

Ultrasmall superparamagnetic Fe_3O_4 nanoparticles: honey-based green and facile synthesis and in vitro viability assay

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Introduction: In the present research, we report a quick and green synthesis of magnetite nanoparticles (Fe_3O_4 -NPs) in aqueous solution using ferric and ferrous chloride, with different percentages of natural honey (0.5%, 1.0%, 3.0% and 5.0% w/v) as the precursors, stabilizer, reducing and capping agent, respectively. The effect of the stabilizer on the magnetic properties and size of Fe_3O_4 -NPs was also studied.

Methods: The nanoparticles were characterized by X-ray diffraction (XRD) analysis, field emission scanning electron microscopy, energy dispersive X-ray fluorescence, transmission electron microscopy (TEM), vibrating sample magnetometry (VSM) and Fourier transform infrared spectroscopy.

Results: The XRD analysis indicated the presence of pure Fe_3O_4 -NPs while the TEM images indicated that the Fe_3O_4 -NPs are spherical with a diameter range between 3.21 and 2.22 nm. The VSM study demonstrated that the magnetic properties were enhanced with the decrease in the percentage of honey. In vitro viability evaluation of Fe_3O_4 -NPs performed by using the MTT assay on the WEHI164 cells demonstrated no significant toxicity in higher concentration up to 140.0 ppm, which allows them to be used in some biological applications such as drug delivery.

Conclusion: The presented synthesis method can be used for the controlled synthesis of Fe_3O_4 -NPs, which could be found to be important in applications in biotechnology, biosensor and biomedicine, magnetic resonance imaging and catalysis.

Keywords: honey, Fe_3O_4 nanoparticles, green synthesis, transmission electron microscopy, magnetic properties, in vitro, viability, MTT assay, WEHI164 cells

Introduction

Recently, rapid advancement in nanotechnology has made synthesis, characterization and improvement of nanoparticles in terms of functional properties possible for various applications. The inspiration for the synthesis of artificial materials for the better quality and quantity comes from nature. The extensive application of metal nanoparticles in different fields, especially in biotechnology, has motivated the metal nanoparticles synthesis.¹ Nanoparticles has attracted incredible attention because of their smaller size and larger surface area compared with bulk materials, in addition to having novel biological and mechanical properties, optical absorption, electrical conductivity and catalytic activity.²⁻⁴

Among the different types of nanoparticles, magnetic nanoparticles, especially magnetite and maghemite nanoparticles with proven biological properties and good biocompatibility, have been the subject of various studies, due to the wide range of important biomedical and environmental applications such as cancer hyperthermia therapy, drug delivery, cell labeling, enhanced magnetic resonance imaging and magnetic

separation. So far, the synthesis and preparation of iron oxide nanoparticle has been an important research priority, and it has been extensively studied and characterized.⁵⁻⁷

Over the past decade, numerous methods have been proposed for the synthesis of magnetite nanoparticles (Fe_3O_4 -NPs), including physical, chemical and biological methods. These include methods such as microemulsions, coprecipitation of ferrous and ferric ions aqueous solution using a base,⁸⁻¹⁰ sol-gel method,¹¹ sonochemistry,¹² colloidal method,¹³ nonaqueous route,¹⁴ pyrolysis reaction,¹⁵ thermal decomposition of organic iron precursor in organic solvents,¹⁶⁻¹⁸ solvothermal synthesis,¹⁹ hydrothermal synthesis,^{20,21} mechano-chemical processing²² and emulsion techniques.^{23,24} Although, bulk chemical synthesis of large amount of nanoparticles is fast and simple, to obtain the effective size stabilization of the nanoparticles, it is important to use capping agents. Furthermore, some of the chemicals used in the synthesis and stabilization are harmful and could produce noneco-friendly, unsafe and hazardous products, and therefore there is a growing demand for the use of green technology in the synthesis of nanoparticles. Thus, new and advanced methods for the growth of nanoparticles must draw inspiration from biological systems.²⁵⁻²⁷

Honey is one of the most beneficial foods accessible, which largely consists of fructose and glucose. Also, it is rich in amino acids, essential minerals, vitamin C and enzymes. Honey has been subjected to broad study throughout the world.⁵⁻⁹ It contains antioxidants, which are important in cancer prevention.²⁰ Recently, aqueous synthesis of silver nanoparticles utilizing natural honey has been reported.²¹

In this work, Fe_3O_4 -NPs are synthesized using iron (III) chloride, iron (II) chloride, sodium hydroxide and natural honey at room temperature through a fast precipitation method. The reaction was completed under optimal conditions, and low cost and energy using friendly environment and fresh, nontoxic materials, solvents and also inert residue materials. The synthesis method is based on green chemistry, which is a main step toward safer synthesis and preparation of nanoparticles. To the best of our knowledge, there is no report on the synthesis and characterization of Fe_3O_4 -NPs using natural honey as a stabilizer and co-reducing agent.

Materials and methods

Materials and reagents

All chemicals used in this study were utilized without further purification and were of analytical grade. Chemicals used for the synthesis of Fe_3O_4 -NPs, such as $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (99.89%), were obtained from Merck (Frankfurt, Germany), natural honey was collected from the unpolluted cold highlands of Northeastern China, and NaOH (99.0%)

was provided by Merck (Frankfurt, Germany). Deionized water was used to prepare all solutions. Glassware was cleaned in HNO_3/HCl (3:1, v/v) solution, washed with deionized water and dried before use.

Preparation of Fe_3O_4 nanoparticles

In the synthesis of Fe_3O_4 -NPs, different amounts of natural honey 0.5, 1.0, 3.0 and 5.0 g were added to 100 mL of deionized water, as the coprecipitation agent and stabilizer to control the particle size. Then $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (with a 2:1 molar ratio) were added under continuous stirring and nitrogen gas bubbling to inhibit oxidation. The mixture was titrated against a NaOH (2.0 M) solution under continuous stirring until the pH reached 10. The Fe_3O_4 -NPs were formed instantly from the reduction procedure. The resulting black suspension was centrifuged, washed three times with the deionized water and ethanol mixture and then dried in an oven at 60°C.

Characterization methods and instruments

The phase structure of the nanoparticles was obtained by X-ray diffraction ($\text{Cu K}\alpha$ 1.5406 Å radiation), using a Bruker D8 Advance diffractometer (Bruker AXS, Billerica, MA, USA) at room temperature between 20°–25°, in the 2 θ scale, with a scanning speed of 0.02°/s and a step time of 3 seconds. Transmission electron microscopy (TEM) was done using a Tecnai G2 F20 transmission electron microscope from FEI (USA), with an acceleration voltage of 200 kV. The size distributions of particles were identified using the ImageJ version 1.46 r program. The morphology of the Fe_3O_4 -NPs was determined through Field emission scanning electron microscopy (FESEM). FESEM and energy-dispersive X-ray (EDX) spectroscopy were performed using Quanta™ 450 FEG, Oxford instrument (USA). The dried samples were coated with gold using a sputter coater. A vibrating sample magnetometer (VSM) was used to study the magnetic properties of the samples utilizing a VSM, Lake Shore Model 7400, Tokyo, Japan, with magnetic fields up to 8 kOe. Fourier transform infrared spectroscopy (FT-IR) was performed between 400 and 4,000 cm^{-1} to characterize possible biomolecules that are responsible for the capping and efficient stabilization of the Fe_3O_4 -NPs. The FT-IR spectra were observed using a Spectrum 400 FT-IR/FT-FIR spectrometer (Perkin Elmer, Waltham, MA, USA).

Results and discussion

As shown in Figure 1, once NaOH solution was added as a reducing agent to the honey aqueous mixture with Fe^{3+} and Fe^{2+} chloride (2:1 molar ratio), the color of suspension turned black and the Fe_3O_4 -NPs were easily separated by a magnet.

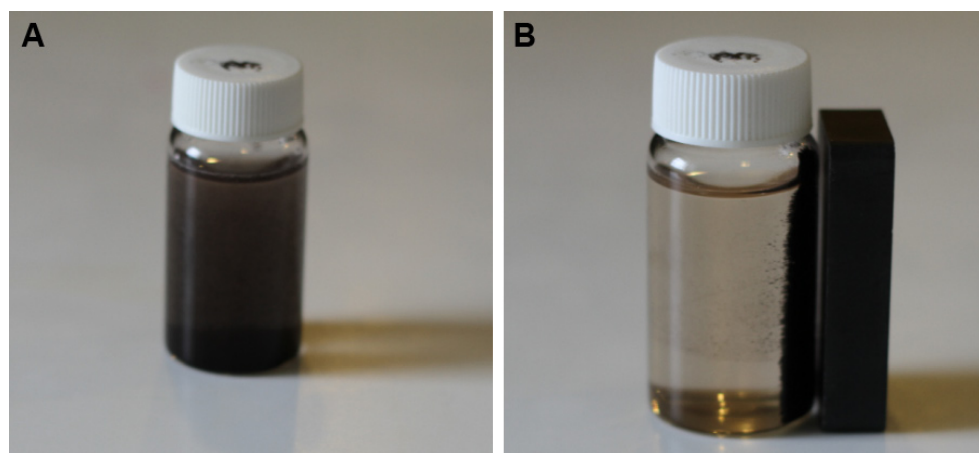
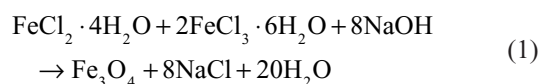


Figure 1 The 0.5% (w/v) honey/magnetite nanoparticles suspension without (A) and with (B) a magnetic field.

The chemical reaction for the precipitation of Fe₃O₄ is as follows:



Phase structure of nanoparticles

The Fe₃O₄-NPs were characterized through X-ray powder diffraction (XRD) and all the peaks were analyzed and indexed using the ICDD database, by comparing with the magnetite standards (Figure 2).²⁸ The peaks were indexed to the (220, 311, 400, 422, 511, 440) and (533) planes, which is attributed

to the 2θ of 30.46°, 35.76°, 43.51°, 53.24°, 56.88°, 63.32° and 71.41°, respectively, with the standard diffraction spectrum (ref. code Fe₃O₄:01-088-0315).²⁹

Generally, the diffraction peaks with less intensity in the XRD pattern show the small size of the Fe₃O₄-NPs. In this case, there is a decreased intensity of the peaks with increasing honey concentration, which also indicates the decrease in the particle size.

FT-IR spectra analysis

Honey contains proteins, minerals, vitamins and natural sugars (mostly fructose, sucrose and glucose).^{30–34} Fourier transform infrared spectroscopy (FT-IR) was performed to

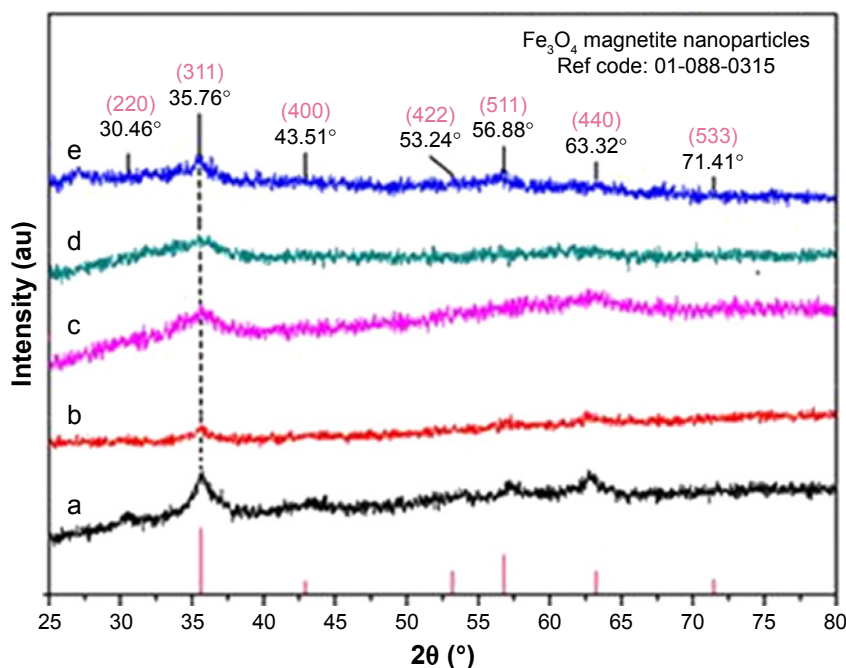


Figure 2 Powder X-ray diffraction patterns of Fe₃O₄-NPs without (a) and with different concentrations of honey (0.5, 1, 3, and 5% [w/v]) (b–e), respectively. **Abbreviations:** Fe₃O₄-NPs, magnetite nanoparticles; ref, reference.

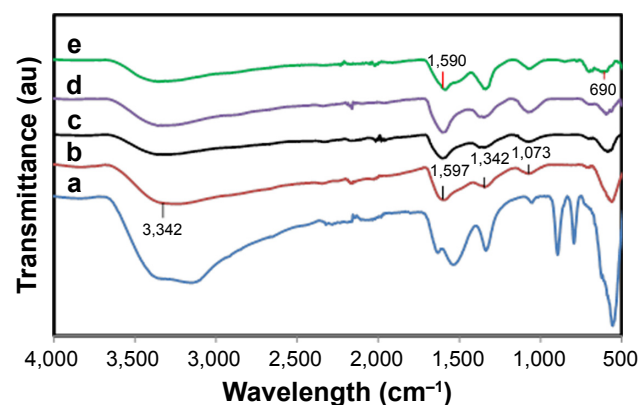


Figure 3 Fourier-transform infrared spectra of honey (a); magnetite nanoparticles with 0.5, 1, 3, and 5% (w/v) honey (b–e, respectively).

determine the potential biomolecules that are responsible for efficient stabilization and also capping of Fe_3O_4 -NPs synthesized using honey. The FT-IR spectrum in Figure 3 shows a strong absorption band at 564.99 cm^{-1} , which is assigned to the Fe–O bond, demonstrates a high grade of crystallinity of the Fe_3O_4 -NPs.³⁵ The absorption band at 609.45 cm^{-1} in the FT-IR spectrum of sample (Figure 3e) indicates the presence of some amount of oxidized maghemite on the surface of magnetite. The characteristic band around $3,200\text{ cm}^{-1}$ is due to the existence of the O–H group.³⁶

The C–O stretching mode merges band of protein in honey arise from the C–O–C symmetric stretching and C–O–H bending vibrations, expected to occur around $1,073\text{ cm}^{-1}$.³⁷ The amide I and II bands of proteins are expected to occur around $1,660$ and $1,535\text{ cm}^{-1}$, respectively,^{38–43} in the current study, these bands occurred around $1,600$ and $1,300\text{ cm}^{-1}$. These bands occur as a result of the carboxyl stretching and N–H deformation vibrations in the amide linkages of protein. Proteins can attach to the Fe_3O_4 -NPs via the free amine group or carboxylate ion of amino acid residues.^{41,44,45} The lack of C=O band because of the stretching mode, the existence of the C–O stretch and amide I and II bands in the FT-IR spectrum (Figure 3) of Fe_3O_4 -NPs represent the stabilization of the system by the $-\text{COO}-$ (carboxylate ion) groups of amino acid remains with free carboxylate groups in the proteins.

Size distribution and morphology of nanoparticles

TEM micrographs of the particle size and distribution of the Fe_3O_4 -NPs are presented in Figure 4. The images reveal significantly smaller nanomagnetite particles with identical particle sizes which have similar shape and are uniformly

dispersed. Also, the Fe_3O_4 -NPs have spherical morphology and a uniform distribution. From the TEM images, it is obvious that the particle size decreases from 3.21 to 2.22 nm for 0.5% and 3.0% (w/v) with the increase in the amount of honey. It is significant to mention that sucrose, glucose (decomposition product of sucrose) and gluconic acid have multiple hydroxyl groups, apart from the carboxylic groups in gluconic acid, in the magnetite synthesis. These functional groups can be absorbed into define crystal planes or chelated with the Fe atoms as a covering material to create steric block, like the common stabilizers and surfactants.⁴⁶

The FESEM images show that the Fe_3O_4 -NPs synthesized using honey have spherical structure (Figure 5). Significantly, no morphological differences were detected with the increase in the honey concentration and confirm that the structure of the nanoparticles remained unchanged. Figure 6 shows the chemical composition of the prepared nanoparticles. From the EDX spectrum, the oxygen and iron peaks reveal the existence of Fe_3O_4 -NPs; the peaks that arise around 0.7 , 6.4 and 7 keV are corresponding to the Fe element.⁴⁷ Besides, the EDX spectra of the Fe_3O_4 -NPs confirm the presence of elemental Fe without any impurity peaks.

Magnetic properties of nanoparticles

The magnetic characterization of the Fe_3O_4 -NPs was performed with VSM. Figure 7 presents the hysteresis loop of all samples measured with a magnetic field of $-8,000$ to $8,000\text{ Oe}$ at room temperature. Samples (a), (b), (c), (d) and (e) display almost immensurable coercivity and remanence, indicating that the magnetic nanoparticles prepared are superparamagnetic.⁴⁸

The saturation magnetization (M_s) of Fe_3O_4 -NPs with 5.0 , 3.0 , 1.0 and 0.5% (w/v) honey are 2.19 , 3.75 , 6.25 and 19.91 emu g^{-1} , respectively, which increases with the decrease in the honey concentration. This might be referring to the decrease of the surface adsorbed species and growth of the particle size.⁴⁹ The VSM shows that the M_s of the Fe_3O_4 -NPs prepared using NaOH (without honey) as the reducing agent is 28.98 emu g^{-1} for sample (a). The presence of organic coating agents on sample (b), (c), (d) and (e) decreased the saturation magnetization value compared with sample (a), which decreases the homogeneity caused by the reducing of the surface moments.⁵⁰ It is widely identified that the size of magnetic particles has an effect on the energy of that particle in an exterior field through the quantity of magnetic molecules in a single magnetic domain. When the energy is converted into the thermal energy, thermal variations

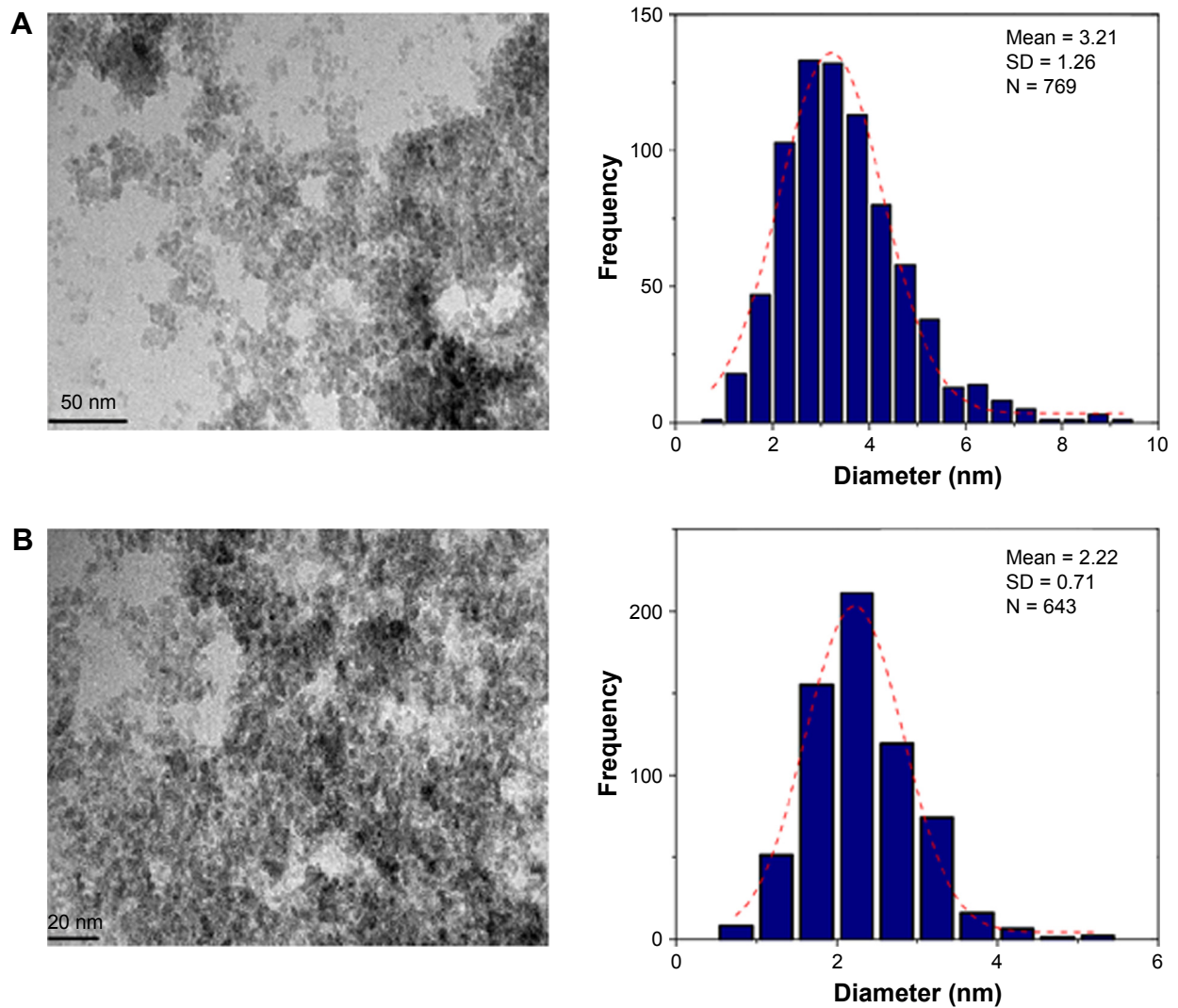


Figure 4 TEM images and histograms of particle size distribution for 0.5% and 3% (w/v) honey/Fe₃O₄-NPs. **(A)** and **(B)**: 0.5% and 3% (w/v), respectively.
Abbreviations: Fe₃O₄-NPs, magnetite nanoparticles; TEM, transmission electron microscopy.

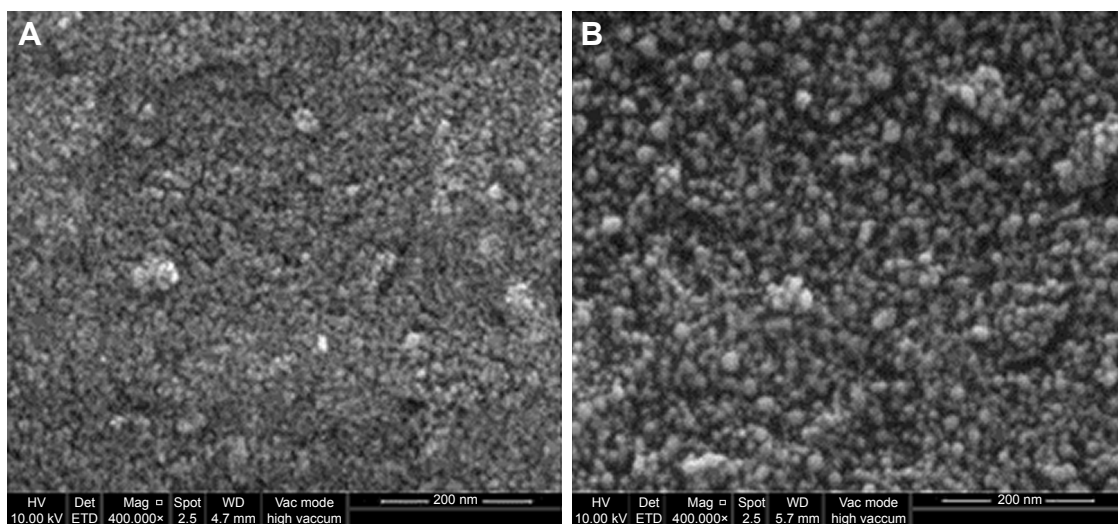


Figure 5 Surface morphology of magnetite nanoparticles with 0.5% and 3% (w/v) honey **(A)** and **(B)**, respectively.

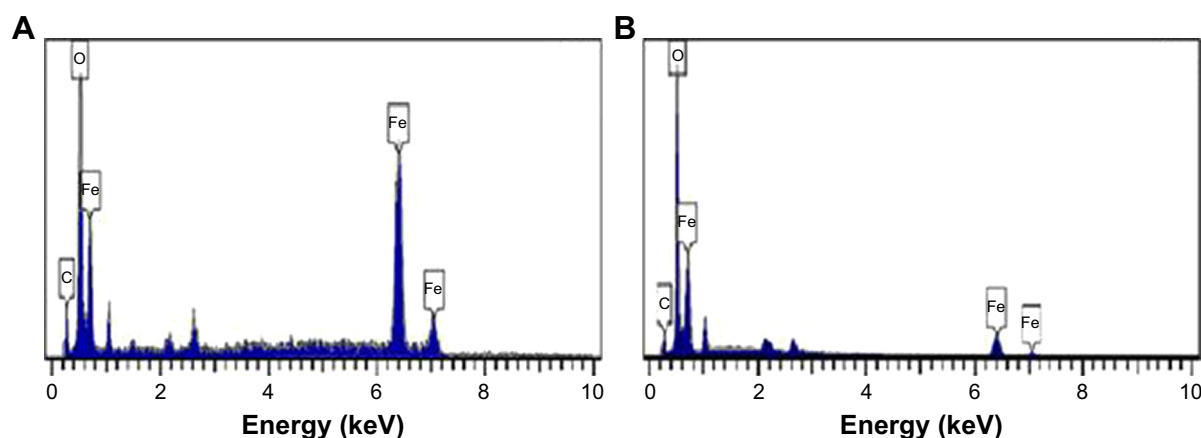


Figure 6 EDX spectroscopy of Fe_3O_4 -NPs with 0.5% and 3% (w/v) honey, (A) and (B), respectively.

Abbreviations: EDX, energy-dispersive X-ray; Fe_3O_4 -NPs, magnetite nanoparticles.

around the prepared Fe_3O_4 -NPs magnetically disturbed surface, which will considerably decrease the overall magnetic moments of a given field.^{51,52} Thus, this circumstance is more critical for smaller nanoparticles because of the more available surface area; consequently the decrease in the saturation magnetization is reasonable. Table 1 gives a comparison between the synthetic methods, particle sizes and saturation magnetization of Fe_3O_4 NPs from the results of other researchers and this work.

Cytotoxicity assay

In vitro cytotoxicity evaluation of Fe_3O_4 -NPs was performed by using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay. The results are shown in Figure 8. Briefly, WEHI164 fibro sarcoma cells (Institute Pastor, Iran) at the density of 1×10^4 cells per well were seeded in 96-well plates and incubated (37°C in 5% CO_2) for 24 hours. Then, various concentrations of Fe_3O_4 -NPs were added and incubated for 24 hours. After 24 hours, 20 μL of 5 mg/mL MTT was added and cells were incubated for another 4 hours. Then the medium was removed and 100 μL of DMSO added in each well to dissolve formazan crystals that produced by living cells. In the following, optical absorbance was measured at 570 (630 used as a reference wavelength) by plate reader (Epoch, BioTech instrument, USA). All experiments were performed in quadruplicate,

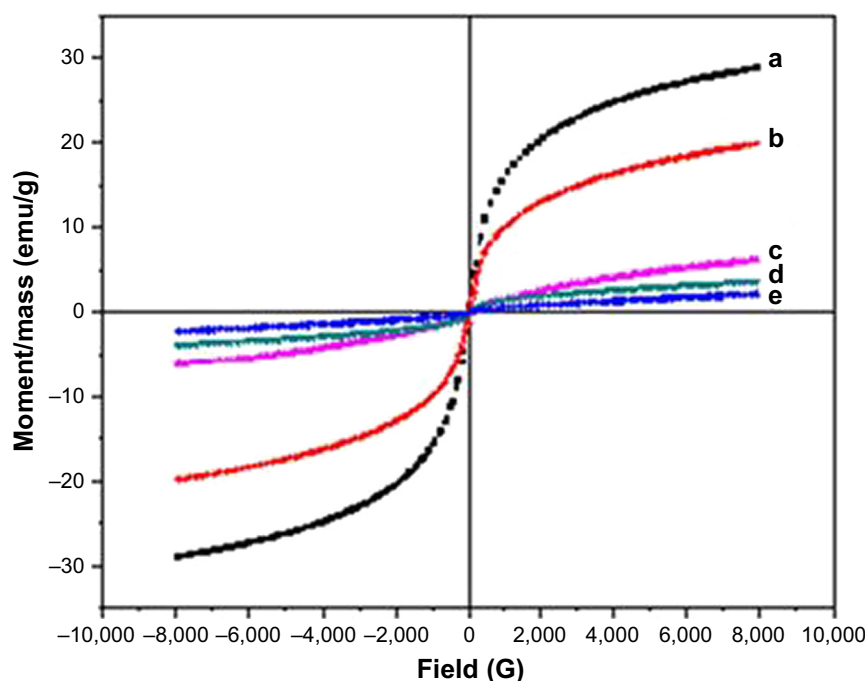


Figure 7 VSM of pure Fe_3O_4 (a) and Fe_3O_4 -NPs with 0.5, 1, 3 and 5% (w/v) honey (b–e, respectively).

Abbreviations: Fe_3O_4 -NPs, magnetite nanoparticles; VSM, vibrating sample magnetometry.

Table 1 Comparison between the synthetic methods, particle sizes and saturation magnetization of Fe₃O₄ NPs from the results of other researchers and this work

Method of synthesis	Particle size (nm)	Saturation magnetization (M _s)	Reference
Coprecipitation method using NH ₄ OH and chitosan	23 nm	15 emu·g ⁻¹	53
Quick precipitation using sodium hydroxide and rice straw	9.93 nm	NA	54
Coprecipitation method using NaOH and montmorillonite	8.24–12.88 nm	12.10–32.40 emu·g ⁻¹	55
Hydrothermal reduction route using sucrose	4–16 nm	14.82–29.55 emu·g ⁻¹	56
Coprecipitation method using NaOH and talc	2.27–8.13 nm	NA	57
Facile technique using diethyl amine	~25 nm	35.76 emu·g ⁻¹	58
Coprecipitation method using NaOH and carob leaf extract	4–8 nm	NA	59
Coprecipitation method using aqueous extract of brown seaweed (<i>Sargassum muticum</i>)	18 nm	22.1 emu·g ⁻¹	60
Coprecipitation method using olive oil (magnetite (Fe ₃ O ₄) and maghemite (γ-Fe ₂ O ₃) nanoparticles)	20 nm	40 emu·g ⁻¹	61
Solvothermal synthesis	~200 nm	78.03 emu·g ⁻¹	21
Coprecipitation method	10 nm	46.7 emu·g ⁻¹	16
Colloidal method at room temperature	4–43 nm	NA	13
Precipitation with forced mixing	~6.0 nm	37.50 emu·g ⁻¹	9
Coprecipitation method using NaOH and honey	3.21 nm	19.91 emu·g ⁻¹	Current study

Abbreviations: Fe₃O₄-NPs, magnetite nanoparticles; NA, not available.

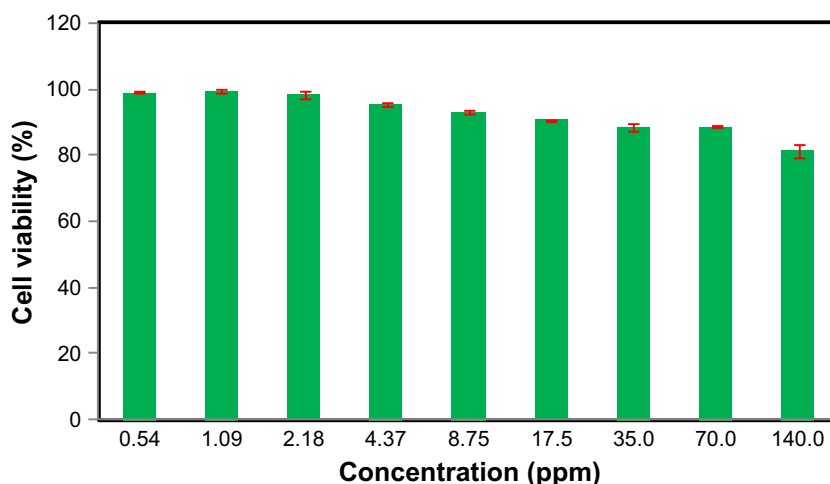
and % cell viability was shown as a percent relative of untreated control cells.^{62–64}

As illustrated in Figure 8, the toxicity of the synthesized Fe₃O₄ Au-NPs to the cells was found to be nonsignificant in higher concentrations up to 140.0 ppm and they were well tolerated by WEHI164 cells in the MTT assay.

Conclusion

This study reports the facile and green synthesis of superparamagnetic Fe₃O₄-NPs using natural honey as the reducing and stabilizing agent. A sharp band at 564 cm⁻¹ in the FT-IR spectra supplementary confirmed the presence of Fe₃O₄-NPs. The particle size of the synthesized material can be

certainly controlled with the change in the concentration of the natural honey. The TEM images showed that the particle size decreased from 3.21 to 2.22 nm with the increase in the amount of honey from 0.5% to 3.0% (w/v), respectively. The VSM analysis shown a super-paramagnetic behavior of the Fe₃O₄-NPs, with a small magnetization of 19.91 emu·g⁻¹ as compared to the Fe₃O₄-NPs prepared in the absence of honey (28.98 emu·g⁻¹), which is related to the nanoparticle size. From the results of these studies, we believe that the present method can be used for the controlled synthesis of Fe₃O₄-NPs, which can find important applications in biotechnology, biosensor and biomedicine, magnetic resonance imaging and catalysis. The most possible co-reducing

**Figure 8** Cell viability of WEHI164 cells measured by the MTT assay (cells were incubated for 24 hours with the indicated concentrations of the magnetite nanoparticles).

agent is fructose and the existence proteins in the natural honey as the capping agent is responsible for stabilization.

Acknowledgments

We would like to acknowledge the financial support provided by University of Malaya under the Equitable Society Research Cluster (ESRC) research grant GC001C-14SBS.

Disclosure

The authors report no conflicts of interest in this work.

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