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Green synthesis of Zinc Oxide Nanoparticles Using Eucalyptus Mellidora Leaf Extract and Evaluation of its Antimicrobial Effects

B. Rafiee (MSc)[*1], S. Ghani (MSc)[2], D. Sadeghi (PhD)[3], M. Ahsani (BSc)[4]

1. Young Researchers and Elite Club, Arak Branch, Islamic Azad University, Arak, I.R.Iran

2.Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, I.R.Iran

3. Biology Research Center, Faculty and Research Institute of Basic Sciences, Imam Hossein University of Technology, Tehran, I.R.Iran

4. Department of Horticulture, Faculty of Agriculture, Arak University, Arak, I.R.Iran

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ABSTRACT

BACKGROUND AND OBJECTIVE: Green production of nanoparticles is an environmentally friendly method in which natural solvents are used. Green nanoparticle synthesis methods have priority over chemical and physical methods due to the reduction of cost, time and energy. The purpose of this study was to biosynthesize zinc oxide nanoparticles and evaluate the antibacterial activity of these nanoparticles.

METHODS: In this experimental study, the extract of *Eucalyptus melliodora* was combined at a ratio of 1: 1 with 0. 1 M zinc sulfate and kept at room temperature for 15 minutes. Synthesis of zinc oxide nanoparticles was confirmed by spectrophotometric methods, mean diameter of nanoparticles meaturement, X-ray diffraction and electron microscopy (SEM). Then, the MIC of zinc oxide nanoparticles was measured on standard strains of Staphylococcus aureus 1431PTCC, Bacillus cereus 1015PTCC, Escherichia coli 1399PTCC and Pseudomonas aeruginosa 1571PTCC.

FINDINGS: The absorption peak of ZnO NPs was observed at of 350 nm. The SEM showed ZnO nanoparticles was spherical shape of size ranging from 30 to 50 nm. The highest MIC was observed for Pseudomonas aeruginosa with 0. 0019 mg / ml and the lowest MIC for Bacillus cereus with 0. 62 mg / ml. Also, in comparison with ZnO nanoparticles, Eucalyptus extract and Zinc sulfate had the most antimicrobial activity for zinc oxide nanoparticles and their MIC had significant difference in the effect on the bacteria tested ($p < 0.05$).

CONCLUSION: The results of this study showed biological nanoparticles of ZnO and aqueous extract of Eucalyptus as well as zinc sulfate have antimicrobial properties, but the most antimicrobial effects were observed by ZnO NPs.

KEY WORDS: *Anti-Bacterial Activity*, *Green synthesis*, *Zinc Oxide Nanoparticles*.

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[*]Corresponding Author: B. Rafiee (MSc)
Address: Young Researchers and Elite Club, Arak Branch, Islamic Azad University, Arak, I.R.Iran
Tel: +98 86 33412251
E-mail: rafiee.17b@gmail.com

Introduction

Nanotechnology has witnessed significant progress in the field of nanomaterials and the use of methods and new materials have been developed and discovered new ways for concerns about environmental pollution by nanoparticles through chemical methods and their products, therefore they require green chemical methods which are healthy, non-toxic and environmentally friendly (2,1). The nanoparticles synthesized biologically plays an important role in modern medicine because of the modern characteristics and environmental sustainability (3). The use of living microorganisms such as fungi, bacteria, yeast, actinomycetes and macro organisms such as plants, algae as an intermediate in the synthesis of nanoparticles of mineral composition, can be another way besides the physical and chemical methods for nano-particle production (4), for example the synthesis of iron nanoparticles by Bacillus megatrium bacteria (5). In general, biochemical methods of nanoparticle production are paramount importance to physical and chemical methods due to lower costs, energy and time. This method does not require the use of toxic solvents and hazardous materials for the environment. Green production of nanoparticles is a friendly nature in which the solvents normally used in the production and assembly of nanoparticles using green chemistry in 1990 were considered and, over time, the use of green plants to produce bio-nanoparticles offer a thrill opportunity and is widely known (6). Therefore, plants and plant products are much more interested in the production of nanoparticles, moreover, nanoparticles produced by medicinal plants with lower risk can be used in some cases, such as drug transfer. Research has also shown that nanoparticles produced by plants are made faster than nanoparticles derived from the activity of other organisms and are more stable (7), the shape and size of nanoparticles made by plants differ from nanoparticles derived from other organisms, so the advantage of plant production of nanoparticles compared with other methods such as use of bacteria, algae, yew and fungi are reliability and safety of plant methods and they are more healthier, cheaper and more renewable (8). These include the biosynthesis of silver nanoparticles by the plant, as well as the biosynthesis of gold nanoparticles using Iranian clover seeds (10, 9). World-class nanoparticles, with their exciting and unique features can be used in areas such as diagnosis, nano-medicine and antimicrobial properties. So that nanotechnology is also in place to solve the antibiotics resistance problem (11-13). One of the most important nanoparticles is zinc oxide used industrially in most countries (14). Zinc nanoparticles in food packaging, in biosensors, in the imaging of cells and in the treatment of cancer, in cosmetics, in water and sewage treatment, as well as zinc oxide metal nanoparticles, are also antimicrobial and have been used for many uses in the last two decades (20-15). Zinc oxide nanoparticles are used to combat gram-negative and gram-positive bacteria, and also as an antifungal agent (22, 21). The mechanism of antimicrobial action of zinc oxide is similar to that of other nanoparticles, and acts mainly through the destruction of the bacterial wall (19). In principle, the mechanism of toxic effects of nanoparticles is not well known, but recent research has shown these toxic effects are mediated by high contact levels of nanoparticles, their permeability to cells and organisms, membrane damage, DNA damage, inflammation in cells, and changes in related cellular interactions (23). Therefore, the aim of this study was to study the green synthesis of ZnO nanoparticles using Eucalyptus Melidoura leaf extract and also to investigate the antimicrobial activity of zinc oxide nanoparticles against a number of common gram-negative bacteria (Staphylococcus aureus, Bacillus cereus, Escherichia coli strains and Pseudomonas aeruginosa). The aim of this study was to synthesize zinc oxide nanoparticles in green and to investigate the antibacterial activity of these nanoparticles.

Methods

Synthesis of ZnO nanoparticles using Eucalyptus Melidoura extract: In order to prepare the Eucalyptus Melidoura extract, the Eucalyptus Melidoura leaves were collected from Dezful's Medicinal Garden, then extracted by boiling method, and finally, 0.45 microns' filters were used to sterilize the extract of the plant. The sulfate solution was then prepared at 0.1 molar and filtered with filter paper. The extracts were then combined with a 1: 1 ratio of zinc sulfate, and the nanoparticles were synthesized at room temperature over a period of 15 minutes.

Specification of zinc oxide nanoparticles using spectrophotometry (Uv-vis): The absorption spectra of nanoparticles were performed by a spectrophotometer (Uv-visSERIES8000CECIL). The zinc nanoparticle absorbance was investigated at a wavelength of 200-700 nm.

Measurement of the Dynamic Light Scattering (Zeta potential) and the Poly Dispersity Index (DLS) and the Zeta potential representing the stability of the nanoparticles, as well as (PDI) from the ZetaSizer (Manufacturing company Malvern-England.

X-Ray Diffraction: A solution containing zinc nanoparticles was centrifuged three times at 20,000 rpm, then the supernatant was discarded and the precipitate dried in the oven. Finally, dried powder was investigated for X-ray diffraction analysis of crystalline nanoparticles.

Scanning Electron Microscopy: The shape and size of the zinc oxide nanoparticles were examined using the HITACHI S-4500 SEM device. So that 15 µl of ZnO nanoparticles were sprayed onto SEM special grains and examined after drying.

Determine the antibacterial properties

Disc diffusion method: To study antibacterial properties, standard strains of Staphylococcus aureus 1431 PTCC, Bacillus cereus 1015PTCC as gram-positive bacteria and Escherichia coli 1399PTCC, Pseudomonas aeruginosis 1571PTCC were used as gram-negative bacteria. 100 µl of each bacterium with a concentration of half MacFarlend's was cultured on a Muller hinton agar medium. For each of the four bacteria, four plates containing a 6 µl impregnated disc from a zinc oxide nanoparticle suspension, a 6 µl diluted solution of 0.1 molar zinc sulfate and a 6 µl disk eucalyptus extract were used. After 24 hours, the non-growth halo diameter was measured and recorded as an antibacterial property index of the specimens. These tests were repeated three times to prevent the occurrence of errors in all stages of operation, and the results were recorded.

Tube dilution method: The minimum concentration of bacterial growth inhibitory (MIC) in Tryptic Soy Broth (TSB) culture media was loaded into 4 set of 10 tubes containing 1 ml sterile TSB environment, into a tube of 1 ml of a Zinc sulfate nanoparticle solution was added. After stirring 1 ml from the first tube into the second tube, then 1 ml from the second tube was added to the third tube, and then until the last tube, and finally 1 ml was poured out of the last tube. All tubes were added 1 ml active bacteria suspension, which was standardized in accordance with the half-MacFarlend tube. All tubes were placed in a suitable temperature incubator for 24 hours. After 24 hours, due to the opacity in the tubes, the MIC was determined. These tests were repeated three times to prevent the occurrence of errors in all stages of operation, and the results were recorded.

Results

Spectrophotometer (UV-Vis): In the analysis of zinc nanoparticles with a spectrophotometric Uv-vis, absorption peak of zinc nanoparticles was observed at nm350 wavelengths, which indicated the presence of nanoparticles of zinc in the reaction solution (Fig. 1). The maximum absorption peak of zinc nanoparticles is at 400-300 nm (24).

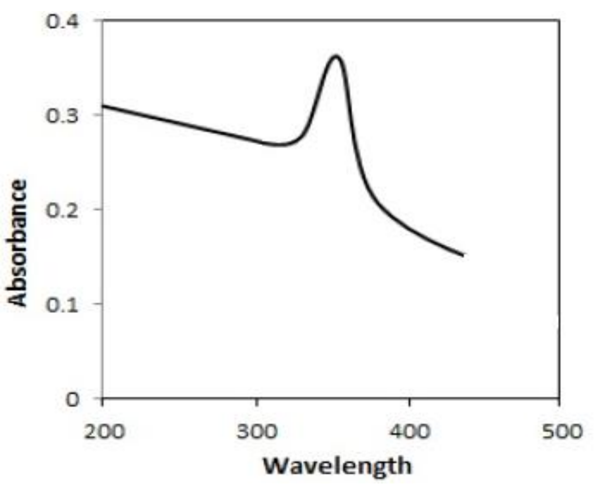

Figure 1. Uv-vis absorption spectra of zinc nanoparticles

The average size of nanoparticle diameter (DLS), zeta potential, and dispersion index of nanoparticles (PDI): In this study, using a zeta-synthesizer device in the range of 8PH, the mean diameter of nanoparticles synthesized were in the range of 37 nm (Fig. 2), also (PDI) was equal to 0.43, indicating a high uniformity of the colloidal solution of the synthesized nanoparticles. Moreover, the zeta potential was recorded as 19.6-millivolt, indicating a very stable zinc nanoparticle (Fig. 3).

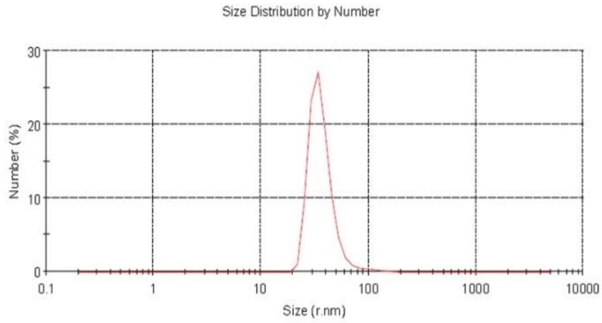

Figure 2. Diagram of average diameter of Zn nanoparticles

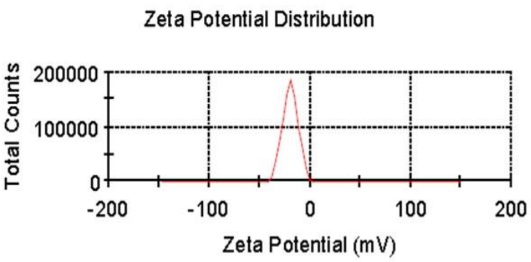

Figure 3. Zeta Potential Chart of Zn Nanoparticles

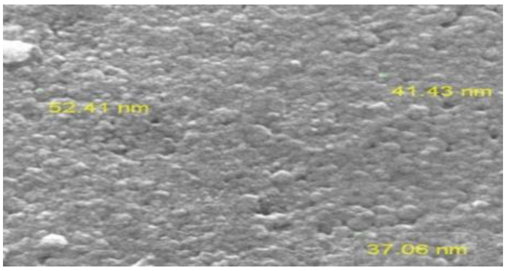

Figure 4. XRD pattern of zinc oxide nanoparticles

X-ray diffraction (XRD): XRD analysis was performed to prove zinc nanoparticles formation. The spectrum obtained from the x-ray editing at the angle θ has peaks (100-002-101-102-110-103-200-112-201) (Fig. 4) which conforms to the X-ray diffraction pattern (ZnO) and no other phase is observed (Fig. 2).

Scanning Electron Microscope (SEM): Images (SEM), Zn nanoparticles are shown in Fig. 3. This is used to confirm the morphology and size of nanoparticles. According to SEM images, nanoparticles were spherical and the size of nanoparticles was between 30-50 nm (Fig. 4).

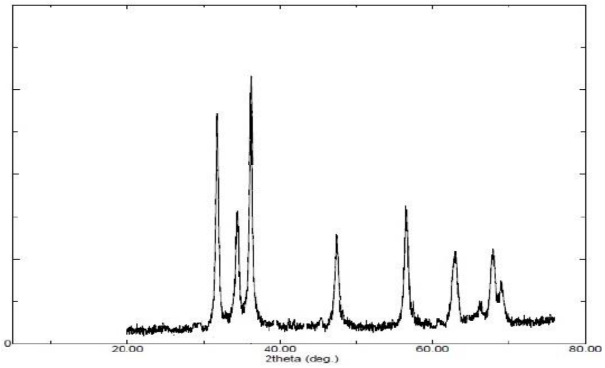

Figure 4. SEM image of spherical nanoparticles of zinc oxide

Disc diffusion: Most antimicrobial activity of zinc oxide nanoparticles was on Pseudomonas aeruginosa with a diameter of inhibition zone of 30 ml and the least antimicrobial activity was on Bacillus cereus with a diameter of inhibition zone of 20 ml (Table 1). According to the results, the antimicrobial properties of zinc nanoparticles were greater than the antimicrobial activity of the eucalyptus extract and zinc sulfate.

Minimum inhibitory concentration (MIC): Results of antimicrobial activity of zinc nanoparticles showed that the highest antimicrobial effect on Pseudomonas aeruginosa with 0.0019 MIC / mg / ml and the least antimicrobial activity on Bacillus cereus with 0.062 mg/ml MIC (Table 2).

Statistical analysis: All statistical tests used in this study were Kruskal Wallis. The evaluation index was mesian in Kroscal Wallis and based on Chi-square (x2) and $p < 0.05$ was considered significant. As for the diameter of the non-growth halos, three antimicrobial compounds were evaluated for effect on the diameter of non-growth halo on the four different microorganisms which there was a significant difference between them (Table 1). In the case of statistical analysis (MIC), the three groups of antimicrobial agents had a significant difference in the effect on all four microorganisms, and zinc sulfate had significantly lower MIC in all four types of microorganisms (Table 2).

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Table 1. The results of measuring the diameter of the non-growth halo

Bacteria \ Repeat	diameter of the non-growth halo (mm): Eucalyptus Extract			Zinc oxide nanoparticles			Zinc sulfate			Statistical analysis
	First	Second	Third	First	Second	Third	First	Second	Third	
Bacillus cereus	6.5	7	7	20	20	20	13.7	14	14	chi-squared=7.579 with 2 d.f. probability=0.0226
Pseudomonas aeruginosa	17.8	18.2	18	30	29.5	30	14.8	14.8	15	chi-squared=7.200 with 2 d.f. probability=0.0273
Staphylococcus aureus	15	15.2	15	24.6	25	25	15.7	15.8	16	chi-squared=7.200 with 2 d.f. probability=0.0273
Escherichia coli	11.8	12	12	27.5	28	28	16	16.2	16	chi-squared=7.200 with 2 d.f. probability=0.0273
Total										X2= 7.538 with 2 d.f.p=0.0231

Table 2. Results of MIC measurements

	Bacteria \ Repeat	Minimum inhibitory concentration from growth (mg/ml): Eucalyptus Extract based on mg/ml			Zinc nanoparticle based on mg/ml			Zinc sulfate based on mg/ml			Statistical analysis
		Third	Second	First	Third	Second	First	Third	Second	First	X2(p-value)
1	Bacillus cereus	0.062	0.062	0.062	0.0078	0.0078	0.0078	0.015	0.015	0.015	Chi-squared =7.23 with 2 d.f. probability=0.027
2	Pseudomonas aeruginosa	0.0039	0.0039	0.0039	0.0019	0.0019	0.0019	0.015	0.015	0.015	chi-squared = 7.200 with 2 d.f. probability =0.0273
3	Staphylococcus aureus	0.0039	0.0039	0.0039	0.0039	0.0019	0.0019	0.015	0.015	0.015	chi-squared =7.200 with 2 d.f. probability=0.0273
4	Escherichia coli	0.0078	0.0078	0.0078	0.0039	0.0019	0.0019	0.015	0.015	0.015	chi-squared =7.24 with 2 d.f. probability =0.0275
Total											X2 = 8.297 with 3 d.f. p=0.0403

Discussion

In this study, it was found that the aqueous extract of Eucalyptus leaves has the ability to produce zinc oxide nanoparticles from zinc sulfate, and it was found that these zinc oxide nanoparticles have antimicrobial activity and, in comparison with the aqueous extract of eucalyptus meliodura and zinc sulfate have more antimicrobial effects. The results of study of Pantidos et al. on the biological synthesis of metal nanoparticles by bacteria, fungi and herbs showed that green nanoparticle synthesis methods require less energy and cost (25). In a study by Mohamed et al., Iron nanoparticles were synthesized by Alternaria fungus (26). In another study by Awwad et al., a green and easy method for the synthesis of magnetic nanoparticles was used (27). Raut et al. used a basal extract of ZnO nanoparticles to study the size of nanoparticles with SEM, such that the size of the nanoparticles synthesized was about 11 to 25 nm and the shape of the nanoparticles was hexagonal (28), while zinc oxide nanoparticles synthesized in our study has a size range of 30 to 50 nm and is a spherical shape. In another similar study by Yedurkar et al., using zinc acetate and Ixora Coccinea leaf extract, nanoparticles of zinc oxide were measured, the size of nanoparticles was

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measured to be 80 to 130 nm, and the shape of the nanoparticles was spherical (29), which is different in size compared with our study but in terms of nanoparticle shape were similar. In another study by Bhumi et al., using zinc acetate and extract of Catharanthus roseu leaves, nanoparticles of zinc oxide were synthesized in the range of 23 to 57 nm in spherical shape, in addition the antimicrobial properties of zinc oxide nanoparticles was evaluated on Pseudomonas, Streptococcus, Escherichia coli and Bacillus thuringiensis bacteria, which had the highest antimicrobial effect on pseudomonas and the lowest antimicrobial activity on Escherichia coli (30), however, our study showed the highest effect on Pseudomonas aeruginosa and the least effect was observed on Staphylococcus aureus, but in general the antimicrobial strength of ZnO nanoparticles was higher in our research based on the diameter of the non-growth zone than the above research.

In another study by Niranjan Bala et al., using Zinc acetate and extract of Hibiscus subdariffa leaves, zinc oxide nanoparticles were made, with size in the range of 12 to 46 nm (30), which was partially similar to the size of synthesized nanoparticles in our study. In addition, the antimicrobial effects of zinc oxide nanoparticles on Staphylococcus aureus and Escherichia coli was investigated which proved that the antimicrobial effects are in line with our research results.

In another study by Awwad et al., using zinc sulfate and olive leaves extract, biosynthesis of zinc oxide nanoparticles was performed, which revealed that nanoparticles form as Nano sheets and Nano flowers of about 500 nm (32), which in our study, this was not observed, and the nanoparticles were spherical at about 30 to 50 nanometers.

On the other hand, in another study, using Costus pictus leaves extracts, nanoparticles of zinc oxide were synthesized. The results of the electron microscope showed that nanoparticles with a mean size of 40 nm with hexagonal and bar geometric shapes. Also, these zinc oxide nanoparticles have antimicrobial effects on Staphylococcus aureus, Escherichia coli and Bacillus subtilis bacteria (33), which are in line with our study, but there is not a significant relation between the shape of nanoparticles, which is undoubtedly related to the plant species and the compounds found in the plant extract.

Another study zinc oxide nanoparticle was made using Parthenium hysterophorus leaf extract which had a size range of 16-45 nm, which were spherical, pseudo spherical and cylindrical forms and had antimicrobial effects against Staphylococcus aureus, Escherichia coli, Bacillus subtilis, and Klebsiella pneumoniae (34), which is consistent with our study. In another study in Iran by Motakef-Kazemi et al., zinc oxide nanoparticles were made using parsley extract that had a nanoparticle diameter of about 50 nm in spherical shape, and the DLS analysis of the nanoparticles had a peak of 50 nm, and in terms of the antimicrobial effect of nanoparticles Zinc has an antimicrobial ability against Escherichia coli (35), which has largely been matched by our study. In another study by Upadhyaya et al., zinc oxide nanoparticles were synthesized using Lawsonia inermis extract and the results of SEM showed that the nanoparticles have a hexagonal shape with a size of 100 nm (36), which is different from our study. It is remarkable that it is unquestionably related to the construction technique and the type of plant used, as well as to the type of its basic material which is nitric oxide. It should be noted that in various researches in the world biosynthesis of zinc oxide nanoparticles was performed by different herbal extracts from Aloe Vera leaves, red clover plants and ginger rhizome (36).

In this study, zinc oxide nanoparticles were made using the Eucalyptus Meliodura aqueous extracts which showed the highest antibacterial effect on Pseudomonas aeruginosa and had the least effect on Bacillus cereus. On the other hand, the antimicrobial activity of ZnO nanoparticles, Eucalyptus aqueous extract and Zinc sulfate was also compared with Zinc oxide nanoparticles that the most antibacterial effect on all tested bacteria was seen by zinc oxide nanoparticles.

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