 ml of Steinberg medium (Brain and Solomon., 2007). Stock cultures were transferred to fresh and sterile medium in every 7-10 days. The plants were cultured under a cold white fluorescent lamp with a light intensity of 6500-10000 lux at a temperature of 24 ± 2 °C in a 16:8 photoperiod (OECD 221, 2006).

For growth inhibition tests, 4 *L. minor* plants, each with 3 fronds were treated in petri dishes with a diameter of 60 mm containing 10 ml (AgNP solution + Steinberg medium) for 7 and 14 days. The solution inside of each petri dish was renewed every 24 hours to allow plants to be exposed to initial AgNP concentrations (Brain and Solomon., 2007). All experiments had four replicates.

2.3. Toxicity parameters

Biologically synthesized AgNPs' toxicological parameters on the *L. minor* plant were determined by vegetative growth data; growth inhibition, fresh-dry weight, and chlorophyll content. To determine the effects of AgNP exposure time on the plant, 7 and 14-day subacute toxicity evaluations were performed with different concentrations.

Short term toxicity of AgNP was determined by exposing the colonies to AgNP (0.1, 0.5, 1, 5, 10, 50 mg L^{-1}) for 7 days. At the end of exposure period 50% growth inhibition concentration (EC_{50}), the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) were determined.

After the initial determination of LOEC, *L. minor* plants were subjected to lower concentrations (0, 0.005, 0.05, 0.5 mg L^{-1}) of AgNPs for 7 and 14 days of to evaluate effect on biosynthetic reactions by measuring fresh-dry weight and photosynthetic pigment content level in details.

2.3.1. Growth Parameters

At the end of the test period, regardless of the size, each frond was counted. The plants in each petri dish were photographed with a Dino Capture camera microscope (Dino-Lite Microscope

USB, Taiwan). Fresh weight measurements were carried out after all colonies in an experimental group were washed with distilled water. Washed colonies were surface dried on filter papers and fresh weights were recorded. The dry weight measurements were performed by drying colonies in the oven for 24 hours at 60°C.

Growth rate: The leaves of the control and AgNP treated groups were counted at the end of the test period. *L. minor* Average Growth Rate (d^{-1}) was calculated according to the formula (OECD 221, 2006) given below:

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

Nj and Ni are the number of leaves at the end and beginning of the experiment, respectively.

Growth Inhibition Rate: To assess the potential toxic effect of the tested materials, the Growth Inhibition Rate (IGR) was calculated based on the number of frond measurements according to the formula given below (OECD 221, 2006): μc and μr are the average number of fronds in the experiments with control and AgNP, respectively.

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

Effective Concentration (EC_X): The EC_X value is the concentration of dissolved test substance in the test environment and represents the x% reduction in L. minor population over a given exposure time. EC_{50} value indicates that the given concentration of test substance had adverse effect on half of the L. minor population over a given exposure time. EC_{50} value of AgNP was calculated from IGRs by Probit Analysis according to OECD 221 (2006) guidelines.

2.3.2. Chlorophyll and total carotenoid content

Photosynthetic pigment content of plants treated with AgNPs were determined according to the modified Arnon (1949) method. 0.1 g of plant leaves are homogenized with 80% (w/v) cold acetone solution inside of an eppendorf with a help of a baguette. The resulting homogenate was centrifuged at 4500 rpm +4 °C for 15 minutes, then absorbance of supernatant at 663, 645 and 470 nm wavelengths were read.

2.4. Statistical analysis

Randomized complete block design was used for experimental design with five replicates. "Statistical Package for Social Sciences (SPSS for Windows 24.0)" program was used to determine the standard error values of the data and to evaluate the differences between means. The differences between the averages were evaluated using one-way ANOVA tests, LSD test with $P < 0.05$ significance level.

3. Results and Discussion

AgNPs constitute the largest group of nanomaterials, covering approximately 55% of all nanoparticles produced (Quadros et al., 2011). After being used in different areas, AgNPs get involved in the environment by mixing with surface waters eg. lakes, streams and rivers. In addition, AgNPs from consumer products may release silver ions (Ag^+), which are highly toxic for aquatic organisms, can be leaked into the environment. Therefore, the production and use of AgNPs raise concerns about their environmental impact due to Ag^+ toxicity. In this study, the subacute toxicity effects of AgNP obtained by phyto-synthesis from laurel plant (*Laurus nobilis*) were investigated on the model aquatic plant *L. minor* by performing growth parameters and photosynthetic pigments experiments.

3.1. Characterization of AgNPs

AgNPs typically exhibit specific surface plasmon resonance at wavelengths between 400–450 nm (Jyoti et al., 2016). According to spectrophotometric measurements, the highest peak values were obtained was between 400 and 435 nm indicating that AgNPs were formed (Figure 1a).

FT-IR analysis provides the information about the organic compounds that are present on the nanoparticles, which may take a part in the reduction of silver ions to AgNPs and capping of the nanoparticles. Figure 1b shows that FT-IR spectra of laurel extract and phyto-synthesized AgNPs. The extract of *L. nobilis* revealed three different peaks at wave number 3300, 2160 and 650 cm^{-1} which exhibited some degree shift in the corresponding AgNPs. These bands may be attributed to C=C=C, –OH and C-Br stretching from polyphenolic compounds (Ahmad et al., 2017). The observed peaks at 600 cm^{-1} comes from laurel extract in AgNP that shows C-Br stretching while C=C bending at 665 and 790 cm^{-1} indicating alkene compounds. Also, a peak at 3271 cm^{-1} shows the shift compared to the laurel extract.

The plant molecules which are involved in reduction of metal elements to metal nanoparticles were analysed by the FT-IR study. Plants produce free radical scavenging molecules and other metabolites that are rich in antioxidant activity (phenolics, vitamins, reducing sugar, terpenoids etc.) (Salama, 2012). The shifts in the absorbance show the changes within the bonds. The decrease in the peaks between 3500–3000 cm^{-1} shows that separated -OH bonds which refer to the hydroxyl groups in phenols and alcohols. The peaks between 1250–1000 cm^{-1} reveals the new C-O bonds were formed that might be the result of covered AgNPs. The results showed that nanoparticles are coated with phytocontent (Edison & Sethuraman, 2012). The negative potential value might be the result of the polyphenolic content of the extract based on FT-IR results.

Zeta size and potential analysis provide the information on particle size and overall charge which effect the dispersion and stability of a nanoparticle (Ahmad et al., 2017). The results showed that the particle size was equal to 102.4 nm and well dispersed (Figure 1c). The zeta potential was –27.7 mV for the phyto-synthesized AgNP which indicated that the silver nanoparticles were capped with negatively charged molecules of plant (Figure 1d).

SEM analysis detects the signals created by electron-sample interactions to reveal the information about crystalline structure, external morphology (texture), chemical composition and orientation of materials in the sample by using a focused beam of electrons with high energy to generate different signals at the surface of solid specimens (Argast & Tennis, 2004). Surface morphology, shape, approximate size, and elemental composition of AgNPs were checked through SEM-EDS analysis technique (Figure 1e, f). The AgNPs were spherical in shape with a mean size of 34 nm (Figure 1e). The silver content was found around 94% by using EDS analysis for the phyto-synthesized AgNP (Figure 1f).

The green synthesized AgNPs showed characteristic AgNP surface plasmon resonance peak which is usually located between 400 and 450 nm indicating the formation of nanoparticles (Arshadi et al., 2018). SEM analysis also showed that

nanoparticles are spherical in shape and have a mean size around 34 nm. AgNP particle size distribution was poly-dispersed and have an average diameter 102.4 nm according to results of Zeta size analysis. The involvement of ions and layers of the solvent in the solution to the measurements of the zeta sizer analysis which can be the reason of the diameter difference between SEM and Zeta results (Costa et al., 2018). The electrical charge of the particle diverging from the 0 (zero) value towards + or - is an important value in minimizing the agglomeration between particles. In this study, the negatively charged AgNPs might be the result of polyphenolic content of the laurel leaf extract and their electrostatic interaction with each other can play a part in preventing the possible aggregation, and provide long-term stability (Chowdhury et al., 2016).

The silver concentration in AgNPs was detected as 0.06717 mg in 1 g of dry sample by ICP-MS analysis. These results showed that the highest concentration of AgNP (50 mg L^{-1}) had $0.034x10^{-3}$ mg of silver while the lowest concentration (0.005 mg L^{-1}) had 3.36 x 10^{-7} mg in 10 ml of medium.

ICP-MS results presented there was only $0.67x10^{-8}$ mg mL^{-1} of silver in NOEC value of phyto-synthesized AgNP while LOEC concentration of AgNP was $0.34x10^{-7}$ mg mL^{-1} silver. Additionally, the amount of silver for EC_{50} was found to be as $0.033x10^{-5}$ mg mL^{-1}. These results shows that the Ag ion release into the growth media was very low.

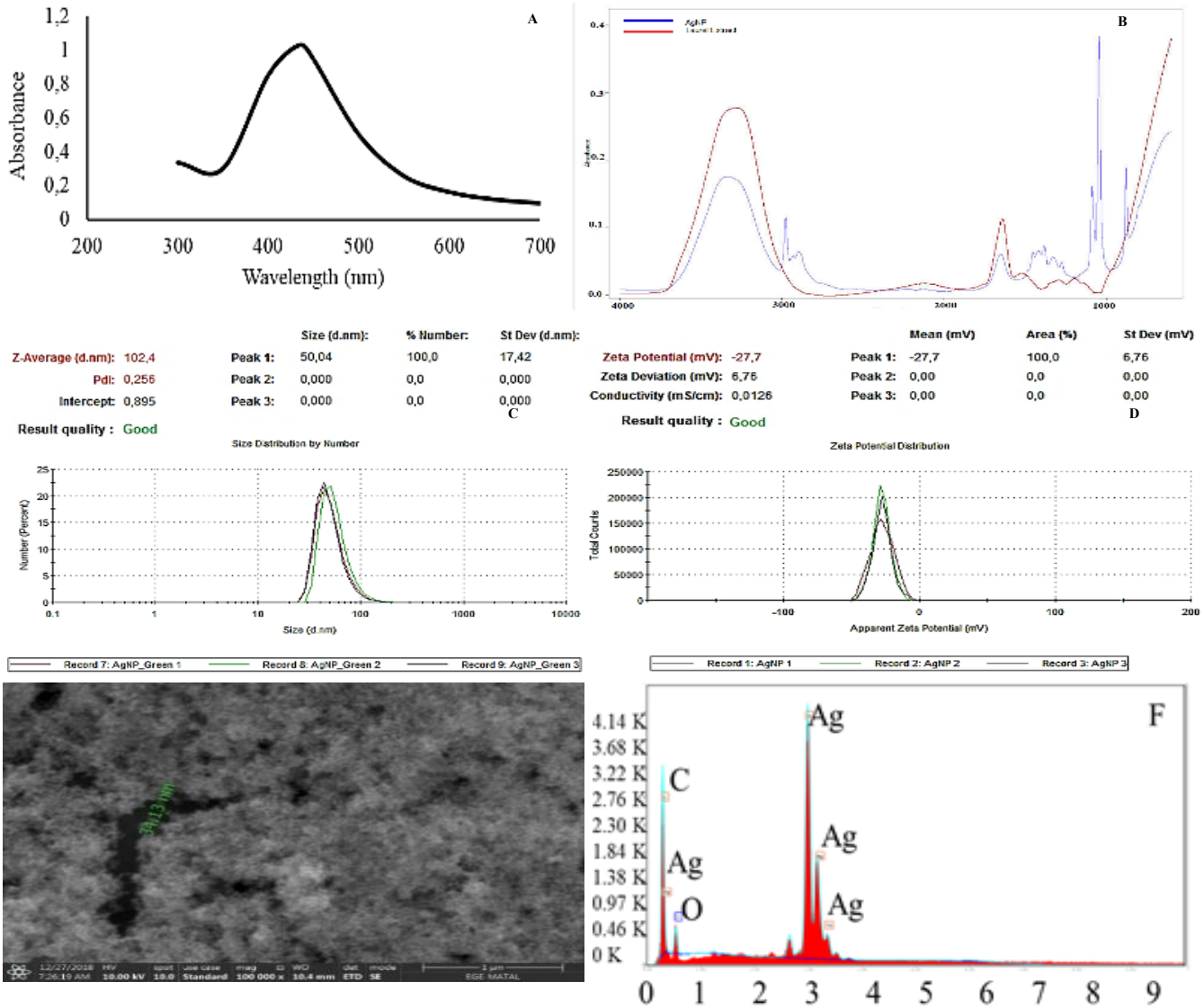

Figure 1. Characterization of phyto-synthesized AgNPs **(a)** UV– VIS spectra and specific SPR peak of AgNPs, **(b)** FT-IR spectra, **(c)** Zeta size and **(d)** zeta potential, SEM images of **(e)** phyto-synthesized AgNPs presents the morphology and size of the nanoparticles, **(f)** EDS results and Ag percent in the sample.

3.2. Toxicity parameters

3.2.1. *Short term toxicity:*

In environmental toxicology researches the aquatic plants of Lemnaceae are often used as a model because of their small size, simple structure, rapid growth, and high sensitivity to pollutants, and for these reasons they are very suitable for toxicity tests (OECD, 2002). One of the most important protocols for the assessment of phytotoxicity is the standardized, 1 or 2-week *Lemna sp.* growth inhibition test (OECD, 2006). Using this protocol, to quantify the effects of AgNP added to the growth medium, the biomass, growth and inhibition rate were measured after 7d exposure.

Short term toxicity of AgNP on *L. minor* assessed in high concentrations (0.1, 0.5, 1, 5, 10, 50 mg L^{-1}) with 7-days exposure

time. In phytotoxicity tests, the frond number is used as an important parameter for determining growth rate and growth inhibition. The growth rate data of the test groups were produced by comparing the number of fronds at the beginning and end of the experiment. 7-day AgNP application at the highest concentration (50 mg L^{-1}) has caused a decrease by 92.8% in growth rate (Figure 2a and b). Additionally, the amount of silver for EC_{50} was found to be as $0.033x10^{-5}$ mg mL^{-1}. These results shows that the Ag ion release into the growth media was very low.

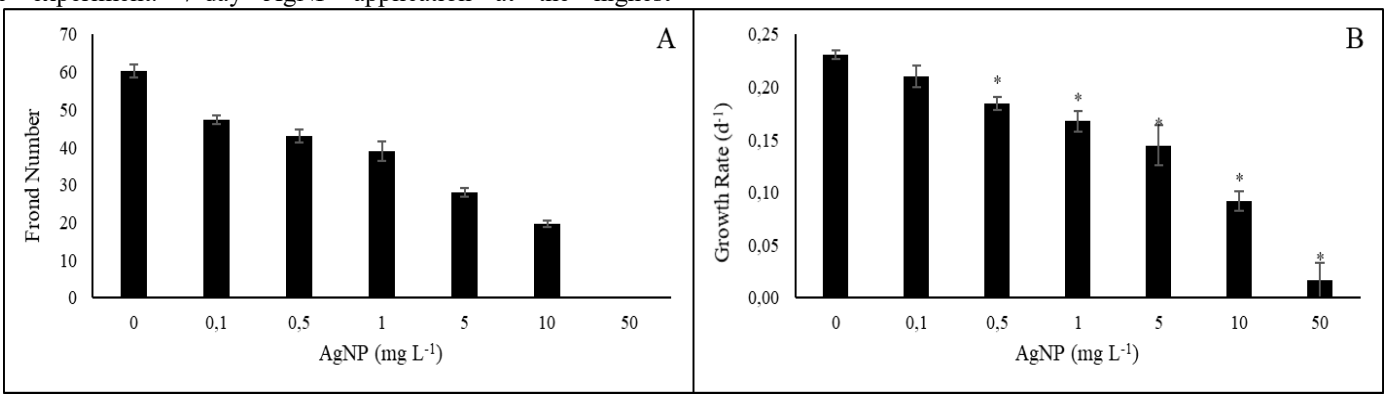

Figure 2. Frond number (a) and growth rate (b) of *L. minor* plants after the treatment with AgNP for 7 days. *Statistically different at $P < 0.05$ according to LSD test

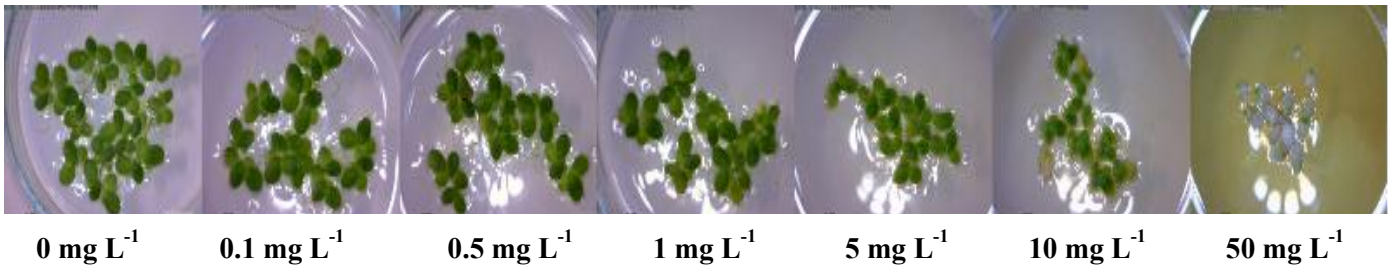

Figure 3. Images of *L. minor* plants after 7-days with high concentrations of AgNP.

The growth inhibition rate data of this experiment were used to define environmental effects of AgNP by calculation of Toxicological Concentration Descriptors like LOEC, NOEC and EC_{50}. The lowest observed effect concentration (LOEC) of AgNP was found as 0.5 mg L^{-1}, while no observed effect concentration (NOEC) was determined as 0.1 mg L^{-1}. The median effective concentration (EC_{50}) of AgNP was calculated as 4.78 mg L^{-1} for 7-days ($R^2 = 0.9171$) (Fig. 4).

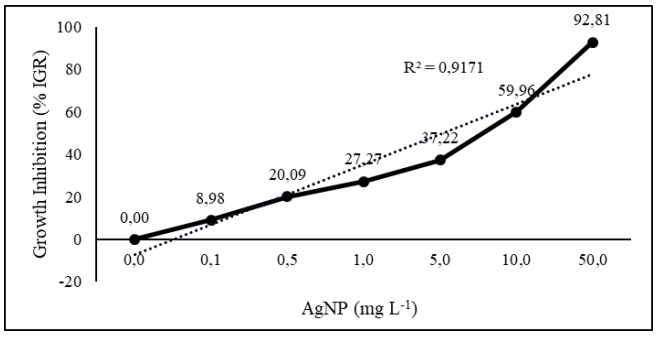

Figure 4. Growth inhibition rate of *L. minor* plants after the treatment with AgNP for 7 days.

The studies with *Lemna gibba* (Oukarroum et al., 2013), *Lemna paucicostata* (Kim et al., 2011), and *L. minor* (Gubbins et al., 2011) showed that growth of these plants was affected after being exposure to AgNPs depending on nanoparticle structure, synthesis method and concentrations. Parallel to previous studies, our data showed that biologically synthesized AgNP also had inhibited the growth of *L. minor* with increasing concentration.

In some of the similar studies, *Lemna sp.* plants were subjected to chemically synthesized AgNPs, EC_{50} was found as 0.026 mg L^{-1} (Üçüncü et al., 2014), 0.12 mg L^{-1} and 0.14 mg L^{-1} (Gubbins et al., 2011). In the study of Mylona et al., (2020) LOEC and NOEC of chemically synthesized AgNP for *Halophila stipulacea* plants were estimated. The LOEC value was found as 0.2 mg L^{-1} where the NOEC was 0.02 mg L^{-1} (Mylona et al., 2020). Also in the study of Khosravi-Katuli et al., (2018), LC_{50} of chemically synthesized AgNPs was found as 0.29 mg L^{-1} for *Cyprinus carpio* (Common carp). Compared to previous studies our results presented higher NOEC, LOEC and EC_{50} values which pointed that phyto-synthesis of AgNPs may lead less environmental toxicity. Different levels of AgNP toxicity can be observed for the different suspensions of AgNPs, due to variations in the quantity of toxic Ag^+ ions released into the aqueous test medium (Dewez et al., 2018) and the different synthesis methods or types/forms of AgNPs used.

After the initial determination of LOEC, *L. minor* plants were subjected to lower concentrations (0, 0.005, 0.05, 0.5 mg L^{-1}) of AgNPs than LOEC for 7- and 14-days. Time dependent toxicological effects of AgNP in low concentrations were assessed by vegetative growth data, fresh-dry weight and chlorophyll content.

Frond number

Fronds were counted to determine growth rate and inhibition of *L. minor* individuals exposed to low doses of AgNP at the end of 7- and 14-days trial periods. AgNP concentrations from 0.05, to 0.5 mg L^{-1} caused significant decrease in the number of fronds 14 and 30%, respectively, compared to the control. However, the

prolonged exposure time (14-days) decreased AgNP impact on frond number by resulting lower decrement rate by 7.6% and 10.9% for 0.05 and 0.5 mg L^{-1}, respectively.

In a previous study, AgNPs were chemically synthesised, characterised and subsequently presented to the *L. minor*. Results showed that inhibition of plant growth was evident after exposure to small (~20 nm) and larger (~100 nm) AgNPs at low concentrations (0.005 mg L^{-1}) and this effect became more acute with a longer exposure time (Gubbins et al., 2011). Frond number data indicated that the toxicity of both NPs increased with time, so greater inhibition of growth was observed after 14d exposure than after 7d (Gubbins et al., 2011). The results we obtained from our study showed this was not the case for phyto-synthesized AgNPs. *L. minor* plants were affected negatively after 7d of exposure, but these adverse effects reduced for the 14d treated plants. Especially, in the lowest concentration of AgNP did not cause any significant decrease in frond number after 14d of exposure.

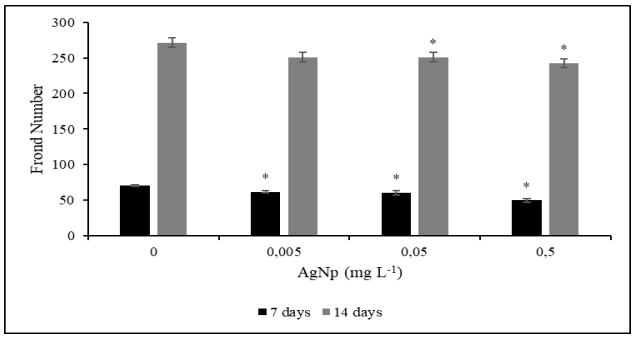

Figure 5. Frond number of *L. minor* plants after the treatment with AgNP for 7 and 14 days. *Statistically different at $P < 0.05$ according to LSD test

Growth Rate

The growth rate of plants was inhibited by 20.07% after 7 days of exposure while it was found 4.03% for 14-days treatment at the highest AgNP concentration (0.5 mg L^{-1}) compared to the control group.

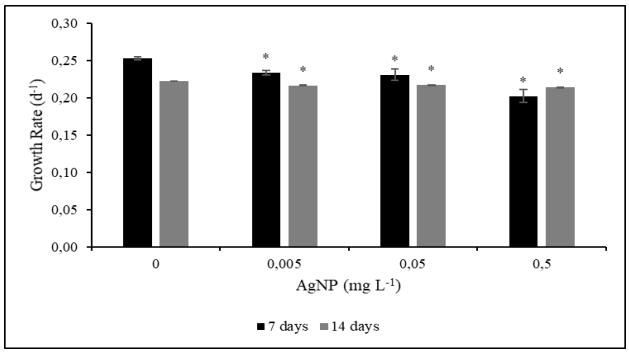

Figure 6. Growth rate of *L. minor* plants after the treatment with AgNP for 7 and 14 days. *Statistically different at $P < 0.05$ according to LSD test

The reduced growth rate of plants agrees with the findings of previous studies in *Elodea canadensis* (Van Koetsem et al., 2016), *L. gibba* (Oukarroum et al., 2013), *L. minor* (Gubbins et al., 2011; Ucuncu et al., 2014) and *Spirodela polyrhiza* (Jiang et al., 2012). In correlation with previous studies, a reduction in the growth rate was observed in our study for both 7 and 14d of treatment to AgNPs. However, the reduction of the growth rate presented a difference for the exposure times, 14d of exposure having a reduced inhibition compared to 7d of exposure which might be the result of an acclimation mechanism of *L. minor* plants to AgNPs.

Fresh-Dry Weight

To observe the effect of 7 and 14 days AgNP application on plant biomass, the fresh and dry weights of the plants were measured. Treatment with 0.005 mg L^{-1}AgNP resulted in a slight reduction in fresh and dry weight of plants while the reduction rate of both parameters was found as 37% and 38%, respectively compared to the control plants after 7-days of exposure to the highest concentration of AgNP (0.5 mg L^{-1}). In a similar trend 14-days of exposure to AgNP led to a decrease in the plant biomass. The reduction rate of fresh and dry biomass reached to 9.14% and 12.85%, respectively at highest concentration of AgNP (0.5 mg L^{-1}).

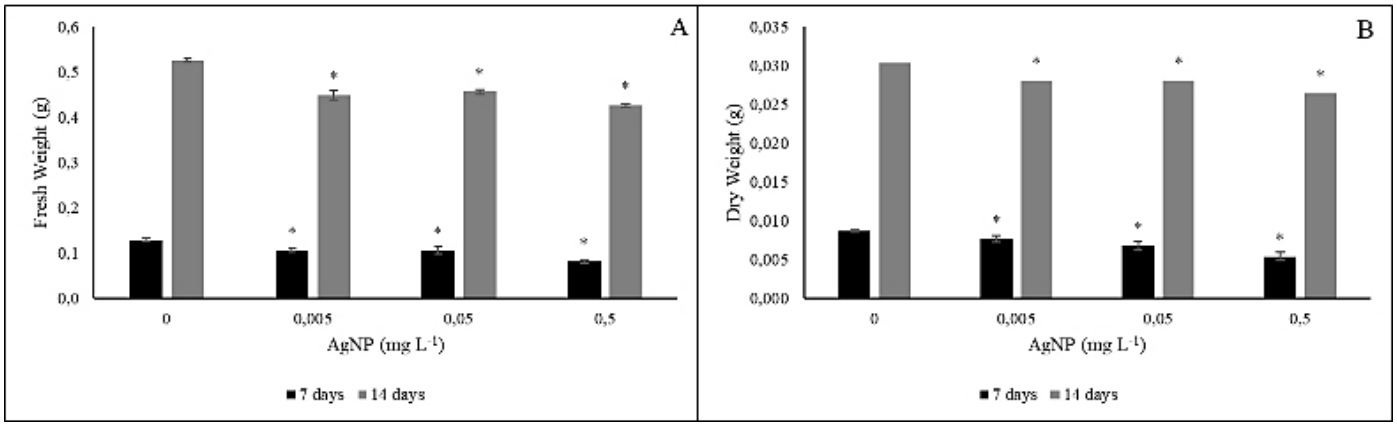

Figure 7. Fresh **(a)** and Dry **(b)** weight measurements of *L. minor* plants after the treatment with AgNP for 7 and 14 days. *Statistically different at $P < 0.05$ according to LSD test

Fresh-dry weight data are important parameters which provide information about whether biosynthesis processes are affected in plants. It has been shown that exposure to AgNP can reduce biomass and leaf area in *Spirodela polyriza* (Jiang et al., 2012). Same study reported that AgNPs significantly reduce plant biomass, inhibit shoot growth and lead to root loss. Kaveh et al. (2013) pointed that exposure of AgNPs to high concentrations (5-20 mg L^{-1}) resulted in a reduction in biomass in *Arabidopsis*. It was suggested that reduction in plant biomass could also be related to the water and mineral uptake required for biosynthesis reactions cannot occur due to the blockage of the apoplastic pathway by AgNPs (Kaveh et al., 2013).

Chlorophylls and Total Carotenoids Content

Recently, the chlorophyll contents of plants exposed to test solutions have taken their place among the parameters controlled in toxicity tests according to OECD guides (OECD 221, 2006). The photosynthetic pigments and chlorophyll a/b ratio can be used as indicator of stress as well as of a plant's photosynthetic capacity (Qian et al., 2013).

AgNP in 0.05 mg L^{-1} concentration did not cause any change in chlorophyll a/b ratio while the higher concentrations led to a significant increase for 7-days of treatment. Differing from 7-days exposure to AgNP, in the prolonged exposure time, chlorophyll a/b content was maintained for all the AgNP concentrations. 7-days of exposure to 0.05 and 0.5 mg L^{-1} of AgNP concentrations led to an increase in total carotenoid content by 34% and 35%, respectively, compared to the control group while no significant changes observed in total carotenoid content of the plants which were exposed to any concentrations of AgNP for 14-days.

Effects on growth reflect changes in the biochemical metabolism of the plant (Lalau et al., 2020). Since chlorophyll content is a significant biomarker that reflects the status of plant growth, the results may indicate AgNP effects on the photosynthetic metabolism. Chlorophyll a oxidation by stress factor was reversed in higher concentrations of AgNP in 7-days could be related with carotenoid content increment in the same concentrations. Carotenoids play a role in the protection of chlorophyll; they also act as antioxidants to scavenge free radicals and reduce the damage to the cell membrane and DNA by removing free radicals. (He et al., 2011, Chew and Park, 2004). Mirzajani et al. (2013) reported that a significant increase in carotenoid content in rice sprouts exposed to AgNP and suggested that plants use carotenoids to reduce the effects of ROS caused by AgNPs. While the chlorophyll a/b ratio and the amount of carotenoid increased in 7-days of AgNP application compared to the control group, the absence of a significant difference in 14 days can be explained as acclimation of *L. minor* plants to AgNP. This situation can be related to the fact that *L. minor* plant is a bioaccumulator plant with high capacity and thus it has developed a tolerance mechanism.

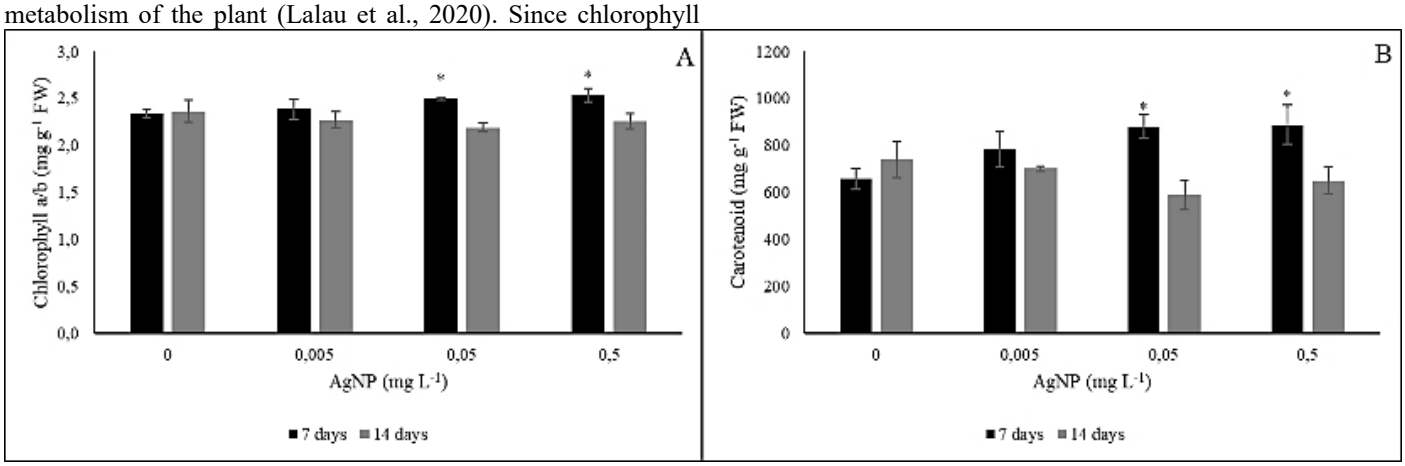

Figure 8. Chlorophyll a/b **(a)** and carotenoids content **(b)** of *L. minor* plants after the treatment with AgNP for 7 and 14 days. *Statistically different at $P < 0.05$ according to LSD test

4. Conclusions and Recommendations

Spherically shaped and 34 nm AgNPs which were phyto-synthesized by using *Laurus nobilis* showed lower toxicity on aquatic plant *L. minor* than chemically synthesized AgNPs based on low EC_{50} value. The result indicates that phyto-synthesized AgNP usage over chemically synthesized counterparts in proper fields may decrease potential AgNP toxicity risk for aquatic environment.

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