Green synthesis of zinc oxide nanoparticles using Seaweed aqueous extract and evaluation of antibacterial and ecotoxicological activity

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Abstract

Green synthesized nanomaterials have garnered much attention due to their economic and eco-friendly benefits over common chemical methods of synthesis. In the present study, zinc oxide nanoparticles with an average diameter of 16.51 nm were successfully biosynthesized using the aqueous extract of the red seaweed *Hypnea musciformis*. The morphology, purity and quality of biosynthesized Hy-ZnONps were highly comparable with its commercial counterpart with less toxicity. The MIC and MBC values were evaluated and the potential ecotoxicity of Hy-ZnO NPs against *Artemia salina* was investigated in various concentrations (0, 1, 5, 10, 15, 25 µg/ml) and mortality rate in 24 hours was evaluated. The findings provide preliminary information for designing cost-effective, eco-friendly green synthesis methods for large-scale production of ZnONps using marine macroalgae.

Keywords: Antimicrobial activities, Green synthesis, *Hypnea musciformis*, Metal oxide, Nanotechnology, Seaweed.

1. Introduction

Nanotechnology, in recent years, has received significant expansion in almost every aspect of life including agriculture, food processing/packaging, pharmaceuticals and cosmetics (Ali et al. 2016). The growing concerns on environmental pollution have encouraged the development of biological approach

for the synthesis of nanoparticles (Elumalai and Velmurugan 2015). In this way, biosynthetic processes of metal oxide nanoparticles are a promising alternative for the synthesis using hazardous chemical solvents. The processes involve using biological materials such as herbal extracts, bacteria, and enzymes as reducing and stabilizing factors for the green synthesis of nanoparticles (Ahmad et al. 2012; Sharma et al. 2018; Siddiqi et al. 2018).

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Zinc oxide (ZnO) belongs to the class of metal oxides that is commercially very important due to the remarkable applications in various industrial fields such as catalysis, solar cells, paints, UV light-emitting devices, electronic devices, biomedicine and cosmetic (Osman et al. 2017; Sangeetha et al. 2011; Sharma et al. 2018).

Seaweeds or marine macroalgae with their diverse bioactive compounds have shown immense potential for using in different aspects of biotechnology. Marine macroalgae cultivated in aquaculture can be a sustainable source of biomass for using in green synthesis of metal oxide nanoparticles. *Hypnea musciformis* is a red macroalgae belonging to the Cystocloniaceae family. It is fast-growing opportunistic seaweed that dominated the macroalgal communities in some eastern coasts of Qeshm Island from April to May (Kokabi et al. 2016). This species is a carrageenan yielding seaweed commercially cultivated throughout the world (Vadlapudi and Amanchy 2017). There are 10 species of *Hypnea* reported from south-east of Iran (Kokabi and Yousefzadi 2015) that some of them could easily culture in the coastal area with no requirement of land and freshwater.

Currently, a number of terrestrial plants have been successfully used for the synthesis of ZnO nanoparticles (Elumalai and Velmurugan 2015; Jafarirad et al. 2016; Vijayakumar et al. 2018), but only limited reports are available for green synthesis of this nanoparticle using seaweeds (Azizi et al. 2014; Pandimurugan and Thambidurai 2016) and Rhodophyta, in particular. Although, the synthesis of silver nanoparticles using *H. musciformis* has been reported before (Vadlapudi and Amanchy 2017), in this study, *H. musciformis* was considered due to its novelty and biomass production capacity for the biosynthesis of zinc oxide nanoparticles. Biosynthesis of ZnO nanoparticles using marine macroalgae has advantages over terrestrial plants since they do not compete with agricultural land or

freshwater resources. Therefore, they are a sustainable source for large-scale production of green synthesis of nanoparticles.

This study describes the green synthesis, characterization and eco-toxicological activity of ZnONPs obtained by biological techniques using *H. musciformis* for the first time. Furthermore, MIC (Minimum inhibitory concentration) and MBC (minimum bactericidal concentration) method against bacterial strains were applied to evaluate the antibacterial activity of biosynthesized NPs.

2. Materials and Methods

2.1. Preparation of the *H. musciformis* extracts

The red algae *H. musciformis* was collected in early May from Qeshm Island, Hormozgan, Iran. The collected biomass was washed thoroughly with tap water to clean up and shade-dried. The algal powder (1.0 g) was obtained by the grinder and extracted with distilled water at 85°C for 15 minutes. After cooling down of solution, until room temperature, the sample was centrifuged (1000 rpm) to remove debris. The obtained extract was stored at 4°C and used in the biosynthesis of ZnO.

2.2. Synthesis of Hy- ZnONPs

An aqueous extract of *H. musciformis* was mixed with the solution of 0.2 M zinc nitrate (1:10 v/v) under vigorous stirring at 60°C for 2 hours in an aqueous bath system to complete the reaction. After adjusting the pH of the solution on six, the appearance of a white precipitate in the reaction vessels suggested the formation of ZnO nanoparticles. Then, the solution was centrifuged at 6000 rpm for 10 minutes and the resulting precipitate washed thoroughly with deionized water. The product was dehydrated in the oven at 80°C overnight and calcinated at 450°C for 2 hours to

completely converted Zn(OH)_2 into ZnO nanoparticles. The prepared biosynthesized ZnO nanoparticles were used for further characterization for their optical and nanostructural properties.

2.3. Characterization of zinc oxide NPs

For evaluating the synthesis of nanoparticles, the optical absorption spectra of biosynthesized nanoparticles were recorded using a UV-vis spectrophotometer (Shimadzu, UV-2501PC) in the wavelength range 200-700 nm. Fourier transform infrared (FT-IR) spectra were obtained for the analysis of functional groups using an FT-IR spectrometer (Tensor 27, Bruker). ZnO nanoparticle powder of *H. musciformis* was homogenized with KBr in a ratio of 1:100 and pellets were prepared for the FT-IR measurements. The IR spectra of alga extracts, ZnONPs obtained from that, and commercial ZnONPs (US-NANO) were analyzed as comparisons. The purity and grain size of the dried powder of ZnONPs was evaluated by X-ray diffraction (XRD) method using $\text{Cu K}\alpha$ radiation in wavelength $\lambda = 0.15406$ nm over the scan range of $2\theta = 1-80^\circ$. Morphology of the Hy-ZnONPs along with the elemental analysis (EDXA) was determined via field emission scanning electron microscopy (FE-SEM with EDXA, MIRA3 TESCAN) using Au element for coating samples.

The mean crystalline size of biosynthesized ZnONPs was estimated using the Debye Scherrer's formula as follows:

$$D = \frac{k \lambda}{\beta \cos \theta}$$

In which D is the mean crystallite size, K is the Scherrer constant (0.93), λ is the X-ray wavelength was used (1.5406 Å), β is the full width at half the maximum (FWHM) of the considered peak, and θ is the Bragg angle (Patterson 1939).

2.4. Antibacterial evaluation

Two standard strains of Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria were used to assess the antibacterial potential of nanoparticles by serial dilution method. Suspensions of both Hy-ZnONPs and commercial ZnO nanoparticles along with alga extract were prepared in Muller Hinton broth medium in various concentrations (from 0.125 to 32 mg/ml) under the sterilized conditions. After incubation for 24 h in 37°C , the lowest concentration of samples that prevented the growth of the bacteria was recorded as MIC.

Those concentrations with inhibition performance in MIC test were sub-cultured into the sterile nutrient agar media for MBC screening. All treatments were exposed for 24 h at 37°C . The lowest concentration of nanoparticles or algal extract with no bacterial growth on the surface of culture media was considered as MBC value (Rajabi et al. 2017).

2.5. Ecotoxicity assay

Artemia cytotoxicity assay was carried out using the newly hatched free-swimming nauplii of *Artemia salina*. The recently hatched larva were isolated from nonhatched cysts based on their positive phototropism and 10 larvae transferred to each well of a 24-well polystyrene plate that contained 1mL of artificial seawater (Gambardella et al. 2014) with suspensions of chosen concentrations (0, 1, 5, 10, 15, 25 $\mu\text{g/ml}$) of commercial ZnONPs, Hy-ZnONPs and DMSO as positive control. Artificial seawater with no chemical was taken as blank or negative control. All of the experiments were performed in triplicate. After 24 h of exposure, the percent mortality of *Artemia* treatments was calculated using the following formula:

$$\text{Mortality (\%)} = [(m-M)/S] \times 100$$

In which m is the mean number of dead nauplii in treatments, M is the mean number of dead nauplii in positive control and S is the mean number of live nauplii in positive control.

3. Results

3.1. Spectrophotometry

The UV-Vis absorption spectrum of obtained biosynthesized nanoparticles showed a sharp absorbance peak at 370 nm (Figure 1). The spectra of Hy-ZnONPs, and the commercial ZnONPs, revealed the same absorption peak at 370 nm while the algal extract and Zn (NO_3)₂ solution was different (Figure 1).

3.2. X-ray diffraction

The phase and crystalline structure of biosynthesized nanoparticles were investigated by X-

ray diffraction measurements as shown in Figure 3. The diffraction pattern showed sharp peaks at 2Theta values of 31.7, 34.3, 36.2, 47.4, 56.5, 62.8, 67.8, and 69.1 proves the good crystalline nature of nanoparticles and corresponds to (100), (002), (101), (102), (110), (103), (112) and (201) reflection patterns respectively for ZnO (Figure 2).

All of the diffraction peaks confirmed the spherical and hexagonal structure of biosynthesized Hy-ZnONPs via comparison with JCPDS card No. 89-7102 (Hong et al. 2009; Rajiv et al. 2013). Furthermore, the strong and narrow diffraction peaks confirm the crystalline nature as well as the high purity of biosynthesized Hy-ZnONPs and the absence of any other impurities (Figure 2). The average particle size of the biosynthesized and the commercial ZnO nanoparticles was found to be 16.51 and 16.83 nm, respectively from their XRD line broadening measurement using Debye-Scherrer formula.

Fig 1: Comparison of UV-Vis spectrum of *Hypnea musciformis* extract, zinc nitrate solution, Hy-ZnO nanoparticles, and the commercial ZnO nanoparticles.

Fig 2: XRD pattern of biosynthesized ZnO NPs using *Hypnea musciformis* extract.

Fig 3: FT-IR spectra of (a) aqueous extracts of *H. musciformis*, and (b) ZnONPs synthesized using alga extract.

3.3. FTIR analysis

The possible functional groups involved in different zinc oxide nanoparticles were identified using FT-IR analysis. The FT-IR spectra of alga extract and biosynthesized zinc oxide nanoparticles in the range of 400–4000 cm^{-1} are shown in Figure 3. The signal at 3421 cm^{-1} corresponds to the O-H group. The absorption band at 2924 cm^{-1} represented C-H stretch of aliphatic compounds (Pandimurugan

and Thambidurai 2016). The C=N group in alga extract showed a sharp band at 1634 cm^{-1} . Moderate levels of absorption in the region covering 1000 – 1300 cm^{-1} implied the presence of C-O group (Figure 3b). The spectrum of Hy-ZnONPs showed a new sharp peak at 430 cm^{-1} which is corresponding to zinc-oxygen stretching mode (Pandimurugan and Thambidurai 2016). Generally, the peak in the range of 400 to 600 cm^{-1} is attributed to Zn-O stretching vibrations (Sharma and Ghose 2015) (Figure 3a).

3.4. FESEM and EDAX analysis

Field emission scanning electron microscopy (FESEM) was used to visualize the topology and size of biosynthesized nanoparticles. Figure 4 represents spherical morphology and agglomeration of commercial ZnO nanoparticles with average size of 29-35 nm in compare to biosynthesized Hy-ZnONPs with uniform distribution and smaller size of 26-35

nm, possibly to reflect less agglomeration in biosynthesized ZnONPs. The agglomeration occurred probably during the process of precipitation and drying. Energy dispersive x-ray analysis (EDXA) of the synthesized nanoparticle confirms the presence of only zinc and oxygen elements in the Hy-ZnONPs powder with no impurities (Figure 5) which was in good agreement with the X-ray diffraction measurements.

Fig 4: FESEM image of comercial ZnO NPs (above) vs *H. musciformis* formed ZnO NPs (below) in two different magnification

Fig 5: EDX analysis plot of ZnONPs synthesized using *Hypnea musciformis* extract

3.5. Antibacterial evaluation of ZnONPs

The MIC and MBC values of the aqueous algal extract, Hy-ZnONPs and commercial ZnONPs against *S. aureus* and *E. coli* bacterial strains are presented in Table 1. No antibacterial activity was observed for aqueous extract of *H. musciformis*, while both zinc oxide NPs displayed good results. The growth of both bacterial strains was completely inhibited at 0.12 mg/ml concentration of commercial ZnONPs whereas Hy-ZnONPs prevented the growth of *S. aureus* and *E. coli* at a concentration of 0.25 and 0.5 mg/ml, respectively. The results indicated that *S. aureus* is more susceptible to ZnONPs. It was also noted that the bactericidal potential of both nanoparticles were lower than that of Ampicillin. However, the MIC test of commercial ZnONPs

showed the same (0.12 mg/ml) and better value (0.12 mg/ml) for *S. aureus* and *E. coli*, respectively compared with the reference. Interestingly, Hy-ZnONPs exhibited higher bactericidal activities than its commercial counterpart (Table 1).

3.6. The ecotoxicity of ZnO NPs against *Artemia salina*

The results of this study revealed that low concentrations of ZnONPs did not induce significant mortalities on *Artemia* after 24 h. The commercial ZnONPs displays a higher toxicity than its biosynthesized equivalent (Table 2). In comparison, DMSO was 5–10 times more toxic to *Artemia* than both kinds of zinc oxide nanoparticles.

Table 1: Assessment of MIC and MBC of ZnONPs (mg/ml) against bacterial strains

Bacteria	Ampicillin		<i>H. musciformis</i> extract		Commercial ZnONPs		Hy-ZnONPs	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>S. aureus</i>	0.12	0.21	< 32	0	0.12	8	0.25	4
<i>E. coli</i>	0.24	0.41	< 32	0	0.12	<8	0.5	8

Table 2. Percent mortality values of *Artemia nauplii* measured for 24 h exposure to different concentrations of DMSO and aqueous suspensions of ZnONPs.

Chemicals	Concentration (µg/ml)	Mortality (%)
commercial ZnONPs	Control	0
	1	0
	5	5±0.5
	10	7±0.5
	15	8±0.7
	25	11±1.7
Hy-ZnONPs	Control	0
	1	0
	5	0
	10	4±0.4
	15	5±0.1
	25	7±0.8
DMSO	Control	0
	1	0
	5	5±0.8
	10	11±0.7
	15	22±1.2
	25	72±2.5

Water temperature and salinity were maintained at 24±2 °C, 29–30‰, respectively. Each exposure is made in triplicate in artificial seawater.

4. Discussion

Recently, various types of algae have been applied in green nanotechnology as reducing agent to form nanometals (Sharma et al. 2015). In current study, zinc oxide nanoparticles with an average diameter of 16.51 nm were successfully biosynthesized using the aqueous extract of the red seaweed *H. musciformis*.

The UV- visible absorption study is one of the most prominent and easy way to find if the ZnO nanoparticles synthesize. Talam et al. 2012, reported two absorption bands at about 355 and 258 nm on the absorbance spectra of ZnO nanoparticles. Our study supported their findings. Elumalai and

Velmurugan (2015), reported the same absorption peak as findings of this study for the green synthesized ZnONPs using aqueous extract of Indian lilac tree (*Azadirachta indica* L.). Ali et al. (2016) also recorded the absorption peak of 375 nm for their biosynthesized zinc oxide nanoparticles by the use of *Aloe vera* extract.

Marine algae extracts contain a variety of functional groups such as amino, sulfate, carboxyl and hydroxyl groups which can take part in the conversion of $\text{Zn}(\text{NO}_3)_2$ to zinc oxide nanoparticles and subsequent stabilization of them (Azizi et al. 2014; Vijayakumar et al. 2018). In this study, the FT-IR spectrums indicate that the O-H and C=N stretchings played major roles during the formation

of Hy-ZnONPs since they are highly shifted. These bands indicate that polysaccharides and imine compounds are abundant in aqueous extract of *H. musciformis*. Since this alga species is carrageenan rich seaweed, it can be postulated that the polysaccharide participated in the reaction is carrageenan. This water soluble macromolecule could have acted as stabilizing agent preventing the aggregation of nanoparticles in reaction solution.

The obtained Hy-ZnONPs are smaller than ZnO nanoparticles reported by Azizi et al. (2014). In their study, FESEM analysis of biosynthesized ZnONPs showed the average size ranged between 30 and 57 nm using the brown marine macroalgae *Sargassum muticum*. It is also smaller than the biosynthesized ZnO nanoparticles (40 nm) from the leaf extract of *Azadirachta indica* (L.) reported by Elumalai and Velmurugan (2015). Ishwarya et al. 2018 reported the average size of 10–50 nm for *U. lactuca* fabricated ZnO NPs with sponge-like asymmetrical shape, while Hy-ZnONPs in this study showed uniform spherical morphology. The morphological properties of shape, size, and crystalline form of nanoparticles depend on various factors such as preparation methods, precursors and organic ligands (usually in the form of surfactants). In reaction solution, ligands (surfactant) affect the growth of the nanoparticles strongly. Generally, stronger chelates with metals will result in smaller particles (Ling et al. 2014). *H. musciformis* is carrageenan yielding seaweed rich in sulphates, carboxyl and hydroxyl groups that gave it an amphiphilic properties (Vadlapudi and Amanchy 2017). These functional groups interact with the zinc surface and form chelation with Zn^{2+} via chemical adsorption. Ultimately, heating and calcination of obtained powder led to the cleavage of Zn-seaweed chelate to form nanozinc oxide (Pandimurugan and Thambidurai 2016). Our results are comparable to commercial ZnONPs in size with less

agglomeration probably due to bioactive compounds in the algal extract act as stabilizers that keeps the particles separated, avoiding aggregation and coalescence.

The results of antibacterial activity were in accordance with previous researches that reported zinc oxide as bacteriostatic at the low concentration and bactericidal at high concentrations (Ali et al. 2016). Two possible mechanisms were suggested to explain the inhibition of bacteria, namely, ROS generation on the surface of this metal oxide and interaction of zinc with cell membrane of bacteria through adhesion of ZnO particle (Ann et al. 2014; Mirzaei and Darroudi 2017).

Artemia assay is a cheap, available, simple and reliable method for ecotoxicity studies. *Artemia* or brine shrimp is a nonselective filter feeder that can normally ingest particles with size of up to 50 μm . Adaptability of *Artemia* specimens to laboratory conditions makes this genus a valuable model organism for a great variety of toxicity studies (Nunes et al. 2006). In current study, less than 10 % mortality was observed in the *Artemia* nauplii exposed to all of the concentrations of the Hy-ZnONPs, including the highest. The results revealed less toxicity of biosynthesized ZnONPs in comparison with commercial ZnONPs as well as DMSO. Soniya et al. 2015 also revealed that the low concentrations of ZnONPs have no toxic effect on *Artemia salina*. Identifying hazardous impacts of nanoparticles on natural organisms is difficult because of their diverse properties and the complexity of biological systems (Contini et al. 2017). Earlier studies revealed that different phylogenetic groups may exhibit various responses and sensitivities towards zinc oxide nanoparticles. For example, Wong et al. 2010 assessed the toxicity of ZnONPs on five selected marine organisms. They concluded that ZnONPs was more toxic towards diatoms, but relatively less toxic towards crustaceans and fish. The toxicity of zinc oxide nanoparticles

could be mainly attributed to dissolve Zn^{2+} ions (Aruoja et al. 2009). As it known, this metal ion is needed as microelements for normal functioning of live cells (Mortimer et al. 2010).

The results of characterization as well as bioassay confirmed that the newly synthesized ZnONPs is potentially an eco-friendly alternative for commercial/ chemical ZnONPs and the *H. musciformis* can provide a cost-effective natural source of raw materials for preparation of this nanoparticle.

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