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Influence of ZnO Nanoparticles on *Candida albicans* of Human Male Pleural Fluid

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Abstract
The utilization of metal oxide nanoparticles, especially zinc oxide, is of a great importance in the medical field because of its physical and chemical properties as well as its antimicrobial potential effects. In our study, the ZnO nanoparticles were synthesized by the precipitation method where pH=14. ZnO nanoparticles were characterized by ultraviolet–visible spectroscopy, X-ray diffraction (XRD), transmission electron microscopy (TEM) and atomic force microscope (AFM). Antifungal activity of the ZnO was tested against *candida albicans*. The results showed that *C. albicans* (15 samples) became resistant to the fungal activity after testing its sensitivity to several types of fungal antibiotics. UV-vis spectroscopy, XRD, TEM and AFM showed that this precipitation synthetic method can produce a good quality of ZnO nanoparticles with sizes in the range of nanometer scale. In ZnO NPs (calcined), the inhibition zone diameter of *C. albicans* was 11 mm at a ZnO NPs concentration of 800 mg/ml, while no inhibition zones were formed at the other concentrations (0.25, 0.5, 1, 2, 10, 50 and 500 mg/ml). Using ZnO NPs (not calcined), the inhibition zone diameter of *C. albicans* was 24 mm at a concentration of 800 mg/ml, while no inhibition zones were observed at the other concentrations (0.25, 0.5, 1, 2, 10, 50 and 500 mg/ml). In addition, a toxicity test was performed on mice and proved that ZnO NPs are effective against *C. albicans* with a toxic effect on liver and spleen cells in rats. The aim of this research was to characterize the in vitro activity of ZnO nanoparticles synthesized by the precipitation method against *C. albicans* of human male pleural fluid using the well diffusion method as well as their toxic effects on both liver and spleen cells in mice.

Keywords: ZnO NPs, *C. albicans*, Pleural fluid, Calcination, Inhibition zone.

تأثير جسيمات اوكسيد الزنك النانوية على مبيضات البيض المعزولة من السائل الجنبي للانسان

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الخلاصة
ان استخدام اكاسيد المعادن النانوية وخصوصا اوكسيد الزنك في المجال الطبي ذو اهمية كبيرة, نظرًا لخصائصه الفيزيائية والكيميائية وله أيضًا خصائص مضادة للميكروبات. في دراستنا هذه, جزيئات اوكسيد الزنك النانوية حضرت بطريقة الترسيب عند الرقم الهيدروجيني =14 . تم توصيف جزيئات اوكسيد الزنك النانوية بفحص المجهر الالكتروني النافذ وحيود الاشعة السنية و مطيافية الضوء المرئي– فوق البنفسجي ومجهر القوة الذرية. تم اختبار النشاط المضاد للفطريات لجزيئات اوكسيد الزنك النانوية ضد المبيضات. أظهرت النتائج ان المبيضات المأخوذة والتي عددها (15 عينة) مقاومة للمضادات الفطرية بعد اختبار

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حساسيتها للعديد من انواع المضادات الفطرية, ويظهر UV–vis Spectroscopy, XRD, TEM و AFM أن طريقة الترسيب يمكن أن تنتج جسيمات نانوية من اوكسيد الزنك ذات نوعية جيدة وأحجامها في نطاق النانو. بالنسبة لجزيئات اوكسيد الزنك النانوية في حالة التكلس، يبلغ قطر منطقة تثبيط 11 ملم عند تركيز ZnO 800 ملغ / مل ، في حين لا توجد منطقة تثبيط عند تراكيز ZnO (0.25 ، 0.5 ، 1 ، 2 ، 10 ، 50 و 500) ملغم / مل. وفي حالة جزيئات اوكسيد الزنك النانوية غير المكلس ، يبلغ قطر منطقة تثبيط 24 ملم عند تركيز 800 ملغ / مل، بينما لا توجد منطقة تثبيط عند التراكيز (0.25 ، 0.5 ، 1 ، 2 ، 10 ، 50 و 500) ملغم / مل. ثم تم اجراء اختبار فحص السمية على الفئران. حيث اثبتت الدراسة ان جزيئات اوكسيد الزنك النانوية فعالة ضد المبيضات وأظهرت أيضًا وجود تأثير سام على خلايا الكبد والطحال في الفئران. الهدف من هذا البحث هو دراسة خصائص جزيئات اوكسيد الزنك النانوية المصنعة بطريقة الترسيب ضد مبيضات البيض المعزولة من السائل الجنبي للانسان بطريقة الانتشار الجيد وكذلك دراسة تأثيرها السام على كل من خلايا الكبد والطحال في الفئران.

Introduction

Nanotechnology is defined as the study of the materials with a size that lies in the nanometer scale, and the characterization of their components, such as fibers, particles, grains, which have dimensions of less than 100 nm [1]. Nanomaterials are categorized according to their dimensions into zero, one, two, and three dimensional [2]. Characteristics of nanoparticles are different from those of the similar atoms that are bonded to form bulk materials and nanoparticles that are bigger than single atoms and molecules but, however, are smaller than bulk materials [3]. Hence, the difference that matters is that the materials at their nano scale behave differently from their bulk counterparts because nanoparticles have a surface-to-volume ratio that is relatively higher than that of the bulk materials [4]. Nanomaterials have been manufactured using different methods, including precipitation, chemical vapor deposition, hydrothermal, milling, etching, sputtering and laser ablation methods [5]. ZnO NPs are one of the most important nanoparticles of metal oxides; it is a unique and inorganic material that appears in a non-water-soluble white powder, with an energy gap of 3.37 electron volts at room temperature [6]. They have been widely used in a wide range of applications in different areas, such as industry (rubber, concrete [7], and textile [8]) and biological applications (anti-bacterial, anti-inflammatory etc. [9]). ZnO NPs appear in different forms, including nano-rods [10] and nanospheres [11]. *C. albicans* is a yeast that grows naturally within the human body and lives in the gastrointestinal, respiratory, and female reproductive tracts as well as on the skin [12]. *C. albicans* is a major fungal pathogen and the main reason of nosocomial infections that can cause both superficial and invasive infections [13]. A mortality rate of 40% was reported for patients who suffer candidiasis caused by *C. albicans* [14]*; C. albicans* is cultured in the lab easily and can be studied *in vitro* and in *vivo* [15].

Materials and methods

Isolation and Identification of *C. albicans*

The clinical samples of *C. albicans* were taken from the pleural fluid of 15-40 years-old patients in Al-Imamein Al-Kadhimein Medical City. The study started in October 2018 and ended in July 2019. The samples were sent to the microbiological lab department to be diagnosed, and then their identification was confirmed in the Department of Microbiology at the Faculty of College of Medicine - Al-Nahrain University.

Morphology Characteristics

Candida species and other yeast colonies were diagnosed depending on the cultural characteristics on Sabouraud Dextrose Agar (SDA); including shape, color, size, and microscopic examination on slides after staining with Gram or lactophenol cotton blue stains in order to determine the morphology of the yeast cells.

Germ Tube Production

Germ tubes production by the cells is a diagnostic characteristic for *C. albicans.* A small portion of the yeast colony to be tested was emulsified with 0.5 ml of mammalian serum in a small test tube. The tube was incubated at 37 °C for 3 hrs. A drop of the serum was removed to a slide and examined microscopically for germ tube production [16].

Resistance of *C. albicans* to various selected antifungal antibiotics

Resistance patterns of *C. albicans* to various selected antifungal antibiotics were determined by Disk Diffusion Test (DDT) [17]. The antifungal disks tested were Amphotericin B (100 units), Fluconazole (25 mg), Ketoconazole (10 mg), and Caspofungin and Itraconazole (25 mg). The *C. albicans* selected in this study were resistant to all these antifungal antibiotics.

Preparation of Zinc Oxide Nanoparticles (ZnO NPs)

The used preparation procedure was described by Koutu *et al*. [18] but applied with some modifications. Zinc acetate dehydrate ($Zn(CH_3CO_2)_2.2H_2O$) and sodium hydroxide were purchased from CDH. ZnO NPs were synthesized by the precipitation method using zinc acetate dehydrate as a source of zinc, sodium hydroxide as a precursor, and deionized water as diluent. 0.1 mole of zinc acetate dehydrate was dissolved in 100 mL of deionized water with stirring using a magnetic bar stirrer for the purpose of complete dissolving and the formation of a transparent solution. After making sure that the zinc acetate dehydrate was completely dissolved, sodium hydroxide (NaOH) was gradually added at different quantities with stirring for the purpose of changing the pH value of the material to be prepared, using a pH meter to measure the pH required. A white solution was obtained and left on the magnetic stirrer for 30 minutes at 75 °C. The solution was then washed and filtered five times with deionized water . A white precipitate was formed and dried in an electrical furnace at 100 ° C. The white precipitate was separated into a part that was calcined at 500 ° C for 3 hours and part without calcination. The resulting material was grinded by a mortar to obtain the final product (ZnO NPs in powder shape), as shown in Figure-1.

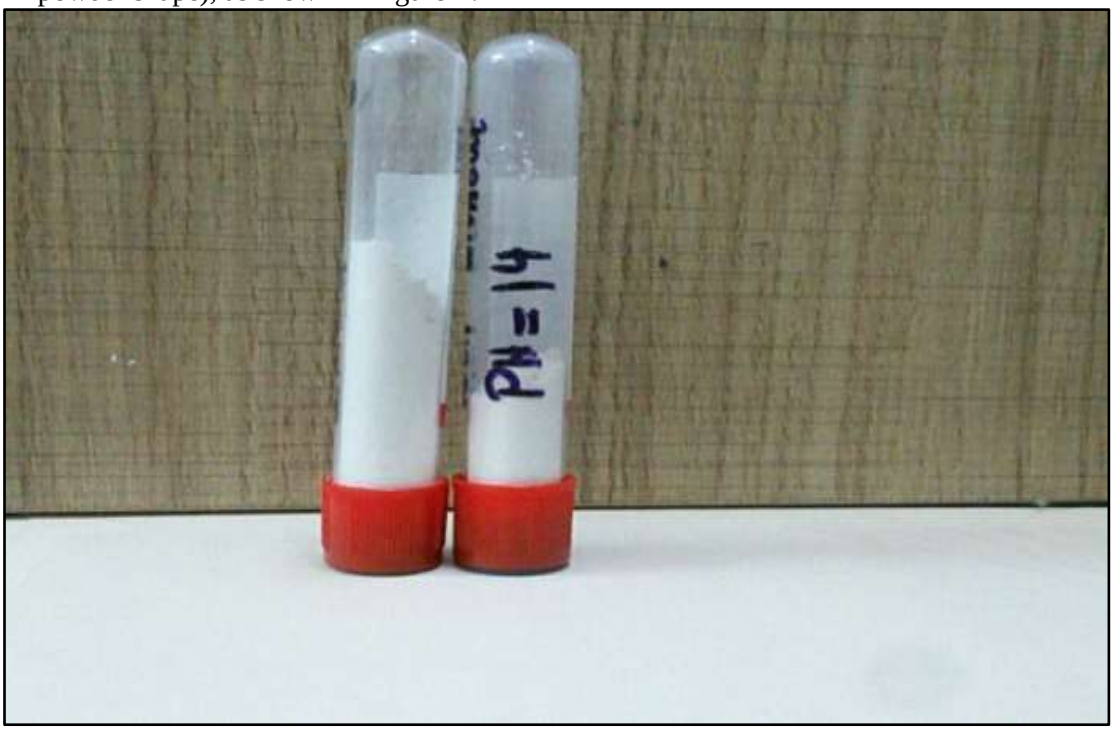

Figure 1-ZnO nanoparticles powder.

Characterization techniques

The synthesized ZnO NPs were characterized by:

- UV-vis Spectroscopy (Shimadzu, UV-1601PC).
- X-Ray Diffraction (XRD) (Shimadzu, XRD-6000).
- Transmittion Electron Microscopy (TEM) (Philips, CM10).
- Atomic force microscope (AFM) (Angstrom Advanced Inc. , AA2000, Contact mode).

Culture media for the isolation of *C. albicans*

Potato Dextrose Agar (PDA): this medium was prepared by dissolving 39 g of PDA in 1 liter of distilled water, heated and sterilized at 121°C in a pressure of 15 pound/inch^2 for 15 minutes according to the instructions of the producing company. Then the medium was dispensed into sterile petri dishes and stored at 4°C until use.

Activity of ZnO NPs *in vitro*

After pouring the PDA into a petri dish, a small swab of the *C. albicans* sample that is most resistant to the antifungal antibiotics was taken and distributed evenly on the surface of PDA, using a special sterile cork borer (5 mm in diameter). Wells were then made on the PDA surface in a petri dish. ZnO NPs of different concentrations (0.25, 0.5, 1, 2, 10, 50, 500 and 800 mg/mL) were dissolved in sterilized deionized water by using an ultrasonic bath, homogenized by a vortex mixer, and poured into the wells in a 0.1 ml volume along with deionized water as a control.. Finally, the dishes were inserted into the incubator for 72 hrs at a temperature of 37 ° C and then the formation of growth inhibitory zones was examined . When the growth inhibition zone was formed, its diameter was measured in millimeters (mm) [19].

Experimental Animals

For the purpose of testing the toxicity of zinc oxide nanoparticles, a histopathological study was conducted. This study was approved by the Animal Research Ethics Committee of the University of Baghdad, Iraq. Thirty mice were divided into 5 groups, each group containing 6 mice aged 5-6 weeks and weighing 20-25 gm. The mice were kept in plastic cages in the animal house of Baghdad Research Center, University of Baghdad at room temperature (22±3 °C) and normal nutrition. The mice were left for 7 days for adaptation before lab investigation. For the histopathological study, one group of mice was injected intraperitoneally with 0.1 ml of a concentration of 800 mg/ml ZnO NPs for 7 days, while another group was used as a control with normal feeding of tab water. All the albino male mice were hair shaved, sacrificed using diethyl ether, and vivisections of liver and spleen were taken. The organs were kept in formalin before lab investigation.

Results and Discussion

From morphology characteristics, hyphae showed distinct points of constriction resembling sausage links, and budding yeast forms (blastoconidia) were often seen. The germ tube production test revealed a cylindrical filament originating from the blastoconidium without any constriction at the point of origin and without obvious swelling along the length of the filaments, indicating a germ tube positive yeast [20].

Figure-2 shows that the optical absorption of ZnO NPs was in the range of 0 – 1000 nm at room temperature. Characteristic absorption peaks were observed at 360-380 nm, while no other strong peaks were observed in the UV spectrum of ZnO [21]. The sharp peaks at 375 and 373.5 nm were observed (at PH= 14) for the calcined and non-calcined ZnO NPs, respectively. Results of the ZnO NPs calcined at 500 ° C for 3 hours are in agreement with those of Ghorbani *et al*. who showed that the peak absorption was 372 nm [22], while the result of non-calcined ZnO NPs are almost compatible with those of Barve *et al*. who demonstrated that the peak absorption of ZnO NPs was 380 nm [23]. From these results, we can conclude that the non-calcination processes produced a blue shift which indicates more energy.

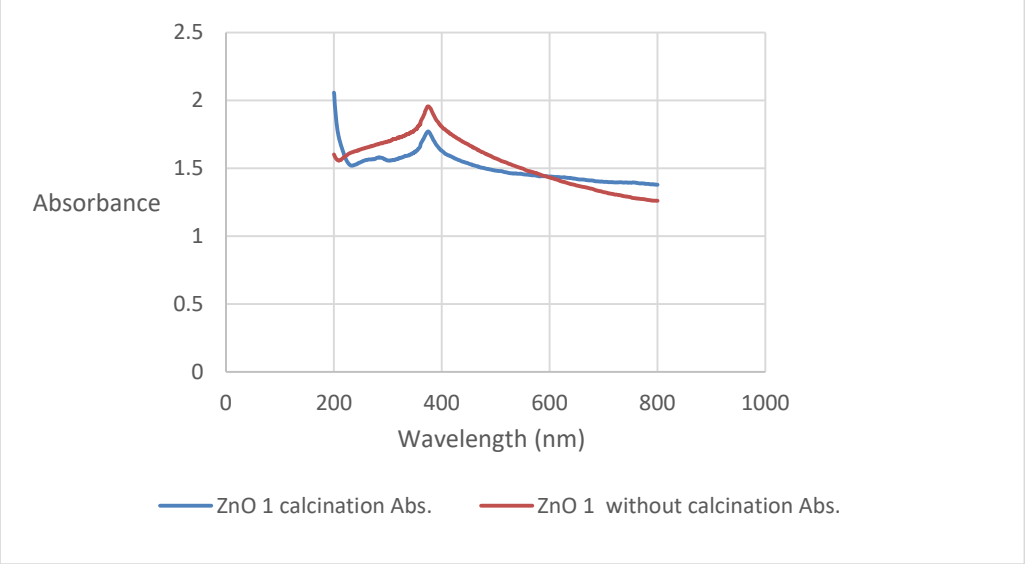

Figure 2-UV-visible absorption spectra of ZnO nanoparticles synthesized in both calcined and non-calcinedconditions.

Figure-3 (a & b) indicates the XRD pattern of ZnO NPs which was located in the 2θ range of 25˚-60˚. The diffraction peaks appeared at 31.81˚, 34.46˚, 36.28˚, 47.56˚, 56.62˚, and at 31.75˚, 34.40˚, 36.22˚, 47.51˚, 56.56˚ for the calcined and non-calcined NPs, respectively. These peaks were identical to the standard zinc oxide peaks (ICDD card: International Centre for Diffraction Data). The average crystallite sizes were calculated by Debye Scherrer's equation [21]:

$$D = 0.9\,\lambda/\beta\cos\theta \qquad \text{.....(1)}$$

Where D is the average crystallite size, λ is the wave length of X-ray equal to 1.54 A˚, β is the peak width at half maximum, and ɵ is the Bragg's diffraction angle.

The D value for the calcined ZnO NPs was equal to 38 nm, while that for the non-calcined ZnO NPs was 37 nm. These results are consistent with the TEM measurements. The results of the ZnO NPs calcined at 500 °C for 3 hours are in agreement with those of Bagheri *et al*. who revealed that the average crystallite size was 35 nm [24], while the results of the non-calcined ZnO NPs are in an approximate consistence with those of Barve *et al*. who showed that the average crystalline size was 23 nm [23]. A line broadening of the X-ray diffraction peaks indicates that the synthesized material consists of particles in the range of nanoscale. It was also clear that the ZnO NPs were free of impurities since they did not show peaks other than the peaks of zinc oxideNPs.

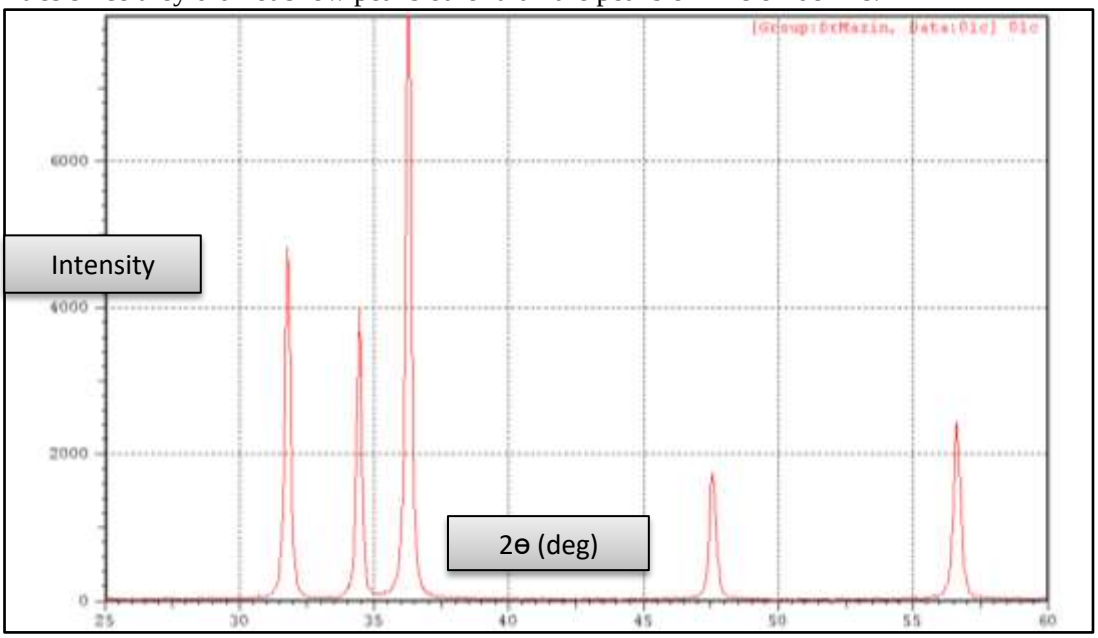

Figure 3a-X-ray diffraction pattern of the calcined ZnO nanoparticles

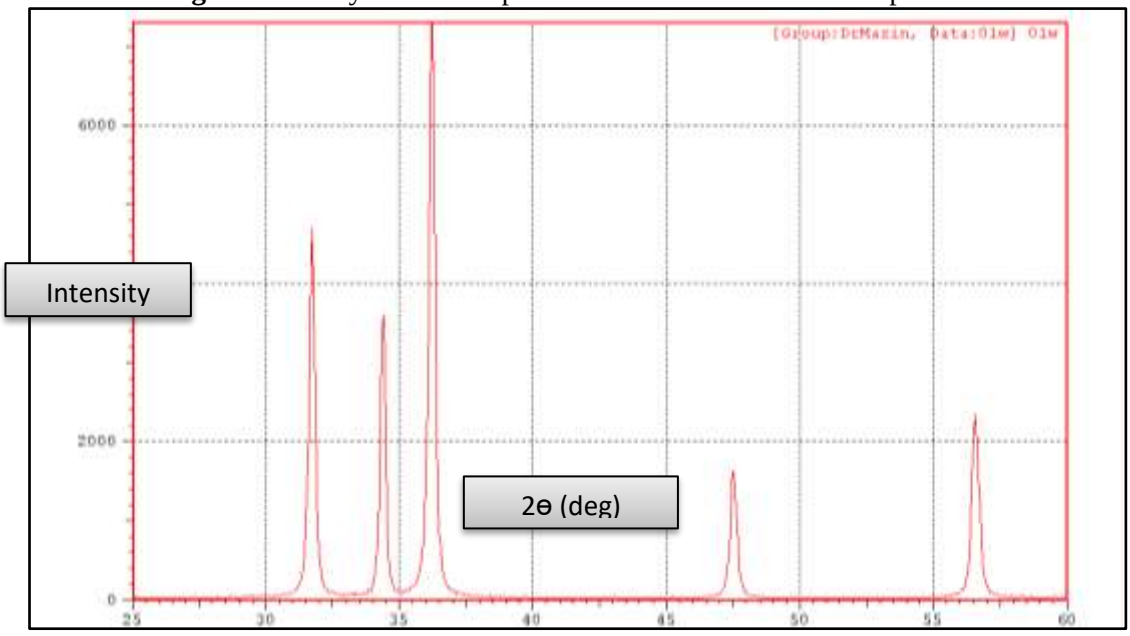

Figure 3b-X-ray diffraction pattern of the non-calcined ZnO nanoparticles

Figure-4(A & B) indicates TEM image of the morphological characterization of ZnO NPs We observe that the shape is spherical and the average sizes are 30 & 28) nm for the calcined and non-calcined ZnO NPs , respectively. The result of the calcined ZnO NPs is in a good agreement with that of Bagheri *et al*. who indicated the formation of spherical-shaped NPs with an average size after heat treatment (500 ˚C for 3 hours) in the range of 30–50 nm [24]. The result of the non-callcined ZnO NPs almost agree with those of Barve *et al*. who demonstrated that the ZnO NPs are of a spherical shape with an average size of approximately 20-25 nm [23].

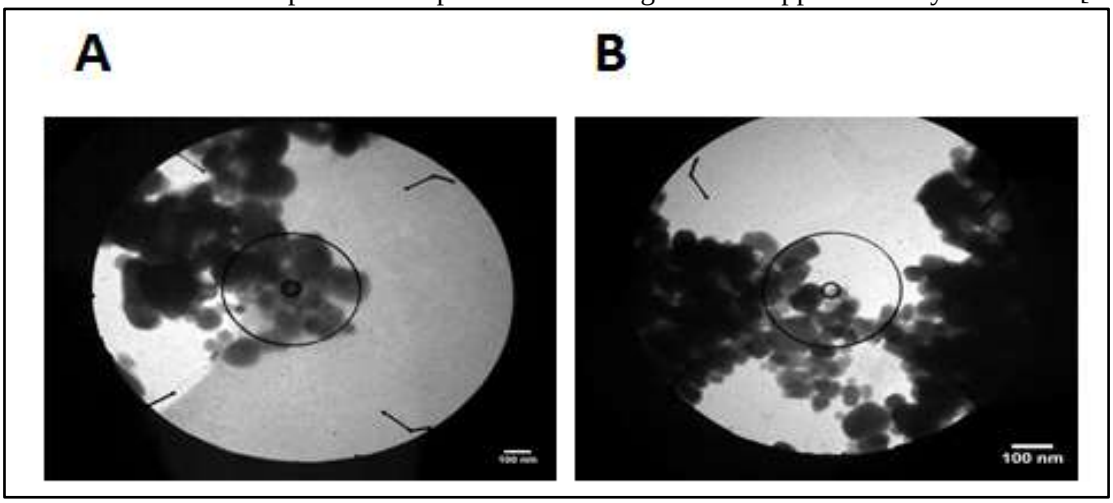

Figure 4-TEM micrographs of ZnO nanoparticles:(A) calcined (B) non-calcined.

Figure-5 (A & B) demonstrates the AFM image of ZnO NPs. It shows that the sizes are equal to 25.9 nm and 30.01 nm and the average diameters are 51.25 nm and 78.43 nm for the calcined and non-calcined ZnO NPs, respectively. This result is not consistent with the result reported by Al-Taie .. who showed that the average particle size was 125.77 nm the ZnO NPs calcined at 500 °C for 3 hours [25].

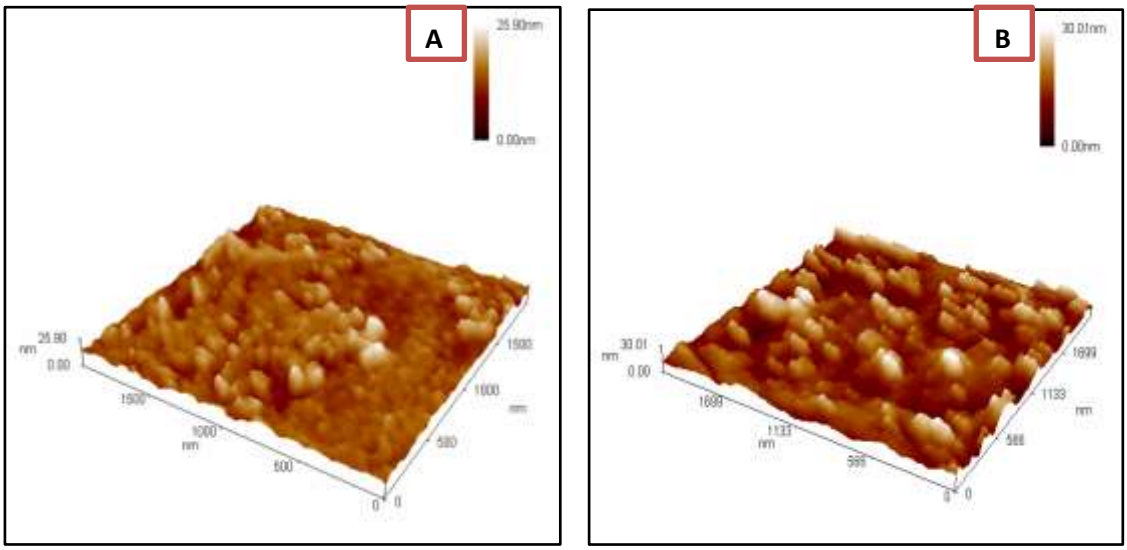

Figure 5-AFM 3D image characterization of ZnO NPs:(A) calcined (B) non-calcined.

In this study, Agar Well Diffusion method was used to evaluate the activity of antimicrobial agents. Figure-6A shows *C. albicans* cultured on PDA. The results indicate a diameter of inhibition zone of 11 mm at 800 mg/mL of calcined ZnO NPs ,as shown in Figure- 6C. Figure 6D shows a diameter of inhibition zone of 24 mm at 800 mg/mL of non-calcined ZnO NPs . However, no inhibition zones were observed when the other concentrations (0.25, 0.5, 1, 2, 10, 50 and 500 mg/mL) and deionized water (control) were applied (Figure-6B).

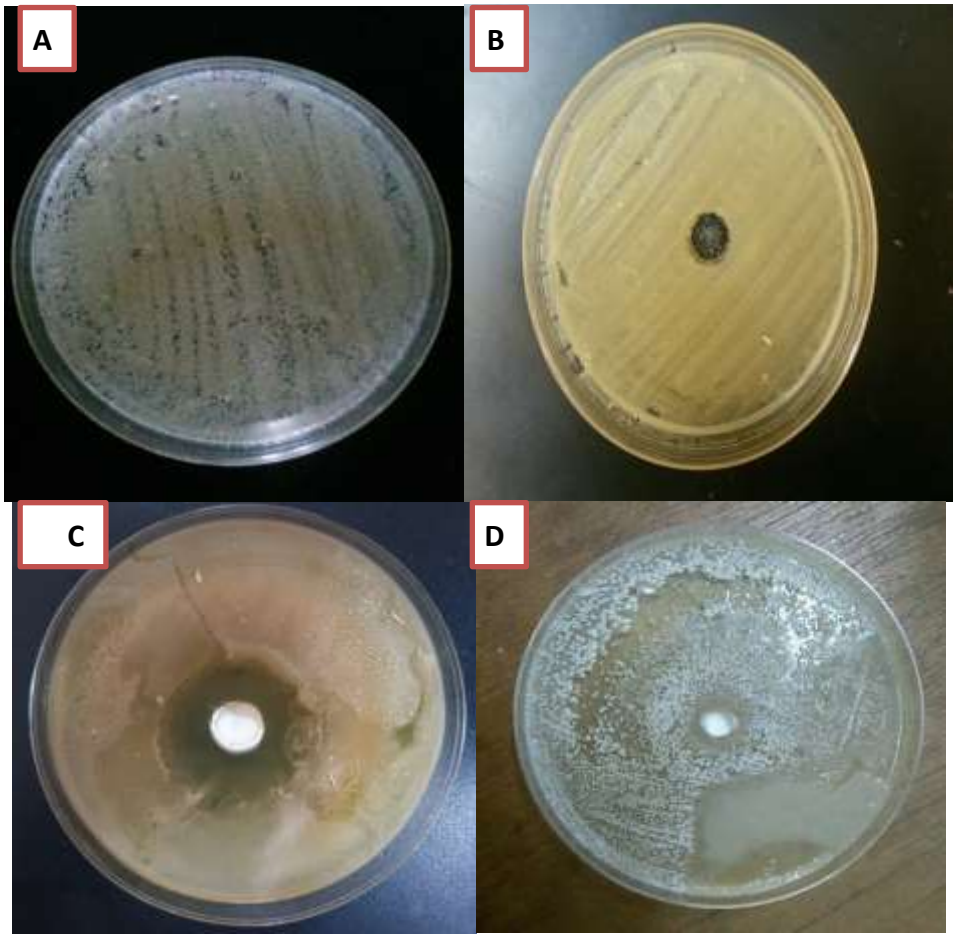

Figure 6- A: *C .albicans* on Potato Dextrose Agar (PDA), B: no inhibition of *C .albicans* in deionized water.
C: Inhibition zone of *C. albicans* = 11 mm at ZnO NPs (calcined) concentration of 800 mg/mL on PDA at 37 ° C for 72 hr.
D: Inhibition zone of *C. albicans* = 24 mm at ZnO NPs (non-calcined) concentration of 800 mg/mL on PDA at 37 ° C for 72 hr.

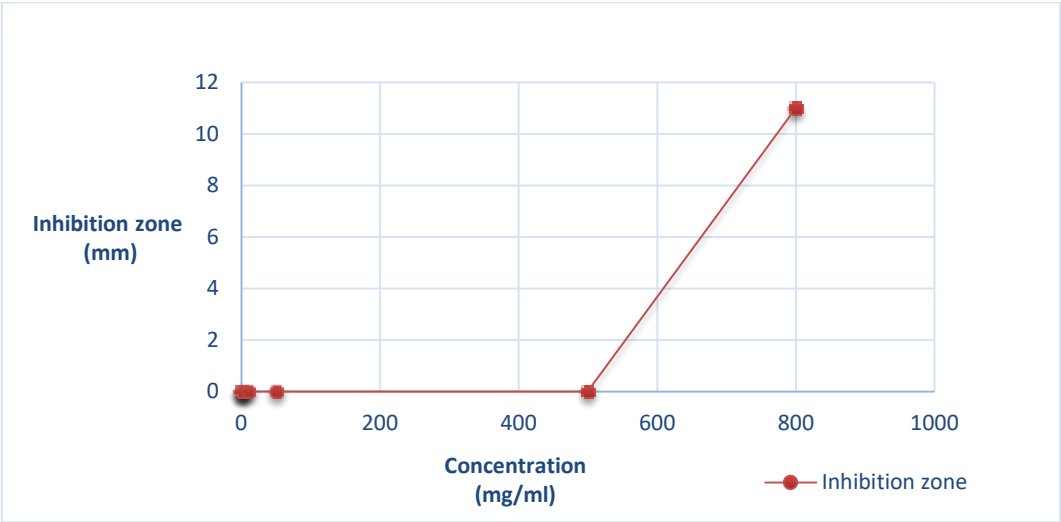

Figure 7-Inhibition zones with various concentrations of ZnO nanoparticles.

Histopathological examination

Different nanoparticles with different physicochemical properties have different toxicological effects. The typical histopathological changes of liver and spleen are shown in Figures-(8, 9),

respectively, where the liver showed a severe congestion of the central veins .

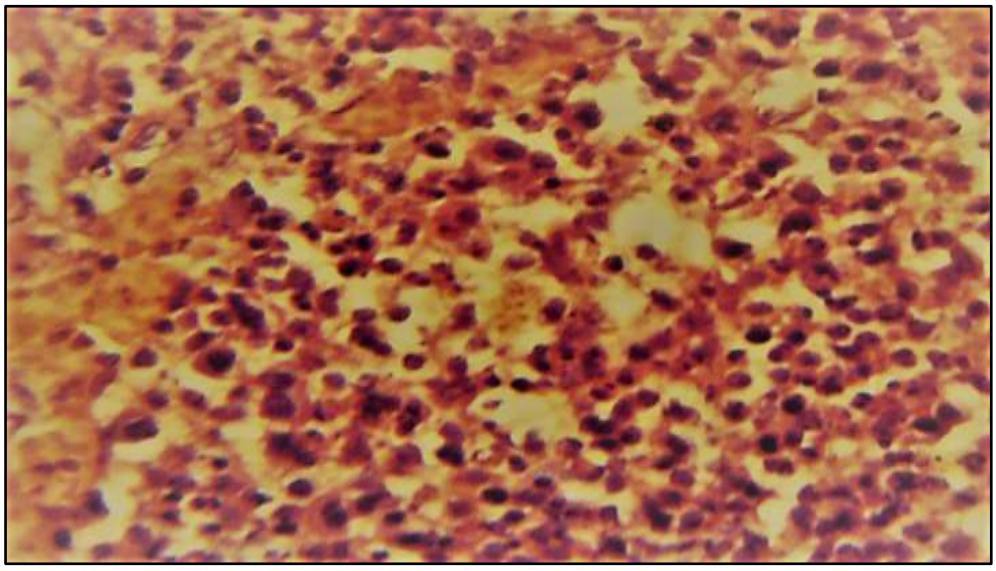

Figure 8-The liver section 7 day post administration showing congestion of the central veins (H & E; 100×). The injection was with 0.1 ml of calcined ZnO NPs at a concentration of 800 mg/ml .

In the spleen, the lesions were more severe with proliferation of megakaryocytes in the red pulp

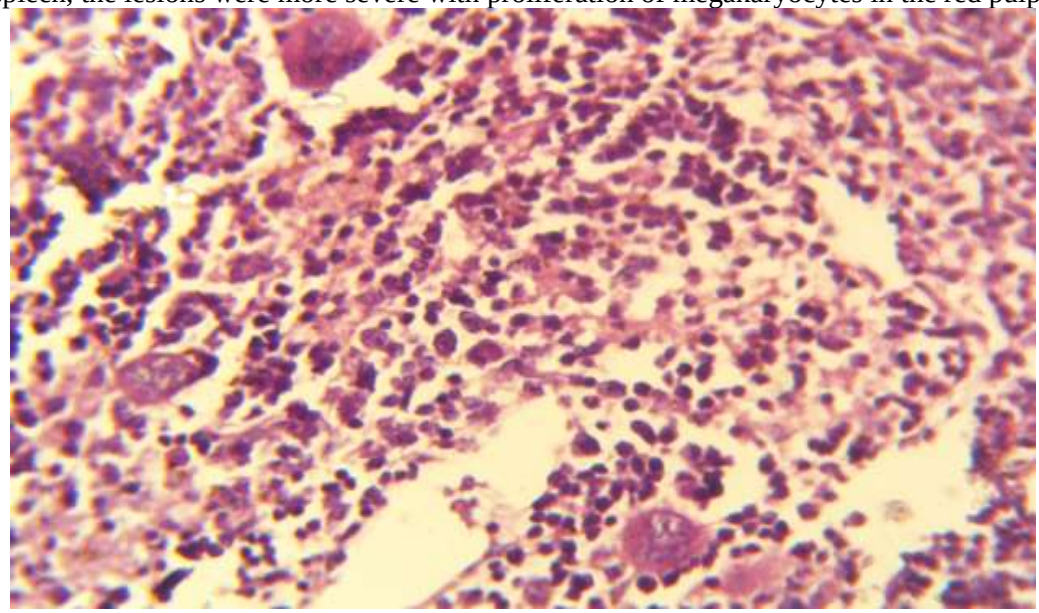

Figure 9-The spleen at day 7 showing proliferation of megakaryocytes in the red pulp (H & E; 400×). The injection was with 0.1 ml of calcined ZnO NPs at a concentration of 800 mg/ml of.

As ZnO nanoparticles are being used increasingly in various commercial products as well as in biological and medical applications, it is becoming more important than ever to study their possible toxicological effects on humans. The results are in agreement with those reported by Line and Wang [26, 27].

The results of the toxic effects of ZnO nanoparticles on many types of cells, such as human liver epithelial cells, have been demonstrated, with few *in vivo* studies in this subject. The pathological observations also confirmed liver damage and these findings are consistent with previous studies.

The results showed histopathological disorders in the liver and spleen. A systematic study of the effects of different sizes, doses, and distribution is critical to the deep understanding of the toxicity mechanisms. There are various factors that render the properties of nanomaterials different

significantly from those of other materials, including increased relative surface area and quantum confinement effect. This is in agreement with the report of Ben-Slama *et al*. [28].

The possible mechanisms through which ZnO nanoparticles exert their toxic effects on human liver cells were investigated by Sharma *et al* [29].

Conclusions

Zinc Oxide NPs were successfully synthesized by a precipitation method where pH was equal to 14. The synthesized NPs showed good sizes and properties, while we observed that the ZnO-NPs synthesized without calcination show better nano and antifungal properties due to the smaller sizes compared to the calcined ZnO-NPs. ZnO-NPs showed strong antifungal activity against *C. albicans* as tested by the well diffusion method. It is observed that, on increasing the ZnO-NPs concentration, the zone of inhibition also increases. Also, the ZnO-NPs have toxic effects as found through the examination of liver and spleen cells of mice. ZnO-NPs causes congestion of the central veins in the liver and proliferation of megakaryocytes in the red pulp of the spleen. The toxicity is dependent on the dose and time exposure to the ZnO nanoparticles.

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