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Orginal Research Article

In *vitro* evaluation of its antimicrobial effect of the synthesized Fe_3O_4 nanoparticles using *Persea Americana* extract as a green approach on two standard strains

Sirous Seifi Mansour[a], Elham Ezzatzadeh[a,*], Roya Safarkar[b]

[a] *Department of Chemistry, Ardabil Branch, Islamic Azad University, Ardabil, Iran*

[b] *Department of Biology, Ardabil Branch, Islamic Azad University, Ardabil, Iran*

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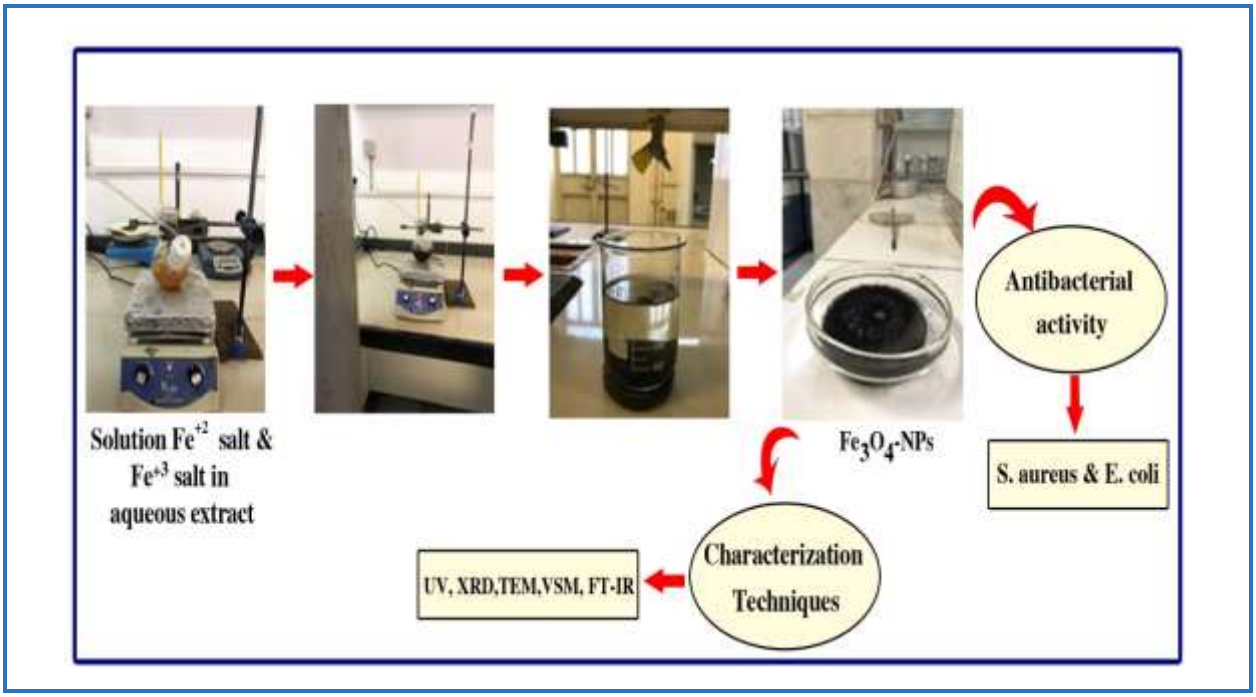
ABSTRACT

The biological synthesis of NPs using plant extracts plays an important role in the field of nanotechnology. In this study, magnetite nanoparticles (Fe_3O_4 NPs) were synthesized using a rapid and single step and completely green biosynthetic method by reducing ferric chloride hexahydrate and ferrous chloride tetrahydrate solution with *Persea Americana* leaf aqueous extract containing flavonoids and phenolic compounds as the main factor which acts as the reducing agent and efficient stabilizer. The structural and properties of the Fe_3O_4 NPs were investigated by UV-visible spectroscopy, fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), scanning electron microscopy (SEM), transmission electron microscopy (TEM) and vibrating sample magnetometry (VSM). Moreover, the antimicrobial properties of Fe_3O_4 NPs were confirmed using the disk diffusion test and minimum inhibitory concentration and minimum bactericidal concentration on *Staphylococcus aureus* and *Escherichia coli*. The obtained results of disk diffusion test indicated that Fe_3O_4 NPs prevented the bacterial growth. Additionally, the obtained results demonstrated that the minimum inhibitory concentration and minimum bactericidal concentration of Fe_3O_4 NPs for *Staphylococcus aureus* were 12.5 mg/mL and for *Escherichia coli* were 6.25 mg/mL. The findings of this study showed that Fe_3O_4 NPs can be used to inhibit the mentioned bacteria.

Corresponding author, email: dr.ezzatzadeh@yahoo.com (E. Ezzatzadeh).
Tel: +98114 765161964, +98912 3234990.

Graphical Abstract



Introduction

Magnetic nanoparticles are the foremost nanoscale materials with many practical applications such as biosensors, catalysts, separation processes and environmental remediation [1]. It plays a central role in a wide range of biomedical applications such as cell therapy, drug delivery, photothermal effect, tissue engineering, regenerative medicine, hyperthermia and diagnosis [2–4]. There are several reports on the synthesis of magnetic nanoparticles where different reducing agents such as hydrazine, dimethylformamide (DMF), sodium borohydride ($NaBH_4$), carbon monoxide (CO) [5, 6] etc., were used. These reducing agents which are highly reactive chemicals have adverse effects on the environment and hinder the biocompatibility of magnetic nanoparticles leading to the limited biomedical applications of the chemically reduced magnetic nanoparticles. In order to use magnetic nanoparticles in biomedical applications, they should be strictly biocompatible. Therefore, the novel and environmentally friendly, biogenic reduction/greener synthesis methods are highly sought. Biogenic reduction methods which include the use of fungi, bacteria and plant extracts, can be one of the best options to opt for the synthesis of nanoparticles (NPs). Such greener methods are environment-friendly, cost-effective, provide good yield, and have decent reproducibility [7]. The availability of biogenic reductive materials in nature makes them a promising candidate for the synthesis of nanoparticles. Such greenly-synthesized biocompatible magnetic nanoparticles are particularly useful for magnetic separation of enzymatic catalysis for reuse [8]. Studies have shown

that bactericidal activity of transition metal NPs can be attributed to many different properties, the most important being the ability to generate ROS and their affinity to associate closely with R-SH groups [9, 10].

Nowadays, drug resistance bacteria have created serious problems in the treatment of many infectious diseases. Consequently, finding new ways to fight against these pathogens are important. Consequently, recent studies have focused on the study of the antimicrobial effects of plant origin [11].

The aim of this study was to synthesize magnetite nanoparticles by a one-pot reaction using leaf extracts of *Persea Americana* and characterization of nanoparticles using UV-visible spectroscopy, fourier transform infrared spectroscopy (FT-IR), X-ray diffraction, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and vibrating sample magnetization (VSM), and also to investigate the antibacterial properties of the nanoparticles on *Escherichia coli* and *Staphylococcus aureus*.

Experimental

Materials and methods

All chemicals were of analytical grade purity and used as received. Ferric chloride hexahydrate, ferrous chloride tetrahydrate and ammonia solution (25%), were purchased from Merck company. Furthermore, the used microorganisms gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and gram-negative bacteria *Escherichia coli* (ATCC 25922) were provided from the Iranian Research Organization for Science and Technology.

Preparation of Persea Americana leaf extract

20 g of dried leaf of *Persea Americana* was powdered and refluxed at 80 °C with 200 mL of sterile distilled water for 1 hour. The mixture was allowed to cool to room temperature and then centrifuged at 6000 rpm. The obtained supernatant was separated by filtration for the further use of the extract.

Preparation of the Fe_3O_4 nanoparticles

100 mL of 5000 ppm *Persea Americana* leaf extract was taken in a 250 two-neck round bottom flask, then 4 mmol ferric chloride hexahydrate ($FeCl_3.6H_2O$) and 2 mmol ferrous chloride tetrahydrate ($FeCl_2.4H_2O$) were added. 10 mL of 9 molar solution of NH_4OH was then injected dropwise into the mixture with vigorous stirring under N_2 atmosphere for 1 h at 60 °C. The resultant solution was a black-color precipitate. The precipitate was separated by applying the external magnetic field and washed with water several times as well as dried in the oven at 60 °C for 24 h.

Investigation of the antibacterial properties of the Fe_3O_4 nanoparticles

The antibacterial effect of Fe_3O_4 nanoparticles against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) was studied using the disk diffusion method. The bacterial suspension was prepared to match the turbidity of the 0.5 McFarland (Approximately 1.5×10^8 CFU/mL) standard and cultured with a sterile swab on Mueller Hinton agar. Then, Fe_3O_4 nanoparticles were sonicated at room temperature for 30 min with a frequency of 28 kHz. Next, 20 µl of different concentrations (10, 20, 40, 60 and 100 mg/mL) of Fe_3O_4-NPs were poured on sterile blank disks. The plates were incubated overnight at 37 °C for 24 h in an incubator. The result was studied by measuring the diameter of the inhibition zone and, then, compared to with the control [12, 13].

Minimum inhibitory concentration method (MIC)

In order to determine MIC, the produced nanoparticles were prepared at different concentrations in Mueller Hinton broth. Afterward, the bacterial suspension containing 1.5×10^8 CFU/mL was added to each concentration. Positive and negative control tubes were prepared as well. The tubes were assessed for turbidity (Growth or non-growth of bacteria) after 24 hours incubation at 37 °C temperature. Then, minimum inhibitory concentration (MIC) was determined as the lowest concentration of each nanoparticle which prevents bacterial growth in the culture medium [13, 14].

Minimum bactericidal concentration method (MBC)

To determine MBC, a loopful of each test tube of non-growth bacteria with the MIC method, was cultured on Muller Hinton agar. The Plates were incubated for 24 hours at 37 °C. The minimum bactericidal concentration (MBC) was determined as the lowest concentration of the nanoparticles which 99.9% killed the bacteria by 99.9% and, besides, no growth was observed [13, 14].

Results and discussion

The essential phytochemicals such as terpenoids, flavones, polyphenols, ketones, aldehydes, amides, and carboxylic acids are mainly responsible for the instant direct reduction of metallic ions as they produce nano-sized particles [15]. In this study, we have developed a new facile route to fabricate magnetic PA/Fe_3O_4 NPs functionalized with active constituents of *Persea Americana* for therapeutic applications [16, 17].

UV-visible spectral studies of Fe_3O_4 NPs

UV–visible spectrum of the synthesized PA/Fe_3O_4 NPs which produced a strong absorbance in the visible region at around 320-350 nm (Figure 1) confirmed that the particles were stable and well-dispersed in the solution. This result also clearly exhibits that the hydroxyl group-containing plant biomaterials are in strong coordination with conduction metals.

FT-IR analysis of synthesized Fe_3O_4 NPs

The FT-IR spectra of the *Persea Americana* leaf extract and Fe_3O_4 nanoparticles are shown in Figure 2. The FT-IR spectrum of the extract Figure **2a** represented a broad peak at 3500 to 3000 which attributed to free OH in molecule and OH group forming hydrogen bonds. The peaks at 1634, 1412 and 1119 cm^{-1} in the spectrum of the extract represented to the carbonyl group (C=O), stretching C=C aromatic ring and C-OH stretching vibrations, respectively. These peaks illustrate the functional groups of flavonoids and other phenolic compounds in the *Persea Americana* leaf extract. Therefore, the FT-IR analysis proved the presence of phenolic compounds as responsible for the bioreduction of metal ions. Actually, the π-electrons of carbonyl group from phenolic compounds in a Red/Ox mechanism can transfer to the free orbital of metal ions and change them to the zero valent. The FT-IR spectrum of Fe_3O_4 nanoparticle (Figure **2b**), in addition to the peaks of the phenolic compounds of the extract, represented the characteristic peak of Fe-O at 583 and 468 cm^{-1} [18].

XRD analysis of synthesized Fe_3O_4 NPs

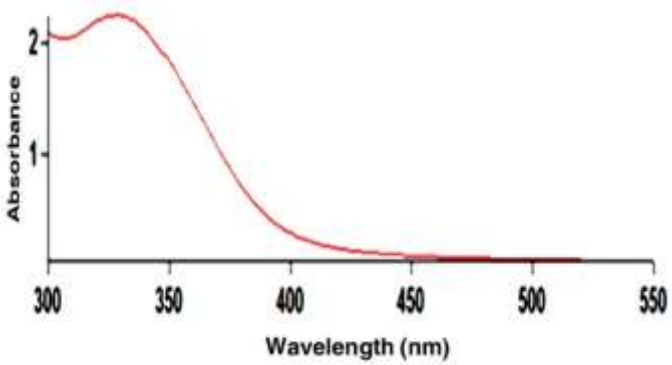
The crystalline structure of PA/Fe_3O_4 NPs was characterized by XRD. As shown in Figure 3, the interplanar distances for biosynthesized PA/Fe_3O_4 NPs were noticed at 2θ = 30.3°, 35.6°, 43.3°, 53.8°, 57.2° and 62.9° corresponding to each Bragg reflection (220), (311), (400), (422), (511) and (440), respectively. They can be well suitably indexed as the inverse cubic spinel structure of magnetite Fe_3O_4 in accordance to the joint committee on powder diffraction standards (JCPDS Card No.75-0499) [19]. Broadening of the peaks in the XRD pattern can be the sign of the small size of magnetite. The crystal size of Fe_3O_4 was calculated using the characteristic peaks of XRD patterns by Scherer equation as follows:

$$D=K\lambda/(\beta_{1/2}\,Cos\,\theta)$$

Where D is the average crystal size; K is a constant (Here chosen as 1); λ is the wavelength of X-ray radiation (1.542 Å); $\beta_{1/2}$ is the half width of the diffraction peak (rad); and θ (°) is Bragg angle [20]. The result of D value using 311 planes is about 12 nm.

Field emission scanning electron microscopy analysis of synthesized Fe_3O_4 NPs

Figure 1. UV-vis absorption spectra of Fe_3O_4 nanoparticles



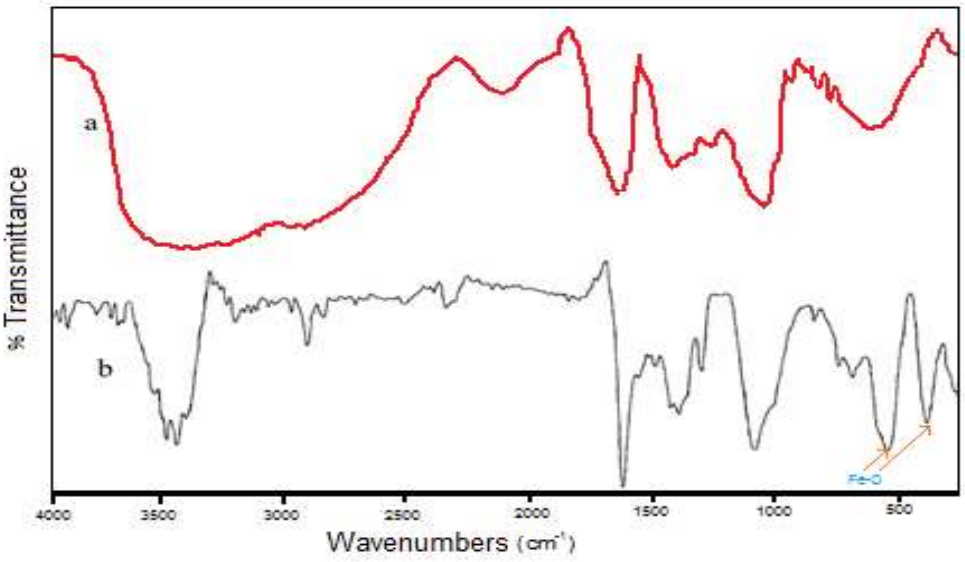
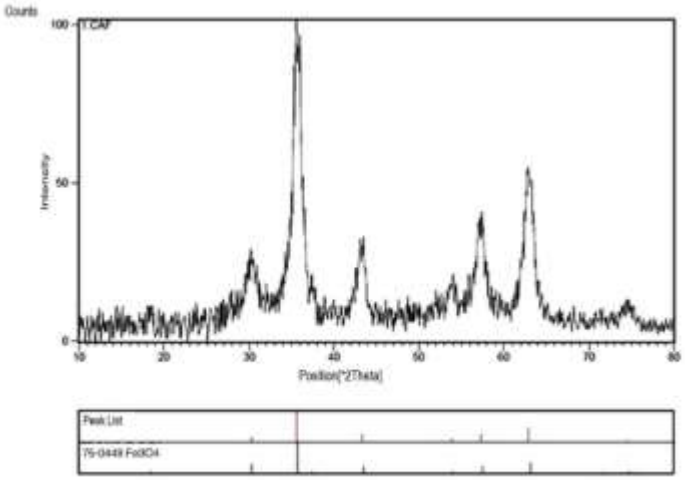


Figure 2. FT-IR spectrum of a) *Persea Americana* leaf extract b) Fe_3O_4 nanoparticles

Figure 3. XRD pattern of synthesized Fe_3O_4 nanoparticles



The morphology of the Fe_3O_4 nanoparticles was revealed by field emission scanning electron microscopy (Figure 4), that shows uniform-sized particles with pseudo-spherical morphology for the product. The size of Fe_3O_4 NPs approximately measured in 12 nm.

Energy dispersive spectroscopic analysis

Elemental analysis of the synthesized iron oxide nanoparticles (Fe_3O_4 NPs) was performed using EDS technique. As shown in Figure 5, Fe and O peaks of iron oxide nanoparticles indicate a successful synthesis. Moreover, the presence of peak carbon in the EDS spectrum indicates the presence of organic compounds in a plant extract at the nanoscale. The presence of excess oxygen and nitrogen can be due to the physical absorption of these elements at the surface of the nanoparticles during the preparation of the sample for analysis [21]. The origin of chlorine and sodium can be attributed to the iron salts used in the synthesis and *Persea Americana* leaf extract, respectively.

Transmission electron microscopy (TEM) analysis of synthesized Fe_3O_4 NPs

To obtain a clear size, shape and structural image of the nanoparticles the sample was analyzed using transmission electron microscopy (Figure 6). Transmission electron microscope image reveals the size of the synthesized iron oxide nanoparticles to be less than 20 nm. Also, nanoparticles are relatively monodisperse and nearly spherical.

Magnetic measurements

In order to study nanoparticles' magnetic behavior, magnetization measurements for Fe_3O_4 were performed. As it can be seen in Figure 7, it has a hysteresis loop with zero coercivity and remanence value. This means that it is single domains with the superparamagnetic characteristic [22]. The saturation magnetization value of magnetic was 22 emu/g.

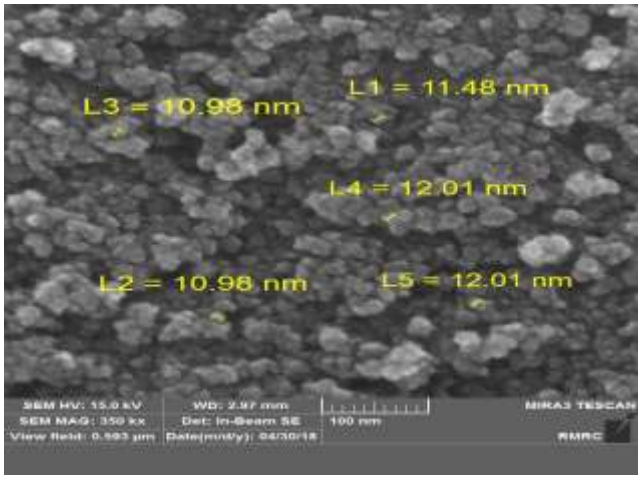
Analysis of the antibacterial activity of Fe_3O_4 nanoparticles

The results of the antimicrobial activity of Fe_3O_4 NPs on bacterial species are shown in Table 1 and Figure 8. The present study indicated that the type of bacteria and Fe_3O_4 NPs concentration are effective on the diameter of the inhibition zone. So that the diameter of the inhibition zone of Fe_3O_4 NPs at concentration of 100 mg/mL has had the maximum effect on *Escherichia coli*.

MIC and MBC results are presented in (Table 2 and 3). The MIC and MBC of nanoparticle demonstrated that the highest antimicrobial activity belongs to the 6.25 mg/mL concentration on *Escherichia coli*. The comparison of the MIC and MBC results between the two bacteria *Staphylococcus aureus* and *Escherichia coli* illustrated that the synthesized Fe_3O_4 NPs had an inhibitory and

bactericidal effect on *Escherichia coli* at the lower concentrations. As a result, it had a better inhibitory effect on this bacterium.

Figure 4. FE-SEM micrographs of the Fe_3O_4 NPs



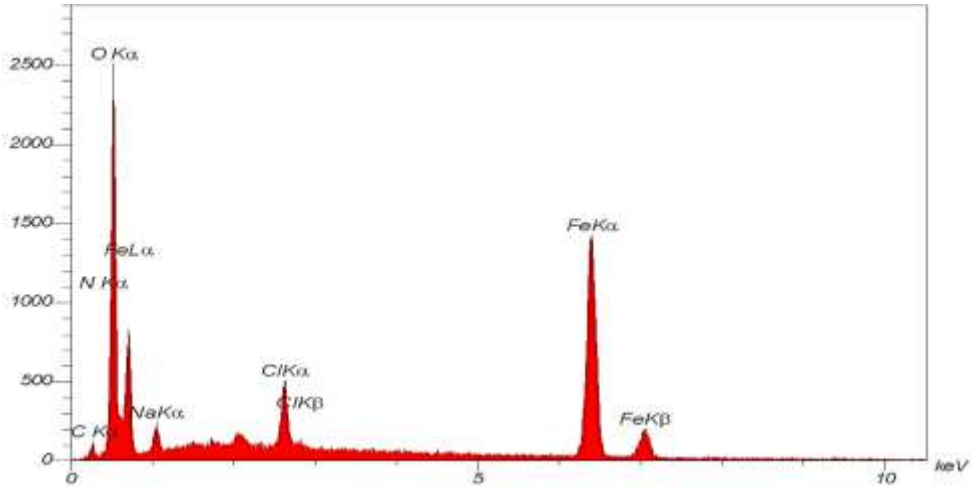
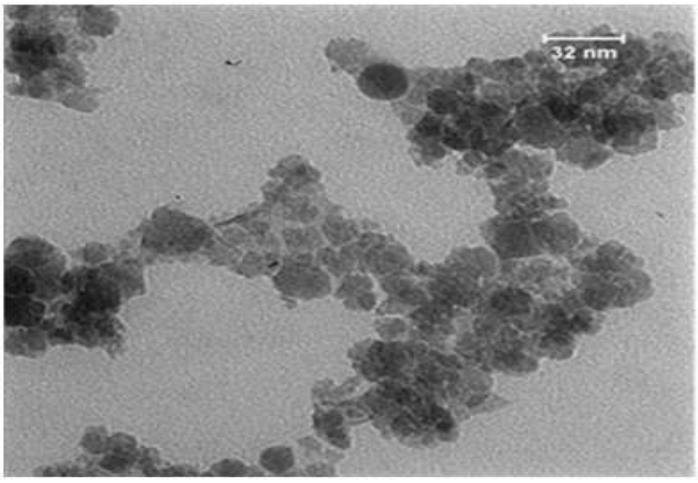


Figure 5. EDS image of the Fe_3O_4 NPs

Figure 6. TEM image of the Fe_3O_4 NPs



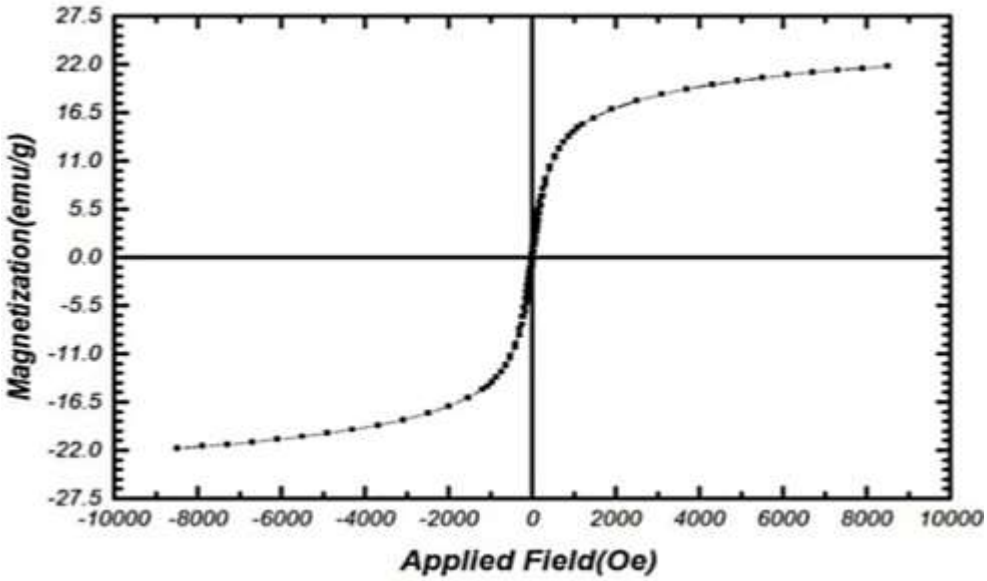


Figure 7. Magnetization curve of Fe_3O_4 NPs

Table 1. Growth inhibition zone diameter in different concentrations of synthesized Fe_3O_4 NPs by disk diffusion method on the selected bacterial strains a mean ± standard deviation

Bacteria	Various concentration of nanoparticle (mg/mL)					
	10	20	40	60	100	Tetracycline (30 µg/mL)
Staphylococcus aureus	0	8±0.2	11±0.1	13±0.5	15±0.3	16±0.4
Escherichia coli	7 ± 0.4	10±0.2	12±0.1	15±0.3	18±0.5	20±0.1

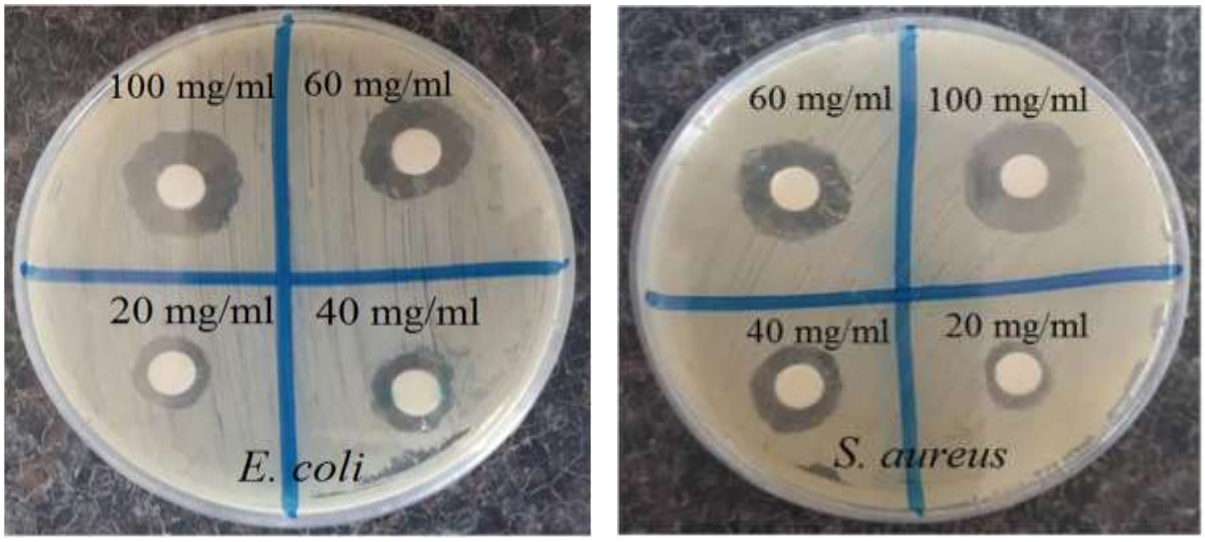


Figure 8. Antimicrobial activity of Fe_3O_4 NPs

Table 2. Minimum inhibitory concentration of synthesized magnetite nanoparticles

Bacteria	Variouz concentration of nanoparticle (mg/mL)							
	1.56	3.12	6.25	12.5	25	50	100	200
Staphylococcus aureus	+	+	+	+	-	-	-	-
Escherichia coli	+	+	+	-	-	-	-	-

+ Lack of the microorganisms

- Growth of the microorganisms

Table 3. Minimum bactericidal concentration of synthesized magnetite nanoparticles

Bacteria	Variouz concentration of nanoparticle (mg/mL)							
	1.56	3.12	6.25	12.5	25	50	100	200
Staphylococcus aureus	+	+	+	+	-	-	-	-
Escherichia coli	+	+	+	-	-	-	-	-

+ Lack of the microorganisms

- Growth of the microorganisms

In this study, the inhibitory growth and bactericidal activity of *Escherichia coli* were 6.25 mg/mL, while the minimum concentration of Fe_3O_4 NPs required for the growth inhibition and bactericidal activity of *Staphylococcus aureus* was 12.5 mg/mL (Figure 9). The presented study found that the synthesized nanoparticles were more effective on gram-negative bacteria such as *Escherichia coli,* whilst were less effective on gram-positive bacteria such as *Staphylococcus aureus*. In thise sense, the results are more consistent with *Shahzadi* and colleagues [12]. This is due to the thick peptidoglycan layer of gram-positive bacteria [23]. Also, this is because of the negative charge of the lipopolysaccharide layer on the outer membrane of gram-negative bacteria. The presence of a negative charge on these bacteria makes them easier to interact with nanoparticles which have negligible positive charge. Thereby, this interaction may make a hole in the cell wall and causing bacteria death [24]. In this regard, we can refer to the work of Jagat *Sen* et al., the study of the antibacterial property of Fe_3O_4 nanoparticles based on green chemistry in 2018. Their synthesized nanoparticles revealed a successful antibacterial result against *Staphylococcus aureus* [25]. Ganyago

and colleagues had offered a novel method for the synthesis of nickel oxide nanoparticles using local herbal medicine. The nanoparticles exhibited a selective antibacterial activity against gram-negative bacteria such as *Escherichia coli* [26]. Nanoparticles are able to damage and destroy the membrane and by penetrating into the cytoplasmic membrane show an antimicrobial activity [27].

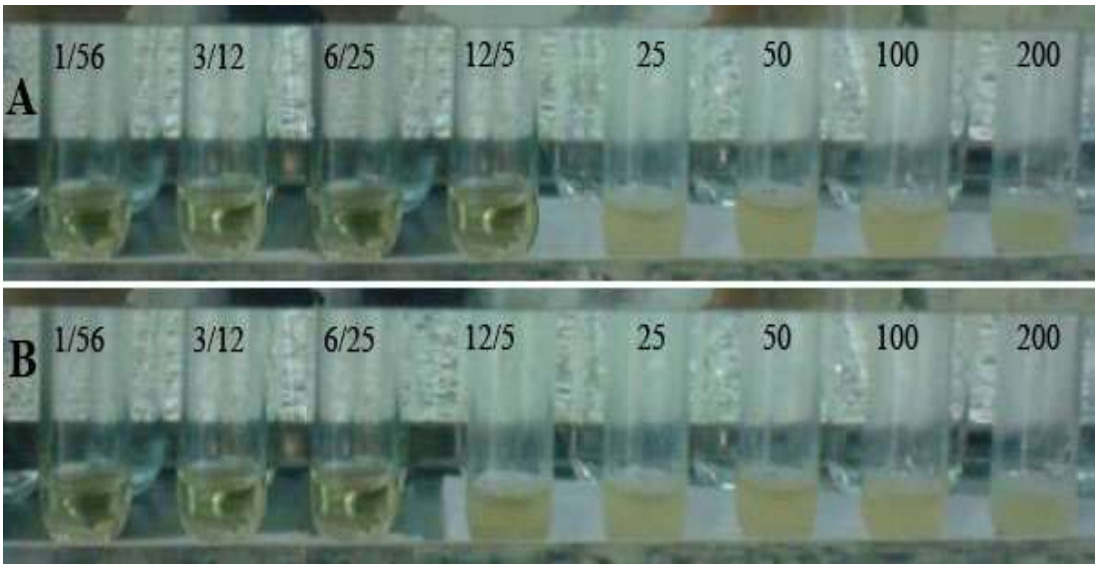


Figure 9. Minimum inhibitory concentration, a) *Staphylococcus aureus,* b) *Escherichia coli*

Conclusion

The rapid biosynthesis of Fe_3O_4 NPs using leaf extract of *Persea Americana* provides an environmentally friendly, simple and efficient route. Our findings demonstrated that the synthesized Fe_3O_4 NPs possess potential antibacterial activity against gram-negative and gram-positive bacteria. Since the risk of increasing infectious diseases threatens human health and the more use of antibiotics can lead to more bacterial resistance to these drugs, it can be claimed that the use of Fe_3O_4 NPs against bacterial infections after clinical examination as an alternative to antibiotics can be effective in the treatment of infectious diseases.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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