Studies on Cancer Cell Cytotoxicity, Antimicrobial Activity of Sol-Gel Synthesized Willemite for Biomedical Applications

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Abstract: ***Background*:** To succeed in biomedical implant surgery, materials must be biocompatible. Willemite nanoparticles can be used in biomedical applications. MTT assay shows that willemite nanoparticles have non-toxic interaction between Hela (Cervical cancer) cell lines and the willemite found to be biocompatible for further applications *in-vivo* systems. In depth cell particle interaction was carried out, by using staining technique coupled with inverted microscopy. The antibacterial test was performed against Gram-negative *Escherichia coli* (*E.coli*, ATCC 8739) and Gram-positive *Staphylococcus aureus* (*S.aureus*, ATCC 6538). The optimal properties with excellent antibacterial ability can be achieved when willemite nanoparticles concentration is between 0.30 ppm and 2.3 ppm. According to the obtained results, the synthesized nano composite powder confirms biocompatibility of willemite, which could be an attractive candidate for biomedical applications

***Method*:** Pure Willemite Nanoparticles were synthesized via the modified sol-gel method. Synthesized powder was studied by Thermo gravimetric analysis (TGA), X-ray diffraction (XRD), scanning electron microscopy (SEM), Energy dispersive X-ray spectroscopy EDS, and transmission electron microscopy (TEM) with SAED techniques. The in vitro biocompatibility of willemite was studied. The biocompatibility of the Willemite nanoparticles was studied using Hela (Cervical cancer) cell lines with MTT assays up to 24 h.

***Result*:** Willemite nanoparticles were synthesized by facile sol-gel method. The synthesis method is cost-effective and easy to scale up. The results of this work demonstrate the applicability of willemite nanoparticles in the biomedical field. The antibacterial rate increased from 65% to 99% while the cell viability decreased from 98% to 70 % when the willemite nanoparticles concentration varied from 0.05 to 190 ppm. Furthermore, the cytotoxicity studies show that the willemite has lower cytotoxicity. Hence, it is revealed that willemite nano crystals are potentially applicable as bone substitution materials in tissue engineering.

***Conclusion*:** Willemite nanoparticles possess effective in vitro noncytotoxicity and antibacterial activity. The favourable properties with excellent cytocompatibility and antibacterial ability can be achieved when willemite concentration is between 0.30 ppm and 2.3 ppm. The antibacterial willemite is non-toxic to the living cells and tissues even if the particles are internalized by cells. Willemite is an excellent candidate in the biomedical field. Novel willemite nano crystals may provide new opportunities for a non-cytotoxic implant with antibacterial ability in bone tissue engineering.

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1. INTRODUCTION

Nowadays, scientists and engineers are actively engaged in developing nanocrystalline ceramic to enhance their biological and mechanical properties for their use in biomedical applications. Developments in materials science research have given a new set of methodologies and systems for applied biomedical research. Calcium based bio ceramic materials have been usually used in the biomedical field for more than 40 years to repair damaged hard tissue [1]. In the health care industry, ceramic materials have been used for a long time for manufacturing chemical ware, diagnostic instruments, eyeglasses, tissue culture flasks, thermometers,

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fibre optics for endoscopy. Only few elements of periodical table are used in the production of bio ceramics from the whole periodic table (alumina, carbon, compounds containing silicon and zirconia).

In modern day pottery, main constituent of crystalline glazes is zinc orthosilicate. Zinc orthosilicate is also known as Willemite [2]. To implant artificial bone, ceramic material should be resorbable, easy to shape, possessing osteoinductive with mechanical properties. Willemite is known as glass ceramics, having strong chemical durability, good mechanical strength, high luminescence efficiency, highly thermal stabilities and high colour purity. These properties of willemite are suitable for different engineering applications and willemite ceramic material has all these properties [3-4]. Willemite subsists in α, β and γ-Zn_2SiO_4 phases, with emission of colours: green, yellow and red colour emission respectively. All the phases of zinc silicate minerals are found abundantly in the earth crust. Recently research on Willemite have been considered to be biologically and technologically important; because of the reliable properties of ceramic material having good thermal conductivity , good mechanical strength , electrical conductivity, low coefficient of thermal expansion and dielectric properties [5].

It has been reported that completely biocompatible external material can be placed within a living body without any side effect. The only autogenous substances are manufactured by the body itself and any other foreign substance initiates various types of reactions [6]. The reactions occurring at the biomaterials surfaces lead to time dependent changes in the surface properties of the implanted biocompatible materials and the tissues at the interface. In order to develop new biocompatible materials, it is essential to study the in vitro and in vivo host responses. Likely bio ceramics and biomaterials react with their surrounding environment and, ideal bio ceramics and biomaterials they should not make any adverse effect on the neighbouring tissues.

Zinc is an important trace element which has different roles in human and different animal's growth such as increasing calcium content, bone protein and alkaline phosphatase activity as well as it plays important role in bone metabolism [7-9]. Deficiency of zinc is collateral with bone defects and bone growth slowness both in humans and animals [10-11]. Moreover zinc has been shown to stimulate osteoclast and bone formation activity inhibition. The bioactive glass containing ZnO could enhance bone formation [12-14]. The *in vitro* study showed that the ceramic supported bone marrow stromal cells adhesion and spreading. Also, from MTT tests demonstrated that cells on zinc silicate showed a significantly higher proliferation rate than on the controls after 7 days of culture [15].

It has been reported many routes, to synthesis of Willemite such as sol-gel synthesis [16], hydrothermal method [15], solid-state reaction method [17] and ball mill [18]. sol-gel synthesis method was performed with a solvent such as, ionic liquid, water and alcohol, advantage of this method is it works at lowest temperature. Sol-gel methods are performed in a solvent at ambient pressure; while in hydrothermal and solvothermal methods tend to use high temperatures and high pressures. It is reported that crystallization of Willemite requires temperatures of around 100°C.The main advantage of so-gel method to providing characteristic such as uniform shapes, spherical particles by varying experimental conditions [19]. It is reported that the nanostucture of a calcined powder depends mainly on the sol-gel synthesis methods conditions [20]. Sol gel methods having some advantages of sol-gel procedure are simple preparation, short time-to product, low cost and the possibility of reducing the operating temperature and preparing different types of materials [21]

In the present study, Willemite was prepared by a modified sol-gel method. This work focus to study the bioactivity of Willemite nanoparticles, under the different physical and biological conditions. The biocompatibility of Willemite was evaluated with different concentration by cytotoxicity assessments. Cytotoxicity of the synthesized nanomaterials has been evaluated with Hela (Cervical cancer) cell lines for 24 and 48 hr using MTT assays method. Hela cervical cell lines and Willemite nanoparticle interactions were found out in depth on inverted microscope, by staining techniques using acidic isopropanol as stain.

2. EXPERIMENTAL

2.1. Chemical

In modified sol-gel synthesis method Tetraethyl orthosilicate (Si $(OC_2H_5)_4$; TEOS, purity ≥99%, Sigma-Aldrich) and Zinc chloride ($ZnCl_2$, purity ≥98.5%, Thomas baker), distilled water and HCl were used. All chemicals used without further purification and all are analytical grade.

2.2. Synthesis of Willemite

Nano crystalline Willemite (WM) was synthesized by using modified sol-gel method. In this method, Tetraethyl orthosilicate ($Si(OC_2H_5)_4$) was partially hydrolysed at a molar ratio of TEOS: water(40 ml:0.25ml) and 4 ml conc. HCl was added as a binder in the solution. After that, 27.256 gm of zinc chloride dissolved in pre-hydrolysed solution. This mixed solution stirred for by a magnetic stirrer to form homogeneous solution. When the mixed solution became homogeneous it is refluxed at 35°C up to gel formation. This Formed gel dried in the oven at 300°C to form powder. The resulting dry gel powder was then annealed in the temperature range of 600°–1200°C for 2 h with a constant heating rate of 10°C min. The synthesis of the Willemite nanoparticles was performed through a modified sol-gel method reported elsewhere [22].

2.3. Characterizations

Crystal phase identification and structural analysis of Willemite was studied by X-ray diffraction (Philip-3710) with Cu-Kα radiation. X-ray diffraction patterns were analyzed by using software X-pert high score plus. To calculate crystalline size gaussian fit of the most intense peak was selected to determine the full width at half maximum for determination of crystallite size (D) by debye-scherrer equation.

$$t = 0.9\alpha/\beta \cos\theta \quad (1)$$

Where, t is crystallite size, α is the wavelength of Cu-Ka radiation, β is FWHM and q is the diffraction angle of strongest peak.

The morphology of particle was determined using scanning electron microscopy (JSM-T330 Jeol) operating at 20 kV and a profilometer (Dektak 6M, Veeco). Particle size was observed by using by using transmission electron microscope (TEM, JEOLJEM-2100) with resolution of 2.4 Å. The elemental analysis was done by energy dispersive spectroscopy (EDS, JEOL JSM 6360).

2.4. Biocompatibility Study

2.4.1. Cell Culture

Cytotoxicity (in vitro) of Willemite nanoparticle was carried out on Hela (Cervical cancer) cell lines by using MTT assay. Cell lines were obtained from National Centre for Cell Sciences, Pune (India). In vitro cytoxicity study was carried out at National Toxicology Centre Pune (ISO10993/ USP 32 NF 27). Target cell lines were grown in MEM culture medium with Antibiotics and 10% FBS [foetal bovine serum, kanamycin (0.1 mg/mL^{-1})], penicillin- G ($100U/mL^{-1}$), and sodium bicarbonate (1.5 $mg\ mL^{-1}$) at 37^0C in a 5% CO_2 atmosphere.

2.4.2. MTT Assay

The Hela (Cervical cancer) cells were incubated with a concentration of 1×10^5 cells/ml in medium in a 96-well microtiter plate (cell count was taken on Neubauer's chamber) for 24 h. Fresh media was replaced by the old media, after 24 h and different proportions of Willemite particles 0.2, 0.4, 0.6, 0.8 and 1mg per ml of cultured media). Then, the total medium was incubated at 37°C in a 5% CO_2 atmosphere for 24 h. After 24 h, to confirm the non-contamination and some other parameters Incubation plates were observed under an inverted microscope. Then, 10 µl of 5 mg/ml MTT solution was added into each well including control wells. The plates were wrapped in aluminium foil and incubated for 4 hr at 37 °C in a 5% CO_2 atmosphere for metabolization of MTT with the nanoparticles and cell media. After 4 h incubation plates were removed and observed under inverted microscope and photographs were taken. The entire medium was then removed by flicking the plates and only the anchored cells remained in the wells. Then the cells were washed with phosphate buffer saline (PBS) and the formazan formed was extracted in 200 µl acidic isopropanol in each well and after 1 hr, absorbance was measured at 492 nm from which cell viability was calculated. The experiments were replicated three times and the mean data were graphically presented as mean ± SD. Obtained relative cell viability (%) compared with control well containing cells without nanoparticles are calculated by the following equation:

$$\text{Relative cell viability (\%)} = [A_{absorbance}]_{tested} / [A_{absorbance}]_{control} \times 100 \quad (1)$$

2.4.3. Antibacterial Activity Assay

The antibacterial activity test of Willemite nanoparticles with the concentration of 0.2, 0.4, 0.6, 0.8, 1.0 ppm, respectively was carried by bacteriological plate counting methods. The antibacterial test was performed against Gram-negative *Escherichia coli* (*E.coli*, ATCC 8739) and Gram-positive *Staphylococcus aureus* (*S. aureus*, ATCC 6538). The bacterial strains were purchased from National Chemical laboratory, Pune. The bacteria were cultured in liquid nutrient broth medium at 35-37°C for 12 h and adjusted to a concentration of about 10^7 CFU/mL. In an autoclave at 121°C for 20 min all laboratory supplies were sterilized. sterile sample (0.5 g of each) was dispersed in a centrifugal tube containing *Buffered sodium chloride-peptone (BECP)* (9 mL) and bacteria suspension (1 mL) are incubated at 30-35°C for 4, 8, 12, 16,32, 48 and 72 h. For subsequent bacteria counting, 100 µL of the suspension was extracted from the centrifugal tube. The extracted suspension was inoculated into solid nutrient agar medium for 24 h incubation at 35°C. Total bacterial colonies were counted.

3. RESULTS AND DISCUSSION

3.1. XRD Analysis

Fig. (**1**) Shows XRD pattern of the pure Willemite recorded in the 2 theta range 10-90 ^{0} and at step size of 0.02. XRD spectra show that the synthesized Willemite material structure corresponds with the rhombohedral structure with space group R-3. All XRD peaks were matched with (JCDPS no. 00-037-1485). The lattice parameter calculated from the reflection of the (113) plane and confirms the formation of phase pure Willemite particles Standard. The intense (113) peak was chosen for calculating the average particle size of the sample using the Scherrer formulae [23]. The most important observation is that the effective broadening of peaks in Willemite shows the fine nano crystalline nature. The result shows that the crystallite size was 38.42 nm, also broadening of the peaks suggest nano crystalline nature of the sample. XRD of Willemite shows similar peaks with previously reported work [24].

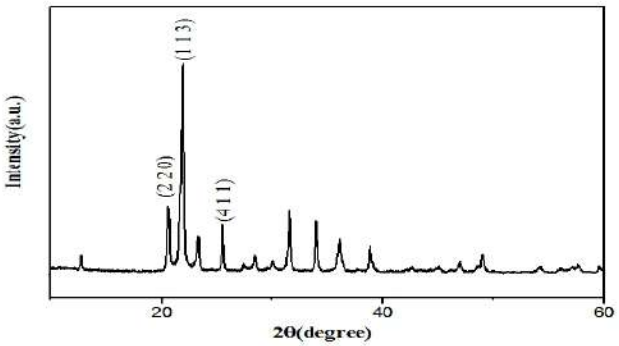

Fig. (1). XRD pattern of Willemite nanoparticles.

3.2. SEM Analysis

Fig. (**2**) shows the SEM images of Willemite nanoparticles. The morphology observed for Willemite nanoparticles reveals that the particles are in good agglomerated and surface morphology is clean. Moreover one should observe the absence of large aggregates. It has been reported that SEM images of Willemite shows an aggregation and irregularity in shape and size [25,26]. It has been reported that crystal size distribution of bone plays an important role in bone fracture [27].

The EDS analysis shows in Fig. (**3**) that the as synthesized Willemite nanoparticles contain Zn, Si and O without any impurity. The EDS spectra show that samples are with

their stoichiometry and elemental signals as expected. This implies that the prepared samples are pure in nature. The elemental composition estimated from EDS analysis is tabulated in Table **1**.

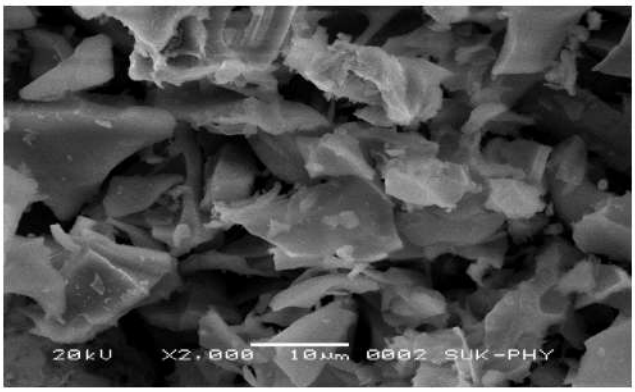

Fig. (2). SEM image of Willemite.

Table 1. Atomic percentage composition.

Name	Zn	Si	O
Willemite	14.23	20.58	58.15

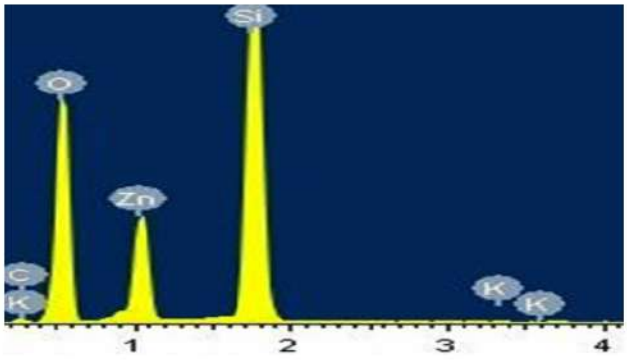

Fig. (3). EDS spectrum of Willemite.

3.3. TEM Analysis

The size and shape of the Willemite nanoparticles was observed in Fig. (**4**). It shows the TEM micrograph of Willemite nanoparticles with uniform particle size and cylindrical rod-like shape. The Willemite nanoparticles with homogeneous microstructure, around 150-160 nm in diameter and length is around 200 nm, particles seem to good aggregates. The diameter of Willemite nanoparticles are slightly larger than the observed crystallite sizes calculated from XRD, Due to the presence of nano crystalline surface layers. Grain growth was obtained due to high calcination temperature [28]. In SAED pattern (inset of Fig. **4**) the diffraction ring pattern are well observed and are match with XRD analysis. The selected area Electron diffraction (SAED) pattern shows bright ring patterns indicating the polycrystalline nature of nano particles which is in good agreement with XRD results.

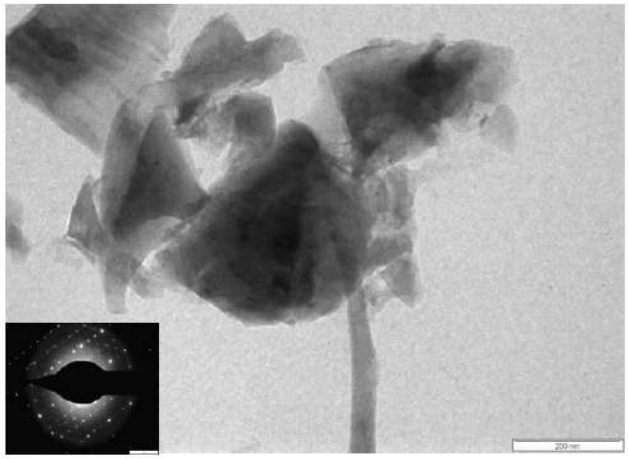

Fig. (4). TEM image of Willemite,(Inset: corresponding SAED pattern).

3.4. Biocompatibility and Cell Particle Interaction Study of Willemite Nanoparticles Cytotoxicity Study

3.4.1. Biocompatibility Study

For possible biomedical applications, toxicity is a very critical factor to consider their potential. Willemite nanoparticles were interact with Hela (Cervical cancer) cell lines, it is important factor to ensure that these nanoparticles are not causing any adverse effect. The cytotoxicity study of Willemite nanoparticles were carried out on varying with different concentrations and incubation time. Fig. (**5**) shows cytotoxicity data obtained for the Hela (Cervical cancer) cell lines were treated with Willemite nanoparticles for 24 h and 48 h, respectively with the concentration of 0.2, 0.4, 0.6, 0.8, and 1 $mg.mL^{-1}$ at 37 °C in a 5% CO_2 atmosphere. The relative cell viability (%) compared with Control (well containing cells without nanoparticles) are calculated by the equation:

$$\text{Relative cell viability (\%)} = [A]_{tested} / [A]_{control} \times 100 \quad (2)$$

From Fig. (**5**) it is observed that the cell viability slightly decreases with increasing the Willemite nanoparticles concentration and incubation time. The cell cytotoxicity effect of nanoparticles at different concentrations is well studied by MTT assay and the mechanism of the cytotoxicity due to presence of nanoparticles is reported in recent literature [29-32]. It is reported that the presence of nanoparticles on the cell surface directly affects the plasma membrane not on the nucleus over a contact period of time and which causes the lysis of the cell [33]. Nanoparticles are removed from cell surface, MTT is used to stain the cells. MTT enters into the cell lines and passes into the mitochondria where it is reduced to an insoluble, colored, formazan product because viable cells reduce the yellow tetrazolium salt to a green dye. The cells are then solubilized with an acidic isopropanol e.g. organic solvent, and the released formazan is measured through spectrophotometrically. In metabolic active cells reduction of MTT can occur, viability of the cells is a measure of the level of activity. In cytotoxicity study two factors should play important roles for the cell viability one is the chemical stability and the other is their grain size. The small size particles have an efficient inter facial interaction with

the cell membrane As compared to larger size particles. It is reported that the large particles exert a stronger impulse on the cell surface and results in the reduction of cell viability [34,35]. From the cytotoxicity study, it can be seen that there was no drastic change in the cytotoxicity of Willemite nanoparticles. Fig. (**6**) sows microscopic images by using stains, Hela (Cervical cancer) cell lines was incubated with Willemite nanoparticles for 24 h. The obtained results from cytotoxicity experiments show better biocompatibility of Willemite nanoparticles with a concentration up to 1 mg.mL^{-1}.

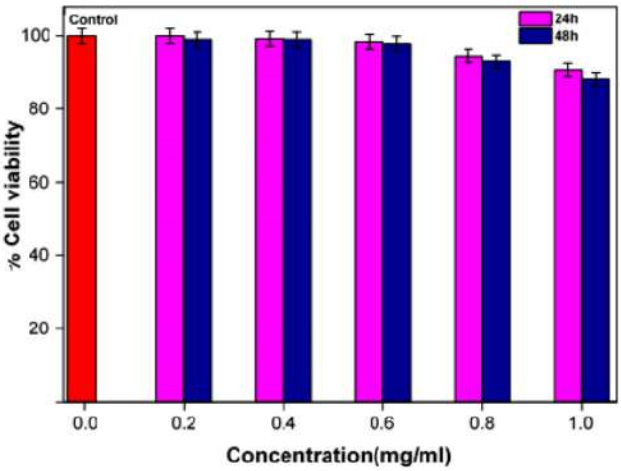

Fig. (5). Biocompatibility and cell particle interaction study on Hela (Cervical cancer) cell lines of Willemite Cell viability study by using MTT assay for 24 h and 48 h.

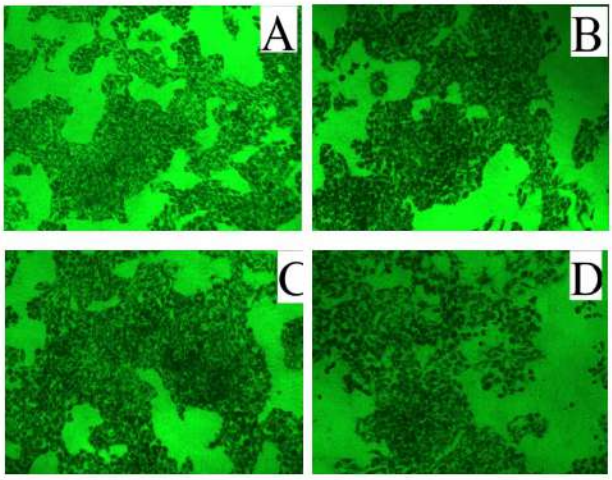

Fig. (6). Microscopic images by using stains, Hela (Cervical cancer) cell lines was incubated with Willemite nanoparticles for 24 h (**A** to **D** represents cells treated with 0, 0.2, 0.6 and 1 mg.mL^{-1} Willemite nanoparticles, respectively).

3.4.2. Cell Particle Interaction Study

The staining technique coupled with microscopic observations gives more accurate results and is able to identify the phenotypically intact cells, cellular morphology and dead cells qualitatively [36,37]. Here, Hela (Cervical cancer) cell lines have been stained with acidic isopropanol and microscopic images are obtained, which have been observed to validate respective MTT data. After 24 h treatment of Willemite nanoparticles with cell lines, it is observed that, the treated Hela (Cervical cancer) cells exhibit good proliferative activity, as that of respective control (0.0 mg.mL^{-1}) cells. It is also observed that cell lines that, at 1 mg.mL^{-1} concentration of Willemite nanoparticles, the percentage of cell staining was found more with isopropanol, which suggests the loss of membrane integrity at higher concentration.

3.5. Antimicrobial activity

The antibacterial effect was evaluated for a Series of time, the gram positive and gram negative bacteria were cultivated with different concentration and by using bacteria counting method they are re-cultivated on agar. The percentage bacterial survival for *E. coli* and *S. aureus* as a function of time are shown in Fig. (**7**). The bacteria reduction of Willemite nanoparticles exhibits time and dose concentration dependent. Antibacterial rates of Willemite (0.05 ppm) are improved from 45% to 55% for incubation time extended from 1 h to 6 h. Moreover, the antibacterial rate increases from 65% to 99% after 6 h incubation when the Willemite concentration is enhanced from 0.05 ppm to 190 ppm. The effective antibacterial ability is defined as a percentage of bacteria reduction above 70% for *S. aureus* and *E. coli*. Antibacterial results shows that the antibacterial rates are 85% and 93% when 0.30 ppm and 2.3 ppm Willemite nanoparticles come in contact with *S. aureus* and *E. coli* bacteria. The maximum concentration that exhibits effective antibacterial is 2.3 ppm. The effective antibacterial activity of Willemite reveals that high doping concentration of Willemite is not necessary to achieve effective antibacterial activity, an appropriate amount of Willemite nanoparticles doping can also be adopted as an ideal option. The physical and chemical characteristics such as surface properties, morphology and chemical composition of surrounding medium can affect the viability of bacteria [38]. The antibacterial activity of Willemite demonstrated the release of Zn^{+}. The released of Zn+ will bind to proteins on the membrane, thereby causing structural changes and damage to the Membranes. It was reported that release Zn^{+} penetrates into cells and interacts with nucleic acids, preventing proliferation process and causing bacteria death. In the present research work, in case of Willemite (2.3 ppm) and (190 ppm), the antibacterial activity shows about 90% and 99% with the accumulated Zn^{+} release amount. However, the Willemite (0.30 ppm) also exhibited 85% bacteria reduction. This is due to the contact antibacterial process that causes bacteria death at the surface of Willemite nanoparticles. When the bacteria come in contact with Willemite nanoparticles, the bacteria were killed due to the high concentration of Zn^{+} at Willemite surface. When the Willemite nanoparticles concentration was 0.05 ppm accompanied by 60% antibacterial when 2.3 ppm, the antibacterial activity was enhanced unceasingly to 90%. When Willemite concentration comes to 190 ppm, 99% of bacteria were killed. The antibacterial properties of Willemite were mainly depending on its concentration. Willemite nanoparticles show good antibacterial activity at low concentration within appropriate time.

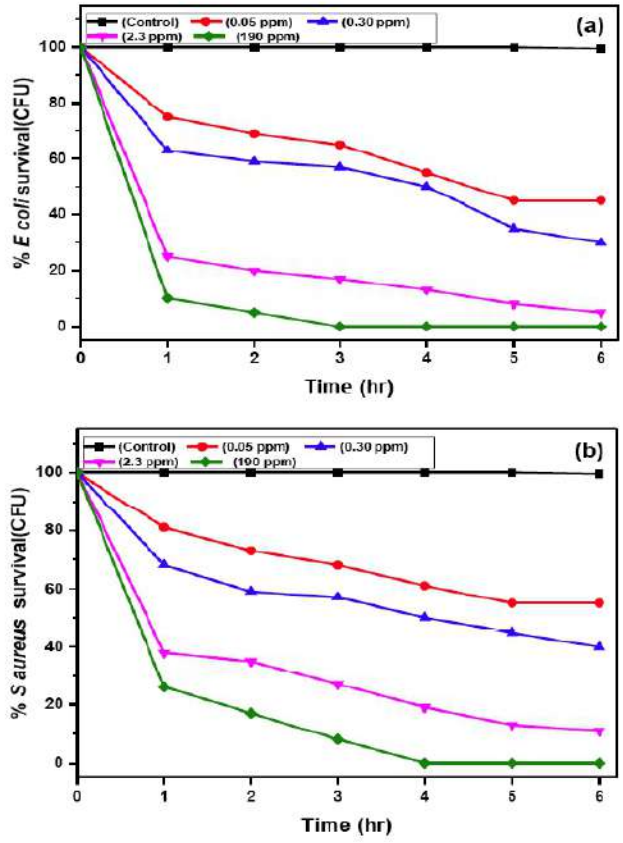

Fig. (7). (**a**) *E. colli* (**b**) *S. aureus* survival (%) as a function of time for Willemite Nanoparticles.

CONCLUSIONS

Willemite nanoparticles were synthesized by facile Sol-Gel method. The synthesis method is cost-effective and easy to scale up. The structural, composition, antibacterial and cytotoxic properties of the Willemite nanoparticles have been studied in great detail. Willemite nanoparticles having pure phase and almost identical particle sizes. EDS analysis confirmed the presence of pure Willemite with stoichiometric ratio. Willemite nanoparticles possess effective in vitro noncytotoxicity and antibacterial activity. The antibacterial rate increases from 65% to 99% while the cell viability decreases from 98% to 70% when the Willemite nanoparticles concentration varies from 0.05 to 190 ppm. The favourable properties with excellent cytocompatibility and antibacterial ability can be achieved when Willemite concentration is between 0.30 ppm and 2.3 ppm. The preliminary cytotoxicity study on Hela (Cervical cancer) cell lines of Willemite nanoparticles synthesised by sol-gel method, here confirmed that the biological effects are not only based on chemical composition, in addition size, aggregation state, shape and surface texture also play an important role. However, obtained results are found to be very satisfactory and no extreme results is noted regarding cell viability. From all these observations, it can be stated that, study of biocompatibility of Willemite nanoparticles towards Hela (Cervical cancer) cell lines, Showed negligible cellular toxicity. The antibacterial Willemite is non-toxicity to living cells and tissues even if the particles are internalized by cells. The results of this work demonstrate the applicability of Willemite nanoparticles in the biomedical field. Furthermore, the cytotoxicity studies show that the Willemite lower cytotoxicity. Hence, it is revealed that Willemite nano crystals are potentially applicable as bone substitution materials in tissue engineering. Willemite is an excellent candidate in the biomedical field. Novel Willemite nano crystals may provide new opportunities for a non-cytotoxic implant with antibacterial ability in bone tissue engineering.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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REFERENCES

[1] Hench, L.L.; Polak, J.M. Third-generation biomedical materials. *Science*, **2002**, *295*, 1007-1014.

[2] Knowles, K.M.; Freeman, F.S.H.B. Microscopy and microanalysis of crystalline glazes. *J. Microsc.*, **2004**, *215*, 257-270.

[3] Cui, H.; Zayat, M.; Levy, D. Nanoparticle synthesis of willemite doped with cobalt ions ($Co_{0.05}Zn_{1.95}SiO_4$) by an epoxide- assisted sol-gel method. *Chem. Mater.*, **2005**, *22*(17), 5562-5566.

[4] Mao, Y.; Huang, J.Y.; Ostroumov, R.; Wang, K.L.; Chang, J.P. Synthesis and luminescence properties of Erbium-doped Y2O3 nanotubes. *J. Phys. Chem. C*, **2008**, *112*(7), 2278-2285.

[5] Chandra Babu, B.; Buddhudu, S. Dielectric properties of Willemite -Zn_2SiO_4 nano powders by sol-gel method. *Phys. Proc.*, **2013**, *49*, 128-136.

[6] Heness, G.; Ben-Nissan, B. Innovative bioceramics. *Mater. Forum*, **2004,** *27*, 104-114.

[7] Ito, A.; Kawamura, H.; Otsuka, M.; Ikeuchi, M.; Ohgushi, H.; Ishikawa, K. Zinc releasing calcium phosphate for stimulating bone formation. *Mater. Sci. Eng.* C, **2002**, *12*, 2221-2225.

[8] Kawamura, H.; Ito, A.; Miyakawa, S.; Layrolle, P.; Ojima, K.; Ichinose, N. Stimulatory effect of zinc-releasing calcium phosphate implant on bone formation in rabbit femora. *J. Biomed. Mater. Res.*, **2000**, *50*, 184-190.

[9] Ito, A.; Otsuka, M.; Kawamura, H.; Ikeuchi, M.; Ohgushi, H.; Sogo, Y. Zinc containing tricalcium phosphate and related materials for promoting bone formation. *Curr. Appl. Phys.*, **2005**, *5*, 402-406.

[10] Kim, J.T.; Baek, S.H.; Lee, S.H.; Park, E.K.; Kim, E.C.; Kwun, I.S.; Shin, H.I. Zinc-deficient diet decreases fetal long bone growth through decreased bone matrix formation in mice. *J. Med. Food*, **2009**, *12*(1),118-123.

[11] Hurley, L.S. Teratogenic aspects of manganese, zinc, and copper nutrition. *Physiol. Rev.*, **1981**, *61*(2), 249-295.

[12] Yamaguchi, M. Role of zinc in bone formation and bone resorption. *J. Trace Elem. Exp. Med.*, **1998,** *11*(3), 119-135.

[13] Parisi, A.F.; Vallee, B.L.; Zinc metallo enzymes: characteristics and significance in biology and medicine. *Am. J. Clin. Nutr.*, **1969**, *22*(9), 1222-1239.

[14] Burch, R.E.; Hahn, H.K.J.; Sullivan, J.F. Newer aspects of the roles of zinc, manganese and copper in human nutrition. *Clin. Chem.*, **1975**, *21*(4), 501-520.

[15] Zhang, M.; Zhai, W.; Chang, J. Preparation and characterization of a novel willemite bioceramic. *J. Mater. Sci: Mater. Med.*, **2010**, *21*, 1169-1173.

[16] Reynaud, L.; Brouca-Cabarrecq, C.; Mosset, A. A new solution route to silicates. Part 3: Aqueous sol-gel synthesis of willemite and potassium antimony silicate. *Mater. Res. Bull.*, **1996**, *31*(9), 1133-1139.

[17] Chundong Li, Zhiqiang Liang , Haiying Xiao, Yiyong Wu, Yong Liu. "Synthesis of $ZnO/Zn_2SiO_4/SiO_2$ composite pigments with enhanced reflectance and radiation-stability under low-energy proton irradiation"*Materials Letters*,**2010,**64,1972–1974.

[18] Pozas, R.; Orera, V.M.; Ocana, M. Hydrothermal synthesis of Co-doped willemite powders with controlled particle size and shape. *J. Eur. Ceram. Soc.*, **2005**, *25*, 3165-3172.

[19] Takesue, M.; Hayashi, H.; Smith, R.L. Thermal and chemical methods for producing zinc silicate (willemite): A review. *Jr. Progr. Cryst. Growth Charact. Mater.*, **2009**, *55*, 98-124.

[20] Clayden, N.J.; Accardo, G.; Mazzei, P.; Piccolo, A.; Pernice, P.; Vergara, A.; Ferone, C.; Aronne, A. Phosphorus stably bonded to a silica gel matrix through niobium bridges. *J. Mater. Chem. A.* **2015**, *3*(31), 15986-15995.

[21] Babu, B.C.; Vengla, R.B.; Ravi, M.; Babu, S. Structural, microstructural, optical, and dielectric properties of Mn2%20: Willemite Zn_2SiO_4 nanocomposites obtained by a sol-gel method. *J. Mol. Struct.*, **2017**, *1127*, 6-14.

[22] Mu-Tsun Tsai, Jun-Min Wu, Yu-Feng Lu, Hen-Chia Chang, Synthesis and Luminescence characterization of manganese-activated willemite gel films. *Thin Solid Films*, **2011**,520,1027–1033.

[23] L.A.Azaroff, L.A. *Elements of X-ray crystallography*. Mc-Graw-Hill, New York, **1968**, 38-42.

[24] Nayeb Pashaee, N.; AAarabi, M.; Sarpoolaky, H.; Vafaeenezhad, H. The effect of zno addition on microstructure, phase and color developments of copper reduction glaze. *Metall. Mater. Eng.*, **2015**, *21*(2), 89-99.

[25] A. M.Hu, M. Li,D. L. M.Dali, and K. M. Liang, "Crystallization and properties of a spodumene willemite glass ceramic," *Thermochimica Acta*, **2005**,437(1), 110–113.

[26] Rao, D.S.; Raju, P.S.; Rao, B.S.; Murthy, K.V.R. Luminescent studies of Zn_2SiO_4Mn (1.1%), Eu (1.5%) phosphor. *Int. J. Luminescence Appl.*, **2014**, *4*, 104-106.

[27] Phillips, M.J.; Darr, J.A.; Luklinska, Z.B.; Rehman, I. Synthesis and characterization of nano-biomaterials with potential osteological applications. *J. Mater. Sci. Mater. Med.*, **2003**, *14*, 875-882.

[28] Kolekar, T.V.; Thorat, N.D.; Yadav, H.M.; Magalad, V.T.; Shinde, M.; Bandgar, S.S.; Kim, J.H.; Agawane, G.L. Nanocrystalline hydroxyapatite doped with aluminium: A potential carrier for biomedical applications. *Ceram. Int.*, **2016**, *42*(4), 5304-5311.

[29] Metanawin, T.; Tang, T.; Chen, R.; Vernon, D.; Wang X. Cytotoxicity and photocytotoxicity of structure-defined water-soluble C60/micelle supramolecular nanoparticles. *Nanotechnology*, **2011**, *22*, 235604-235609.

[30] Yin, H.; Casey, P.S.; McCall, M.J.; Fenech, M. Effects of surface chemistry on cytotoxicity, genotoxicity, and the generation of reactive oxygen species induced by ZnO nanoparticles. *Langmuir,* **2010,** *26*, 15399-15408.

[31] Reddy, L.H.; Arias, J.L.; Nicolas, J.; Couvreur, P. Magnetic nanoparticles: design and characterization, toxicity and biocompatibility, pharmaceutical and biomedical applications. *Chem. Rev.*, **2012,** *112*, 5818-5878.

[32] Sharifi, S.; Behzadi, S.; Laurent, S.; Forrest, M.L.; Stroevee, P.; Mahmoudi, M. Toxicity of nanomaterials. *Chem. Soc. Rev.*, **2012**, *41*, 2323-2343.

[33] Barick, K.C.; Singh, S.; Jadhav, N.V.; Bahadur, D.; Pandey, B.N.; Hassan, P.A. pH-responsive peptide mimic shell cross-linked magnetic nanocarriers for combination therapy. *Adv. Funct. Mater.*, **2012**, *22*, 4875-4884.

[34] Thorat, N.D.; Khot, V.M.; Salunkhe, A.B.; Ningthoujam, R.S.; Pawar, S.H. Functionalization of $La(0.7)Sr(0.3)MnO_3$ nanoparticles with polymer:studies on enhanced hyperthermia and biocompatibility properties for biomedical applications. *Colloids Surf. B. Biointerfaces*, **2013**, *104*, 40-47.

[35] Gupta, A.K.; Gupta, M. Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. *Biomaterials*, **2005**, *26*, 3995-4021.

[36] Blechinger, J.; Bauer, A.T.; Torrano, A.A.; Gorzelanny, C.; Br¨auchle, C.; Schneider, S.W. Uptake kinetics and nanotoxicity of silica nanoparticles are cell type dependent. *Small*, **2013**, *9*, 3970-3980.

[37] Clarke, J.M.; Gillings, M.R.; Altavilla, N.; Beattie, A.J. Potential problems with fluorescein diacetate assays of cell viability when testing natural products for antimicrobial activity. *J. Microbiol. Methods*, **2001**, *46*, 261-267.

[38] Kang, S.; Herzberg, M.; Rodrigues, D.F.; Elimelech, M. Antibacterial effects of carbon nanotubes: size does matter. *Langmuir*, **2008**, *24*, 6409-6413.