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Research Article

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Toxicity study of silver nanoparticles synthesized using seaweed *Sargassum angustifolium* in common carp, *Cyprinus carpio*

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ABSTRACT

In this study, the lethal concentration and acute toxicity of silver nanoparticles synthesized using seaweed Sargassum angustifolium that is produced by biological methods were investigated at static renewal condition during 96 hours in common carp in accordance with standard methods OECD. TEM analysis showed that the average size of the bio nanoparticles were found to be 32.54 nm and spherical in shape. Since the exact amount of lethal concentration of this silver nanoparticle has not been determined in fish previously, at first determining the lethal concentration range, then the preliminary experiments was carried out at several concentrations. Once the lethal concentration, LC50 tests were done at different concentrations. The results showed that the LC50 at 24, 48, 72 and 96-h after exposure were 79.54 ± 0.007, 52.1 7 ± 0.006, 30.62 ± 0.008 and 11.34 ± 0.016 mg/L res pectively. According to these results, the mortality rates of common carp showed an increasing trend with increasing concentration and exposure time, that is indicating the toxicity of biological synthesized AgNP in high concentration for common carp.

Keywords: toxicity, silver nanoparticles, seaweed, common carp

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INTRODUCTION

In recent years, advancement of technology from micro [1] to nanoscales [2], many theoretical phenomena [3] have found their importance in emerging applications in chemical engineering [4]. One of these phenomena is the using nanoparticles [5] that could be controllable by MHD forces [6-10] in microchannel [11] and micro polar fluids [12]. Many applications are open to this area such as power plant industry [13-20], heat conversion [21-25] and management [26-28] ,and tribology [29-35].

Among nanoparticles, the silver nanoparticles (AgNps) are widely investigated because of wide range applications such as antibacterial, catalyst, medical devices, photonics, optoelectronics and biosensors [36-37]. Usually metallic nanoparticles are synthesized by chemical, mechanical and electrochemical methods [38]. In these methods for the synthesis of silver nanoparticles used toxic chemicals compounds that can have negative effects on the environment and water ecosystems. Due to the devastating effects of these methods on the marine environment, at present several methods for the synthesis of these nanomaterials are necessary. One of these methods that's more compatible with the environment and create less pollution, are biological methods. In this method to synthesis metal nanoparticles, used microorganism and plant materials that exist in nature instead of toxic chemicals [39]. One of the resources that can be used in nanotechnology and synthesis of nanoparticles are seaweeds that have variety types of phytochemical compounds such as proteins, carbohydrates, alkaloids, steroids, phenols, saponins and flavonoids [40] play key role in bio reduction of the metal ions into Nano form. Toxicity of silver ion has been known for centuries but silver nanoparticles toxicity may be dependent on particle concentration, particle size and shape and surface chemistry

[41]. The common carp (*Cyprinus carpio*) is species of cyprinidae family, native to Asia and a high economic importance farming fish in khuzestaan province of Iran. According to our knowledge the toxicity of silver nanoparticles synthesized using chemicals methods is better understood than silver nanoparticles synthesized using biological methods because the most of the current research on the toxicity to aquatic organisms has focused on chemical synthesized nanomaterials. In recent years, several studies have been conducted regarding to the effect of silver nanoparticles synthesized by chemical methods on fish toxicity by Alishahi et al. [42], Asharani et al. [43], and Bilberg et al. [44-45]. The present study is the first study on the toxicity of silver nanoparticles synthesized using biological methods on common carp. The purpose of this study is biological synthesis of silver nanoparticles using seaweed *Sargassum angustifolium* and determines its toxicity in common carp.

EXPERIMENTAL SECTION

Seaweeds were collected from the intertidal region of Bushehr coast, Iran. The samples were brought to laboratory, cleaned and washed thrice with fresh water followed twice by distilled water to remove the adhering salts and other associated contaminants. Then the shade-dried was used for 5 days and powdered using mixer grinder [46]. Later, aqueous extract was prepared by dissolving 10g of powdered seaweed in 100ml of sterile distilled water. The mixture was heated at 60°C for 10mins, centrifuged twice at 4,000 rpm for 25mins and filtered through Whatmann no. 1 filter paper. For the biosynthesis of silver nanoparticles, 90ml of 1mM silver nitrate (AgNO3) was added to 10ml of seaweed extract [47]. After that a color change from yellowish brown to reddish or blackish brown, visually confirms the formation of AgNPs [47]. Also the sample have been characterized for the construction of silver nanoparticles by using Ultra Violet –Visible Spectrum (Perkin-Elmer *Uv*-Vis, Lambda 12) , Transmission Electron Microscopy (LEO 906E).

Juvenile specimens of common carp with a mean total body weight of 50-100g were purchased from a commercial farm of Shooshtar, Iran and transferred to laboratory. For adaptation, fish were kept in 150L aquarium at temperature ranging between 24 and 25^0C, with natural light/dark cycle for 2 weeks and fed twice per day with commercial diet at a rate of 2% of their body weight in advance of exposure. After end of adaptation period, all fish were fasted during exposure and then for each treatment 15 (5 fish in each replicates) healthy fish were kept in 50L aquaria with aeration. Toxicity test of manufactured silver nanoparticles were investigated at static renewal (renewed every 24h) condition for 96h according to standard methods OECD [48]. Since the exact amount of lethal concentration of this silver nanoparticle has not been determined in fish, at first to determining the lethal concentration range, preliminary experiments (14 treatment with 3 replicates) were carried out at several concentrations (0.5, 1, 2.5, 5, 15, 25, 35, 45, 55, 65, 75, 85, 95, 105 mg/L). Control group was kept in dechlorinated tap water without any add-on material. After preliminary experiment, LC50 test were examined with different AgNP treatment groups (2.5, 5, 10, 20, 40, and 80 mg/L) in 3 replicate for each treatment. During the 96-h static exposure, dead animals were recorded at 24, 48, 72 and 96h for the calculation of median lethal concentration (LC50) values.

Then LC_{50} values were calculated from the data obtained in acute toxicity bioassays, by Finney's method of ''probit analysis'' [49] and with SPSS computer statistical software. The $LC_{1,10,30,50,70,90,99}$ values (with 95% confidence limits) were derived using simple substitution probit (a unit of probability based on deviation from the mean of a standard distribution.) of 1,10,30,50,70,90 and 99 respectively for probit of mortality in the regression equations of probit of mortality vs. synthesized silver nanoparticles. Maximum acceptable concentrations calculated based on the proposed formula T.R.C. (1984) (LC50 96h divided by 10).

RESULTS AND DISCUSSION

After adding silver nitrate to *Sargassum anustifolium* extract, the brownish-yellow color in mixture turned in to dark brown color after 110 min (Fig 1). To make sure the synthesis of silver nanoparticles, nanoparticle absorption peak was measured using UV-visible spectrometer (UV-Vis) in the wavelength range of 200-700 nm. The best peak was observed after 2h of reaction time in the range of 406 nm, which corresponds to plasmon excitation of the AgNPs.

Fig.1. Change of color from pale yellow to dark brown by the addition of silver nitrate

The peak formed in this range represented reduction of silver ions and after synthesis of silver nanoparticles using extracts of seaweed *Sargassum* (Fig 2).

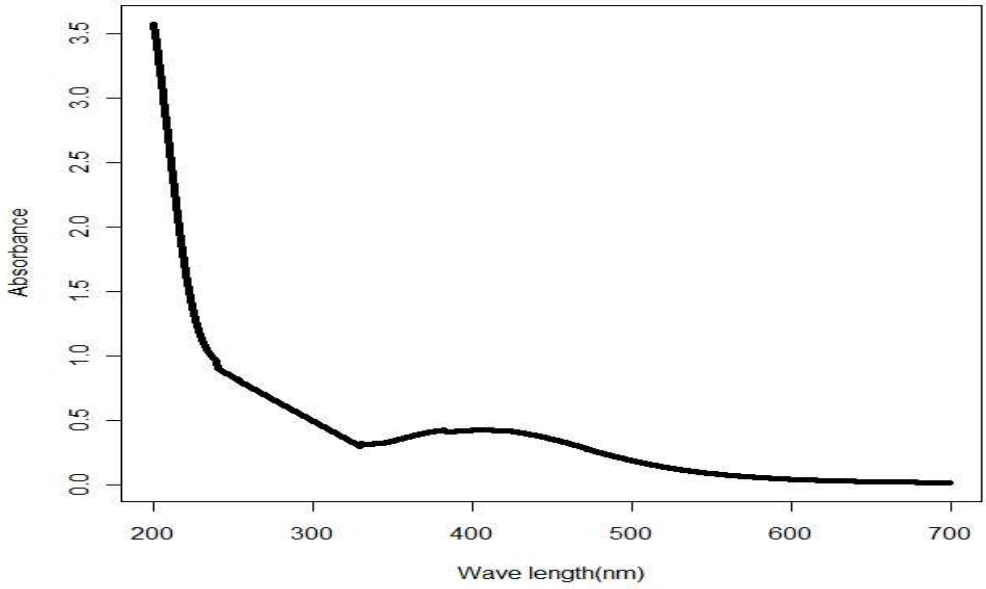

Fig. 2. UV-Vis absorption spectrum of silver nanoparticles synthesized by treating 1mM AgNO3 solution with *Sargassum angustifolium* extract

According to the TEM analysis, average size of synthesized silver nanoparticles was 32.54nm and predominately spherical in shape (Fig 3).

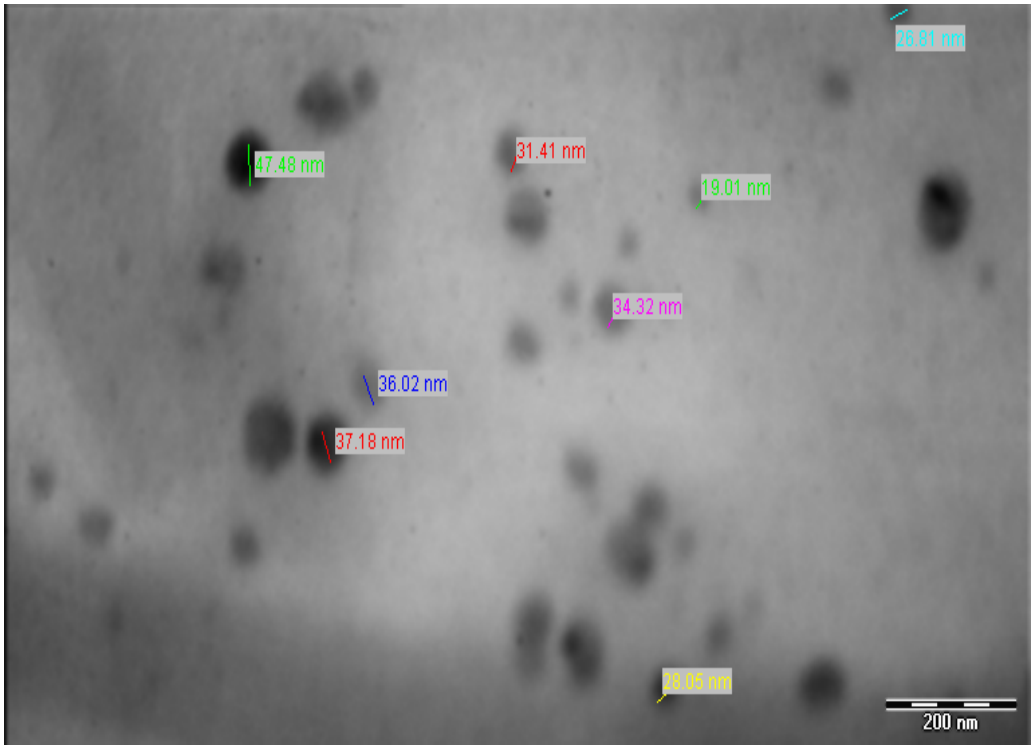

Fig. 3. TEM images of AgNPs synthesized by *Sargassum angustifolium*

As shown in Figure 3, apart from the perfect spherical shape and size all particles dispersion was good and well–distributed in solution and is not in contact with each other.

According to preliminary test results that performed in ascending concentrations of 0.5, 1, 2.5, 5, 15, 25, 35, 45, 55, 65, 75, 85, 95 and 105 mg/L AgNP, first mortality in fish exposed in high concentration were observed after 7h. At concentrations of 85, 95 and 105mg/L, all fish were dead after 16, 18 and 68h respectively. According to experimental test, lethality range of these nanoparticles in common carp was determined 2.5-80 mg/L. Therefore LC50 test were studied at concentrations of 2.5, 5, 10, 20, 40 and 80 mg/L. Fish mortality during 24, 48, 72 and 96h of exposure were recorded. The results showed that fish mortality increased with increasing concentration and exposure time. After exposure to high concentrations (105 and 95 mg/L) fish were showed immediately some abnormal behaviors such as gill cover movements, abnormal swimming and jumping out of the water and their activity were reduced gradually and stayed at the floor of water in steady state, then came to surface of water and lost their balance finally died. The dead fish has a natural color. In control treatment, all fish showed normal behaviors and any signs of abnormal behavior were not observed. Also at low concentrations abnormal behaviors were lower than higher concentrations.

The first mortality was observed at 24h in a concentration of 40 mg/L. The major losses were observed at high concentration of AgNP (table 1). The concentration of 80mg/L after 96 hours of exposure, 100% mortality was observed. At a concentration of 2.5mg/L, the lowest concentration for LC50 were determined in this study, mortality have been observed simply at 96h after exposure to silver nanoparticles. The results showed that the LC50 levels in common carp after exposure to silver nanoparticles synthesized from seaweed Sargassum at 24, 48, 72 and 96h were 79.54 ± 0.007, 52.17 ± 0.006, 30.62 ± 0.008 and 11.34 ± 0.016 mg/L respectively. The values of LC10, LC50 and LC90 within 96 hours were determined 0.75 ± 0.016, 11.34 ± 0.016 and 28.42 ± 0.016 mg/L respectively, which represents more toxic at higher concentrations of the nanoparticles than lower concentrations. According to Table 2 the LC50 level was decreased with increasing exposure time (LC50-24= 79.54 ± 0.007, LC50-96= 11.34 ± 0.016), which indicates the lower concentration of silver nanoparticles are needed for 50% fish mortality with increasing in time exposure.

Table 1. Mortality rate in common carp exposed to different concentrations of silver nanoparticles at different times

Concentration (ppm)	No. of mortality			
	24h	48h	72h	96h
Control	0	0	0	0
2.5	0	0	0	3
5	0	0	2	7
10	0	1	4	9
20	0	3	7	12
40	5	8	11	14
80	6	11	14	15

Table 2. Lethal Concentrations (LC_{1-99}) of synthesis silver nanoparticles (mean ± Standard Error) depending on time (24-96h) for common carp

Point	Concentration (ppm) (95 % of confidence limits)			
	24h	48h	72h	96h
LC_{10}	36.06 ± 0.007	15.84 ± 0.006	1.50 ± 0.008	0.75 ± 0.016
LC_{30}	61.75 ± 0.007	37.30 ± 0.006	18.70 ± 0.008	4.35 ± 0.016
LC_{50}	**79.54 ± 0.007**	**52.17± 0.006**	**30.62 ± 0.008**	**11.34 ± 0.016**
LC_{70}	97.34 ± 0.007	67.03 ± 0.006	42.54 ± 0.008	18.32 ± 0.016
LC_{90}	123.03± 0.007	88.49 ± 0.006	59.75 ± 0.008	28.42 ± 0.016
LC_{99}	158.48 ± 0.007	118.10 ± 0.006	83.49 ± 0.008	42.34 ± 0.016

The maximum acceptable toxicant concentration (MATC) of silver nanoparticles for common carp at intervals of 24, 48, 72 and 96 hours was determined 7.95, 5.22, 3.06 and 1.13mg/L respectively. The lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) of silver nanoparticles with maximum allowable toxicant concentration *(*MATC*)* of LC50 values were calculated and compared to the results of a study of the factors in the LC50 as follows:

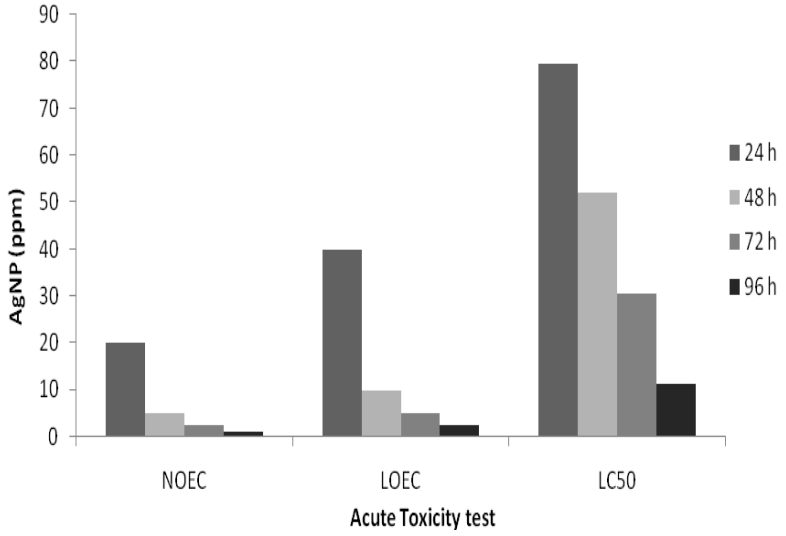

Fig. 4. Acute toxicity testing statistical endpoints of AgNP

Table 3. Values LOEC, NOEC and MATC of silver nanoparticles synthesized from seaweed *Sargassum angustifolium* in common carp

LOEC (mg/L)	NOEC (mg/L)	MATC (mg/L)
2.5	1	**1.13**

Toxicity of synthesized silver nanoparticles by chemical methods on fish and other aquatic animals has been confirmed in many studies by various researchers [50-51]. Recently, synthesis of silver nanoparticles using plants and marine macro algae to adapt this approach to the environment, is getting more popular [52-53]. Therefore, understanding the toxicity of silver nanoparticles synthesized using biological methods is very important. In this study, extracellular biosynthesis of silver nanoparticles using aqueous extract of seaweed, *Sargassum angustifolium*, was performed. Silver nanoparticles were formed by adding *Sargassum angustifolium* extract to 1 mM silver nitrate, after that the colorless solution of AgNO3 turned into dark brown color, indicating the reduction of Ag^{+} into Ag° and formation of silver nanoparticles. Reduction of silver ions in the mixture is done by some compounds in seaweed including flavonoids, enzymes, proteins, etc [39]. It is well known that the color of silver nanoparticles synthesized using brown seaweed, gradually turned in to brown or dark reddish color [47]. Therefore, in this study,

the solution color change from brownish pale yellow to dark brown that indicates the formation of silver nanoparticles (Fig. 1). In this study, after 2 hours of reaction, nanoparticle absorption peak wavelength was detected at 406nm (Fig. 2). This peak represents reduction of silver ions and forming the silver nanoparticles using extracts of seaweed *Sargassum angustifolium*. Singaravelu et al. [46] carried out the synthesis of gold nanoparticles using extracts of seaweed *Sargassum wightii* within 24h of incubation. In a study by Kumar et al. [39] synthesis of silver nanoparticles using extracts of seaweed *Sargassum tenerrimum* done within 20 minutes, which is visible through color changing. According to TEM images, nanoparticles have an average size of 32.54nm and spherical in shape. The suitable distribution of particles in solution in TEM images indicating the role of protein involved in the biosynthesis of silver nanoparticles [54]. In preliminary toxicity experiments, first mortality was started in 7 hours after exposure at highest concentration (105 mg/L). In the acute toxicity test by WU and Zhou [55] in medaka fish that exposed to silver nanoparticles with 29.9 nm average size, all the fish died after 12h at concentration of 4.8 mg/L and the value of LC50-96 was determined 0.87 mg/L. In this study LC50 value in common carp after exposure to silver nanoparticles synthesized from seaweed at 24, 48, 72 and 96h, were determined 79.54 ± 0.007, 52.17 ± 0.006, 30.62 ± 0.008 and 11.34 ± 0.016 mg/L respectively. In a study by Bilberg et al [44] in zebra fish LC50-48 h of silver nanoparticles was in the range of micrograms per liter (84 micrograms per liter), which indicates greater toxicity of nanoparticles synthesis by chemical methods compared to this research, because in the synthesis of silver nanoparticles using chemical methods, several chemicals used that are very toxic to environment, in addition, the toxicity of nanoparticles can vary according to the species tested [56]. LC50 results at different times showed that in the first 24h of exposure, LC50 value is always higher than the end of the 96h. Therefore it can be suggested that the duration of exposure to silver nanoparticles has been also one of the factors affecting toxicity in common carp which indicating that when the fish is exposed to constant concentration of silver nanoparticles with time, nanoparticles will have more opportunity to influence fish. In various studies, the toxicity of silver nanoparticles in different fish species was reported different [44,55]. Asharani et al. [43] were reported the LC50 value of silver nanoparticles in zebra embroy, 20-50 mg/L at 72 h. Alishahi et al. [42] were reported the LC50 value of silver nanoparticles in four species of *Cyprinus carpio*, *Barbus barbulus*, *Herotilapia multispinosa* and *Poecilia reticulata* guppies, respectively, 1.12, 0.77, 5.7 and 7.35 µg/cc. Shahbazzadeh et al. [57], LC50 96h of silver nanoparticles for rainbow trout fry was determined 2.3 mg/L. In a study by soltani et al [58], LC50 value of rainbow trout during 96h of chemical synthesized silver nanoparticle was determined 5 mg/L. LC50 value of common carp exposed in two different size of silver nanoparticles (Nanosil (less than 100 nm) and Nanocid (18nm)) were 73.8 and 0.43mg/L respectively [59]. One of the reasons for the LC50 differences is differences in the size of nanoparticles. In a study by Johari et al. [60] LC50 value of silver nanoparticles with average size 16.6nm in embryos, larvae and juvenile of rainbow trout were reported 0.25, 0.71 and 2.16 mg/L respectively. The toxicity of nanoparticles not only depend on the chemical form, particle size and synthesis method but also a variety of other factors such as the type of species, physiological state, nutrition and dietary interactions, and route of administration [56]. Therefore it is necessary that the toxicity of nanoparticles in order to have a better understanding of their effects on the aquatic environment that should be assessed separately in different species. In the present study the different species of fish, the synthesis method of examined nanoparticle was different compared with other studies, there it is expected that the results of the present study will be vary in toxicity with other studies.

CONCLUSION

Toxicity results in this study compared with other researchers on common carp [42, 59] have been shown that silver nanoparticles synthesized using biological methods are less toxic than chemically synthesized nanoparticles. One of the other reason for the differences in toxicity, in addition to differences in particle size, condition and size of the fish species, can be due to greater stability of biologically synthesized silver nanoparticles that have many biomolecules compounds and resulting in less release of silver ions in water. Also for biological synthesized nanoparticles toxicity is generally lower. In some studies higher toxicity of silver nanoparticles compared with silver ions in addition to the physicochemical properties of the particles have been reported resulting the release of silver ions from silver nanoparticles [61-62]. Comparison of mortality in fish exposed to silver nanoparticles treatments with a control group that did not cause any mortality. It shows that the only cause of fish mortality in the different treatments was the addition of silver nanoparticles in water. Results LC1-99 at 96h obtained in the present study at different times show a direct correlation between toxicity and concentration of silver nanoparticles (LC1-96: -19/67 and LC99-96: 42/34 mg/L). As well as according probit analysis, the upper and lower 50% lethal concentration of silver nanoparticles within 96h with 95% confidence were calculated respectively 24.90 ± 0.016 and 2.5 ± 0.016 mg/L. Behavioral signs including of extreme activity of fishes, jumping from the water immediately after exposure to high concentrations of silver nanoparticles suggest that the extreme stress and rapid toxic effects of silver nanoparticles at high concentrations compared with low concentrations. So fish in response to these factors and escape from this condition exhibit such behavioral signs. In a study conducted by the Bilberg et al. [44] on the zebra fish exposed to silver nanoparticles, abnormal behavioral signs and stress appears immediately 30min after exposure, reason for this is cited due to the rapid effect of silver nanoparticle toxicity. So that the fish remained

motionless at the bottom of the tank and their breathing rate increased and then come to the water column, they lost balance and fell on the floor. Also some fish before loss of balance exhibit jump and swim rotational movements showed that the behavioral symptoms are somewhat similar to this study.

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