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Original Article

# EVALUATION OF BIOLOGICAL ACTIVITIES OF NANOCRYSTALLINE TETRAGONAL ZIRCONIA SYNTHESIZED VIA SOL-GEL METHOD

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**ABSTRACT**

**Objective:** The objective of the following study was a synthesis of nanocrystalline tetragonal zirconia ($ZrO_2$) using simple sol–gel method and evaluation of its structural and biological properties.

**Methods:** The sample was characterized by X-ray powder diffraction (XRD), Field Emission Scanning Electron Microscopy (FESEM), Transmission Electron Microscopy (TEM) and evaluated for cell growth study using 3T3 mouse fibroblast cells and for degradation using Phosphate Buffered Saline (PBS) solution. The synthesized materials were also evaluated for their antibacterial activity against *Escherichia coli (E. coli)* and S*taphylococcus aureus (S. aureus)* bacterial strains.

**Results:** The XRD pattern shows that the tetragonal phase of nanocrystalline zirconia was obtained at relatively low temperature i.e. 300 °C. The FESEM images showed that the prepared sample consists of particles in the range of 35-69 nm and homogenous particle size distribution. The TEM images confirmed the results shown by FESEM images. The sample of zirconia has excellent tissue biocompatibility, higher cell growth and does not show the toxicity towards normal 3T3 mouse fibroblast cells. The result of qualitative antibacterial tests revealed that the nanocrystalline zirconia had an important inhibitory activity on *E. coli* and *S. aureus.* The sample shows stability at the physiological condition and does not show degradation.

**Conclusion:** Nanocrystalline tetragonal zirconia shows higher cell growth and efficient antibacterial activity against *E. coli* and *S. aureus* bacterial pathogen and also it is stable at the physiological condition. Hence, it can be used for various biomedical applications.

**Keywords:** Nanocrystalline zirconia, Sol-gel route, Antimicrobial action, Biomedical application



## INTRODUCTION

Zirconia ceramics has been increasingly used as implant biomaterials [1-3]. The excellent electrical, mechanical, optical and thermal properties of zirconia, makes it a good choice for application such as: structural materials [4], dental crowns [5], femoral heads for total hip replacement [6], solid oxide fuel cell electrolytes [7], air-fuel ratio sensors for automotive applications [8], Catalytic application [9]. Pure zirconia exhibits three polymorphs of monoclinic, tetragonal and cubic symmetries. The monoclinic phase is stable at room temperature and transforms to the tetragonal phase at 1170 °C during heating while this phase transforms to the cubic one at 2370 °C [10, 11]. Zirconia and yttria stabilized zirconia (YTZP) are very attractive material for orthopedic applications. It has excellent biocompatibility, high fracture toughness; high strength and low wear rates. But they have some limitation such as fatigue failure and case studies show that delayed failure can occur *in vivo* due to crack propagation [12].

To overcome these limitations, nanocrystalline materials are expected to show improved mechanical properties, and the nanometric features in the surface of prostheses seem to reduce the risk of rejection and enhance the proliferation of osteoblasts (bone-forming cells) [13, 14]. It is well known that nanostructured materials exhibited unique physiochemical properties that are unseen in conventional bulk materials. Nano-$ZrO_2$ exhibits much better chemical and physical properties as compared to normal $ZrO_2$ powder, due to the small particle diameter which will result in better sintering ability [15, 16]. The nanocrystalline tetragonal zirconia has not only high strength and fracture toughness but also a long-term biocompatibility. This makes it of major benefit for use in prosthetic hip, knee bearings and as superior dental material for crown and bridges [7]. Addressing the above issues, researchers have focused on developing the nanocrystalline structure of tetragonal zirconia for better performance. In recent years, inorganic antimicrobial agents are increased widely for control of microorganisms in various areas especially in the textile field [17-19]. The key advantages of inorganic antimicrobial agents are improved safety and stability compared with organic antimicrobial agents [20]. The growing importance of biomaterials that prevent microbial growth is leading to the development of new biomaterials exhibiting antibacterial action, which create a bacteria-free environment while healing and repairing the defect area [21]. In the field of biomedical, many failures in the implantation are may be due to the formation of microbes in the implanted site. If the implant material has the capability of antimicrobial activity within them, then the problem of failure may be reduced. Moreover, microbes which cause a wide variety of infections in humans can be controlled by the antimicrobial materials [22].

There are many synthesis routes have been employed to obtain nano-sized tetragonal zirconia particles likes co-precipitation [23], Glycothermal Processing [24] Solid-State Reaction [25], Pechini Method [26], microwave-assisted sol-gel synthesis [27], bio-phase protocol [28], hydrothermal method [29] and sol-gel [30] processing. Among this Sol–gel is one of the best methods for synthesizing the nanoceramics e. g. zirconia, titania, hafnia, etc. Crystallite phase, crystallite size and other properties of zirconia nanoparticles are dependent on diverse parameters such as the type of precursor, pH during hydrolysis and thermal treatment [31]. In sol–gel route, purity, homogeneity, and physical properties of zirconia are manageable at a low temperature [14, 32-34].

The present paper, however, proposes Hydroxypropyl methyl-cellulose as organic additives. Also, the suggested additives are non-toxic, easily available to store at a low temperature, adequately supplied and water-soluble. The sol–gel method is environmentally friendly and only takes 15–20 h in total are two considerable advantages. Organic additives make particles of spherical shape; reach the fairly uniform size and a decrease in the crystallite size

(35-69 nm) with the pure tetragonal phase of zirconia. We report the antimicrobial study of zirconia against *E. coli* and *S. aureus* bacterial pathogens.

## MATERIALS AND METHODS

### Sol-gel synthesis

The entire precursor was taken of AR grade (99.99% pure) procured from SD fine scientific which included Zirconium n-propoxide, n-propanol, Hydroxypropyl methyl cellulose, and ammonia. The $ZrO_2$ nanomaterial was synthesized using the method described by Heshmatpour *et al.* [4] with some modifications. Initially, n-propanol was added to Zirconium n-propoxide (70 wt %). Then resulting solution was hydrolysed using drop by drop addition of ammonia and distilled water with a pH value of 9 to 10. Hydroxypropyl methyl cellulose (3 g) was added to the solution under vigorous stirring. After homogenization, the solution was stirred at room temperature for an hour, so the resulting gel was polymerized. The gel was dried in an oven at a temperature of 100 °C for 12 h and sintered by using microwave furnace at 300 °C for 2 h. The resulting powders were then compacted into the mold and made pellets with the help of die and punch in a hydraulic dry press at a load of 4 Ton for 10 min with a diameter of 10 mm and thickness of 2 mm. Each pellet consists of 0.25 g powder without any binder added and given the name P1, P2, and P3. The flow chart of Sol-gel synthesized zirconia as shown in fig 1.

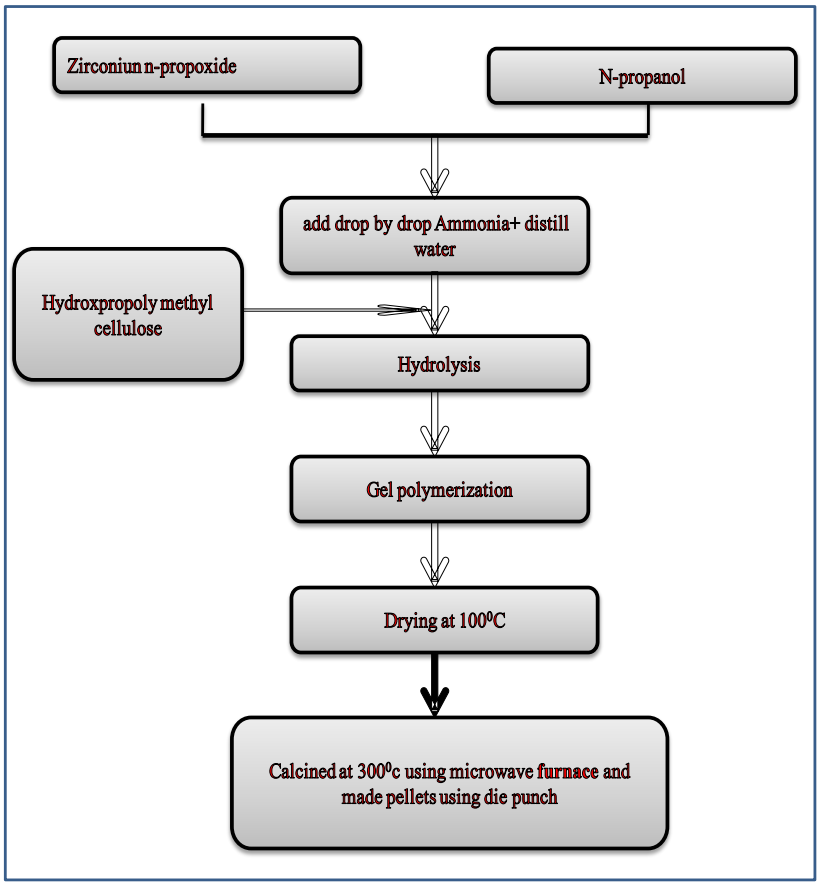


**Fig. 1: Flow chart of $ZrO_2$ synthesized by Sol-Gel method**

### Evaluation of cell growth

### Cell culture

The growth of cells in the presence of zirconia powder pellets was evaluated using 3T3 mouse fibroblast cell line which is obtained from National Center for cell Science, Pune. 3T3 cells were cultured in DMEM (Gibco, USA) containing 10% FBS and 1% antibiotics and antimycotic solution (Himedia, Mumbai) in $CO_2$ incubator (37 °C, 5% $CO_2$). DMEM was replaced every two days. When 3T3 cells reached 80% confluence, they were trypsinized and counted using a hemocytometer. A density of 2.5 ×$10^5$ cells/well was seeded on each pellet in 24-well plates for cell counting, morphology and cell cytotoxicity tests for day 1 to 6.

### Evaluation of cell growth

The zirconia powder pellets were sterilized by autoclaving at 120 °C and immersed in culture medium and cell growth was evaluated by cell counting on day 1 to 6. 3T3 cells in Dulbecco modified Eagles medium (DMEM) were seeded in 24 well plates as described above and were cultured in presence of pellets at 37 °C in 5% $CO_2$ atmosphere for day 1 to 6 so that cells were grown near pellets. At the end of incubation, the pellets were removed. The cells were washed with PBS and trypsinized using 0.25 % trypsin in PBS. The growth of cells was determined by counting cells using heamo-cytometer and morphology of cells near pellets was observed with an inverted phase contrast microscope (Magnus).

### Inhibition of biofilm formation by zirconia nanomaterial

The synthesized zirconia nanoparticles were evaluated for inhibition of biofilm formation against both Gram positive and Gram negative bacterial strains such as *S. aureus* (ATCC 33591) (Gram positive) and *E. coli* (ATCC 14948) (Gram negative) was obtained from Microbial Biotechnology Laboratory, Department of Biotechnology, Sant Gadge Baba Amravati University, Amravati.

Bacterial inoculums were prepared by subculturing microorganisms into Hilton Muller agar (HMA) slants at 37 °C for 18 h. The zirconia nanoparticles were added in nutrient broth, and starting stock solution was of 20000 µg/ml concentration. The quantitative assay of the antimicrobial activity against microbial strains was performed using the liquid medium microdilution method, in 96-multiwell

plates, in order to establish the minimal inhibitory concentration (MIC). For this purpose, two-fold serial dilutions of the zirconia material ranging between 2000 to 1.95 µg/ml were performed in a 200-µl volume of broth, and each well was seeded with 50 µl of microbial inoculum. Sterility control (wells containing only culture medium) and culture controls (wells containing culture medium seeded with the microbial inoculum) were used. The plates were incubated for 24 h at 37 °C [35-38]. At the end of the incubation period, the plastic wells were emptied, washed three times with Phosphate-buffered saline, fixed with cold methanol, and stained with 1% violet crystal solution for 30 min. The biofilm that formed on plastic wells was resuspended in 30% acetic acid. The intensity of the colored suspensions was assessed by measuring the absorbance at 490 nm. The last concentration of the zirconia nanomaterials that inhibited the development of microbial biofilm on the plastic wells was considered the minimum inhibitory concentration of biofilm development and was also expressed in micrograms per milliliter [39, 40].

**Study of degradation**

Degradation test of Zirconia pellets was done by taking Phosphate Buffered Saline solution. The pH of the solution was 7.4 at 37 °C. Initially, took the weight of pellets P1, P2 and P3. Then pellets were soaked in phosphate buffered saline solution. The pellets were dried at 120°C after every one week, and final weight of the sample was taken. This process was repeated for five weeks.

$$\%Weight\ loss = \frac{Wo-Wt}{Wo} * 100 \ldots\ldots\ldots \text{Eq. 1}$$

Where, Wo = initial weight of pellet

Wt = final weight of pellet after soaking in phosphate buffered saline solution

**RESULTS AND DISCUSSION**

Powder X-ray Diffraction analysis of sintered samples was carried out in order to study the structural properties of zirconia using a rigaku diffractometer (XRD, miniflex rigaku), and then analyzed, using Ni-filtered CuKα radiation (λ = 0.1542 nm) in the step scanning mode, with tube voltage of 40 kV and tube current of 40 mA. The XRD patterns were recorded in the 2$\theta$ range of 20 to 70 °, with a step size of 0.02 ° and step duration of 1 s. Field effect scanning electron microscopy (FESEM) technique was also used to observe the surface morphology. For this, a very small amount of powder was placed on a carbon adhesive tape, coated with gold/palladium and then observed in a FE-SEM (HITACHI S-4800). The sample was tested for transmission on Philips CM 200 TEM machine operated at a voltage of 200 kV and having a resolution of 0.23 nm.

**XRD analysis**

The structural properties are studied by X-Ray diffraction technique. The XRD pattern of $ZrO_2$ as shown in fig. 1(a), which is well matched with standard ICCD file no, 01-079-1764 of $ZrO_2$ as shown in fig. 1(b). Tetragonal phase formation starts by the loss of OH ions [41]. Tyagi *et al.* reported that transformation of amorphous Zr $(OH)_4$ gel to a metastable tetragonal phase occurs at about 400 °C [31] and Heshmatpour *et al.* obtained zirconia in both phases that is monoclinic and tetragonal at 500 °-700 °C. Using sol–gel method, Suciu *et al.* made use of sucrose and pectin to synthesize YSZ nanoparticles [41, 42]. They used zirconium chloride ($ZrCl_4$) and zirconium nitrate Zr $(NO_3)_4$) to synthesize the nanopowder of YSZ. Their materials were mainly in cubic phase. Heshmatpour *et al.* made a use of glucose and fructose as organic additives to synthesize zirconia nanoparticles. They obtained zirconia in both phases that are monoclinic and tetragonal [3]. Through the presented organic additives in this paper, the sample that was calcined at low temperature that is 300 °C showed relatively high crystallinity with small particle size as observed by the intensity of the characteristic peaks of the tetragonal phase. The diffraction peaks in zirconia crystal structure, as shown in fig. 2(a), viz., (101), (110), (200), (211), (212), are matched well with the peaks shown in fig. 2(b) and Structural parameters of phase were estimated in table 1.

**Table 1: Structural parameters of Zirconia**

| Phase | Space group | Crystal | Lateral parameters and angles | |
|---|---|---|---|---|
| | | | a/α | b/β c/γ |
| t-$ZrO_2$ | P42/nmc | Tetragonal | 3.596/90 °C | 3.596/90 °C 5.184/90 °C |

Crystallite size of the $ZrO_2$ was calculated by well-known Scherrer formula [43-45].

$$D = K\lambda/\beta\cos\Theta \ldots\ldots\ldots \text{Eq. 2}$$

Where K=0.9 is the shape factor, λ is the X-ray wavelength of Cu Kα radiation (0.1542 nm), Θ is the Bragg angle, and β is the full-width at half-maximum (FWHM) of the respective diffraction peak (in unit of radian). The average crystallite size of the $ZrO_2$ was calculated to be 13.49 nm.

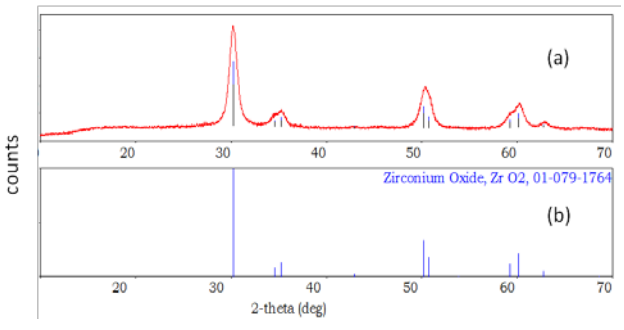


**Fig. 2: (a) XRD patterns of $ZrO_2$ sintered at 300 °C and (b) standard ICDD file of $ZrO_2$**

**FESEM analysis**

Field Effect scanning Electron Microscope (FESEM) is well known and reliable technique to analyze nanoscale samples. It gives surface morphology of samples. The FESEM image of $ZrO_2$ fig. 3(a-c) at different magnification shows a uniform distribution of particles and spherical like morphology. The image 3(a) shows the particles in the range of 35-69 nm. The formation and composition of crystalline of $ZrO_2$ nanoparticles are confirmed from EDX analysis, fig. 3 (d) which reveals that the Zr and O as the only elements in the sample, high purity and no any other impurity in the sample.

**TEM analysis**

Transmission Electron Microscopy micrograph, as shown in fig. 4 (a-b), confirm the shape of particles as that of FESEM image of fig 3(a-c), with average particles size 5.84 nm which is good agreement with the one calculated by Scherrer formula. The selected area electron diffraction (SAED) pattern in fig. 4 (c) shows the concentric rings of crysatalline planes.

**Cells growth assessment OF $ZrO_2$**

Cells growth and morphological characterization for $ZrO_2$ pellets were studied. The fig.5 shows cells growth in the presence of nanocrystalline $ZrO_2$ pellets along with control (in the absence of pellets). The growth of cells was measured using hemocytometer for day 1 to 6. This shows that the growth of cells along with pellets of $ZrO_2$ is similar to that of control. It means that nanocrystalline $ZrO_2$ powder does not show any sign of cytotoxicity. fig 6(a-c) shows images of 3T3 fibroblast cells near $ZrO_2$ pellets which show that after

2 d the growth of cells was found in control as well as in the presence of nanocrystalline $ZrO_2$ pellets. After 6 d the cell growth was found to be higher as compared to 2 d which indicates that nanocrystalline $ZrO_2$ pellets did not cause any toxic effect on cells. Fig. 6 shows a regular pattern of cell growth of fibroblast cells which is characteristic of a test conducted on $ZrO_2$ pellets. The *in vitro* study conducted on cytotoxicity of $ZrO_2$ shows very high biocompatibility of materials produced. Nanocrystalline $ZrO_2$ can be considered as non-toxic. Also, the numbers of living cells near the $ZrO_2$ pellets were found to be increased as compared to controls (fig. 6). The present data reveals the no signs of cytotoxicity and the rate of cell growth was higher in the case of nanocrystalline $ZrO_2$ pellets.

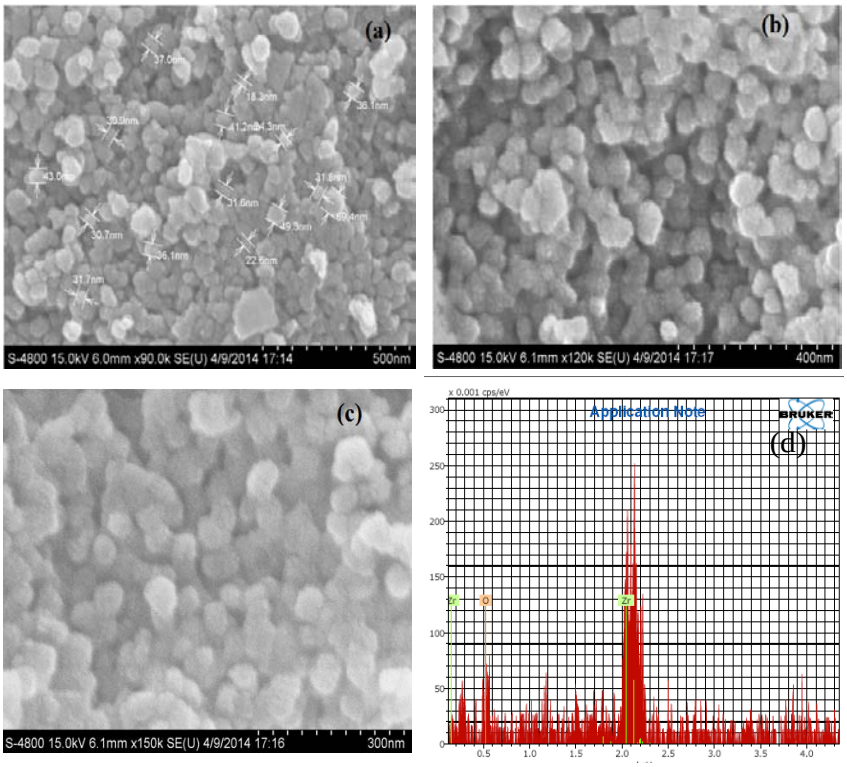


**Fig. 3: FE-SEM images of (a-c) $ZrO_2$ at different magnification, (d) EDAX spectrum of $ZrO_2$**

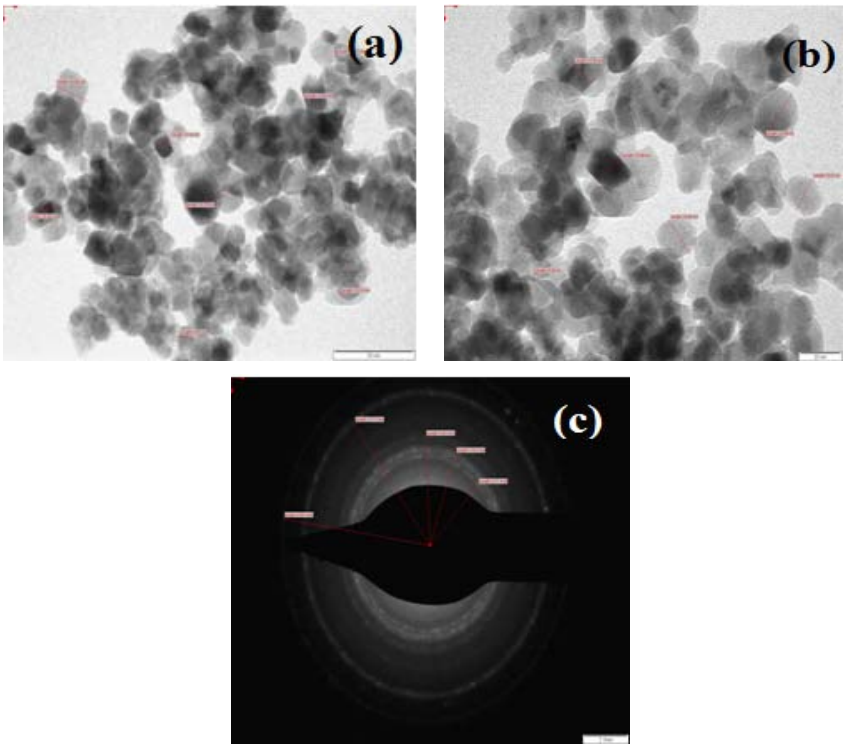


**Fig. 4: (a-b) TEM images of $ZrO_2$ (c) selected area electron diffraction pattern (SAED) of $ZrO_2$**

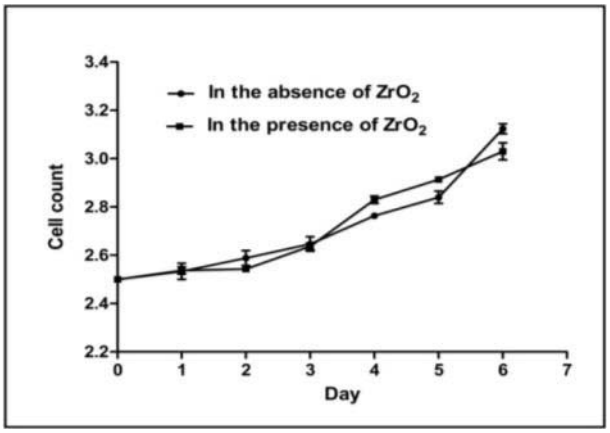


**Fig. 5: Growth of mouse fibroblast (3T3) in the presence of $ZrO_2$ pallets**

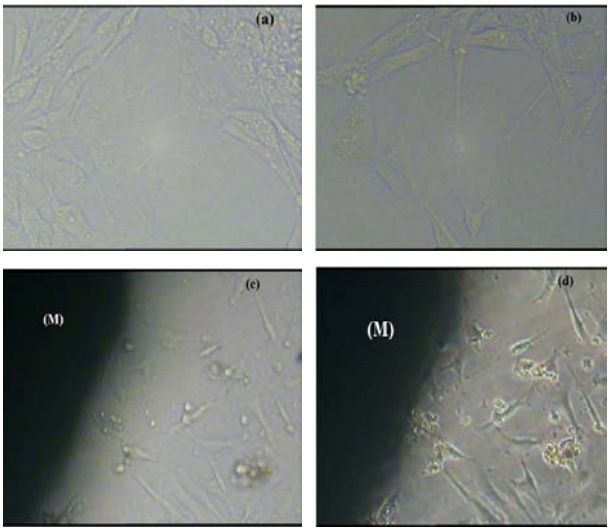


**Fig. 6: (a-d) optical microscope images of a culture of mouse fibroblasts after 2 d and 6 d contact with the $ZrO_2$ pellet, (M) area of pellet**

### Inhibition of biofilm formation by zirconia nanomaterial

Gram-positive *S. aureus* and Gram-negative *E. coli* were widely used for bacterial experiments. *S. aureus* and *E. coli* live on the body surface of mammals and sometimes occur infection to them [46]. Therefore, *S. aureus* and *E. coli* strains were selected for this antibacterial study. Fig. 7 shows the decrease in the population growth of *E. coil* and *S. aureus* with an increase in the concentration of zirconia nanoparticles. The growth of bacteria in the absence of zirconia nanoparticles (control) was considered as 100 % and the concentration required to inhibit the formation of biofilm due to the growth of bacteria to 50% was calculated. The inhibitory concentration that inhibited the development of microbial biofilm of *S. aureus* and *E. coli* was found to be 15.62 µg/ml and 7.81 µg/ml respectively and considered as the minimum inhibitory concentration. The antimicrobial performance of $ZrO_2$ nanoparticles due to the following assumptions: active oxygen species generated from the $ZrO_2$ nanoparticles actively inhibit the growth of *S. aureus* cells by accumulation or deposition on the surface of *S. aureus* cells. It is also suggested that $ZrO_2$ nanoparticles are able to slow down *E. coli* growth due to disorganization of *E. coli* membranes, which increases membrane permeability leading to accumulation of nanoparticles in the bacterial membrane and cytoplasmic regions of the cells. From the above discussion we clearly came to know about the enhanced antimicrobial activity of $ZrO_2$ nanoparticles [28]. In dentistry, restoration failure is generally attributed to a combination of oral bacteria and inappropriate features of dental materials. Efficient dental restorative materials are important for an adequate recovery of masticatory and esthetic functions. However, these materials are prone to biofilm formation, affecting oral health [47]. So, $ZrO_2$ nanomaterial inhibiting biofilm formation can be used in biomedical especially in dentistry applications.

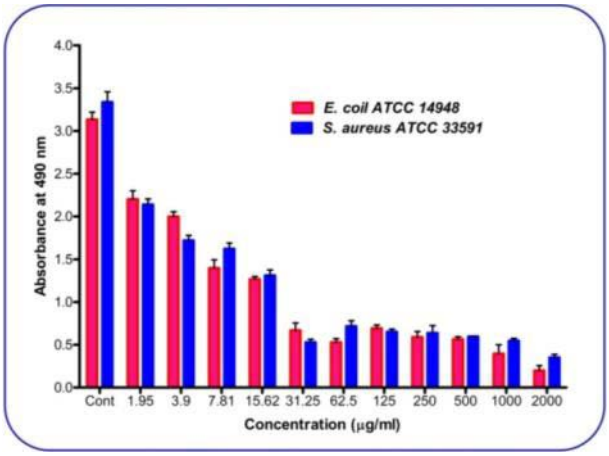


**Fig. 7: Effect of $ZrO_2$ powder on the bacterial growth (*E. coli* and *S. aureus*)**

**Data is represented as mean±SEM (n=3)**

### Degradation of $ZrO_2$

Fig. 8 shows that degradation of $ZrO_2$ nanomaterials was 0.0021% for five weeks which is very negligible. It means that a zirconia nanomaterial does not show any degradation and stable at physiological condition in PBS solution.

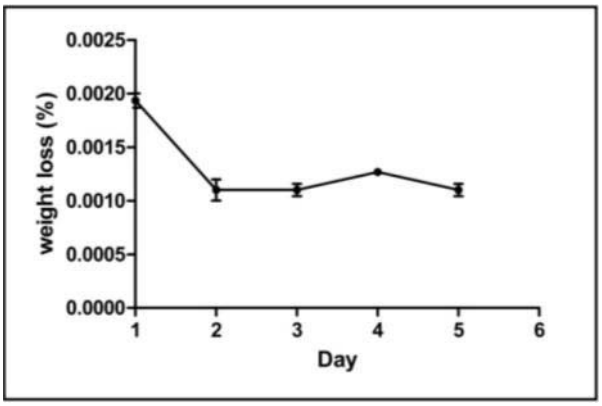


**Fig. 8: degradation of $ZrO_2$ pellets in PBS**
**Data is represented as mean±SEM (n=3)**

## CONCLUSION

In this study, our aim was to synthesize nanocrystalline zirconia by using the simple sol-gel method with organic additive and to study its antibacterial property. The formation of the tetragonal crystalline phase of $ZrO_2$ was confirmed by powder XRD analysis at relatively low temperature using microwave furnace. The morphology, particle size, and nanostructure were analyzed using FESEM. TEM data also confirmed the formation of the nanostructure. TEM micrograph and SAED pattern confirmed the crystalline perfection in the prepared zirconia nanoparticles. Nanocrystalline zirconia does not inhibit cell growth of normal 3T3 mouse fibroblast cells which indicated its nontoxic nature. The zirconia nanoparticles

showed the significant antibacterial activity against *E. coli* and *S. aureus* bacterial pathogen and also it is stable at the physiological condition. Hence, it can be used for various biomedical applications.

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**CONFLICT OF INTERESTS**

Declared none

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