Evaluation of silver nanoparticles toxicity in *Daphnia magna*: Comparison of chemical and green biosynthetic productions

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Abstract

Recently nanoparticles, particularly silver nanoparticles, are broadly used in industry, hence the contamination of the environment with AgNPs has caused considerable concern. In this study, the toxicity of biosynthetic nanosilver produced by two macroalgae: *Sargassum boveanum* and *Ulva flexuosa* extracts were compared with chemical nanosilver in *Daphnia magna*. Size and quality of nanoparticles evaluated by TEM[1], FT-IR[2] spectrum, and Particle size analyzer. The acute toxicity test was evaluated following the OECD[3] and Test guideline No: 211. *D. magna* were reproduced using parthenogenesis from a single individual according to OECD guideline. Then *Daphnia* exposed to eight serial dilutions of each nanosilver in triplicates for 48 hours. The mortality rate after 12h, 24h, 36h, and 48h were recorded and analyzed using probit software.

Results showed that all nanosilver (regardless of their synthesis origin) were toxic in Daphnia and acute toxicity of this nanosilver was different ($p<0.05$). The 48h LC_{50} of SPN, UPN, and CPN in Daphnia were 1.03, 3.24 and 0.03 mg L^{-1} respectively. The mortality rate in *D. magna* enhanced in all tested groups, along with increasing nanosilver concentration and exposure time duration. Highest toxicity belongs to chemical nanosilver (LC_{50} = 0.03 mg L^{-1}), which was 30 and 100 times more toxic than SP (LC_{50} = 1.03) and UP (LC_{50} = 3.24 mg L^{-1}) respectively. According to the high toxicity of chemosynthetic nanosilver compare to biosynthetic ones, biosynthetic nanoparticles are highly recommendable and environmentally friendly alternative to chemical oriented nanoparticles.

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Keywords: Nanosilver, *Daphnia magna*, *Sargassum boveanum*, *Ulva flexuosa*, Biosynthetic.

[1] Transmission Electron Microscopy
[2] Frustrated Total Internal Reflection
[3] Organization for Economic Cooperation and Development

Introduction

Silver nanoparticles (AgNPs) are widely used as spectrally selective coatings for solar energy absorption, chemical catalysts and especially for antimicrobial sterilization. After their discharge, AgNPs will most likely enter the ecosystems and may produce a physiological reaction in various animals, maybe changing their health, and finally changing their densities or community populations. Open access literature regarding the toxicity of nanoparticles is still developing, and gaps still exist in our information of this area (Hedayati, Kolangi, Jahanbakhshi & Shaluei 2012).

Biosynthesis of metallic nanoparticles is a fairly innovative emerging extent of nanotechnology that has commercial and environmentally friendly advantages in compare to chemical and physical approaches to nanometal synthesis. With increasing interest in the minimization of waste and implementation of sustainable processes in the adoption of all important principles of 'green' chemistry, the development of eco-friendly, simple, and economical methods for the preparation of advanced materials is necessary. Moreover, biological synthesis of metal nanoparticles has several important advantages over chemical synthesis such as larger quantities and lower costs of production (Parikh, Singh, Prasad, Patole, Sastry & Shouche 2008).

Information on the use of extracts of marine algae to synthesize metallic nanoparticles, silver particularly is scant. Because of its abundance and ready availability, marine algae are good and economical sources of phytochemicals that can be exploited for the synthesis of metallic nanoparticles, but they have been less studied in comparison with other biosources such as plants and microbes. The increased popularity of these particles in marketable products is because of their discharge of free silver ions (Ag^+), that are identified to be antibacterial substances (Luoma, 2008). Recently, Wang, Chen, Li, Shao & Peijnenburg (2012) indicated quantitatively that Ag^+ contributed to the toxicity of nanosilver colloids in 3 aquatic species at dissimilar trophic stages due to alterations in mechanisms of action.

Due to antibacterial and antifungal properties of silver nanoparticles (AgNPs), they have been used broadly as a biocide, water purification, food packaging and in many individual care products (Sotiriou & Pratsinis, 2010). Previously, colloidal silver such as collargol (protein-stabilized nanosilver) was used for several medical purposes (Fung & Bowen, 1996). Benn & Westerhoff (2008) showed that AgNPs can be released into domestic wastewater by laundering of socks that are treated with AgNPs. According to Mueller & Nowack (2008), around 500 tons of nanosilver is produced each year. Therefore, due to its leaching from consumer products, besides through industrial waste streams, there is a high risk of environmental pollution by nanosilver. Up to now, the acute test design has been applied globally for the screening of possibly hazardous chemicals and the

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examination of industrial effluents. Benefits of using Daphnia sp. toxicity test methods contain: easy to use in a laboratory situation, well-established culturing and test procedure, strong toxicity database, economical observation regime, small sample volume and quality control (Baird, Barber, Bradley, Calow. & Soares 1989).

Daphnia magna, a freshwater zooplankton species, is a standard test organism for the toxicity protocols of the U.S. Environmental Protection Agency (EPA), International Standards Organization (ISO) and Organization for Economic Cooperation and Development (OECD 202 2004; OECD 211 2008). Comprehensive toxicity researches about *Daphnia* sp. were started and technique adjustment was advanced in the 1980s (Baird et al. 1989).

Sargassum boveanum is one of the species of marine plants that have distribution in the Indian Ocean (Iran, Saudi Arabia, Qatar and Kuwait) (silva, Basson & Moe 1996). The sea lettuces comprise the genus *Ulva*, a group of edible green algae that is widely distributed along the coasts of the world's oceans. *Ulva flexuosa* is a species of seaweed in Ulvaceae family that can be found worldwide (Asia, Africa, Europe, Americas) (Hardy & Guiry 2003, Burrows 1991). Sam, Palanichamy, Chellammal, Kalaiselvi & Subramanian (2015) reported that Biosynthesis of silver nanoparticles was done with 11 species macroalgae green, red and brown algae. *Ulva lactuca* is one of the best seaweed for biosynthesis of silver nanoparticles, is known to have antimicrobial effects and its toxicity (LC_{50}) for *Artemia salina* was 1µg mL^{-1}.

Many researchers stated that biosynthetic nanoparticles can be less toxic for aquatic animals and are more friendly to the environment (Sam et al. 2015; Kumar, Senthamil selvi, Lakshmi prabha, Selvaraj, Macklin Rani, Suganthi, Sarojini Devi & Govindaraju 2012). Then in this study, the acute toxicity of biosynthetic nanosilver's produced by seaweeds (Sargassum and Ulva) were compared with commercial chemosynthetic nanosilver.

Materials and Methods

Two methods were used for preparing silver nanoparticles:

1. Chemical nanoparticle (Trade name = Nanosid L2000) that purchased from Nanonasbe Pars co, Iran.
2. Biosynthetic nanosilver with seaweeds (*S. boveanum* and *U. flexuosa*) that were made in the laboratory. This process was done according to Kumar method in two different steps including:

Preparation of seaweed extract

The seaweed *S. boveanum* and *U. flexuosa* were collected manually from southeast offshore of Persian Gulf, Chabahar city, Sistan and Baluchestan province, Iran, and thoroughly washed in sea water to remove detritus and cleaned algae then washed in distilled Water and shade dried for five days. Fifty grams of powder sample was mixed into 1 liter of deionized water and the mixture was boiled for ten minutes. After cooling, the extract was

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filtered with Whatman no.1 filter paper. The extract was stored at 4 °C for further use.

Nanosilver biosynthesis

900 mL of 1mM silver nitrate (AgNO3, Nanonasb Pars co.) solution was prepared and added to 100 mL of seaweed extract in a conical flask. The extract was incubated at room temperature (25 ˚C) for 24 hours exposed to direct natural sunlight. The reaction solution color changes were observed for the characterization of silver nanoparticles. Then, biosynthetic nonosilvers were purified by centrifuging at 8,000 rpm and rinsed with redispersed with same aliquot of deionized water (Kumar, Senthamil Selvi & Govindaraju 2012). Transmission Electron Microscopic (SEM) analysis was done by using a transmission electron microscopy (Philips, CM-30) for characterizing size and shape of biosynthesized silver nanoparticles.

Evaluation of nanoparticles physicochemical specifications

Characteristics of chemosynthetic colloidal nanosilver (Nanocid L2000), was as follow nanosilver concentration 4000 mg L^{-1}, size of silver particles 8 ± 0.78 nm. Zeta potential 53/33 ± 7/86 Mv average, pH 4.2 and geometric mean diameter 12/65 ± 1/46 nm (Asghari, Johari, Lee, Kim, Jeon, Choi, Moon & Yu 2012).

Biosynthetic production of nanosilver was done using *S. boveanum* and *U. flexuosa* extraction and AgNO3 salt, then the size and quality of nanoparticles evaluated by Particle size analyzer apparatus (model: Scatterscope I quit), TEM (LEO, Co. model: 906E)[4].

Experimental design

Three silver nanoparticles including one chemical origin (CPN) and two biosynthetic origins (SPN and UPN) were evaluated and compared for their environmental toxicity using *D. magna* (environmental pollution indicator) through Organization for Economic Cooperation and Development (OECD), Test guideline No: 211. *D. magna* was purified using parthenogenesis in one individual according to the guideline. Then purified daphnia transferred to 6-Well Cell Culture Plates, exposed to eight concentration (Table 1) of each nanosilver in triplicates for 48 hours. Water temperature was 25 ± 1˚C.

Table 1. The selected concentrations of *Chemo* and Biosynthetic nanosilver to determine their acute toxicity for *D. magna*

Treatment	Total *D. magna*	Concentration (mg L^{-1})								
Chemosynthetic Nanosilver	30	0	0.006	0.013	0.025	0.05	0.1	0.2	0.4	1
Biosynthetic Nanosilver by *Sargassum*	30	0	0.5	1	2	3	4	5	10	_
Biosynthetic Nanosilver by *Ulva*	30	0	1	2	5	10	20	25	30	_

[4] Transmission Electron Microscopy

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Statistical analysis

Maximum Acceptable Concentration (MAC) determined for each nanosilver. The mortality rate after 12h, 24h, 36h, and 48h were recorded and analyzed using Probit analysis software, SPSS 16.0 version (Statistical software package - SPSS Inc., Chicago, IL, USA), then LC_{10}, LC_{50}, LC_{90} were measured to find the regression equation values. Results with $p<0.05$ were considered to be statistically significant.

Results

The results of TEM images of silver nanoparticles biosynthesized from *S. boveanum* showed shape spherical nanoparticles (Fig. 1). The apparatus of particle size analyzers estimated the average size of *S. boveanum* and *U. flexuosa* nanoparticles 2.9 nm and 5.39 nm, respectively (Table 2).

Acute toxicity of this nanosilver was different in *D. magna*. The LC_{50} in 48h of biosynthetic nanosilver (*S. boveanum* and *Ulva. Flexuosa* origin) and chemical nanosilver in Daphnia were 1.03, 3.24 and 0.03 mg L^{-1} respectively. The mortality rate in biosynthesized nanosilver from *U. flexuosa* (LC_{50} = 3.24 mg L^{-1}) was lower than other nanosilver and was 108 times lower than chemosynthetic nanoparticle (LC_{50} = 0.03 mg L^{-1}) (Fig 4). The mortality rate of *D. magna* enhanced in the chemosynthetic nanosilver, along with increasing concentration and exposure duration (Fig 2). In biosynthetic nanoparticles of Ulva (Fig 4), the maximum mortality rate at LC_{90} 12h was 25.72 mg L^{-1}, while in chemosynthetic nanoparticle was 1.32 mg L^{-1} (Fig. 2).

Table 2. The results of particle size of Chemo and Biosynthetic nanosilver product by two algae, *S. boveanum* and *U. Flexuosa*

Type of nanoparticles	Mean ± Span*	d (10)**	d (50)***	d (90)****
Biosynthetic Nanosilver by *S. boveanum*	2.9 ± 1.99	1.42	2.90	7.2
Biosynthetic Nanosilver by *U. flexuosa*	5.39 ± 0.98	3.49	5.39	8.77
Nanosilver L2000	7.1 ± 1.49	3.66	7.1	11.92

*Span shows how the emission of particles and is obtained from d50 (d90-d10) and has the role of standard deviation (STED) in statistical calculations.
**The average particle diameter of the first decile (10% of particles with the smallest diameter).
***The average particle diameter of the half (50%) of particles.
****The average particle diameter of the ninth decile (90% of particles).

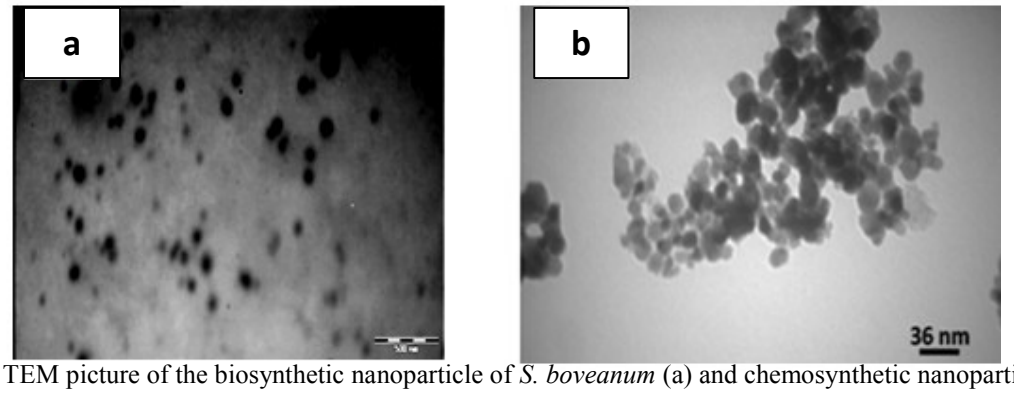

Figure 1. TEM picture of the biosynthetic nanoparticle of *S. boveanum* (a) and chemosynthetic nanoparticle (b).

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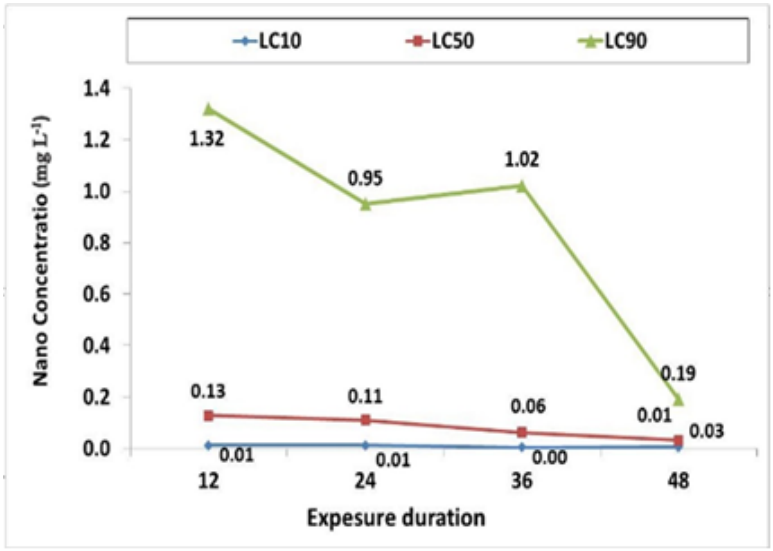

Figure 2. Mortality rate of *D. magna* in chemosynthetic nanosilver treatment.

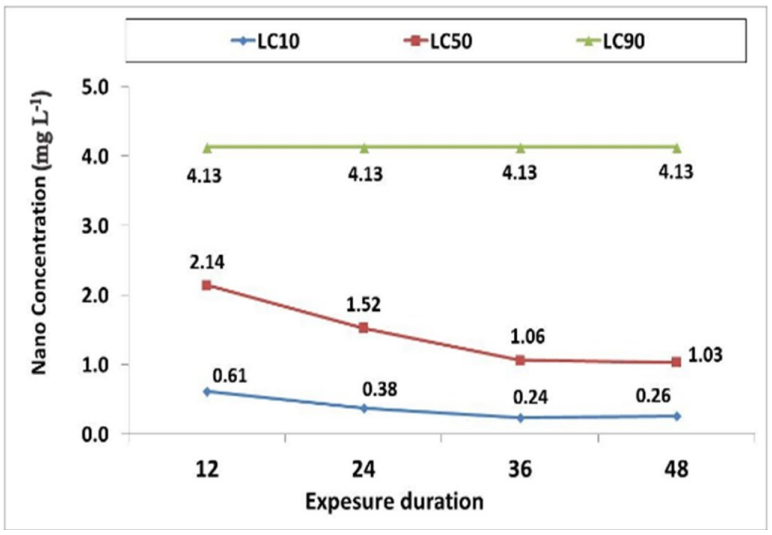

Figure 3. Mortality rate of *D. magna* in biosynthetic nanosilver treatment by *S. boveanum*.

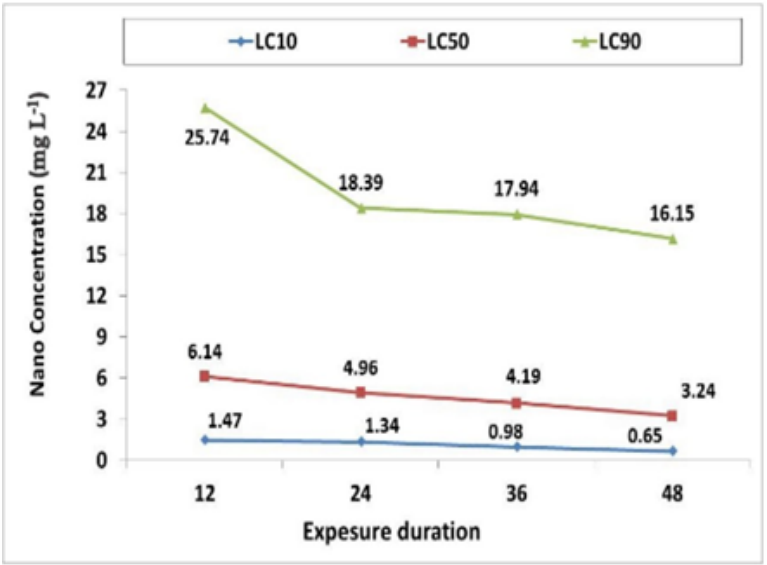

Figure 4. Mortality rate of *D. magna* in biosynthetic nanosilver treatment by *U. flexuosa.*

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Figure 5. Left picture is *D. magna* that absorbed nanosilver (note to accumulation of nanoparticles in gut), right picture is *D. magna* without nanosilver, (Taken by light microscope).

Discussion

One of the common methods of toxicity assessment test (Toxicity bioassay) is the usage of standard model organisms such as *Daphnia magna*, zebrafish and *Artemia spp.* (Nunes, Carvalho, Guilhermino & Stappen 2006). Both size biogenic (biosynthesized by *Sargassum boveanum*) and chemical of silver nanoparticles were in the range of 7 ± 1nm (fig1 b) and a spherical shape. Many types of research were founded that function of biosynthesized nanoparticles toxicity depends on the size, physicochemical properties (electrical charge, crystal structure, solubility, area surface material, shape, and morphology) and their in vivo behavioral (Lapresta-Ferna´ndez, Ferna´ndez & Blasco 2012; Gatoo et al. 2014). So basically, particle size influence distribution and penetration of nanoparticles in biological systems (Lovri´c, Bazzi, Cuie, Fortin, Winnik & Maysinger 2005) and future hazards of nanoparticles with sizes smaller than 10 nm, are more disturbing (Gatoo, Naseem, Arfat, Dar, Qasim & Zubair 2014).

Moreover, biosynthesis of silver nanoparticles extracts of *U. flexuosa* seaweed has medium size range 5.39 nm, the circular shape and the size of the average diameter of between 2 nm to 32 nm (15 ± 1/5). In addition, Vijayan, Santhiyagu, Singamuthu, Kumari Ahila, Jayaraman and Ethiraj (2014) were obtained the size and shape silver nanoparticles extract of *Turbinaria conoides* seaweed ranging between 2-17 nm and spherical shape respectively. Nanoparticles extracted from *U. flexuosa* showed the medium size of 5.39 nm (Fig. 1a) spherical shape (Fig. 2) while having consistent with the relevant studies.

Nano Particles can enter through the cell membrane by simple diffusion process or cell wall proteins and receptors. These materials create a membrane electrochemical gradient interference, cause disruption in electrons generation in the respiratory chains, which cause generation of ROS[5]. ROS cause lipid peroxidation, changes in proteins, antioxidant enzymes generation, DNA damages, and

[5] Reactive Oxygen Species

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ultimately creating a break in the path of ion transport, membrane changes and increasing its permeability, lead to entrance NPs to the cells inside, which lead to apoptosis and cell death (Lapresta-Ferna´ndez, Ferna´ndez & Blasco 2012). Hence the increase in mortality rates of *D. magna,* along with increasing the Nps concentration and exposure duration, can be caused by the production of ROS and oxidative stress effects.

Griffitt, Luo, Gao, Bonzongo & Barber (2008) reported that LC_{50} of silver nanoparticles (AgNPs) with size of 20 to 30 nm and spherical shape in two Daphnia species (adult *Daphnia pulex* and neonates *Ceriodaphnia dubia*) at 48 hours, were 0.040 and 0.067 mg L^{-1} while in silver metal solution (Ag^{+}) were 0.008 and 0.16 mg L^{-1} respectively. We were obtained LC_{50} of chemical silver nanoparticles (AgNPs) around 0.03 mg L^{-1} in *D. magna* at 48 hours (fig 3), while its value was close to adult *Daphnia pulex* (0.04 mg L^{-1}).

Kumar et al. (2012) assessed the effects of silver nanoparticles biosynthesized from extracts of *Sargassum ilicifolium* by *Artemia salina* mortality test. They reported LC_{50} at 48 hours 1.08 mg L^{-1} which its value is close to the toxicity of biosynthesized nanosilver from *Sargassum boveanum* in *D. magna* in our work (1.03 mg L^{-1}) (fig4).

Due to the test species, the toxicity of nanosilver is measured varied. For instance, the latest review by Kahru & Dubourguier (2010) revealed that AgNPs were toxic to algae and crustaceans even at very low concentration (EC50<1 mg L^{-1}), however the toxicity to protozoa was fairly low, EC5 = 40 mgAg L^{-1}. The mechanism of Ag^{+} toxicity in aquatic organisms is ion regulatory disturbance or failure related to competitive or noncompetitive inhibition of sodium or potassium ion-dependent adenosine triphosphates (Na^{+}, K^{+}-ATPase) activity. This inhibits Na^{+} uptake at the gills, that leads to an arrangement of actions ending in cardiac arrest and death (Hogstrand & Wood 1998, Bianchini & Wood 2003) Zhao and Wang, (2013) indicated that soluble Ag^{+} released from AgNPs through preventing sodium uptake was toxic to *D. magna*. Moreover, Shen, Zhou, Yang, Chao, Liu & Liu (2015) were calculated the LC_{50} of AgNO3 in *Daphnia magna* at around 0.58–2.51 µg L^{-1}. which is lower than our results. This different results can be related to the difference in Daphnia strain, water physicochemical parametersicle and even nanoparticles characteristics. Ribeiro, Gallego-Urrea, Jurkschat, Crossley, Hassellöv, Taylor, Soares & Loureiro (2014) were calculated 24 h LC_{50} of AgNP and AgNO3 in *D. magna* as: 11.41 µg L^{-1} and 1.36 µg L^{-1}, respectively, while the 48 h-LC_{50} for AgNP and AgNO3 were 11.02 µg $L^{-1}$and 1.05 µg L^{-1}, respectively.

Kumar et al. (2012) showed that *Artemia salina* and heterotroph bacteria have less mortality in the silver nanoparticle biosynthesized by *Sargassum iliifolium.* In the current research, we obtained that less mortality in the silver nanoparticle biosynthesized by S. *boveanum* and *U. flexuosa* on the *D. magna* (fig 4 and 5).

Zhao & wang (2012) have compared the toxicity of three surface coated nanosilver's including lactate, polyvinylpyrrolidone, and sodium dodecyl benzene sulfonate nanosilver (as

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AgNPs-L, AgNPs-P and AgNPs-S, respectively) in *Daphnia magna*. They reported LC_{50} 48h concentration of AgNPs-L, AgNPs-P and AgNPs-S around 28.7, 2.0 and 1.1 µg L^{-1} respectively, While AgNPs showed less mortality rate. In this research calculated AgNPs of *Sargassum boveanum* (0.03 mg L^{-1}) (fig 4) was similar to AgNPs lactate (28.7 µg L^{-1}).

As a conclusion, nanosilver from *U. flexuosa* was less toxic and more environmental friendly than AgNPs synthesized from *Sargassum boveanum,* although both biosynthesized AgNPs showed significant lower toxicity than chemical nanosilver, then it is highly recommended to produce biosynthesized nanoparticles from marine algae as a proper alternative to chemical nanosilver.

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بررسی سمیت نانوذرات نقره در دافنی ماگنا (*Daphnia magna*): مقایسه تولید شیمیایی و سنتز سبز

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چکیده

اخیراً نانوذرات به ویژه نانو ذرات نقره (AgNPs) به طور گسترده در صنعت استفاده می‌شود، از این رو آلودگی محیط زیست با نانو ذرات نقره موجب نگرانی زیادی شده است. در این مطالعه سمیت نانوذرات نقره بیوسنتزی تولید شده توسط دو ماکروجلبک، سارگاسوم بوونوم و اولوا فلکسوزا با نانو نقره شیمیایی در دافنی ماگنا مقایسه شدند. اندازه و کیفیت نانوذرات توسط TEM، FT-IR و تجزیه کننده اندازه ذرات ارزیابی گردیدند. آزمون سمیت حاد با استفاده از OECD و راهنمای تست ۲۱۱ بررسی شد. دافنی ماگنا به روش بکرزایی از یک دافنی با توجه به استاندارد OECD تکثیر داده شد. سپس دافنی‌ها در معرض هشت رقت متوالی نانونقره در سه تکرار به مدت ۴۸ ساعت قرار گرفتند. میزان مرگ و میر پس ۱۲، ۲۴، ۳۶ و ۴۸ ساعت ثبت شد و با استفاده از نرم افزار پروبیت مورد تجزیه و تحلیل قرار گرفت. نتایج نشان داد که تمام نانوذرات نقره (بدون در نظر گرفتن منشاء سنتز آن‌ها) در دافنی سمی و سمیت حاد آن‌ها متفاوت بود. LC_{50} ۴۸ ساعته نانو ذرات بیوسنتزی سارگاسوم، اولوا و نانو نقره شیمیایی در دافنی به ترتیب ۱/۰۳، ۳/۲۴ و ۰/۰۳ میلی‌گرم در لیتر بود. میزان مرگ و میر در دافنی ماگنا همراه با افزایش غلظت نانو ذرات نقره و مدت زمان در معرض قرار گرفتن در تمام گروه‌ها، افزایش یافت. بالاترین سمیت متعلق در گروه نانو نقره شیمیایی (LC_{50}=۰/۰۳ میلی‌گرم در لیتر) مشاهده گردید که به ترتیب ۳۰ و ۱۰۰ برابر از نانو ذرات بیوسنتزی *سارگاسوم بوونوم* (LC_{50}=۱/۰۳) و *اولوا فلکسوزا* (LC_{50}=۳/۲۴) بیشتر بود. با توجه به سمیت بالای نانو ذرات نقره شیمیایی نسبت به بیوسنتزی، جایگزین شدن نانوذرات بیوسنتزی به دلیل دوستدار محیط زیست بودن پیشنهاد می‌گردد.

کلمات کلیدی: نانو نقره، *دافنی ماگنا*، *سارگاسوم بوونوم*، *اولوا فلکسوزا*، بیوسنتز.

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