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PROOF
RESEARCH PAPER

Acute Toxicity of Zinc Oxide Nanoparticles from *Satureja hortensis* on Rainbow Trout (*Oncorhynchus mykiss)*

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Abstract

Nanoparticles are commonly synthesized chemically and because of their risk for living organisms and environment, safer synthesis methods are highly considered. This work was carried out to evaluate the acute toxicity of zinc oxide nanoparticles synthesized from *Satureja hortensis* on rainbow trout (*Oncorhynchus mykiss)* by static bioassay test. The average weight and length of trout fish used in the study were 20±3 g and 15±2 cm respectively. Three groups of the experimented fish were exposed to different concentrations (1, 10 and 100 mg/L) of ZnO nanoparticles. All the exposed fish were daily observed and dead fishes were immediately removed and recorded. The obtained data were statistically analyzed using probit regression method. The LC_{50} value at 96 h was found to be 25.50 mg/L for trout fish which makes this nanoparticle as a low toxicity substance in terms of toxicity classification. According to the results lifetime reduced with increasing the concentration of ZnO. Based on the obtained LC_{50} for the synthesized ZnO from Satureja plant in this study and comparison with that of the chemically fabricated in others work, it was revealed that the green nanoparticles synthesis is much less hazardous and a good alternative technique.

Introduction

In last decade, nanotechnology has been an attractive subject in nearly all fields from research to production and application. But the aspect of nanomaterials impact on human and animals has not been widely considered in many part of the world (Krysanov, Pavlov, Demidova, & Dgebuadze, 2010).

One of the most important nanoparticle is zinc oxide (ZnO) that is widely used in industries as an additive in various products (Fakhari, Jamzad, & Kabiri Fard, 2019). The increased production of nano-products results in an increase in their release to environment, particularly aquatic environments (Keller, McFerran, Lazareva, & Suh, 2013; Skjolding *et al.,* 2016). Hence, assessment the risk of these nano materials and especially their toxicity is of great importance and necessity (Skjolding *et al.,* 2016). Inadequate or lack khorFof data on the fate of nanoparticles in aquatic environments and also their interaction with aquatic life have raised serious concerns (Ahmed, Chaudhry, & Ikram, 2017). In recent years, the production of nanoparticles has become an attractive topic in science and industry (Fakhari *et al.,* 2019). In most cases, chemical methods are used to synthesize nanoparticles. These chemically synthesized nanomaterials are adversely harmful for human health and the environment (Ahmed *et al.,* 2017; Roy & Barik, 2010).

Given this issue, the demand for the production of nanoparticles with safer methods, such as biological methods (also known as green method), is increasing (Agarwal, Kumar, & Rajeshkumar, 2017; Song & Kim, 2009). In these methods, plants or microorganisms or substances already exist in nature are commonly applied for nanoparticles synthesis (Agarwal *et al.,* 2017). Iran is very diverse in terms of the native flora and more studies are needed in this regard (Baharara J & T., 2014). One of Iran's most abundant herbs is savory (Satureja) plant, which is widely used in food and pharmaceutical industries and plays a special role in traditional medicine. It mainly contains carvacrol, thymol, parasmine and terpinene compounds (nooshkam. A, majonhoseini. N, hadian. J, jahansos.M, & khavazi, 2015). Although the savory is considered to be among the most important medicinal plants, but very few studies on nanoparticle synthesis from this plant, the toxicity and safety in the environment has been reported before (Afshar & Sedaghat, 2016).

Since the routine physical and chemical analysis cannot completely reveal the adverse effects of environmental pollutants, bioassay tests are commonly used to determine their toxicity (Clesceria, Greenberg, & and Eaton, 1998). These tests are generally used to find out the concentrations that can cause mortality in half of the examined organisms over a specific period of time (Prinsloo *et al.,* 2017). These tests indicate that which pollutants have the greatest potential to impact on the environment and whether the pollutants exceed the standard limits or not (Azimi. Z, Sadeghpor, Porgholami, & Rofchai, 2015). Nowadays, for a variety of reasons, sea products play a significant role in supplying human food. As the nutritional value of sea foods are recognized, they receive more attention and are more consumed (Ardebili, 2017). Among sea products, fish is of great importance because of its high economic value and sensitivity to pollutants. Rainbow trout is among the most important cold-water fish belonging to the Salmonidae family and is one of the main species of fish cultivated in Iran (Kamani, Mortazavi, Safari, & Mehraban, 2016). Apart from its food value, this fish is also economically important. Studies have shown that salmon and trout farming projects in different provinces of Iran are considered as one of the most important production sectors contributing to job creation (Hoseinpour, Souri, Ghaemi, & 1, 2017).

Rainbow trout fish is one of the most sensitive species to presence of contaminants in water even at low concentrations. Therefore, it can be considered as an appropriate indicator for pollutants assessment (Khodabakhshi, Porbagher, & Hosseini, 2017). Given the mentioned issues regarding to the importance of nanoparticles in industries, as well as the economic and nutritional importance of salmon and trout in our lives, this study aimed to synthesize green zinc oxide nanoparticles from the savory plant and assess its toxicity.

Materials and Methods

In this work, zinc oxide nanoparticle was prepared biologically from the *satureja plant* as follows. At first the savory plant was purchased from farms around Khorramabad, Iran. After confirmation the characteristics by the University Herbarium, the leaves of the plant were removed and dried in shade and then crushed.

Biosynthesis of ZnO Nanoparticles and Characterization

Biosynthesis of ZnO nanoparticles of S. *hortensis* was mainly prepared according to the method presented Arinjoy Datta (Datta *et al.,* 2017). To prepare the plant extract, 100 ml of double- distilled water and 10 g of dried savory plant were put into a beaker and then the beaker was put in an electric stirrer (speed of 300 rpm) at 150°C and a for 1 h. After cooling down, the extract was filtered using a Whatman paper filter (No 40) at room temperature and finally a uniform red solution was obtained. To synthesize zinc oxide nanoparticles, 100 mL of the savory extract was mixed with 7 g of zinc nitrate and 10 mL of sodium hydroxide, then the mixture was placed in an electric stirrer with a speed of 400 rpm at 150°C. Subsequently, the samples were centrifuged at 5,000 rpm for 5 min. The obtained precipitate was dried at 85°C for 90 min. The dried samples were then put into a porcelain mortar and completely crushed. The obtained powder was passed through a sieve (with a pore size of .015 mm) to complete the nanoparticle synthesis (Figure 1). The powder samples were analyzed and characterized using UV-vis (T80-UV-vis, England), Scanning electron microscope (SEM) (FE-SEM, tescan company, Czech Republic), energy-dispersive X-ray analysis (EDX) (mira3-tescan device, Czech Republic), and X-ray diffraction (XRD) (Stoe company, Germany).

Experimental Fish and Acclimatization

Rainbow trout *(Oncorhynchus mykiss*) was purchased from a fish cultivation filed around Khorramabad city, Iran. The weight of the fish was 20±3 g and their mean length was 15±2 cm. For proper transferring the fish to the laboratory, a 60-L plastic container equipped with an air pump, and an oxygen cylinder was used. Furthermore, to control the water temperature during the transferring, ice pieces were used.

Water Analysis and Quality

The water used in this study was from public drinking water of Khorramabad city. Water was aerated for three days (72 h) in order to complete removed of residual chlorine (Jahanbakhshi, Hedayati, Pirbeigi, & Javadimoosavi, 2015). Before the experiments, water quality was analyzed for common quality parameters using standard methods (Clesceria *et al.,* 1998) as shown in Table 1.

Data Analysis

Data analysis was carried out using, probit regression method) SPSS -19)(trial version) and the graphs were presented by SPSS and MS-Excel.

Acute Toxicity Test

In laboratory, the fish were kept for three days for adaptation to new lab condition. Experiments were planned and carried out based on the standard methodology of the Organization for Economic Cooperation and Development (OECD, 2009). Accordingly, the experiment was carried out statically, i.e. the concentration of nanoparticle was constant in each container and the water was not replaced during the experiment. Additionally, the feeding of the fish was stopped 24 h before the start of the experiment. The fish were randomly divided into four groups of nine, placed in 40-L plastic containers. An air pump and a water pump were placed in each container to circulate and aerate the water. One group was kept as control and three other groups were exposed to different concentrations of zinc oxide nanoparticle, (1, 10 and 100 mg/L). To reduce the stress of the fish and also to reduce the effect of their excrements on the results of the experiments, they were not fed during the experiment period. The water temperature was controlled between 18 and 20 ^{o}C., by placing plastic ice bags into the containers. To add the nanoparticles, a uniform suspensions were prepared based on the certain amount of each concentration and consequently added to the containers. The duration of the experiment was 96 h and the mortality rates were recorded at 24, 48, 72 and 96 hours (Helfrich, Weigmann, Hipkins, & Stinson, 2009). During the experiment, all apparent symptoms and behaviors of the fish were carefully monitored and the observations were recorded.

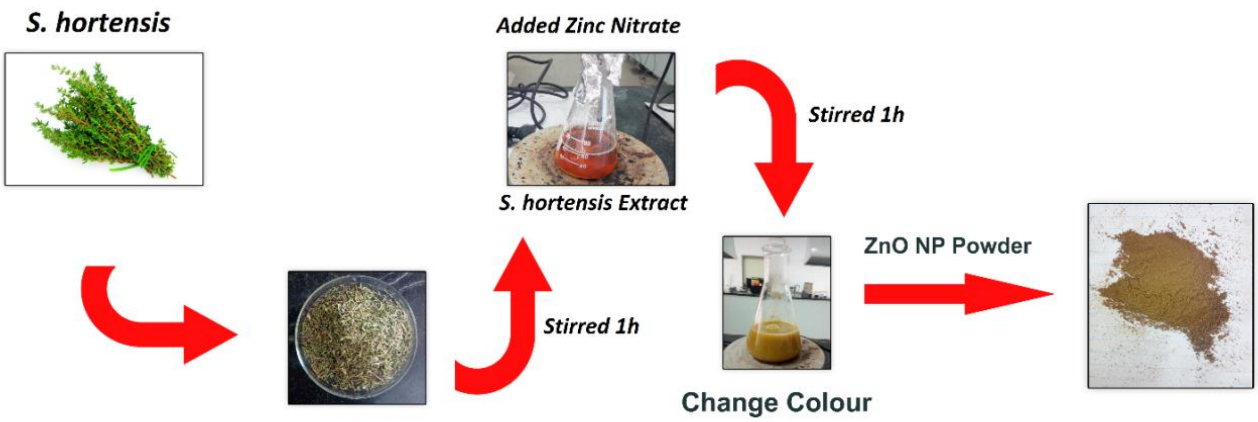

Figure 1. Preparing zinc oxide nanoparticles produced from Satureja plant.

Table 1. Chemical parameters of the water used for the experiments

Parameter	Unit	Min - max	Mean	Method of analysis
pH	-	7.1-7.9	7.5	pH meter
Total Hardness	mg/L(as $CaCO_3$)	152-336	244	Titration with EDTA
Electrical conductivity	µSiemens/cm	330-803	566.5	Conductivity meter
Total dissolved solids	mg/L	204-488	346	TDS meter
Chloride	mg/L	8-48.5	285	Argentometry
Nitrate	mg/L	1.3-28.3	14.8	Spectrophotometry
Nitrite	mg/L	0.03-2.2	2.1	Spectrophotometry
Dissolved oxygen	mg/L	6-7	6.5	DO meter
Ammonium	mg/L	0	0	Titrimetric method

Results

Characterization of ZnO NP

The synthesized zinc oxide nanoparticle powder, was brown in color. This powder was characterized and the synthesis of the nanoparticles was confirmed with the available instruments. UV spectroscopy revealed that the absorption peak of the powder was at the 330 nm. SEM images clearly showed the existence of zinc oxide nanoparticles in spherical and crystalline form, that confirmed the presence of zinc oxide nanoparticles in the sample. EDX analysis also demonstrated the presence of zinc and oxygen elements as the main elements in the sample. XRD analysis showed a particle size of 35.88 nm, and the obtained peaks showed that the most frequent synthesis phases assigned to zinc, confirming the synthesis of zinc oxide nanoparticles from the savory plant (Figure 2).

Toxicity

In the control group and at a concentration of 1 mg/L, no mortalities were observed. The first mortality in this study occurred at concentration of 10 mg/L, with one death in the first 24 h. At concentration of 100 mg/L, a high mortality rate was observed in the fish population, so that in the first 24 h of the experiment, and in the first 48 h, 3 and 8 fish died, respectively. The mortality rates of the tested fish at the pre-determined times and the concentrations are presented in Table 2. The results of LC_{10}, LC_{30}, LC_{50} and LC_{90} are presented in Table 3. Based on the results (Table 3), the four-day LC_{50} (96h) was calculated to be 25.50 mg/L. The probability deaths of the fish at different concentrations of nanoparticles for 24, 48, 72 and 96 h are shown in Figure 3. LC_{50} values at the above periods were obtained 2127.99, 19.03, 25.50, and 25.50, respectively. Figure 4 shows the logarithm of the concentration of ZnO nanoparticles synthesized from the Satureja plant on trout. As it is evident, nanoparticle concentration decreases with the elapsing the time. Given the results of the lethal concentration at different periods, the maximum allowable concentration of zinc oxide nanoparticles can be determined. According to the OECD standard (OECD, 2009), the allowable lethal concentration, which is also called the maximum ineffective concentration, is 10% of LC_{50} 96h, and since LC_{50} 96h is 25.50 mg/L, the maximum allowable concentration is 2.55 mg/L. Based on the same standard, the minimum effective concentration is equal to LC_{50} 10h which is 4.69 mg/L. The results of no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) and their comparisons with LC_{50} 96h, are presented in Figure 5. As noted before, the LC_{50} 96h value in this study was obtained 25.50 mg/L. Therefore, based on the toxicity levels as determined by the Globally Harmonized System of Classification and Labelling of Chemicals (Table 4), zinc oxide nanoparticles biologically prepared from the savory plant belong to the low toxicity category (Helfrich *et al.,* 2009).

Behavioral Effects

Observation of the behavior of the fish during the experiment indicated that they showed abnormal movements and behaviors during the first hours of exposure to the nanoparticles. These behaviors included rising to the surface of the water, fast swimming, restlessness in moving round and round the container, gathering in one part of the aquarium, and the quick opening and closing of the gill slits. At concentrations of 1 and 10 mg/L, the fish quickly adapted to the nanoparticles in the water, and their abnormal behaviors subsided after 24 h and they began slow swimming in the water. At high concentrations, the fish that remained alive, even on the fourth day, were restless when responding to external stimuli. As soon as the researcher approached the container, they began to swim swiftly. The fish went towards air bubbles and opened and closed their gills. Mucus secretion was observed in small amounts and backward swimming was also observed.

Discussion

In this study the characteristics of the obtained zinc oxide nanoparticles from the Satureja plant, are highly consistent with other works. The synthesized zinc oxide nanoparticles are often brown like as the present work found out. Devasenan et. al. reported that the color of the zinc oxide nanoparticle powder was brownish green (Devasenan.S, Beevi.N, & Jayanthi.S, 2016). while, Joel reported that chemically fabricated zinc oxide nanoparticles were commonly white and the green-synthesized nanoparticles were brown in color (Joel.C & Badhusha, 2016). The results of existing studies indicate that zinc oxide nanoparticles often have adsorption peaks between 305-375 nm (Dhanemozhi, Rajeswari, & Sathyajothi, 2017; Senthilkumar & Sivakumar, 2014; Sutradhar & Saha, 2016; Yedurkar, Maurya, & Mahanwar, 2016). The 330 nm absorption peak obtained in this study is in complete agreement with the previously published works. The nanoparticles synthesized in the present study are predominantly spherical and crystalline in shape. Other studies have also reported the same shapes especially spherical, for fabricated zinc oxide nanoparticles (Anvekar, Chari, & Kadam, 2017; Hoseinpour *et al.,* 2017; Jamdagni, Khatri, & Rana, 2018). Furthermore, XRD spectrum showed that the zinc oxide nanoparticles have hexagonal structures and their average diameter is 35.88 nm. Various studies have confirmed the hexagonal structure of the zinc oxide nanoparticle, with nearly the same diameters, depending on the experimental conditions, plant type, region type, and genetic properties (Joel.C & Badhusha, 2016; Taghavi A & Entezari M, 2017). The results of

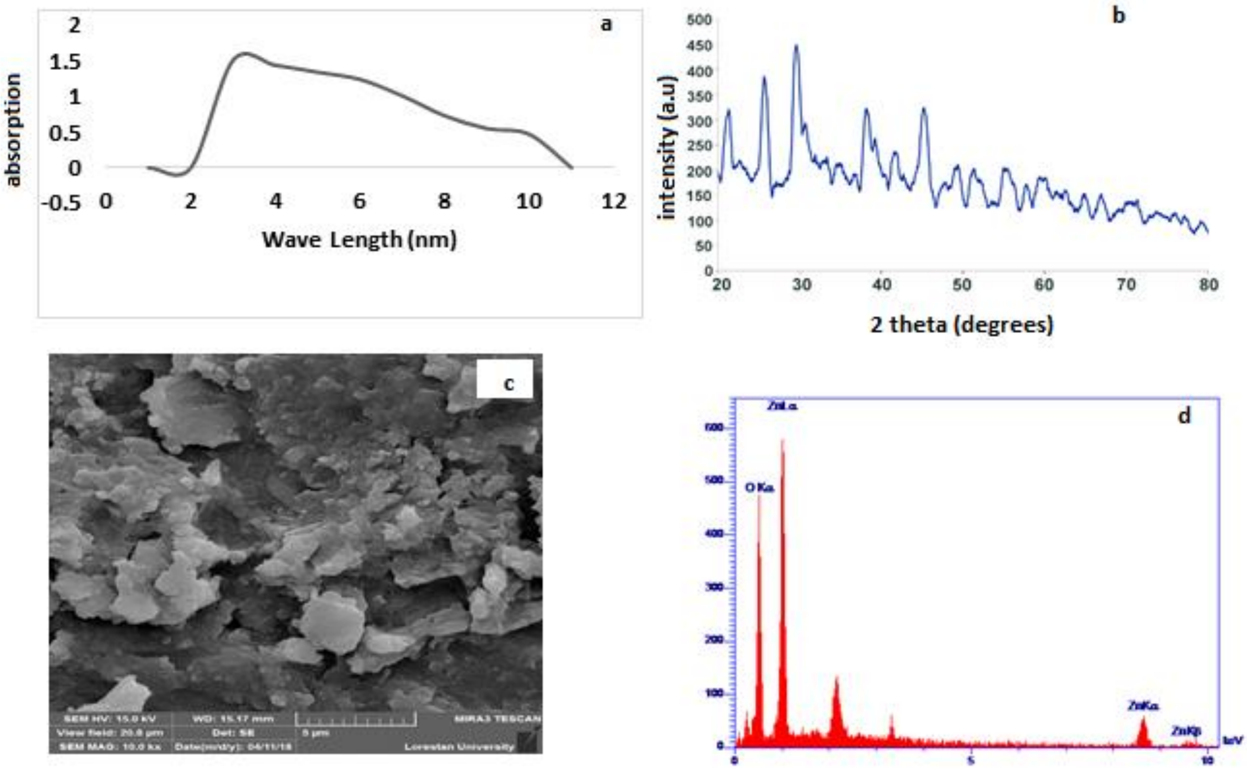

Figure 2. Charactrisation of ZnO NP by satureja plant, a: UV-vis pectrum, b: XRD analysis, c: SEM image, d: EDX analysis.

Table 2. mortality rate in trout fish exposed to different concentrations of ZnO nanoparticles by Satureja plant at different times (24-96h)

Treatment	Concentration Of ZnO(mg/L)	24h		48h		72h		96h	
		Live fish	mortality n(%)	Live fish	mortality n(%)	Live fish	mortality n(%)	Live fish	mortality n(%)
Control	0	9	0	9	0	9	0	9	0
Group1	1	9	0	9	0	9	0	9	0
Group2	10	8	1(11)	8	1(11)	8	1(11)	8	1(11)
Group3	100	6	3(33)	1	8(89)	1	8(89)	1	8(89)

Table 3. lethal concentration (LC_{1-99}) of ZnO nanoparticle by Satureja plant depending on time (24-96h) for trout fish

LC	24h		48h		72h		96h	
	Concentration(mg/L)	Log Con.	Con.	Log Con.	Con.	Log Con.	Con.	Log Con.
LC_{10}	4.69	0.672	0.92	0.32	1.28	0.108	1.28	0.108
LC_{30}	174.19	2.24	5.57	0.74	7.50	0.87	7.5	0.87
LC_{50}	2127.99	3.32	19.03	1.28	25.50	1.40	25.50	1.40
LC_{70}	25996	4.41	66.81	1.825	86.71	1.93	86.71	1.93
LC_{90}	964486	5.98	401.32	2.60	507.53	2.70	507.53	2.70

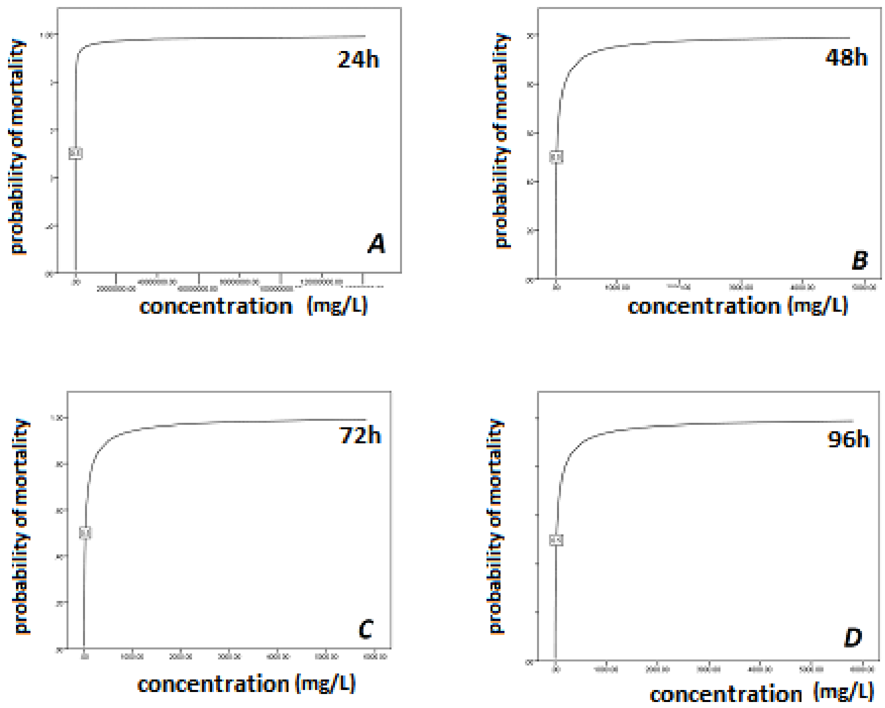

Figure 3. The probability of trout fish mortality at different concentrations of ZnO nanoparticles from Satureja , A- 24h, B- 48h, C- 72, D- 96h.

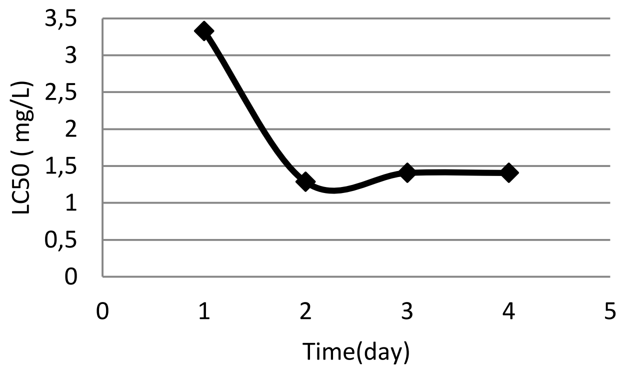

Figure 4. Effect of ZnO nanoparticles from Satureja on Trout fish.

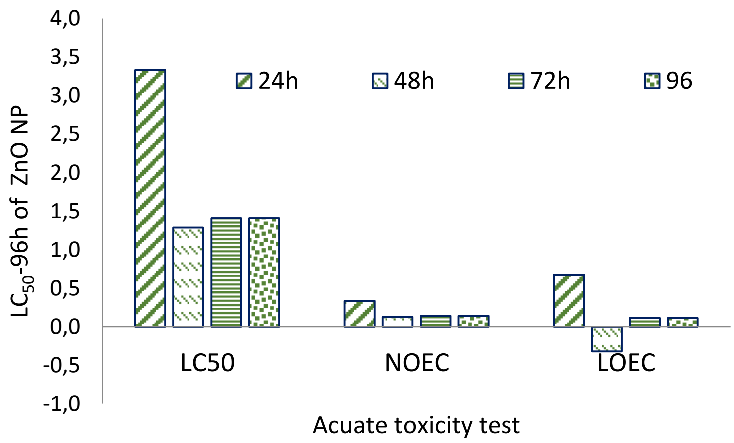

Figure 5. Acute toxicity testing statistical endpoints of ZnONP.

Table 4. Categorization of Toxicity Levels of Materials according to the Global Harmonized Classification (GHS) (Louis et al.,2009)

Degree of toxicity	LC_{50} (mg/lit)
Highly toxic	< 0.1
Very toxic	0.1-1
Medium toxicity	1- 10
Low toxicity	100 — 10
Relatively toxic	>100

bioassay studies are acceptable only when the maximum deaths in the control group are not more than 10% (Helfrich *et al.,* 2009; OECD, 2009). In this study, no deaths were observed in the control group. Based on the results obtained in this study, the amount of LC_{50} 96h of zinc oxide nanoparticles prepared from savory on trout, was 25.50 mg/L. In addition, the LC_{50} 24h value of the zinc oxide nanoparticles was calculated as 2127.99 mg/L.

The obtained results indicated that as the exposure time elapsed, lower concentration of zinc oxide nanoparticles is needed to kill 50% of the fish population. It has been confirmed that one of the most important factors affecting mortality in fish is exposure time (Bazari Moghaddam *et al.,* 2015; shamlofar, Jorjani, & ghelichi, 2015). When the fish are gradually exposed to a constant concentration of nanoparticles, their resistance to the pollutant decreases, and the nanoparticle gets enough time to affect the fish (shamlofar *et al.,* 2015). On the other hand, the gradual accumulation of a toxin in fish body also increased the adverse effects within the 4 days of the experiments and led to a decrease in LC_{50} value (Bazari Moghaddam *et al.,* 2015). Khodabakhshi revealed that LC_{50} 96h value of chemically synthesized zinc oxide nanoparticles on rainbow trout was 0.75 mg/L (Khodabakhshi *et al.,* 2017). The value obtained in present study was 25.50 mg/L that indicates the low risk of biologically produced nanoparticle in comparison with chemically prepared one. The same finding has been reported for other nanomaterials. for instance, Ghobadi et al. showed that chemically synthesized silver nanoparticles had a severe effect on salmon and trout at a very low concentration of 50 µg/L and destroyed all gonadal cell lines (Ghobadi, Farahmand, & Mirjalili, 2014). While Bita *et al.,* reported that the LC_{50} value of biologically produced silver nitrate nanoparticles from the Sargassum algae was 11.34 mg/L. However, this finding indicates the superiority of biological methods to chemical methods (S Bita, Mesbah, Shahryari, & Ghorbanpoor Najafabadi, 2017). Fish Abnormal behaviors resulting from pollutant exposure have also been reported in many works. For example, exposure to thyme, was reported to have similar behaviors on salmon, such as backward swimming, purposeless fast swimming, irritability, mucus secretion (especially in high concentrations), respiratory problems and death (Sharif Rohani, Haghighi, & Assaeian, 2011). The effects of silver nanoparticles on zebrafish, also showed the occurrence of abnormal behaviors, such as swimming at the surface of the water, gathering at a specific point in the aquarium, rapid gill movements and mucus secretion (Mazarei, Sajadi, Sorinejad, Johari, & Asadi, 2016). The above symptoms, as well as muscle contractions and abnormal curving of the spine have been reported in studies by Jegede (Jegede, 2007). These observations are consistent with the behavioral outcomes observed in the present study. In general, different fish show nearly similar symptoms in exposure to nanoparticles.

Conclusion

This study mainly aimed to synthesize zinc oxide nanoparticles using Satureja and evaluate its toxicity on rainbow trout. The characterization analysis confirmed the proper fabrication of the nanoparticle.

Considering the obtained LC_{50} values, the findings of this study indicated that biologically synthesized nanoparticles are less hazardous for the environment and aquatic organisms than chemically prepared nanomaterials. Plants can be considered as the main option for the synthesis of nanoparticles due to their species diversity and they should be considered as an alternative to chemical methods.

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