



Original Research Article

The study of antibacterial properties of iron oxide nanoparticles synthesized using the extract of lichen *Ramalina sinensis*

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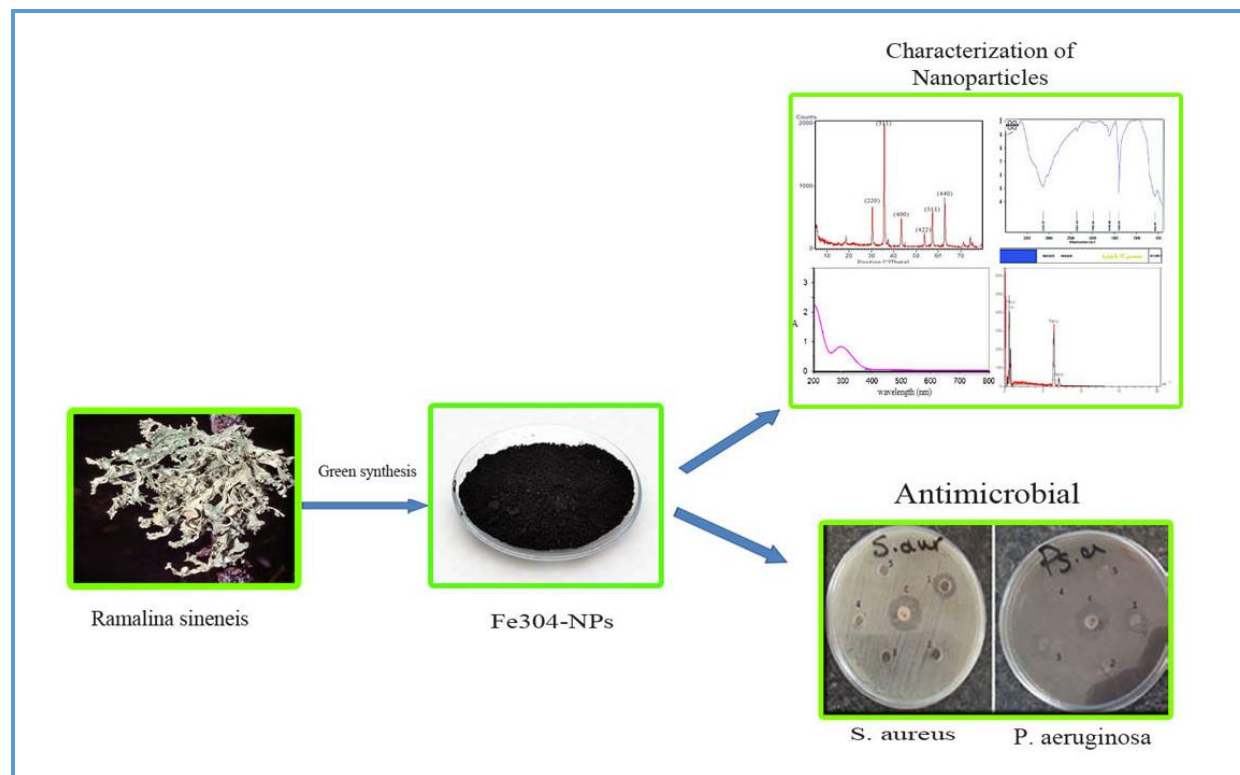
Antibacterial

ABSTRACT

In this work, the synthesis of iron oxide nanoparticles (NPs) was done by the extract of *Ramalina sinensis*. Co-precipitation method was used to synthesize the nano-particles, which is very fast, low cost, and eco-friendly. For the green synthesis of the iron oxide nanoparticles, the distilled extract of *Ramalina sinensis* was utilized. Also, to confirm the synthesis of the nanoparticles, some techniques such as UV-Vis spectrophotometry, XRD, FT-IR, and FESEM-EDX were used. In addition, the antimicrobial activity of biosynthetic magnetite nanoparticles against bacterial strains was evaluated. The uniform spherical nature of the iron oxide nanoparticles with the particle size 31.74 to 53.91 nm was observed in the FESEM images. In the spectrum obtained from the spectrophotometer, the peak at 310±5 nm indicated the electron transfer from oxygen to the iron. The iron oxide NPs depicted effective antibacterial probably against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Pseudomonas aeruginosa*. Based on the obtained results, it can be stated that the powerful approach of green synthesis of magnetic iron oxide NPs using *Ramalina sinensis* has led to producing the nanoparticles with high antimicrobial potential.

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Graphical Abstract



Introduction

Following the recent advances in nanotechnology, the antibacterial effects of some nanoparticles such as iron oxide have been considered by biology and chemistry researchers. In general, nanoparticles are considered as superior antibacterial compared with that of the bulk materials due to their smaller size, high surface area, and faster kinetics [1]. There are no existing studies concerning the synthesis of iron oxide nanoparticles using the extract of *Ramalina sinensis*.

Iron oxide nanoparticles were synthesized following a simple co-precipitation approach using *Ramalina sinensis* extract as the green reducing agent [2]. Moreover, the iron oxide nanoparticles have the innate antibacterial and antifungal properties [3]. Properties of the magnetic nanoparticles rely majorly on their

method of synthesis, reaction conditions such as time, temperature, use of surfactant, and use of reducing agent [4]. A few conventional methods have been used for synthesis of magnetic iron nanoparticles including, co-precipitation [5], hydrothermal [6], pyrolysis [7], electrothermal synthesis [8], and microemulsion [9]. However, these methods make use of harmful chemicals as reducing agents which are a potential environmental burdens and not eco-friendly. Against these chemical methods, green synthesis of iron oxide from plant extract can be considered. Magnetic nanoparticles that are synthesized using the green methods are non-toxic as compared to nanoparticles synthesized using other toxic methods.

Despite a large number of available antibiotics, bacterial infections are still a leading cause of many diseases and mortality worldwide. The current overuse of antibiotics

has led to creation of antibiotics resistant strains that have increased the severity of their pathogenicity. Therefore, there is a need to develop different types of bactericides in the medical field [10]. Since, iron oxide nanoparticles are non-toxic to the human body at low concentrations, they are a good alternative to antibiotics. These substances inhibit bacterial growth at lower concentrations compared with that of the antibiotics and have much fewer side effects [11]. Lichens are organisms that arise from the community of fungi and algae [12]. Lichens, due to their abundance can be a good choice for producing low-cost nanoparticles. The secondary metabolites produced from lichens have antimicrobial activity. Lichens also can demonstrate antibacterial activity due to their chemical structure of components [13]. About 800 secondary combinations of fungal lichens have been discovered which are unique with antibacterial properties against the human pathogenic bacteria [14]. So, the current study aimed at biosynthesizing the superparamagnetic nanoparticles and investigating their maximum antibacterial capacity for some bacteria [15]. These superparamagnetic nanoparticles have already been studied for the antibacterial effects on Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Pseudomonas aeruginosa* [16]. Despite a large number of available antibiotics, bacterial infections are still a leading cause of many diseases and mortality worldwide [17]. The current overuse of antibiotics has led to creation of antibiotics resistant strains that have increased the severity of their pathogenicity than in the past [18]. For this reason, there is a need to develop different types of bactericides in the medical field [19].

Experimental

Materials and methods

All the chemicals used in the experiment were analytical grade, and solutions were prepared with deionized water. The reagents used in this work included: $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (99%) from Merck Company, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (99%) from Sigma Aldrich Company, NaOH (96%) from Tianjin Yongda chemical reagent company, China, HCl (36%) from Chongqing Dongchuan Chemical Company, and NH_3 (28%) from Merck Company. Native lichen *Ramalina sinensis* was collected from the Fandoqlu Forest in the southeastern of Ardabil, Iran. X-ray diffraction pattern of the dried iron oxide nanoparticle powder was obtained using the X'PERT HighScore-PANalytical diffractometer using $\text{Cu-}\alpha$ radiation ($\lambda=0.154$ nm). Fourier-transform infrared spectroscopy (FTIR) spectra of the dried iron oxide NP_s was recorded using an IR BRUKER spectrometer. The UV-visible spectra of the NP_s was obtained using a UV-2450 Shimadzu spectrometer. The surface morphology of the NP_s was observed using a Philips field emission scanning electron microscope (FESEM, XL-30). Also, the used microorganisms Gram-positive bacteria *S. aureus* (ATCC 25923) and Gram-negative bacteria *P. aeruginosa* (ATCC 9027) were provided from the Iranian Research Organization for Science and Technology.

Synthesis of iron oxide NP_s

Iron oxide NP_s were synthesized using the co-precipitation method [20]. First, 100 mL solution of $\text{Fe}^{2+}/\text{Fe}^{3+}$ with the ratio of 1:2 was added to 10 mL extract solution obtained from distillation of 10 g lichen *Ramalina sinensis* into the 250 mL volumetric flask. The prepared solution was stirred at 70 °C for 1 h. During this time, 1 mL of ammonia solution (0.1 M) was dropwise added into the flask to complete the NP_s synthesis reaction. After decantation and

filtration, the obtained brownish NPs were rapidly washed with double distilled water until the pH of the solution reached a neutral range of around 7. The synthesized NPs were then washed with acetone and dried in an oven at 50 °C for 24 h, then sintered at 500 °C for 3 h. This gray powder product was stored in a desiccator for later use.

Antibacterial activity of iron oxide nanoparticles

To investigate the antibacterial properties of the produced iron oxide NPs on *P. aeruginosa* and *S. aureus*, the well diffusion method was used [21]. First, wells of 6 mm diameter were made on Mueller Hinton agar. Next, the microbial suspension equivalent to 0.5 McFarland standard (1.5×10^8 CFU/mL) was cultured with a sterile swab on Mueller Hinton agar. The iron oxide NPs were sonicated at room temperature for 30 min at 28 kHz. Then, 20 μ L (0.075 to 0.00046875 mg/mL) of the synthesized iron oxide NPs were added into the wells. Control wells contained the standard antibiotic positive control tetracycline. Afterward, the cultured plates were then incubated at 37 °C for 24 h and finally, the diameter of the zone of inhibition was measured.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of iron oxide nanoparticles

Broth Macrodilution method was performed to determine the MIC, and also the concentrations of 0.075 to 0.0000014648 mg/mL of NPs were prepared. Afterward, the microbial suspensions containing 1.5×10^8 CFU/mL of microorganisms were added into the tubes of Mueller Hinton broth medium and then incubated at 35 °C for 24 h. Then, the microbial growth was investigated by turbidimetric measurement using a spectrophotometer. The

tests also included a positive control (test tube containing nanoparticle and Mueller Hinton broth medium, devoid of inoculum) and negative control (test tube containing inoculum and Muller Hinton broth medium, devoid of nanoparticle) [22]. In the following, in the test tubes without turbidity, the MBC was determined by a subculture on Mueller Hinton agar medium [23].

Results and Discussion

UV-Vis spectrophotometric technique

This technique can be considered for detecting the conversion of iron (II, III) to iron oxide NPs. For this analysis, a UV-2450 Shimadzu spectrophotometer with double beam irradiance was used at the wavelength range of 200-800 nm. The obtained curve (Figure 1) showed that for the iron oxide NPs dispersed in the solution, a peak appears in the range of 280-320 nm. This peak shows the formation of magnetic NPs of iron oxide.

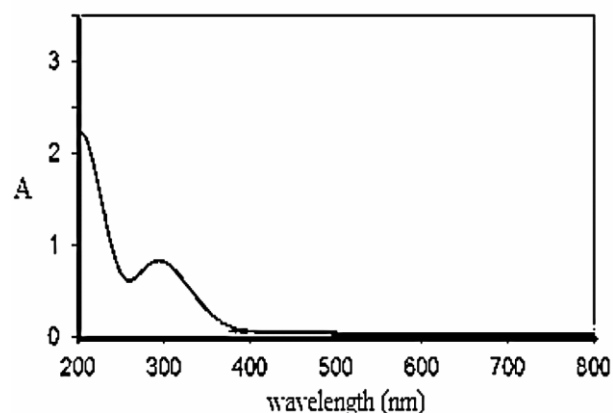


Figure 1. The visible-ultraviolet spectra obtained for the iron oxide nanoparticles synthesized from *Ramalina sinensis*

X-ray diffraction (XRD) analysis

The X-ray diffraction analysis was performed to confirm the crystalline nature of the iron oxide nanoparticles. In X-ray

diffraction analysis, a cathode film coating was used to determine the type of crystals of NPs of iron oxide. For this reason, A X'PERT HighScore-PANalytical diffractometer using Cu- α radiation ($\lambda=0.154$ nm) device with the diaphragm mechanism ($2\theta=0-80$) was used. X-ray diffraction with the wavelength of 1.540598 Å was used on this device.

The spectrum obtained by the XRD method (Figure 2) for the synthesized iron oxide NPs from the lichen extract was well suited to the

patterns registered for this nanoparticle in the scientific literature [24]. Peaks appearing at the $2\theta=30.5^\circ$, 36.1° , 43.3° , 53.9° , 57.5° , and 63.3° positions, confirmed this conclusion very well. The 2θ values of the observed diffraction peaks were compared with the corresponding data of JCPDS (Joint Committee on Powder Diffraction Standards) for the inverse cubic spinel structure of the magnetite iron oxide (JCPDS card no. 19-629)[25].

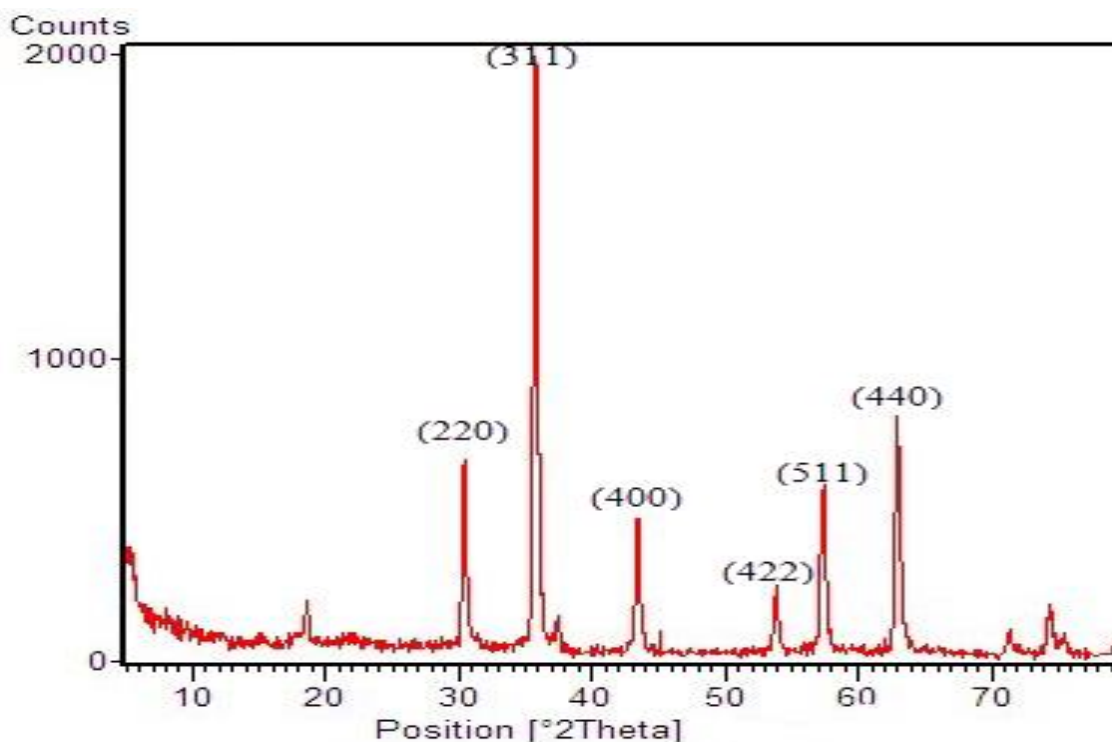


Figure 2. X-ray diffraction pattern recorded by XRD for the iron oxide nanoparticles synthesized from the *Ramalina sinensis* extract

The Scherrer equation was used for measuring the particle size iron oxide NPs synthesized by *Ramalina sinensis* extract. The Scherrer equation can be written as:

$$D = \frac{K\lambda}{\beta \cos\theta} \quad (1)$$

where D is the mean size of the crystalline, which may be smaller or equal to the NPs size; K is adimensionless shape factor, with a value

close to 0.9; λ is the X-ray wavelength; β is the line broadening at half the maximum intensity (FWHM); and θ is the Bragg angle. The estimated value of D was obtained at 27.83 nm for the (311) plane by applying the Scherrer equation.

FT-IR spectroscopy technique

The FT-IR spectrum was obtained using a Bruker-8 FT-IR spectrophotometer. In this

technique, the synthesized iron oxide NPs and KBr powder were mixed and measured in compressed pills. The FT-IR spectrums

obtained in the range of 400-4000 cm^{-1} are shown in Figure 3.

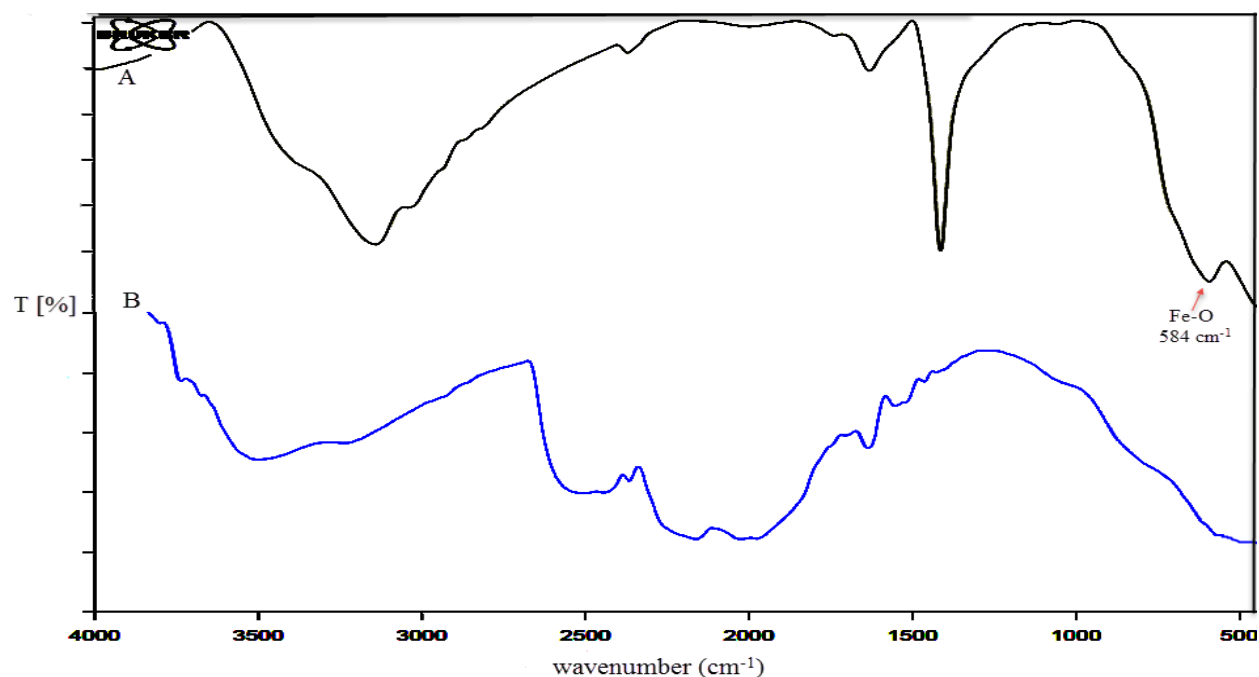


Figure 3. The FT-IR spectra of iron oxide nanoparticles synthesized from the extract of the a) *Ramalina sinensis*, and b) *Ramalina sinensis* extract

The peak appearing at 584 cm^{-1} in the infrared spectrum perfectly showed the new chemical bond between the iron and oxygen. This was while the peak appearing in this position did not appear in the spectrum of iron and *Ramalina sinensis* extract solution. The results obtained from the infrared spectroscopy indicated that, the vibrations were due to the bonds created by chemical reactions, such as the tensile vibrations caused by the new bond between oxygen and iron. The tensile vibrations in the wavenumber of 1613 cm^{-1} was related to the vibration of carbonyl spherical elasticity ($\text{O}=\text{C}<$). The peaks appeared at (1021), and 1410 cm^{-1} were attributed to the symmetric C-O vibration of the C-O-SO₃ and C=C stretching vibration, respectively. The broad peaks at around 3400 cm^{-1} are correspond to the O-H stretching vibrations. The peaks at 2825 cm^{-1}

are attributed to the symmetric and asymmetric C-H stretching vibrations. The appearance of these peaks in the *Ramalina sinensis* extract spectrum reinforces the presence of flavonoid derivatives and phenolic compounds. So, the bioreduction of iron ions can be done by the π -electrons of carbonyl groups.

Field emission scanning electron microscope technique (FESEM)

The iron oxide NPs that synthesized from the aqueous extract of *Ramalina sinensis* lichen were prepared for FESEM analysis. These technique is used very effectively in microanalysis and failure analysis of solid inorganic materials such as iron oxide NPs. The FESEM analysis was performed using a Philips electron microscope and the image was magnified 200,000 times (Figure 4).

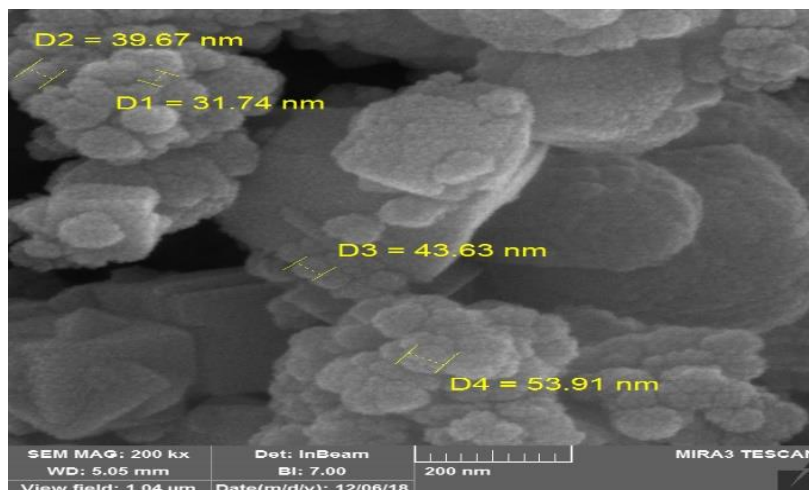


Figure 4. The FESEM image obtained for iron oxide nanoparticles synthesized from the extract of the *Ramalina sinensis* with a magnification of 200,000

The FESEM image obtained from iron oxide (III) nanoparticles synthesized from lichen extract confirmed the particle size distribution to be at the range of 31.74–53.91 nm. Also, the FESEM image revealed formation of pores in the iron oxide NP_s structure.

Energy dispersive X-ray spectroscopy (EDX) results

Structural analysis or chemical properties were used to investigate the interaction between an X-ray excitation source and a sample. The descriptive capabilities of this method are generally based on the principle that each element has a unique atomic structure that enables a unique set of peaks in its X-ray spectrum. The number and energy of X-rays emitted from a sample can be measured using an energy-dispersive spectrometer. Since the energy of X-rays reflects the energy difference between the two layers as well as the atomic structure of the element from which they are emitted, it is possible to measure the composition of the sample [26]. The EDX spectrum (Figure 5) obtained for the synthesized iron oxide NPs using lichen extract

was well fitted to the patterns registered for this nanoparticle in the scientific literature [27].

The EDX analysis suggested that only two atoms (Fe and O) were the main constituents in the iron oxide NP_s synthesized using the extract of the lichen. The peaks at 0.8, 6.5, and 7.2 keV were related to the binding energies of iron.

Analysis of antibacterial activity of iron oxide nanoparticles

The results of this study indicated that the iron oxide NP_s had the highest effect on the standard strains at 0.075 mg/mL. Diameter of the zone of inhibition at 0.75 mg/mL was determined 13 mm for *S. aureus*. The diameter of the zone of inhibition at 0.75 mg/mL was determined by 11 mm for *P. aeruginosa*. This study depicted that the type of bacteria and iron oxide NP_s were effective on the diameter of the zone of inhibition. So that the diameter of the zone of inhibition of iron oxide NP_s at 0.75 mg/mL had the most effect on *S. aureus* and prevented its growth on culture medium. Based on the results, it can be concluded that iron oxide NP_s have significantly higher effects on inhibiting the *S. aureus* compared with that of *P. aeruginosa*.

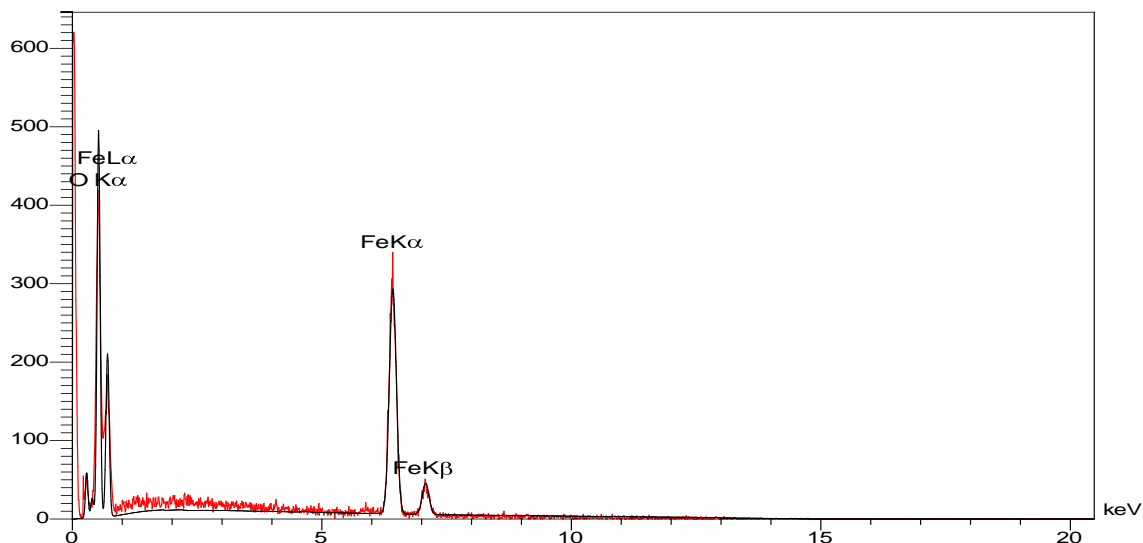


Figure 5. EDX spectra to determine the constituents in iron oxide nanoparticles synthesized from the extract of the *Ramalina sinensis*

It was also found that, both the bacteria were sensitive to tetracycline disc. The antimicrobial activity in comparison with tetracycline-positive control discs and iron oxide NPs showed that our synthesized iron oxide NPs had an antimicrobial activity almost close to the tetracycline antibiotic.

In this study, the lowest inhibitory concentration of NPs for *S. aureus* and *P. aeruginosa* was determined to be 0.0075 and 0.000234375 mg/mL, and the lowest bactericidal concentration of nanoparticles for

S. aureus and *P. aeruginosa* was determined to be 0.0075 and 0.000117188 mg/mL, respectively. Comparing the results of the minimum inhibitory concentration and minimum bactericidal concentration between the *S. aureus* and *P. aeruginosa* indicated that the synthesized iron oxide nanoparticles at lower concentrations have inhibitory and bactericidal properties for *P. aeruginosa*. As a result, it showed a significant effect on the bacteria (Figure 6).

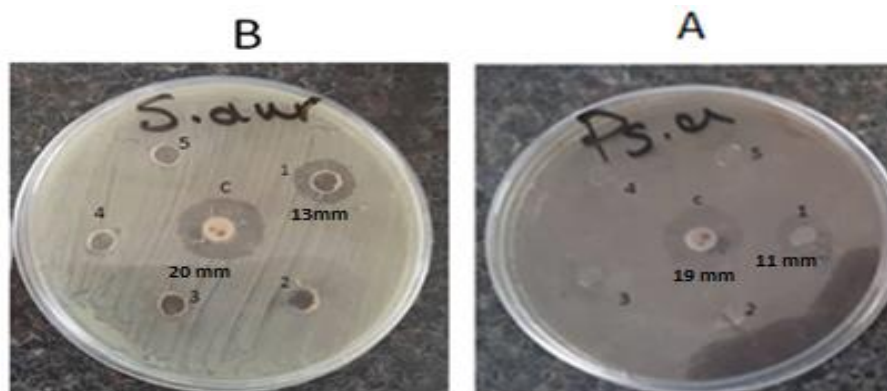


Figure 6. Antibacterial assay of iron oxide nanoparticles (20 μ L per well) against *Staphylococcus aureus*, *Pseudomonas aeruginosa*. (1 to 5: 0.075 to 0.00046875 mg/mL and C: positive controls, a) *Pseudomonas aeruginosa*, b) *Staphylococcus aureus*)

Based on the obtained results, the synthesized iron oxide NPs had a bactericidal activity on both Gram-positive and Gram-negative bacteria. Among the few reported studies in this field, we can refer to the Senthil and Ramesh studies [28], which they biosynthesized magnetite NPs using the aqueous leaf extract of *Tridax procumbens* and found that it had an antimicrobial effect on the *P. aeruginosa*. They have also reported the strong antibacterial activity against bacteria *B. subtilis*, *E. coli*, and *S. aureus* via biosynthesized magnetite NPs from *Desmodium gangeticum* plant [29]. The results of our study indicated that the synthesized iron oxide NPs had a relatively high antimicrobial activity against two *S. aureus* and *P. aeruginosa*, which was consistent with the mentioned above studies. So far, no research study was conducted on evaluating the antibacterial effects of biosynthesized magnetite nanoparticles from lichen. The possible mechanism of the bactericidal activity of the biosynthetic magnetite nanoparticles can be affected by the occurrence of an electrostatic adsorption potential between the magnetite nanoparticles (positive charge) and pathogenic bacteria (negative charge) [30]. This interaction leads to oxidation of the bacterial membrane upon release of the iron ions by the NPs which is able to interact with the thiol groups of the membrane proteins. Therefore, this process can increase the potential of nanoparticles to induce the oxidative stress reactions and produce reactive oxygen species (ROS). The whole of this process disrupts the function, permeability and respiration of the cell membrane. Ultimately, it causes cell breakdown and death of microorganisms [31]. Overall, the results revealed that, the NPs synthesis method with plant extract seems to be efficient and can be used to remove the toxic and hazardous substances [32].

Conclusions

Based on the obtained results, it can be stated that the powerful approach of green synthesis of magnetic iron oxide NPs using *Ramalina sinensis* has led to production of the NPs with high antimicrobial potential. Also, the antimicrobial property of iron nanoparticles produced from *Ramalina sinensis* can be due to the production of reactive oxygen species and the effects of oxidative stress. It can open up a safe and clean solution in the field of health, medical, and pharmaceutical. These NPs were found to be environmental eco-friendly with relatively high effects. Finally, it is suggested that the side and secondary effects of using iron oxide NPs should be studied.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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