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Antifungal Effect of Colloidal Iron Oxide Nano-particles on the *Candida albicans*

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One of the important human pathogens that can cause systemic infections is *Candida albicans*. Iron oxide Nano-particles with unique magnetic properties have a high potential for use in several biomedical, bioengineering and in vivo applications. In this study, we were investigated antifungal property of Iron Oxide Nano-particles. *Candida albicans* was undertaken to evaluate the dose- and time-dependent toxic potential. MTT assay showed 65% viability of *Candida albicans* in lower concentrations (5-30 μg/mL) and up to 12 hours of exposure, whereas at higher concentrations (30-100 μg/mL) and prolonged (24-48 hours) exposure viability reduced to 80%–95%. Our results suggest that antifungal activity of colloidal Fe_3O_4 NP that can be useful for medical equipment such as catheters, implants, denturescan be inhibited especially growth of *Candida albicans*.

Key words: Colloidal iron oxide Nano-particles, Cytotoxicity, MTT assay, *Candida albicans*.

Catheter-related infections pursue to be a significant source of morbidity and mortality in patients requiring catheterization and increment medical overheads by lengthening hospitalization[1]. One of the most common etiologies of catheter infections is staphylococci, coagulase-negative staphylococci or S. aureus, P.aeruginosa and fungal infarction spatially in biofilms formation. There are many investigations trying to indicate the productivity of different materials as anti-biofilm coated agents in reduction the incidence of catheter associatedbiofilm infections (i.e.,cefazolin, teicoplanin,vancomycin, silver sulfadiazine, chlorhexidine-silver sulfadiazine, minocycline-rifampin, lysostaphin, ciprofloxacin, and protamine sulfate combinations). Furthermore, there are a lot of examinations reporting the potency of antibiotic-bonded catheters in preventing microbial biofilms from progressing. Itwas displayed that the submersion of central venous catheters and arterial catheters in a 50 mg/mL cefazolin solution decreased the catheter colonization with Staphylococcus epidermidis from 40% to 2%, confirming that antibiotic bonding is an effective, secure, and cost-effective method of reducing intravascular catheter infections in patients who are in intensive care units[2-3]. Furthermore, other research group exhibited that catheter covering with lysostaph in might be more appropriate than antimicrobial joining, due to the quick covering time of catheters with minimal insitu catheter preparation, and the rapidity of kill would annihilate sticky bacteria within a very short amount of time, removing the risk of infections[4] . Nano-silver coverings have been executed to several medical implements ofwhich catheters, drains, and wound

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dressings are the most eminent[4] . The incident of microorganisms living in association aggregated communities is called a biofilm. Biofilms can form on many types of exteriors and interfaces, comprising the human body[4-5]. The Candida as a genus of the fungi, is the most common biofilms that are associated with human disease events, most remarkably Candida albicans, which origins both superficial and systemic disease. C. albicans biofilm is commonly found in children and aging persons with a insufficient immune system, and it is the etiological agent of oral moniliasis; the mortality of patients with incursive candidiasis can be as high as 40%[5-6]. Fungal infections are difficult to annihilate requiring high quantities and longtime disclosure to an antimycotic agent to efficiently eliminate the etiological pathogen[5]. The use of modified nanostructured surfaces for the design of film-coated surfaces of solid and fiber-based materials give a new approach to prevent or disrupt the formation of microbialbiofilms[5] .*Candida albicans* associated wound infections are frequently associated with burns(28%)[6], and less frequently with non-surgical epithelial injuries (0.8%)[7] . A significant increase was seen in infections attributable to *C. albicans* in surgical site postoperative infections[8]. One of the major difficulties in *C. albicans* wound infections is biofilm formation, since microorganisms inserted in biofilms are hundred times more resistant to antifungal compounds[9-10] the infection being therefore, difficult to annihilate[11-12]. Iron oxide based nanosized materials are of great interest for the biomedical field due to their superior properties[13] derived from their innate magnetic nature, as well as from the enhanced physico-chemical properties, such as ultra small and controllable size, large surface area to mass ratio, high reactivity, and functionalizable structure[14] . Magnetite (Fe_3O_4) has been widely surveyed for biomedical applications in biological separations[15-16] drug delivery and targeting, magneticresonance imaging[17] hyperthermia[13] cancer treatment[18-19] stabilization of essential oils[20] repression of microbial colonization and ferrofluids[21-22]. The magnetic nanoparticles as delivery nanosystems are considered powerful new tools to attack the current challenges in treating infectious diseases, by edifying the therapeutic index of antimicrobial drugs, and diminishing the local and systemic side effects, including cutaneous irritation, peeling, scaling and gut flora Reduction[23] In this research, we investigated effect of the antifungal potential of Fe_3O_4 NPs against the growth of C. albicans (ATCC10231) and its potential to remove the fungal biofilm.

MATERIALS AND METHODS

Iron oxide nanoparticle synthesis

Fe_3O_4 nanoparticles were combined in situ by way of a matrix mediated method applying PVA (Poly(vinyl alcohol)) alike to previously mentioned processes. Fleetingly, an aqueous solution of PVA (Sigma, Germany) was mixed in equal volumes of ferrous/ferric aqueous solutions under circumfused conditions. The iron-loaded PVA gels were diped in stoichiometric amounts of warm aqueous solutions of NaOH. The resulting ferrosoferric hydroxide dehydrates reached brownish sedimentations in the polymer solution. Delineation of iron oxide/PVA A droplet of IO nanoparticles was located on a transmission electron microscopy (TEM) copper grid and permitted to dry (Figure1). The imaging was carried out at 100 kV on a Philips EM420 TEM and size calculations were carried out with Image J. To determine the hydrodynamic size of the nanoparticles,100 µL of the particle solution was diluted with 1.5 mL of water and located into a cuvette of a Zetasizer-nano device (Malvern Instruments Ltd).tests were administered in triplicate to obtain an average number-size dispensation (Figure 2).

X-ray diffraction (XRD) on dried Fe_3O_4 NPs

X-ray diffraction analysis was executed on a Siemens D500 within a 2è range of 20–80 degrees using Cu Ká radiation. The magnetic properties of the dried NPswere obtained using vibrating sample manometer at RT-Condition (room temperature) (Figure3).

In Vitro Fungal Biofilm Development

Biofilm formation was sized up in six multi-well plates (Nunc, USA), applying a static model for monospecific biofilms evolvement. Control WD and MNP18-SH covered WD pieces of 1 cm × 1 cm was sterilized by exposure to direct UV light for 20 min and divided into six multi-well plates (one per well). Two milliliter of the C. albicans inoculants with standardized density were supplemented in each well, to entirely cover the WD pieces, then

specimens were incubated at 37 °C for 24 h. Biofilms were analyzed by viable cell count assay. Briefly, in order to remove unattached bacteria after 24 h incubation, the culture medium was ejected and the pieces of WD washed with sterile PBS (phosphate-buffered saline). WD specimens were located in fresh medium and incubated for other additional 24 h, 48 h and 72 h. After the incubation period wound dressing pieces were gently washed with sterile PBS for not disturbing the biofilm and located in 1.5 mL tubes containing 750 ìL PBS. Specimens were forcefully mixed by vortexing for 30 s and sonicated for 10 s in order to break up biofilm cells from the suspension. Serial ten-fold dilutions were obtained and plated on Sabouraud Agar for viable cell counts assay. Tests were carried out in triplicate and repeated on three separate occasions.

Antifungal activity of Fe_3O_4 NPs against *C. albicans* growth (MTT assay)

C. albicans (ATCC 10231) was come by from the American Type Culture Collection, (Pasteur Institute of Iran). The antifungal effect of Fe_3O_4 NPs on C. albicans growth was settled applying a (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, Germany), following the guidances from the manufacturer. C. albicans was grown in Trypticase™ Soy Broth (TSB) at 37°C, overnight in aerobic conditions. The fungal cells were counted, applying a Neubauer chamber, and 1×10^4 cells were inoculated in 100 ìL of TSB medium, in a 96-well polystyrene plate. Three wells with only TSB medium were used as controls for growth of C. albicans. Ketoconazole was used as a positive antimicrobial control. The 96-well plate was incubated at 37°C overnight. Subsequent, 20 ìL of MTT was supplemented to each well, and the plate was guarded against light and incubated at 37°C for four hours. Following, 100 ìL of DMSO (dimethyl sulfoxide) was supplemented to break up the reduced MTT species. The quantity of live cells was determined utilizing a microplate absorbance reader (Rayto EKISA reader, USA), at 572 nm. The trials were repeated three times, and the deliberate OD (optical density) was evaluated by illustrative statistics (Figure 4).

Cytotoxicity of Fe_3O_4 NPs on mammalian cells (3T3) by MTT assay

In this scrutiny, we used mouse embryo fibroblast (3T3) cells to evaluate the cytotoxicity effect of Fe_3O_4 NPs. A merging monolayer of 3T3 cells (NCBI Code:C155) was grown in RPM 1640 (Gibco, UK) supplemented with 10% of Fetal Bovine Serum (FBS) (Gibco, UK), at 37°C with 5% of CO_2, in a 96 well mammalian culture pleat. This was naked to 5, 10, 30, 50,100µl of Fe3O4NPs for 12, 24, 48 hours, and the possible cytotoxicity effect was discerned by MTT assay and invert microscopy. For the negative control, cells without NPs were operated (Figure 5,6).

Minimal inhibitory concentration (MIC) of Fe_3O_4 NPs

Five tubes in the McFarland scale, with 1×10^9 CFU was acquired (McFarland scales are fungal solutions, For MIC determination. There are 1–9 ranges in this scale with a precise number of microbes in each one. C. albicans was grown in TSB agar and incubated at 37°C for 24 hours. One colony was inoculated in five mL of TSB medium and incubated at 37°C for 24 hours. The fungal enumeration was concluded with a Neubauer chamber. Tubes with a final concentration of 1×10^6 CFU were obtained by dilution of the five tubes in the McFarland scale. The Fe_3O_4 NPs suspension was diluted to final concentrations of 0.25, 0.5, 1, and 1.5, from the two mM stock. Subsequently, one mL of each Fe_3O_4 NPs scattering was mixed with a fungal culture medium and afterwards, incubated at 37°C for 18 hours. The MIC was settled by the presence or absence of murkiness in the different tubes containing the NPs.

Statistical Analysis

Data were examined applying Microsoft office Excel software and SPSS, by applying One-way Analysis of Variance (ANOVA) test. *P* values lower than 0.05 were considered significant.

RESULTS AND DISCUSSION

Iron oxide (IO) has been greatly used for biomedical research because of its biocompatibility and magnetic properties[1-2]. Already, have been advanced as contrast agents for magnetic resonance imaging (MRI)[3,4], as hyperthermia agents[5-6] and as transporters for targeted drug delivery to treat several kinds of cancer by using Nanoparticle of IO that its size was less than 100 nm[7-8]. It is further conceived that through the employ of magnetic NPs, an desirable drug delivery

system can be advanced by using an external magnetic field to direct such as NPs to eligible sites for immediate therapy[9] simultaneously this line, some have hypothesized that reactive oxygen species (ROS) made by Fe_3O_4 NPs could kill bacteria without damaging non-bacterial cells[17]. particularly, Pareta *et al* cultured osteoblasts (bone-forming cells) with IO NPs (at a concentration of 4.25 mg/mL) and discovered that cell density was extremely enhanced in the presence of IO NPs contrasted with cells cultured without NPs[9].

Nanoparticle combination and characterization TEM images of the synthesized Fe_3O_4 revealed that the size of the nanoparticles was 83 nm ± 3 nm (Figour 1). The nanoparticles constructed necklace-like chains with an average length of approximately 83–100 nm. A similar construction was reported in an earlier study, in which IO nanoparticles were supposed to sediment along the polymer chain of PVA[20-21]. The nanoparticle solution was a clear, and no major forms of accumulation were detectable after Several days from synthesis. The hydrodynamic diameter calculation results demonstrated that with the PVA coating, the IO chain-like particles had a typical size of 83-110 nm.

In this scrutiny, we demonstrated proof of the antimycotic activity of Fe_3O_4 NPs. Their

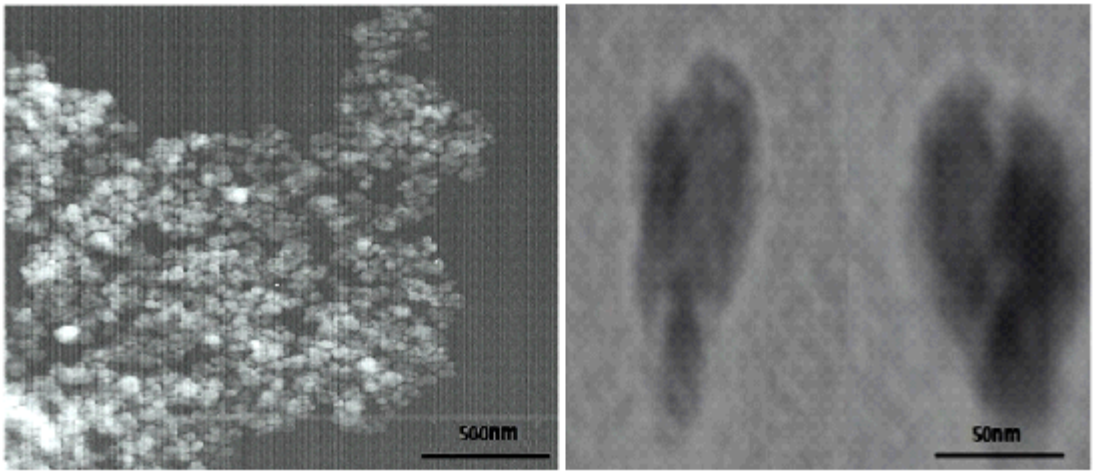

Fig. 1. TEM images of the synthesized Fe_3O_4 nanoparticles in different bars (a: 500nm and b:50 nm)

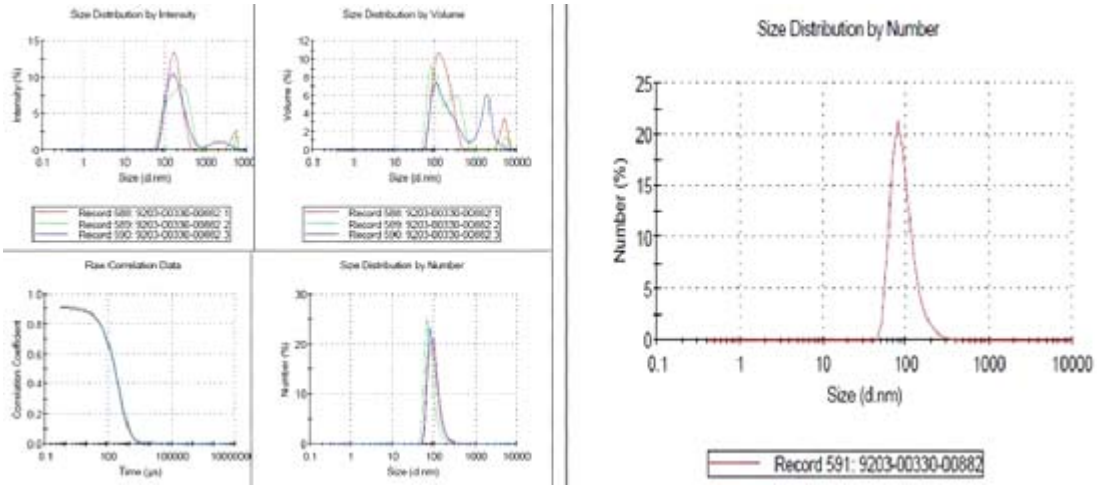

Fig. 2. Fe3O4 NPs size distribution as measured by dynamic light scattering. DLS Results show that average of NPs present in 80-100nm

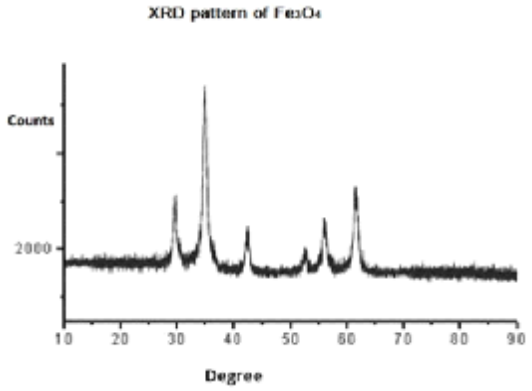

Fig. 3. Fe_3O_4 NPs X-ray diffraction (XRD) pattern. This sample was synthesized in water, as described in the Materials and methods section

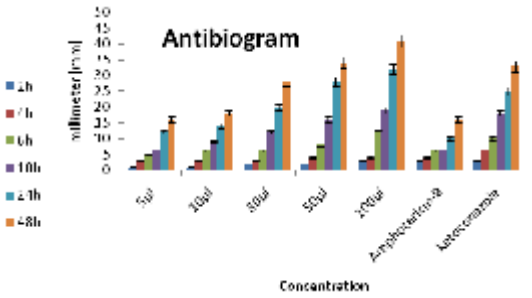

Fig. 4. Antifungal activity of Fe_3O_4 NPs against *Candida Albicans* growth and comparative with amphotericin-B and Ketoconazole at the antibiogram study

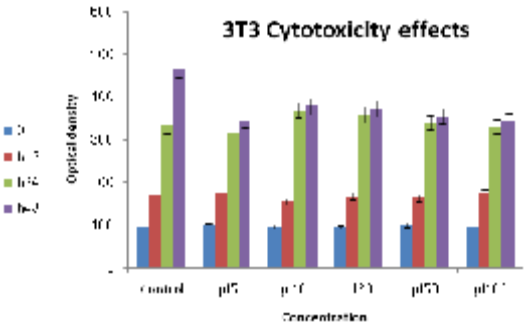

Fig. 5. Cytotoxicity of Fe_3O_4 NPs detected by MTT assay. The possible cytotoxicity effect of Fe_3O_4 NPs was evaluated in 3T3 cell line by MTT assay

advantage in repressive C. albicans growth was considerably better than that of Ketoconazole and amphotericin-B. These results reveal that Fe_3O_4 NPs are best antifungal agents than the most commonly used oral antiseptic and trading antifungal agents. To be confirmed that the antifungal effect was due to the nanostructured Fe_3O_4, this was compared with the antifungal effect of bulk Fe3O4. This result was very important because it demonstrated the efficacy of the nanostructured material against the same bulk compound[20] recommending that it is the nanostructured particle of Fe3O4NPs that is discussed with the antimicrobial properties expressed earlier[15]. These results indicate that Fe3O4NPs could be very attractive antifungal agents. Previously, it was reported that zinc, titanium, and silver NPs have very good antifungal activity[22, 24] however, it is impossible to confirm any quantitative antibiofilm activity comparison, due to the type of published results.

Inorganic nanostructures have many implementations in fields resembling the biological sciences and medicine. NPs have been directed as coating materials and in treatments and diagnosis[25]. NPs of titanium dioxide, silver, diamonds, iron oxides, carbon nanotubes, and biodegradable polymers have all been studied for their benefit in diagnosis and treatments. NPs of silver, copper oxides, and selenium have been described to have

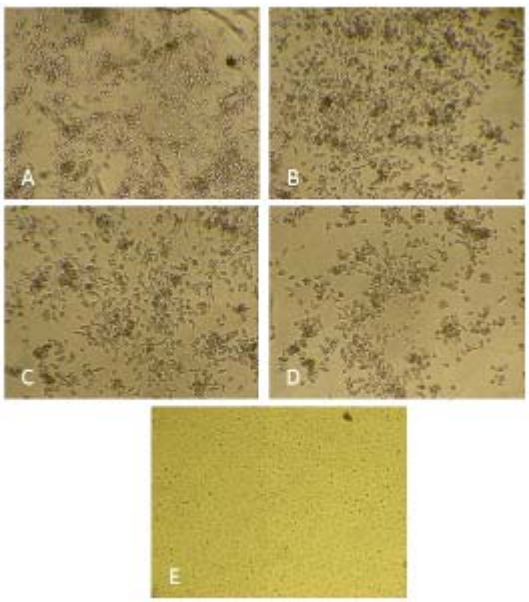

Fig. 6. invert image microscopy from minimal inhibitory concentration (MIC): 10µl: 0.25mM of Fe_3O_4 NPs at 12h (A), 24h (B), 48h (C), 72h (D), control after 72h prolong culture of 3T3 cell line(E)

antimicrobial activity[26-27].The benefits of inorganic NPs are their high surface-to-volume ratios, different shapes, many structural deficiencies and of course, their nanoscale size, which permits more active sites to interact with biological systems such as bacteria, fungi, and viruses. This is the most significant difference between NPs and the conventional organic molecular antimicrobial agents, and could decrease the risk of developing antimicrobial resistance.

The mechanism of the antimicrobial activity of inorganic NPs is not fully implied, and their precise mechanism of activity against bacteria and fungi remains to be fully understood. Repression of Candida albicans biofilm revealed by invert microscopy and MTT assay, after 12, 24, 48 hours. As a growth control, C. albicans was added to culture media.

It has been elucidated that positive charges on the metal ion are critical for the antimicrobial activity, letting for the electrostatic attraction between a negatively charged cell membrane and the positively charged NPs[28]. It has as well as been reported that silver NPs can damage DNA, change gene expression, and affect the membrane-bound respiratory enzymes[29-30].

Simultaneous to their antifungal activity, the Fe_3O_4 NPs had the capability to interfere with biofilm construction of C. albicans. This antibiofilm activity of the Fe_3O_4 NPs was also studied. Startlingly, the antibiofilm construction effect was complete in the media with the Fe_3O_4 NPs. This effect was unforeseen since Fe_3O_4 NPs only lowered cell growth and did not completely repress it. To illustrate this finding, we propose that 85% of cells were inactivated during inoculation with Fe_3O_4 NPs and that outliving cells were deficient to build a biofilm. It may be that they went into planktonic and stress states due to the presence of the Fe_3O_4 NPs, and it is probably that they were lost during the wash of dye excess.

It is remarkable that there are published reports of fungal growth repression, comprising inhibition of C. albicans, where inorganic NPs, such as diamond, silver, gold, platinum, and palladium-based NPs, were used as antifungal agents[31]. especially, silver (Ag) NPs have also been shown to suppress yeast growth[28] and their antifungal activity against specific species of Candida is well attested[32]. These studies elucidate proof for the molecular mechanism of Ag NPs activity, whereby Ag NPs act on and repress a number of oxidative enzymes, such as the yeast alcohol dehydrogenase, through the generation of reactive oxygen species[28]. Ag NPs have been illustrated to a eligible and promising antifungal effect in several examinations, with no serious side effects to the host[33]. However, the outcomes and the experimental conditions used in the mentioned before studies are not comparable with those of this investigation.

The potential cytotoxic effect of Fe_3O_4 NPs was investigated in 3T3 cells by invert microscopy and MTT assay. No alteration was revealed in the morphology of the nuclei. In fact, proposing the lack of cytotoxicity effect after 24 hours of exposure. Earlier, the toxicity of metal oxide NPs, especially Ag oxide (Ag_2O), zinc oxide (ZnO), and iron oxide (Fe_2O_3),was declared[34]. The possible toxicity of Fe_3O_4 NPs was examined applying the same concentration of NPs as was employed in those antifungal assays.

For particle toxicity, three factors are critical: size, shape, and chemical constitution. A diminution in the size of a nanosized particle leads to an increase in the specific surface area of the nanostructured powder. consequently, more chemical species may stick to its surface, which elevates its reactivity and leads to an increase in its toxic effects[35-36].

In this case, the colloidal Fe_3O_4 NPs had a proportionately small size; however, the number of nanoclusters introduced into the 3T3 cell culture medium, was considerably low compared with the total loads of the other two derivatives of IO.

In this work, we attended on the efficiency of Fe_3O_4 NPs in repressive the growth of C. albicans. All together, the experimental data propose that Colloidal Fe3O4NPs could be an interesting alternative to battle the fungal infections at the origin of biofilms. The property of Fe_3O_4 NPs could be used in oral health, supporting the antifungal activity of oral antiseptics. Further investigations will be necessary to determine the possible toxicity of Fe_3O_4 NPs and analyze the genotoxicity (apoptosis, DNA fragmentation, DNA damage) in human fibroblasts cultures and examine their potential use in humans[37, 38].

In summary, Fe_3O_4 NPs of 83 nm average size has an antimycotic activity repressive the growth of C. albicans, as well as an antibiofilm

activity. This conduct is different from one of the related bulk materials; so, the observed activity is a size-dependent property, an explanation that was proposed first time. Moreover, our results suggest that the Fe_3O_4 NPs, under the experimental tested conditions and concentrations, do not display cytotoxicity.

CONCLUSIONS

Fe_3O_4 NPs were characterized with TEM, dynamic light dispersing and XRD. A live/dead assay (MTT assay) demonstrated that at the lowest doses of iron oxide (5-30µl), the growth of C. albicans was inhibited significantly compared with the positive control samples (Amphotericin-B and Ketoconazole). Further studies should investigate the Microbial effect of iron oxide NPs on other types of invasive microbial for potentially widening such antimicrobial applications.

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