Instantaneous Green Synthesis of Zerivalent Iron Nanoparticles by Thuja orientalis Extract and Investigation of Their Antibacterial Properties

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(Received 15 Oct. 2014; Final version received 18 Dec. 2014)

Abstract
The applications of zerivalent iron nanoparticles in environmental remediation have led to the development of green methods in their synthesis. This study for the first time reports on the green synthesis of zerivalent iron nanoparticles using Thuja orientalis extract as the reducing and capping agent at room temperature. The synthesized nanoparticles were characterized with different techniques such as XRD, ICP/AES, UV-Vis spectroscopy, SEM, and EDX. The reaction was completed within the first seconds. It was found that phenols and reducing sugars present in the extract have an important role in the production of nanoparticles. Complete degradation of methylene blue as a model pollutant was performed using these nanoparticles. Zerivalent iron nanoparticles showed a strong antibacterial effect on B. subtilis and E. amylovora bacteria. As a consequence, this green and rapid method can compete with chemical synthesis methods, and the synthesized nanoparticles can be applied to the degradation of pollutants and bacteria.

Keywords: Zerivalent Iron Nanoparticles; Antibacterial Properties; Green Synthesis; Reductive Degradation.

Introduction
In recent years, zerivalent iron nanoparticles (ZVINs) have attracted a growing attention in groundwater remediation of heavy metals and environmental halogenated pollutants due to a large surface area and a high potential of reducing ability [1, 2, 3]. Owing to the environmental applications of ZVINs, there is

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a need to develop green and environmentally-friendly methods in ZVINs synthesis. At present, the most common method of ZVINs synthesis is the use of Sodium borohydride (NaBH₄) as a strong reductant [4] where NaBH₄ is a very toxic material. This non-green method is a threat to human health and leads to environmental pollutions, too. Nevertheless, few studies have investigated the green synthesis of ZVINs. Njagi et al. synthesized ZVINs by the use of sorghum bran extract. They used these nanoparticles in the H₂O₂-catalyzed degradation of bromothymol blue as a model organic contaminant [5]. Also, a research group used green tea extract to produce ZVINs at room temperature [6]. They found that the high level of polyphenols available in the extract may be responsible for the ZVINs synthesis as the reducing agent. Furthermore, Mazumdar et al. have investigated the production of ZVINs by Pleurotus sp [7]. The results showed the involvement of the proteins in biosynthesis process. Recently, eucalyptus leaf extract was used for ZVINs production in the 20–80 nm diameter range. These nanoparticles showed a high potential for the treatment of swine wastewater and the removal of total nitrogen and COD from water [8].

In the present study, the aqueous extract of Thuja orientalis leaves was used for the biosynthesis of ZVINs. Thuja orientalis is a species of evergreen cypress trees which grows widely in Iran. The leaves of this plant are available in all seasons and no individual application has ever been reported for these leaves in Iran. For this reason, this synthesis method is a cost-effective approach which may be suitable for large-scale production of ZVINs. The synthesized nanoparticles were used in the complete removal of Methyl Blue (MB) as a chlorinated model contaminant from water. Also, the antibacterial properties of these nanoparticles against Bacillus subtilis and Erwinia amylovora were investigated as representatives of gram-positive and gram-negative bacteria, respectively. It is important to note that the bacterial plant pathogen, E. amylovora, causes the serious disease known as fire blight in some Rosaceous plants [9]. Furthermore, B. subtilis, which is among biofilm forming bacteria, is a public health problem in food and pharmaceutical industries [10] because of its high growth rate and its resistance to antimicrobial treatments. To prevent or control the causal agent of the fire blight disease or the film forming bacteria, an improvement is needed in current strategies. In this study, ZVINs were used to completely inactivate the bacterial strains. Several studies have investigated the antibacterial effects of ZVINs produced by chemical methods [11-13] but this is the first study on the antimicrobial properties of ZVINs produced by biosynthesis methods.
Experimental

Materials
Ferric nitrate, potassium sodium tartrate, ferric chloride and MB obtained from Merck. Dinitrosalicylic acid (DNS) and Bradford reagent were purchased from Fluka and Biorad, respectively.

Preparation of the extract and ZVINs synthesis
The leaves of *Thuja orientalis* were washed with distilled water and dried at room temperature for three days. Powdered leaves (20 g) were mixed with 200 mL of distilled water in an Erlenmeyer flask. The extraction was done on a magnetic heater-stirrer at 80°C for 30 min. The solution was filtered and stored at 4°C until the time of usage. For ZVINs synthesis, 1 mL of ferric nitrate solution (0.1 M) was added to 10 mL of the extract at room temperature. The color of the solution changed immediately to deep black, which is indicative of ZVINs formation.

Ferric chloride test
20 μL of 10% ferric chloride solution was added to both the extract and the reaction supernatant. The supernatant of the reaction was prepared by separating the produced ZVINs from the reaction mixture by the use of centrifuge instrument (14,000 rpm, 25 °C, 10 min).

Preparation of DNS reagent and the measurement of reducing sugars
Accurately, 1 g of DNS material was weighed into a beaker. Then, 1.6 g of sodium hydroxide pellets, 30 g of potassium sodium tartrate and 100 mL of distilled water were added, and the mixture was placed on the heater-stirrer and warmed gently to dissolve. 1 mL of the sample was added to 3 mL of DNS reagent and the mixture was placed on the boiling water bath for 15 min. The absorptions of the extract and supernatant of the reaction were determined in 540 nm by the use of UV-Vis spectrophotometer.

Preparation of MB and its decolorization
A 0.01 M solution of MB was prepared in water. 10 mL of MB solution was added to the total of ZVINs suspension and the mixture was placed on a magnetic stirrer. Decolorization was done at room temperature. After 30 minutes, the mixture was centrifuged (14,000 rpm, 25 °C, 10 min) to separate particles from the supernatant. The supernatant was pale yellow which is the extract color. UV-Vis absorption spectroscopy was used to indicate the removal and degradation of MB from solution. For this purpose, 1 mL aliquot of the mixture was subjected to scanning in the range of 500-800 nm.

Characterization of ZVINs
XRD experiment was done by XRD (XPERT Philips) with CuKα radiation (1.54Å) in
the 2θ range of 5–90 operated at a voltage of 40 kV and a current of 30 mA. ZVINs were centrifuged (14,000 rpm, 25 °C) for 15 min, washed several times with distilled water, and then freeze-dried and subjected to XRD experiment. Energy dispersive X-ray spectroscopy (EDX) was done on TESCAN SEM instrument equipped with an EDX system. The conversion value of ferric iron to ZVINs was determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES) by Varian Vista Pro instrument. For this purpose, ZVINs were separated by centrifuge (14,000 rpm, 15 min). Then, the supernatant was subjected to ICP test to measure the concentration of residual ferric ions. This amount was subtracted from the initial concentration of ferric ions to determine the yield of the reaction. The FT-IR analysis was carried out by Bruker Equinox 55 spectrophotometer in the range of 400–4000 cm\(^{-1}\). The same sample preparation of XRD experiment was used for FT-IR analysis. Average particle size and size distribution were determined by particle size analyzer (Particle Metrix PMX-200CS). Size measurement was performed 8 times for each sample and relative standard deviations were less than 5%. The morphology of ZVINs was determined by SEM instrument (TESCAN).

**Bacterial strains and culture conditions**

The bacterial strains used in this study were *B. subtilis* g6l and *E. amylovora* 273 (representative of gram-positive and gram-negative bacteria) obtained from Microbial Gene Bank of the Agricultural Biotechnology Research Institute of Iran (ABRII). The *B. subtilis* g6l and *E. amylovora* 273 strains were grown in Nutrient Broth and NBS (Nutrient Broth + 5% Sucrose) media for 24 hours at 28°C and 180 rpm, respectively.

**Determining the antibacterial effect by Microtiter plate assay**

The overnight cultures of bacterial strains *B. subtilis* g6l and *E. amylovora* 273 were diluted to an OD\(_{600\text{nm}}\) of 0.2, and then, 100 µL of bacterial culture was added to each well of 96-well PVC micro titer plates. To measure the antimicrobial effect of ZVINs, these nanoparticles were synthesized in the wells. For this purpose, different amounts of the extract and 0.1 M iron (III) salt (142.5 µL cypress extract and 7.5, 15 or 30 µL iron salt) were added to the wells which contain bacteria and the content of each well was immediately black. Also, in order to evaluate the antimicrobial effect of the cypress extract and the ferric iron separately, different values of the cypress extract (50, 100 and 250 µL) and the iron salt (7.5, 15 and 30 µL) were added to the wells containing bacteria and were kept at 28°C for 48 hours. All the samples were kept in a constant volume of 300 µL. The inhibition effect of the cypress extract on bacterial growth was determined.
extract, iron salt and ZVINs against the two bacterial strains was evaluated by measuring the absorbance of each well at 600 nm. The data were analyzed using one-way ANOVA and the Duncan multiple test (P < 0.05).

**Results and discussion**

*Synthesis and characterization*

Immediately after mixing the extract with ferric iron, the color change occurred from pale yellow to black (Figure 1), which is an indication of ZVINs formation as previously reported by other researchers [5, 6]. The reaction yield was measured by the use of ICP technique and it was found to be 85% at room temperature. Research studies in this field have not reported the yield of the reaction. This reaction was completed within a few seconds and a high reaction yield was obtained at this short time. As a result, this synthesis method as a rapid, green and inexpensive technique can be used instead of chemical methods in ZVINs production.

![Figure 1](image1.png)

*Figure 1*. The color change of the extract from pale yellow to deep black which is indicative of ZVINs formation.

Figure 2 shows the XRD spectrum of the produced ZVINs. No diffraction peak is observed in XRD pattern which indicates ZVINs are amorph materials. A similar result was obtained in the XRD spectrum of ZVINs produced by green tea and Surghum bran extract [5, 6]. The peaks of iron can be observed in the EDX spectrum of ZVINs (Figure 3). This confirms the formation of ZVINs. The peaks of carbon and oxygen in this spectrum have probably originated from the biomolecules available in the extract that have been bound to the surface of the nanoparticles and have led to the stability of the suspension for 3 weeks. Weak oxygen peak can also be related to partial oxidation of iron nanoparticles which were later reported in synthesis of ZVINs [3, 14]. An Aluminum peak is observed which corresponds to SEM Al grid.
FT-IR spectroscopy was used to identify the functional groups that were bound to the nanoparticles surface. The FT-IR spectrum of ZVINs (Figure 4) shows the bands at 3451, 2919, 1624, 1371 and 1104 cm\(^{-1}\) which originated from the biomolecules present in the extract. The broad absorbance at 3451 cm\(^{-1}\) can be attributed to stretching modes of hydroxyl (-O-H) and (-N-H) functional groups [15]. The band at 1624 cm\(^{-1}\) is assigned to stretching vibrations of -C=C [16]. Also, the band at 2919 cm\(^{-1}\) is characteristic of the stretching vibration of C-N functional group which may indicate the presence of amines on the surface of nanoparticles. The absorption bands arise from (-C-O) functional group.
and germinal methyls at 1104 cm$^{-1}$ and 1371 cm$^{-1}$, respectively. These observations are indicative of successful functionalization of ZVINs which leads to their stability.

Several rapid tests were performed to identify the compounds likely to be responsible for the reduction of ferric iron and the formation of ZVINs. These experiments were ferric chloride, Bradford and DNS tests. Ferric chloride test was used to detect the presence of natural phenols before and after the reaction. According to this test, when a sample contains phenols, its color changes to blue or violet after adding a few drops of ferric chloride to the solution [17]. It can be observed that the color of the supernatant (reaction mixture after separating the nanoparticles) did not change after treatment with ferric chloride, whereas the extract color changed from pale yellow to violet (Figure 5). This indicates complete consumption of the phenols present in the extract as reducing or capping agents in the synthesis reaction of ZVINs. Bradford [18] and DNS [19] tests were performed to estimate the variations in the amounts of proteins and reducing sugars before and after the reaction. Figure 6 shows that the intensity of reducing sugars absorption in the wavelength of 540 nm which is characteristic of reducing sugars decreased after the reaction. This indicates that reducing sugars can also be responsible for the reduction of ferric ions and the formation of ZVINs. Shankar et al. have reported the role of reducing sugars in the synthesis of metallic...
nanoparticles synthesized by neem extract [20]. There is no significant difference between absorption of proteins in the wavelength of 590 nm before and after the reaction (i.e. the difference is 0.05) (Figure 7). As a result, proteins were not involved in the reduction of ferric ions.

Figure 5. a) Supernatant of the reaction after adding ferric chloride. b) Thuja orientalis extract after adding ferric chloride.

Figure 6. The absorption of reducing sugars of the extract before (a) and after (b) ZVINs formation.
SEM images (Figure 8) show that the produced nanoparticles are almost spherical in shape. Some aggregations can be observed in ZVINs, which is due to magnetic interactions of ZVINs. Earlier studies have reported similar aggregations in ZVINs [1, 3].

The average particle size and size distribution of the ZVINs were obtained by particle size analyzer. The average size was found to be 92 nm and the size distribution was in the range from 50 nm to 740 nm.
**Reductive degradation of MB**

Halogenated organic compounds constitute an important group of pollutants of international concern due to their high toxicity, persistence, and various sources of distribution in the environment [21]. The potential of produced ZVINs in degradation of halogenated pollutants was investigated by the use of MB as a model contaminant. To ensure the reductive degradation of MB, UV-Vis spectroscopy was used. Figure 9 shows that the absorption peak of MB disappeared after 30 min, indicating complete degradation of MB. To make sure that the extract does not play a role in degradation of MB, this experiment was performed by the use of the extract instead of ZVINs. No color change was observed in this experiment. Research studies have demonstrated that when both the ZVINs and the hydrogen peroxide solution are used in degradation of halogenated contaminants, the degradation reaction proceeds through oxidation by free radicals as a result of Fenton reaction in which ZVINs participate as a catalyst [15, 16]. In our study, ZVINs are involved as a reagent, not as a catalyst. This indicates that the decomposition of halogenated pollutants can occur without the use of hydrogen peroxide through the reduction mechanism due to the reductive ability of ZVINs. The reductive dechlorination of Alachlor herbicide has been previously reported [22]. Also, reductive properties of ZVINs have been used in the removal of hexavalent chromium from chromium-spiked soils [23]. The reductive degradation of MB as a halogenated model contaminant shows that ZVINs produced by this method can be applicable to complete destruction and removal of halogenated pesticides from polluted soils and water in a short time.

![Figure 9](image_url)

*Figure 9.* UV-Vis spectrum of MB before (a) and after (b) reaction with ZVINs.
Antibacterial activity of ZVINs, cypress extract, and ferric iron (iron salt)

There are several reports on antibacterial properties of ZVINs produced by chemical methods [11-13]. Lee et al. reported that the inactivation of E.coli by ZVINs could be because of the penetration of the small nanoparticles into E.coli membranes and its reaction with intracellular oxygen, which leads to oxidative stress and disruption of the cell membrane [11]. The results of the present study (Figure 10) showed that all of the treatments have significant different inhibitory effects against two bacterial strains. The most important antibacterial effect belonged to ZVINs, iron salt, and cypress extract, respectively (Figure 10). The different values of ZVINs had the maximum inhibitory effect against B.subtilis, although there was not a statistic difference between different treatments. Also, the biggest antibacterial effect against E.amylovora was observed by different concentrations of ZVINs but the treatment including 142.5 µL of cypress extract and 7.5 µL of iron salt exhibited the maximum inhibitory effect compared with the two other treatments (i.e. 142.5 µL of cypress extract and 15 or 30 µL of iron salt).

Iron salt is an inexpensive compound in comparison with silver ion compounds. Moreover, Thuja orientalis which is a species of cypress is easily available in all seasons in many parts of the world and no individual application has been reported for its leaves in Iran. This indicates that the production of ZVINs by the use of this green method is cost-effective compared to silver nanoparticles synthesis, and the produced ZVINs by this method can be used as a strong antibacterial instead of silver nanoparticles.

According to the results, the survival rate of two bacterial strains decreased when different values of iron salt were applied. Although different treatments of iron salt had similar effects on E.amylovora, B.subtilis was the most affected by the value of 30 µL (Figure 10). In a previous study, antimicrobial properties of ferric iron against several bacteria were investigated and the mechanism of antibacterial effect was demonstrated. According to this study, the bacteria can rapidly reduce ferric ions to ferrous ions. Then, Fe$^{2+}$ ions react with H$_2$O$_2$ (Fenton reaction), which is a normal metabolite in aerobic organisms. One of the products of this reaction is hydroxyl free radicals. These radicals are very reactive and have a strong biocidal effect [24]. It is notable that our results revealed that the gram-positive bacterium, B.subtilis, is more susceptible to the increase in the concentration of iron salt (Fig 10). This is in accordance with the fact that the sites responsible for metal binding in gram-positive bacteria are probably the carboxyl sites within the peptidoglycan, as well as the phosphoryl groups of the teichoic and teichuronic acid secondary polymers [25].
Although it appears that most of the metal-binding capacity of gram-positive organisms is generated by the thick peptidoglycan layer, it is unlikely that the same layer provides the same binding capacity in a gram-negative organism, since gram-negative peptidoglycan is much thinner than gram-positive peptidoglycan and is shielded by an outer membrane [26]. However, the lipopolysaccharide (LPS) layer can be highly anionic and extends beyond the outer membrane proteins; this layer has been found as the major source of metal binding in gram-negative bacteria [27].

The use of cypress extract against *B. subtilis* and *E. amylovora* in microplate showed that three different values of plant extract (i.e. 50, 100 and 250 µL) had significant inhibitory effects on bacterial growth. In addition, the results demonstrated that increase in the cypress extract value caused an increase in antibacterial activity (Fig 10). Bissa et al. have reported antibacterial potential of aqueous extract of *Thuja orientalis* leaves against *Ecoli, Salmonella typhi*, and *Enterobacter aerogenes* by disc diffusion method [28]. Also, antimicrobial activity of *Thuja orientalis* against *Streptococcus mutans* has been previously investigated [29]. Antibacterial properties of the aqueous extract of *Thuja orientalis* are attributed to the presence of high levels of flavonoids and triterpenoids in leaves [28].

![Figure 10](image-url)
Conclusions
To conclude, the aqueous extract of *Thuja orientalis* was used in green synthesis of ZVINs which have a high potential in remediation of environmental pollutions. It was found that phenols and reducing sugars present in the extract are responsible for the reduction of ferric iron to ZVINs. ZVINs were successfully used for the complete degradation of a common model pollutant, methylene blue. The produced ZVINs showed a strong antibacterial effect on *B. subtilis* and *E. amylovora* bacteria. In this synthesis method, there is no need to buy the reducing and capping agents from companies that sell chemicals because a benign extract was used as the reducing and capping agent, which indicates this method is a cost-effective technique. The reaction rate is high and takes only a few seconds to complete. As a result, this green method can easily be used instead of expensive and hazardous chemical synthesis methods in the production of ZVINs.

Acknowledgments
The authors would like to thank the Nanotechnology Department of Agricultural Biotechnology Research Institute of Iran for financing this study.

References


