Antimicrobial Activity of Zirconia Nanostructures and Zirconium Complexes

By

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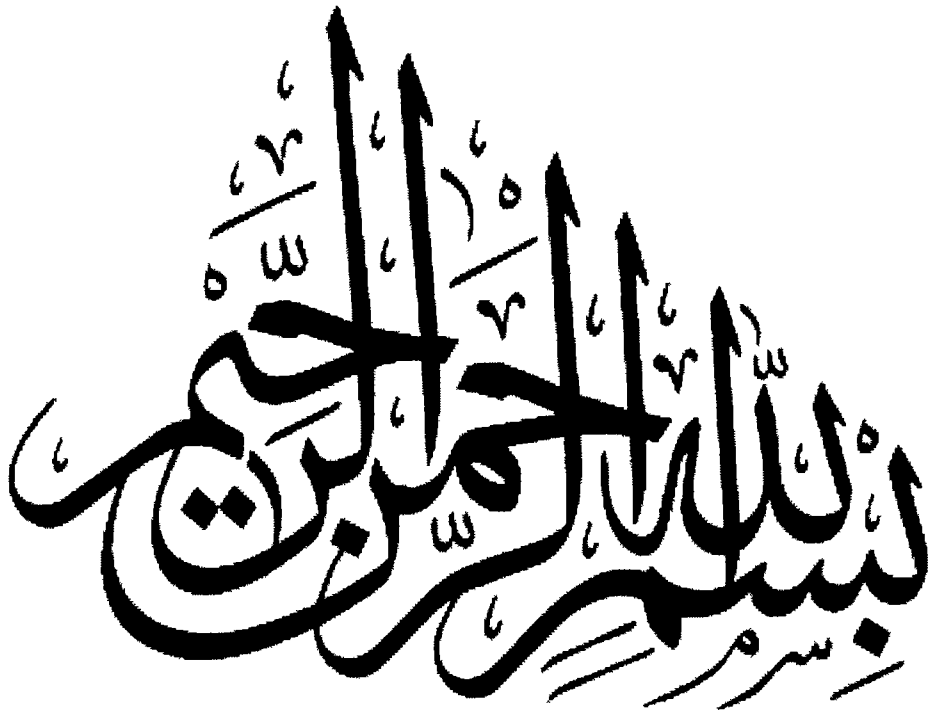

"In the name of ALLAH The Most Gracious and The Most Beneficent"

Department of Bioinformatics and Biotechnology

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Dated: 26-08-2016

FINAL APPROVAL

It is certified that we have read the final thesis entitled **"Antimicrobial Activity of Zirconia Nanostructures and Zirconium Complexes"** submitted by **Mr. Aftab Rafiq** and it is our Judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

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Master in Sciences of Biotechnology
(MSBT)

This humble effort is

Dedicated

To

My beloved

Parents

Brothers, Sisters

and

Teachers

Who inspired me for higher ideals of Life

DECLARATION

I hereby solemnly declare that the work **"Antimicrobial Activity of Zirconia Nanostructures and Zirconium Complexes"** presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: 26-8-2016

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Aftab Rafiq

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ABSTRACT

ZrO_2 nanoparticles have gotten an extraordinary response for their appealing scientific and innovative perspectives in various fields because of mechanical and electrical properties. These mechanical and electronic properties were found and concentrated for the most part in crystalline zirconia, yet they have likewise been examined for antimicrobial properties. The present study was led to prepare zirconia nanoparticles and zirconium complexes with various amino acids as ligands by co-precipitation method and to decide their conceivable antimicrobial properties. The morphology and crystalline nature of the prepared items was affirmed by X-RAY diffraction (XRD) and SEM. The antibacterial activity of the ZrO_2 nanoparticles and zirconium complexes containing primary ligand (8-hydroxyquinoline) and secondary ligand (glycine, L- alanine and L-serine) was tested separately against bacterial strain of *S. aureus, E. coli* and fungi sp. *A. niger*. According to current study, the zirconia has shown minor activity against *E. coli* only, in comparison to the Zr (IV) complexes that have shown activity not only against both the bacteria: gram negative *E. coli* and gram positive *S. aureus* but also against fungal strains. Zr-G produces clear zone of inhibition against *S. aureus* (14.3 mm) *E. coli* (9.6 mm) and *A. niger* (6.3 mm). *Zr-A* produced zone of inhibition against E. *coli* (14.3 mm), *S. aureus* (8.6 mm) and *A. niger* (6.3 mm). Zr-S produces zone of inhibition against *S. aureus* (14.6 mm), *E. coli* (15.6 mm) and against *A. niger* (7mm). The outcomes are showing antimicrobial action of zirconia nanoparticles and complexes that depend on their crystal plane. The comparison of different antibacterial action of ZrO_2 nanoparticles and Zr (IV) complexes a might be attributed to the atomic arrangement of various exposed surfaces. On the premise of the study comes about, the ZrO_2 nanoparticles with the same surface regions yet distinctive shapes i.e., diverse dynamic features and active facets may prove effective in antimicrobial activity.

INTRODUCTION

1. INTRODUCTION

Nanostuctures are small clusters of atoms, which are about 1-100 nanometer in dimensions. The term nano derives from Greek word nanos, which means verysmall. The nano means one billionth (10^{-9}), so a nanometer is one billionth of a meter. Nanoparticles are larger than individual atoms or molecules but are smaller than bulk solid. Hence they are obeying neither absolute quantum chemistry nor laws of classical physics and have properties that differ markedly from those expected from the bulk materials. Nanotechnology is the creation and utilization of materials, devices and system through the control matter at length scale less than 100 nm. It is a interrelated with principle of Chemistry, Physics and Engineering, which are involves the processing , manufacturing and their applications at nanometre scale. Nanomaterials are the materials with at least one dimension less than 100 nm (Powers *et al.,* 2006). There physical and chemical characteristics are unique which differ from others individual molecules or atoms and also the same material at a bulk scale (Banfield and Zhang, 2001). These differences are due to their very small sizes and higher specific surface area and reactivity, which enable them to have novel application.

The products of nanotechnology are applicable in the fields of medicine, energy, environmental remediation and electronics. (Aitken *et al.,* 2006; Baruah and Dutta, 2009; Luther, 2004; Rickerby and Morrison, 2007; Schmid, 2001).

The human society and microorganism co-exists, the modern life is facing the problem of increasing infections. Microorganisms are becoming resistant to the majority of antibiotics and antiseptics during last ten years. The focus is on combating multidrug-resistant (MDR) microorganisms especially on gram-positive bacteria, like methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *enterococci* (Schist 2006). On the other hand, according to current study not much work is done on gram-negative bacteria, hence no antibiotic from a new class has been developed against MDR gram-negative bacilli. (Rubinstein and Vaughan, 2005).

Number of cases reporting infections with gram negative bacteria have grown, for which no adequate therapeutic options exist (Falagas *et al.*, 2005) pushing back humanity to pre antibiotic era, which is now a reality in many parts of the world (Paterson and Bonomo, 2005).

Nanotechnology developed rapidly toward manufacturing and application of nanoparticles (NPs) in various sectors. Nowadays biosynthesis of nanoparticles has been intensively studied in investigations into biological systems like bacteria, fungi, yeast, algae and plants with much success, but the impact of nonmaterial's on organisms and the ecosystem is not yet sufficiently known (Thamilselvi and Radha, 2013). The experiences gained from different fields as well as the rapid progress in nanotechnology, developments and innovations in imaging and analytical technologies suggest that an immense variety of other biological applications are possible. Another promising area where nanoparticles can be used is agriculture which could result in potential improvement in strategies for better current crop. Already, nano-encapsulated pesticides are an example, their application in field with targeted release of chemical have been a great success that provide easier and safer control against pests (Beddington, 2010) Another possible application of nanotechnology of nanotechnology In the future may provide smart soil monitoring device, that will result in timely delivery of chemicals which plant needs (Rosa *et al.*,2010; Nair *et al.*, 2010).

Thus, an ultimate & balanced fusion of nanotechnology, inorganic chemistry, microbiology and biotechnology can design novel anti-microbial agents by using atomic scale tailoring of materials which can serve to fulfill the current relevant problems. However, nanotechnology can be simply referred as a fabrication of some miniature machines having the ability to travel through human body systems and repair the damaged systems. Whenever it comes for nanoparticles and antibiotic properties it has been found that metal nanoparticles are more therapeutic compare to others.

Nanostructure materials have promised several breakthrough applications in the field of nanomedicine and biomedical sciences due to their exceptional physical and chemical properties. Among nanostructures materials, nanostructured metal oxides and metal complexes have emerged as a class of materials which is increasingly being studied for health-related applications. Metal oxides with high ionic character are not only known for their wide variety of chemical and physical properties, but also for their antibacterial activity. (Karnik *et al.*, 2007). The nanostructures of metal oxide such as MgO, TiO_2, and ZnO are reportedly superior antimicrobial agents in terms of durability. Safety and heat resistance as of conventional organic antibacterial agents. (Makhlufi *et al.*, 2005) but

according to current study not much efforts regarding antibacterial activity of zirconia nanoparticles are done. However, zirconium Zr (IV) mixed ligand complexes have shown significant antifungal and antibacterial activity (Jangra *et al.*, 2012).

Zirconia is an oxide of zirconium element, a transition metal in the periodic table that has relative mass 91.224 grams per mole. Zirconia exhibits different shapes like monoclinic, tetragonal and cubic. At room temperature and pressure, zirconia has unstable crystalline structure. To maintain its crystalline structure, it requires the addition of stabilizer agents including other compounds. The formation of monoclinic structure of zirconia requires temperature range between 4000 °C to 11700 °C. While tetragonal structure forms between 11700 °C to 23700 °C, and cubic structure is obtained between higher temperatures range, which is 23700 °C to 26000 °C, (Dercz *et* al., 2008) .These changes in crystalline structures at different temperatures result in change in density and physical properties of zirconia (Callister and Rethwisch 2007). Zirconia with tetragonal and cubic structures has higher density and higher crystallization temperature than. Zirconia with the monoclinic structure has low density and lower crystallization temperature then tetragonal and cubic structure (Dercz *et al.*, 2008).

There are different methods of preparation of zirconia nanoparticles that include physico-chemical methods and hydrothermal mehods. Physico-chemical method include sole gel synthesis (Dercz *et al.*, 2008)], aqueous precipitation method (Callister and Rethwisch, 2007) , thermal decomposition and hydrothermal methods (Suciu *et al.*, 2006) requires very high temperatures, on the contrary biological synthesis skips most of costly procedures with low cost, more beneficent and easily attainable mild conditions ,proceeds the synthesis beneficially at low cost in mild condition . In past few years, inorganic antimicrobial agents have been used widely especially in field of textile to control microorganisms (Milman *et al.*, 2009). The success of inorganic antimicrobial agents lies in are increased safety and stability which prove it more useful than conventional antimicrobial agents. ZrO_2 nanoparticles have also shown antimicrobial property to greater extant. (Piconi and Maccauro 1999). Previously biosynthesis of TiO_2, SnO_2 and MgO is reported. Biosynthesis of ZrO_2 nanoparticles was reported by utilizing a natural hydrolyzing agents *A. vera* leaf extract. Antibacterial studies were carried out for both ZrO_2 nanoparticles and treated cotton against *Staphylococcus aureus* (*S. aureus*)

and *Escherichia coli* (*E. coli*) bacterial pathogens and antifungal activity was demonstrated against *C. albicans* and *A. niger* for only ZrO_2 nanoparticles. This kind of biobased synthesis of zirconia nanoparticles and their application on cotton with non toxic chemicals is an eco-friendly cost effective approach (Gowri *et al.*, 2014).

Few studies have reported on the role of zirconia nanoparticles as anticancer materials according to current study, this is only one report available in the literature of the *in vitro* anticancer effect of sulphated zirconia nanoparticles against three cancer cell lines. Specifically, the toxicity of sulphated zirconia nanoparticles against human breast cancer MCF-7, human colon cancer HT29 and human liver cancer HepG2 cell lines was observed, showing encouraging results. Moreover, it is reported here that these novel nanoparticles hold promise not just for anticancer applications but also for anti-infection applications. The steady increase in the antimicrobial resistance of microorganisms represents a great public health concern. This requires the search for new unconventional antimicrobial agents. Nanotechnology provides promising nanomaterials to irradiate these infectious diseases without disturbing the functionality of normal cells. The same materials in their report was also used for the antimicrobial properties which were determined using the agar diffusion method against different Gram-positive and Gram-negative bacteria (Mftah *et al.*, 2015).

The exact mechanism for the enhancement in antimicrobial activity is not yet clear and is hot issue of current research. However, it can be due to the photo-degradation, particles accumulation in cells and electrostatic interactions between nanostructures and cell walls.

1.1 Aims and Objectives

The present study is designed to achieve the following objectives:

- Synthesis of nanostructures of zirconia and Zr (IV) complexes with amino acids as ligands by chemical method
- To confirms the formation of desired nanostructures by diffraction and spectroscopy
- To study the detail antimicrobial activities of zirconia nanostructures.
- To study the comparison of antimicrobial effect of the synthesized ZrO_2 and Zr (IV) complexes against specific microorganisms including gram positive , gram negative bacteria and fungus.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Shivankar and Takkar (2003) observed the antimicrobial activity of Co(II) and Ni(II) complexes.The antibacterial and antifungal activity of the ligands and the complexes were assayed against some of the bacteria and fungi. The cup-plate method was used to assay antibacterial activity against C. *diphtheria, E. coli, S. typhi, S. dysenteriae, S. aureus* and *V. cholerae*. The results indicate the moderate activity of mixed ligand Co(II) complex against selected bacteria, while all of the selected microorganisms except *S. dysenteriae* and *S. aureus*, show general resistance to the Ni(II) complex. Both the complexes show antifungal activity to a moderate extent against the two fungi.

Malghe *et al.*, (2009) experimented the zirconium complexes against the pathogenic microbes for antibacterial *(Staphylococcus aureus, Enterococcus faecium)* and experiment results were contrasted with standard vancomycin while antifungal action was tried against (*Candida albicans, Candida krusei, Aspergillus fumigatus*) and results were compared with amphotericin B. All the complexes show antifungal and antibacterial movement against chosen strains

Sadeek *et al.*, (2011) studied spectroscopic, structure and antimicrobial activity of new Y (III) and Zr(IV). The ligand and also their metal complexes were assessed for their antibacterial properties against a few bacterial species, for example, *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). Antifungal screening was performed against two species *Penicillium* (*P. rotatum)* and *Trichoderma* (T. sp.) This study demonstrated that the metal complexes are more antibacterial in comparison with free ligand and no antifungal activity was observed for ligand and their complexes.

Saengmee *et al.*, (2013) demonstrated that silver inorganic materials including AgZ, AgZrPSi and AgZrP have antimicrobial effects on S. *mutans*, L. *casei*, C. *albican* and S. *aureus*. AgZrPSi and AgZrP showed higher antimicrobial ability than AgZ. It seems that crystal structure, the rhombohedral structure, not particle size, influenced the antimicrobial activity

Lubasova and Barbora, (2014) observed that the nanofiber membrane with SnO_2 exhibited antimicrobial properties only against *S. aureus* whereas nanofiber membranes with ZnO or ZrO_2, exhibited antimicrobial properties against both bacteria, particularly

E. coli and *S. aureus*. These results suggest that PVA nanofiber membranes with incorporated TiO_2, ZnO, ZrO_2 or SnO_2 nanoparticles have a potential to be used for many different environments where bacteria are harmful such as an air conditioning system in hospitals, variable antibacterial products for medicine, sanitary and antibacterial textiles etc.

Hakam *et al.*, (2014) prepared the series of ZnO and ZrO_2 doped ZnO catalyst and carried out antibacterial activity test using disk diffusion method, and the result indicated antibacterial activity of the prepared catalysts.

Pradhaban *et al.*, (2014) worked on prevention of bacterial invasion on biomedical implants. Bacteria secretes extracellular matrix which is resistant to antibacterial agents. In his study, recently, he used surface coatings such as zirconia (ZrO_2) to improve implants durability. From his study it could be also speculated that ZrO_2 coatings exhibited antibacterial activity against only *E. coli*, whereas coatings with Ag–ZrO_2 composite showed superior activity against *E. coli* and *S. aureus* strains.

Gowri *et al.*, (2014) investigated antimicrobial and antifungal properties of zirconia nanoparticles by agar diffusion method against *Staphylococcus aureus* and *Escherichia coli* bacterial pathogens and fungal strains *Candida albicans* and *Aspergillus niger*, respectively and ZrO_2 nanoparticles shown remarkable antimicrobial property. Biosynthesis of ZrO_2 nanoparticles was reported for the first time. *A. vera* leaf extract utilized as a hydrolysing agent replacing of synthetic chemicals. Both ZrO_2 and cotton treated with same nanoparticles was used to determine Antibacterial studies against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) bacterial pathogens and only ZrO_2 nanoparticles were used to evalute Antifungal activity against *Candida albicans* (*C. albicans*) and *Aspergillus niger* (*A. niger*). For this kind of biobased synthesis of zirconia nanoparticles and their application on cotton with non toxic chemicals is an eco-friendly cost effective approach.

Obidi and Nwachukwu, (2014) observed that the ZrO_2 nanoparticles yielded 65 % inhibition of *B. sphaericus* and 80% of *B. choshinensis*. Their antibacterial activity results revealed that ZrO_2 nanoparticles were very effective against the tested organisms compared to the conventional biocides because of high surface to volume ratio of atoms

in their sizes. However, more sophisticated antimicrobials nanostructured are needed to explore to get rid from these conventional biocides.

Mftah *et al.,* (2015) reported that the antimicrobial activity sulphated zirconia nanoparticles against Gram-negative and Gram-positive bacteria. It was found that the sulphated zirconia nanoparticles showed the enhanced antimicrobial activity against *P. aeruginosa* and methicillin-resistant *S. aureus*, followed by *B. subtilis* and *S. choleraesuis.* In contrast, the sulphated zirconia nanoparticles did not show any activity against *C. albicans*, suggesting a lack of antifungal activity. Other fungal species may be used to detect any possible antifungal effect of the sulphated zirconia nanoparticles. All of these microorganisms are responsible for a range of serious infections in human and animal populations. Therefore, the sulphated zirconia nanoparticles could find various biomedical applications of therapeutic importance to counteract such highly resistant microorganisms. The Study showed one of the initial report about the exposure of cancer cells to sulphated zirconia nanoparticles. The inhibition of cell growth was dose-dependent inhibition. Similar promising results were also observed for reducing bacteria functions. In this manner, this study demonstrated that sulphated zirconia nanoparticles with bronsted acidic sites should be further studied for a wide range of anticancer and antibacterial applications.

MATERIALS AND METHODS

3. MATERIALS, METHODS AND TOOLS

The present study was conducted at Infectious Diseases Laboratory, Department of Bioinformatics and Biotechnology, International Islamic University Islamabad.

3.1 Materials

3.1.1 Chemicals

All chemicals used for the study were of analytical grade. The detail is mentioned in Appendix.

3.1.2 Microbial strains

The bacterial strain *E. coli* was collected from KRL Hospital Islamabad and *S. aureus* used in experiments was collected from the DBIBT, IIU Islamabad and the fungal strain *Aspergillus niger* was collected from infectious diseases lab International Islamic University, Islamabad.

3.2. ZrO_2 synthesis

ZrO_2 nanostructures were synthesized by a Co precipitation method using 20 ml aqueous solutions of $ZrO(NO_3)_2 \cdot xH_2O$ (0.5 M), and surfactant (Gowri *et al.*, 2014). The solution pH was maintained at 9. The temperature of solution is maintained at 358 K at hot plate for 1 h. After the stipulated time the solutions were allowed to cool down to room temperature. The white colored precipitate was washed with absolute ethanol and distilled water several times, and dried at 80 °C, annealed and then used for various characterizations.

3.2.1 Zr(IV) complex synthesis

Complexes were prepared from $ZrO(NO_3)_2 \cdot xH2O$ (0.5 M) using HoQ as a primary ligand and amino acids as a secondary ligand. 10 ml of (99.90 mM aq. sol.) $ZrO(NO_3)_2 \cdot xH_2O$ (0.5 M) and 10 ml (199.77 mM ethanolic solution) HoQ were mixed (Jangra *et al.*, 2012) The mixture was stirred and kept in a boiling water bath for 10 min. 10 ml (1 mmol) aqueous solution of amino acid i.e., L-serine or L-alanine or glycine was added separately under constant stirring. The complexes were obtained by raising the pH (from 2.8 to 6.5) of the reaction mixtures. The mixtures were cooled, filtered and washed with water. The solid complexes thus prepared were dried under vacuum.

3.3 Antimicrobial Activity

The antimicrobial activity of ZrO_2 and Zr(IV) mixed ligand complex nanostructures against the bacterial strains and fungal strains were evaluated by the agar well diffusion test method. For *E. coli* and *S. aureus* nutrient agar medium and for *A. niger*, potato dextrose agar (PDA) was used.

In a laminar flow chamber, 20 ml of the molten agar medium was poured into sterile petri plates. After solidification of the culture medium, the fresh bacterial and fungal cultures were inoculated onto the respective medium. 5 mm diameter wells were dug on each plate at equidistant points using a sterile cork borer, and were immediately filled with the test samples. 80 µl methanolic solution (500 g/ml) of Zr(IV) complex and aqueous solution (80 mM) of ZrO_2 were used for pouring in the wells after proper sonication. Stock concentrations of the standard drugs were prepared in sterile distilled water to give a final concentration of 100 g/ml (Jangra *et al.*, 2012).

The plates containing bacterial and fungal strains were incubated separately in controlled temperature incubator for 24 to 72 h. After incubation, the diameter of the zones of bacterial and fungal growth inhibition formed around the wells was measured using a scale and expressed in mm

3.3.1 Types of media

Two types of media were used for the growth of bacteria and fungus such as Mueller-Hinton agar media and potato dextrose agar media respectively.

3.3.1.1 Mueller-Hinton (MH) agar media

MH Media has been used for the growth of bacteria. The composition of this media is consisting of Trypton, Yeast extract, Agar and NaCl. Trypton 10 g, yeast extract 5 g, agar 15 g and NaCl 10 g are used for 1 liter media preparation. For the preparation of 500 ml media take 500 ml distilled water in which the composition of trypton 5g, yeast extract 2.5g, agar 7.5g and NaCl 5g were added. Maintain the pH till to 7.2 with help of pH meter. The media were sterilized in autoclave for 15 min. at 121 °C under the pressure of 15 pcal. The media was cool down and pour 25ml in each petri plates.

3.3.1.2 Potato dextrose agar media

Potato dextrose agar has been used for the growth of fungi. The composition of media is consisting of Potato extract, yeast extract, glucose dextrose and agar. For the

preparation of 1 liter media the recipe of media is 500 ml distilled, 500 ml potato extract, 5g of agar, 10 g of Yeast extract, 10 g of dextrose glucose and 250 mg tablet of ciprofloxacin to protect of the media from bacterial contamination. So for the preparation of 200 ml media the composition of media is 100 ml distilled water, 100 ml potato extract, 1gof agar, 2 g of yeast extract, 2 g of dextrose glucose extract and 50 mg of ciprofloxacin. The media has been mixed with hot plate and pH maintains till to 7.5. The media were sterilized in autoclave for 15min 121 ^{O}C under the pressure of 15 pcal and cool down till to 37 ^{o}C pour 25 ml in each petri plates in laminar hood.

3.3.2 Microorganism stock preparation

Two types of media were used for the stock of microbes. Mueller-Hinton (MH) media was used for the stock of bacteria and Potato Dextrose Media for stock of fungi.

3.3.2.1 Bacterial stock preparation

Inoculum of 100 ml was prepared for bacterial stock by the help of Mueller-Hinton (MH) media without agar addition. The media containing Trypton 1 gram, Yeast extract 0.5 gram and NaCl 1 g was poured in 100 ml distilled water and mixed gently with help of hot plate and maintained the pH till to 7.2. The media were sterilized in autoclave for 15 min 121 ^{o}C under the pressure of 15 pcal. The media was cool down and pour in different falcon tubes. The bacterial colony was added in media to produce culture then those cultures were incubated at 35 ^{o}C until the achievement of bacterial growth. The growth stocks were determined by growing bacteria on MH agar media in petri plates.

3.3.2.2 Fungal stock preparation

Inoculum of 50 ml was prepared for Fungi stock by the help of Potato dextrose media without the addition of agar. The media contain 50 ml of Potato extract, Yeast extract 1g and 1g of dextrose was poured in 50 ml distilled water and mixed gently with help of hot plate and maintain the pH of media till to 7.2. The media were sterilized in autoclave for 15min 121- ^{o}C under the pressure of 15 pcal. The media was cool down and pour in different falcon tubes. The A. *niger* spores were added in media to produce culture then those cultures were incubated at 35- ^{o}C until the achievement of fungal spores Stock. The growth stocks were determined by growing spores on Potato dextrose media in petri plates.

3.3.3 Well preparation

5 mm wells were dug on solidified agar medium by the help of sterile cork borer approximately.

3.3.4 Streaking process

Before starting the Streaking of microorganisms the laminar hood was sterilized by UV for 15 min and wash completely with 70 % ethanol. Petri plates were label kept inside the laminar hood along with glass tube for swabbing of microbes, tissue paper, burner, micropipette and spatula. Then sterilized with UV for 15 min. the media were pour in each petri plates wait for half hours to solidify the media. With help of micropipette take 100µl of microbes pour on each petri plate and spread on the surface of entire petri plates with help of glass tube. The streaking process is repeated several times and rotates the plates to ensure the strain is distributed completely over plates.

3.3.5 Antimicrobial solutions application

5 separate solutions were made including zirconia, zirconium complex and antibiotics. The zirconia and zirconium complex were taken in the form of aqueous and methanolic solutions. With help of micropipette into the wells on each petri plate separately and 3 wells were dug and filled on each plate with respective after pouring of wells all the plates were placed in incubator at 37- ^{o}C for 24 h.

3.3.6 Observations

After 24 h of solutions application each and every plates were examined. If the result is satisfied and it has produced a circular inhibition zone then the inhibition zone is measured with reading scale along the diameter of discs. In case of Fungus the reading was noted after 48 h of application.

3.4 Characterization tools

3.4.1 XRD

X-ray diffraction is now a common technique for the study of crystal structures and atomic spacing. X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. These X-rays are generated by a cathode ray tube, filtered to produce monochromatic radiation, collimated to concentrate, and directed toward the sample. The interaction of the incident rays with the sample produces

constructive interference (and a diffracted ray) when conditions satisfy Bragg's Law ($n\lambda=2d \sin\theta$). This law relates the wavelength of electromagnetic radiation to the diffraction angle and the lattice spacing in a crystalline sample. These diffracted X-rays are then detected, processed and counted. By scanning the sample through a range of 2θangles, all possible diffraction directions of the lattice should be attained due to the random orientation of the powdered material. Conversion of the diffraction peaks to d-spacings allows identification of the mineral because each mineral has a set of unique d-spacings. Typically, this is achieved by comparison of d-spacings with standard reference patterns.

All diffraction methods are based on generation of X-rays in an X-ray tube. These X-rays are directed at the sample, and the diffracted rays are collected. A key component of all diffraction is the angle between the incident and diffracted rays. Powder and single crystal diffraction vary in instrumentation beyond this

3.4.2 SEM

Accelerated electrons in an SEM carry significant amounts of kinetic energy, and this energy is dissipated as a variety of signals produced by electron-sample interactions when the incident electrons are decelerated in the solid sample. These signals include secondary electrons (that produce SEM images), backscattered electrons (BSE), diffracted backscattered electrons (EBSD that are used to determine crystal structures and orientations of minerals), photons (characteristic X-rays that are used for elemental analysis and continuum X-rays), visible light (cathode luminescence–CL), and heat. Secondary electrons and backscattered electrons are commonly used for imaging samples: secondary electrons are most valuable for showing morphology and topography on samples and backscattered electrons are most valuable for illustrating contrasts in composition in multiphase samples (i.e. for rapid phase discrimination). X-ray generation is produced by inelastic collisions of the incident electrons with electrons in discrete orbital's (shells) of atoms in the sample. As the excited electrons return to lower energy states, they yield X-rays that are of a fixed wavelength (that is related to the difference in energy levels of electrons in different shells for a given element). Thus, characteristic X-rays are produced for each element in a mineral that is "excited" by the electron beam. SEM analysis is considered to be "non-destructive"; that is, x-rays

generated by electron interactions do not lead to volume loss of the sample, so it is possible to analyze the same materials repeatedly.

RESULTS

4. RESULTS

4.1 Antibacterial activity

The antibacterial activity of the ZrO_2 nanostructures and zirconium complexes containing primary ligand (8-hydroxyquinoline) and secondary ligand (glycine, L- alanine and L-serine) was tested separately against bacterial strains of *S. aureus, E. coli* and fungal sp. *A. niger.* 80 µl of methanolic solution of Zr (IV) complexes (500 g/ml conc.) and aqueous solution of zirconia nanostructures (80 mM conc.) was used as shown in table 4.1. The antimicrobial solutions were applied by well diffusion assay method on nutrient agar and PDA plates with spreaded respective microbes. The plates were incubated in incubator for 24 h at 30 °C. After 24 h of incubation time, the plates were observed for zone of inhibition. The zirconium complexes have shown zone of inhibition at all tested sample for bacteria and fungus but ZrO_2 nanoparticles have shown minimum antibacterial or antifungal activity.

The antibacterial activity of the zirconia nanostructures was tested separately against bacterial strain of *S. aureus, E. coli* and fungal sp. *A. niger.* The 80 µl aqueous solution of Zirconia (500 µg/ml concentration) was used by well diffusion method as shown in table 4.1. Minor zone of inhibition were observed against *E. coli* as figure 4.1.

Table 4.1 Zone of inhibition by zirconia nanoparticle against *S. aureus, E. coli and A. niger*

Zr nanostructures	Microbial strains	Zone of inhibition. (mm)
ZrO_2 (80 µl) Water and Methanolic solution	*S. aureus*	—
	E. coli	—
	A. niger	—

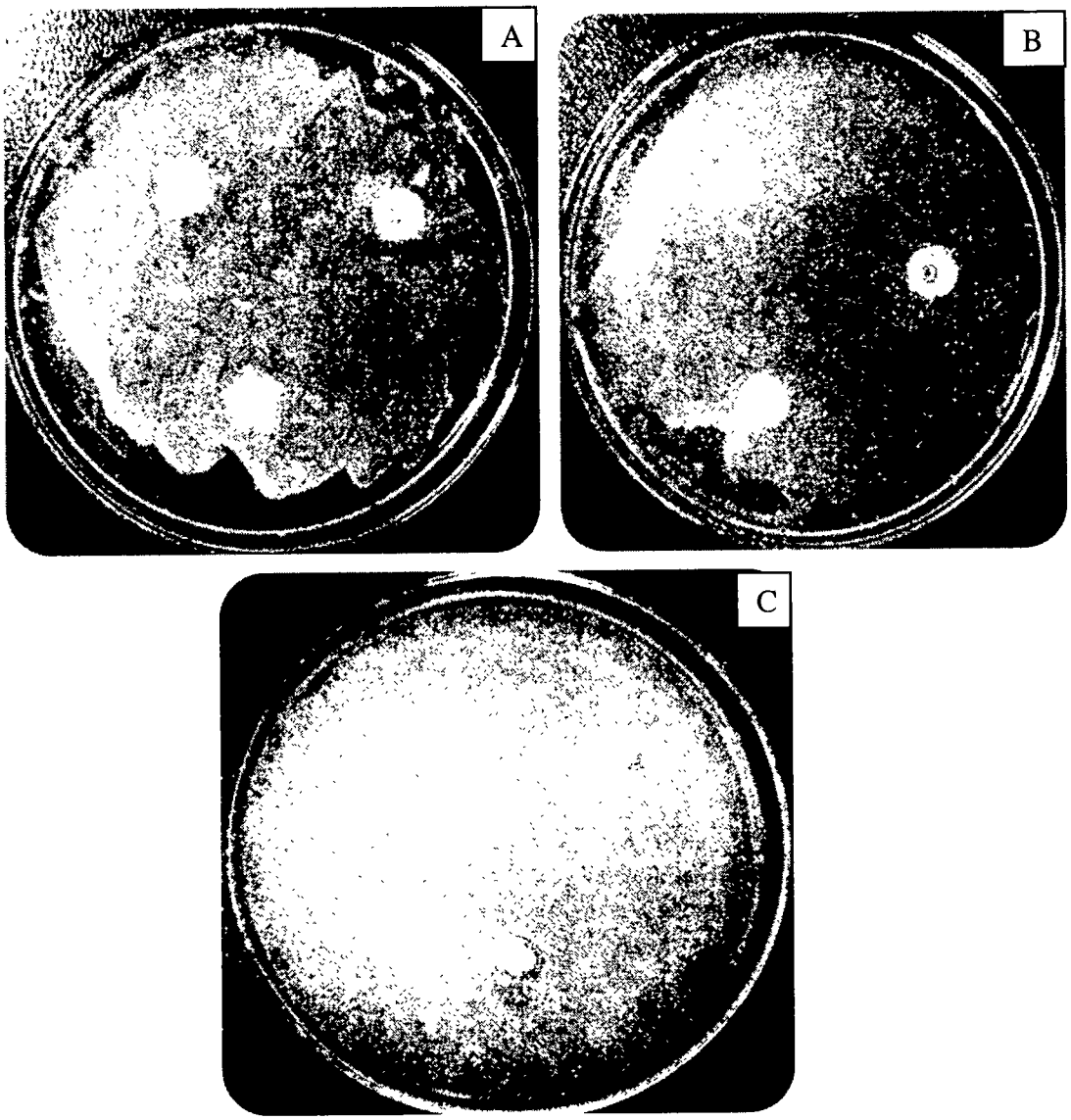


Figure 4.1 Zone of inhibition by ZrO_2(80 µl) against A) *S. aureus* B) *E. coli* C) *A. niger*

The antibacterial activity of the zirconium complexes containing primary ligand (8-hydroxyquinoline) and secondary ligand glycine, was tested separately against bacterial strain of *S. aureus, E. coli* and fungal sp. *A. niger*. The 80 µl methanolic solution of Zr-G complex (500 µg/ml concentration) was used by well diffusion method as shown in table 4.2. Zone of inhibitions were observed against all microbes.14.3 mm clear zone was obtained against *S. aureus* 9.6 mm clear zone was obtained against *E. coli* and 6.3 mm clear zone was obtained against *A. niger* as shown in figure 4.2 and 4.3.

Table 4.2 Zone of inhibition by Zr_G against *S. aureus, E. coli and A. niger*

Zr complexes	Microbial strains	Zone of inhibition. (mm)
Zr-G 80 µl Methanolic solution	*S. aureus*	14.3
	E. coli	9.6
	A. niger	6.3

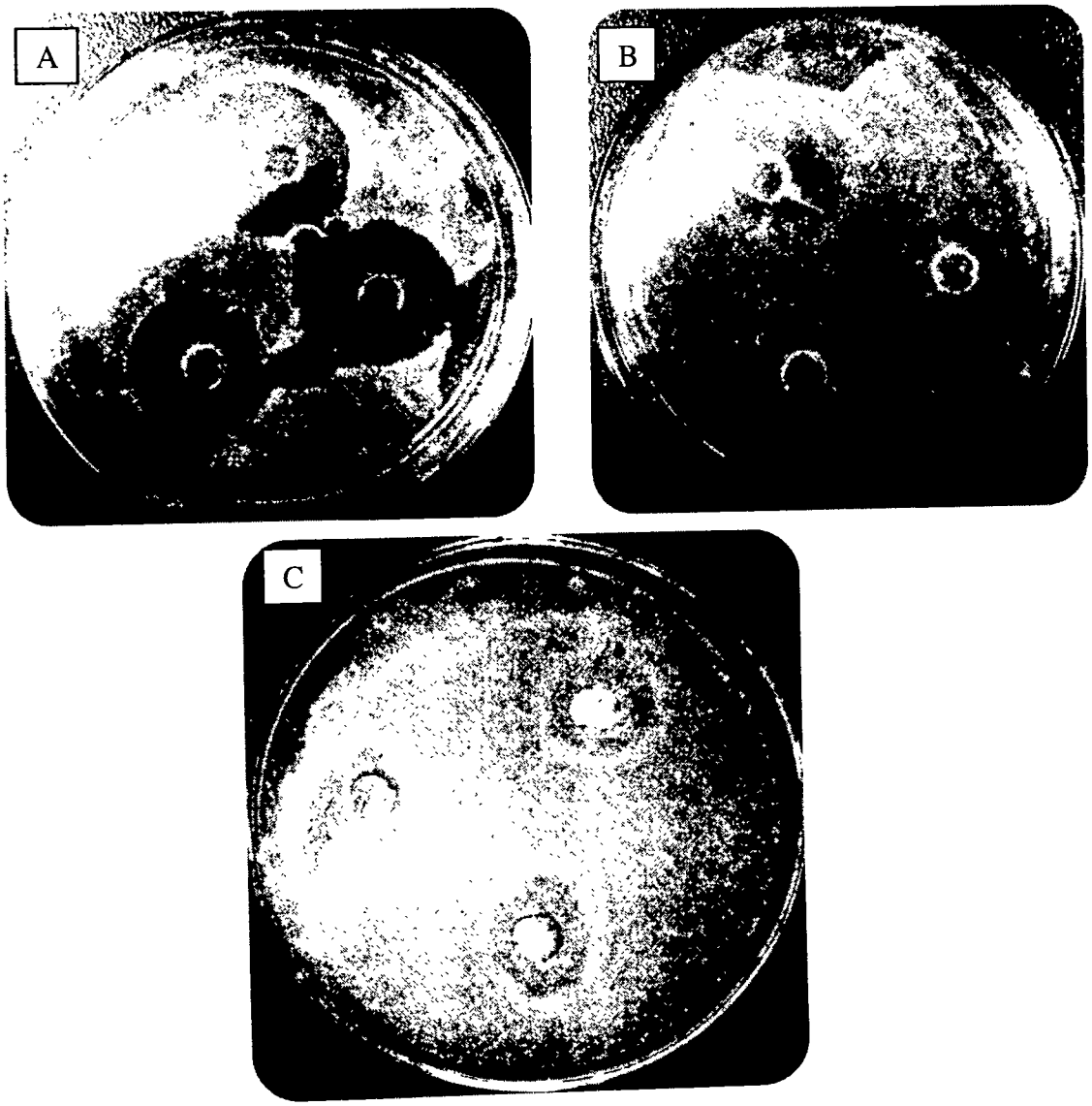


Figure 4.2 Zone of inhibition by Zr-G(80 µl) against A) *S. aureus* B) *E. coli* C) *A. niger*

Table 4.3 Zone of inhibition by Zr-A against *S. aureus, E. coli and A. niger*

Zr complexes	Microbial strains	Zone of inhibition. (mm)
Zr_A 80 µl Methanolic solution	*S. aureus*	14.3
	E. coli	8.6
	A. niger	6.3

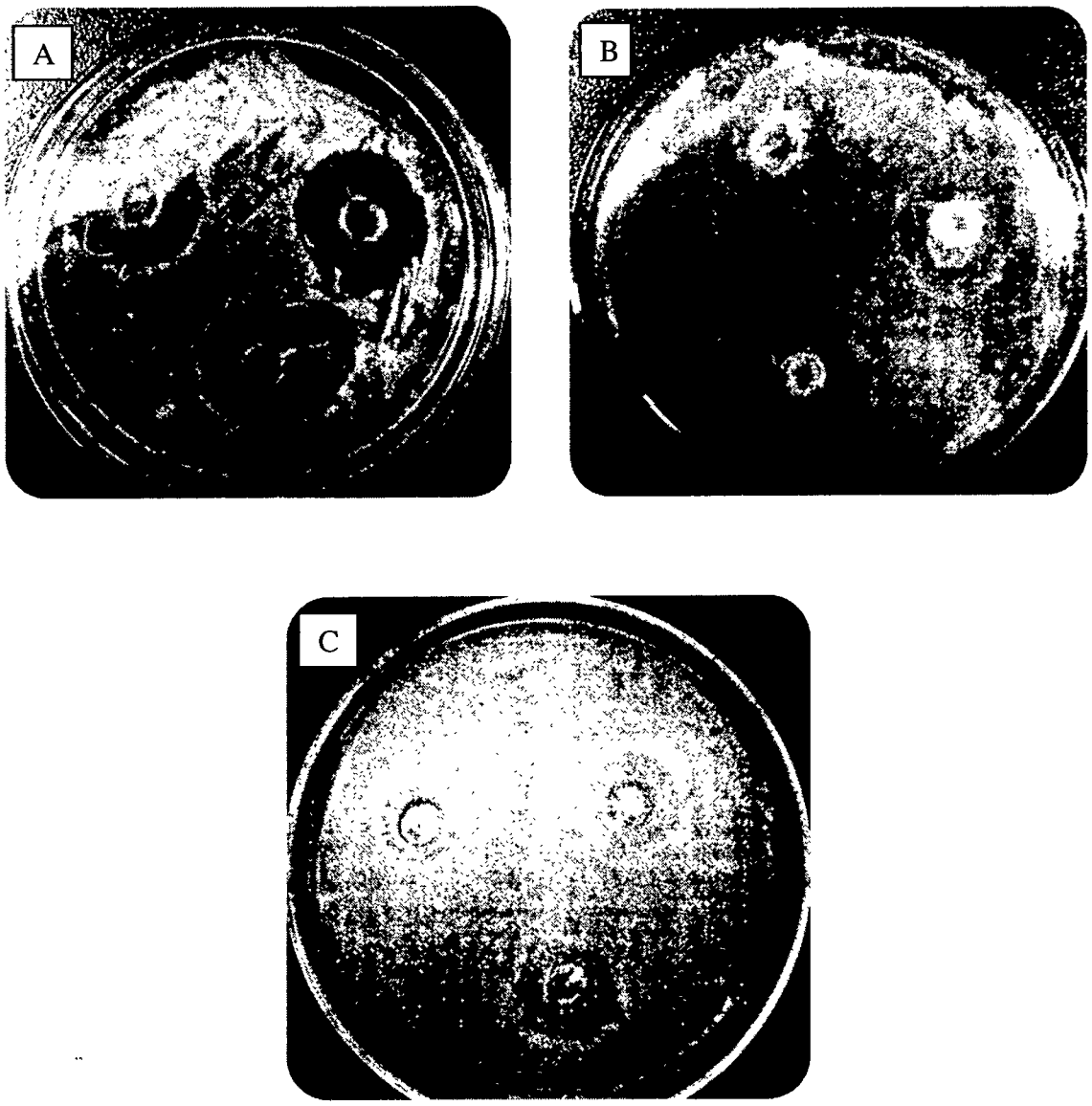


Figure 4.4 Zone of inhibition by Zr-A (80 µl) against A) *S. aureus* B) *E. coli* C) *A. niger*

The antibacterial activity of the zirconium complexes containing primary ligand (8-hydroxyquinoline) and secondary ligand allanine, was tested separately against bacterial strain of *S. aureus, E. coli* and fungal sp. *A. niger*. The 80 µl methanolic solution of Zr-A complex (500 µg/ml concentrations) was used by well diffusion method as shown in table 4.3. Zone of inhibitions were formed against all microbes.14.3 mm clear zone was obtained against *S. aureus* 8.6 mm clear zone was obtained against *E. coli* and 6.3 mm clear zone was obtained against *A. niger* as shown in figure 4.4 and 4.5

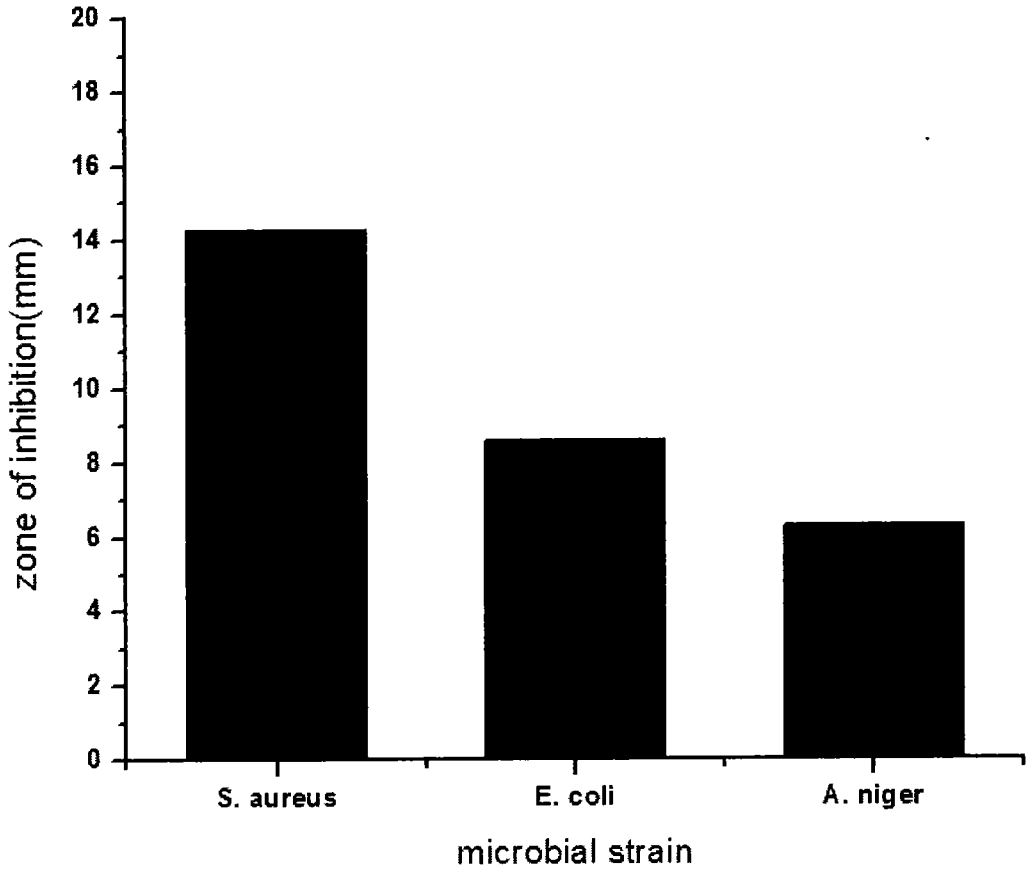


Figure 4.5 Antibacterial activity (80 µl) of Zr-A complex.

Table 4.4 Zone of inhibition by Zr-S against *S. aureus, E. coli and A. niger*

Zr complexes	Microbial strains	Zone of inhibition. (mm)
Zr_S 80µl Methanolic solution	*S. aureus*	14.6
	E. coli	15.6
	A. niger	7

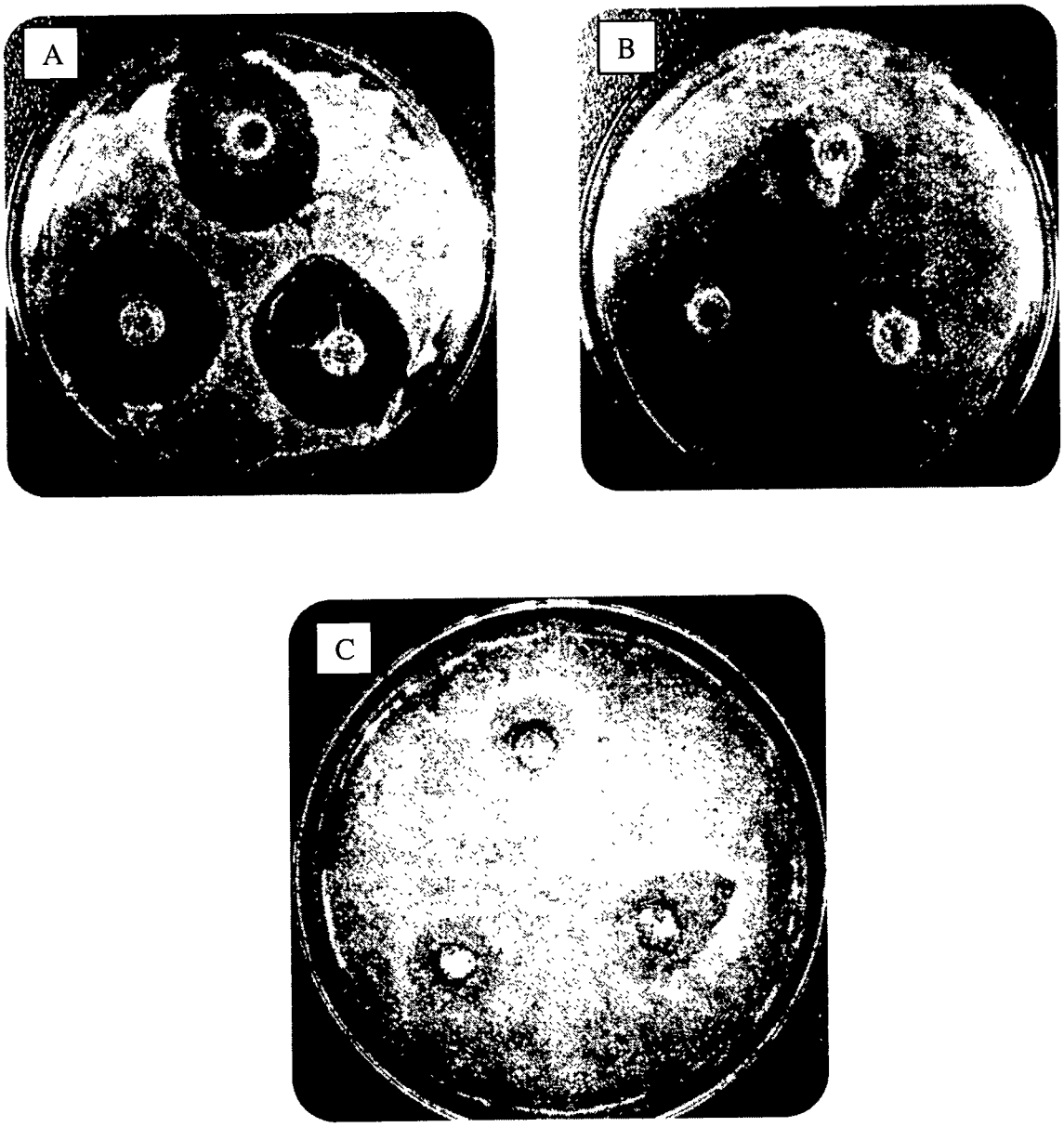


Figure 4.6 Zone of inhibition by Zr-S (80 µl) against A) *S. aureus* B) *E. coli* C) *A. niger*

The antibacterial activity of the zirconium complexes containing primary ligand (8-hydroxyquinoline) and secondary ligand glycine, was tested separately against bacterial strain of *S. aureus, E. coli* and fungal sp. *A. niger*. The 80 µl methanolic solution of Zr-S complex (500 µg/ml concentration) was used by well diffusion method as shown in table 4.4. Zone of inhibitions were formed against all microbes.14.6 mm clear zone is obtained against *S. aureus,* 15.6 mm clear zone was obtained against *E. coli* and 7 mm clear zone was obtained against *A. niger* as shown in figure 4.6 and 4.7

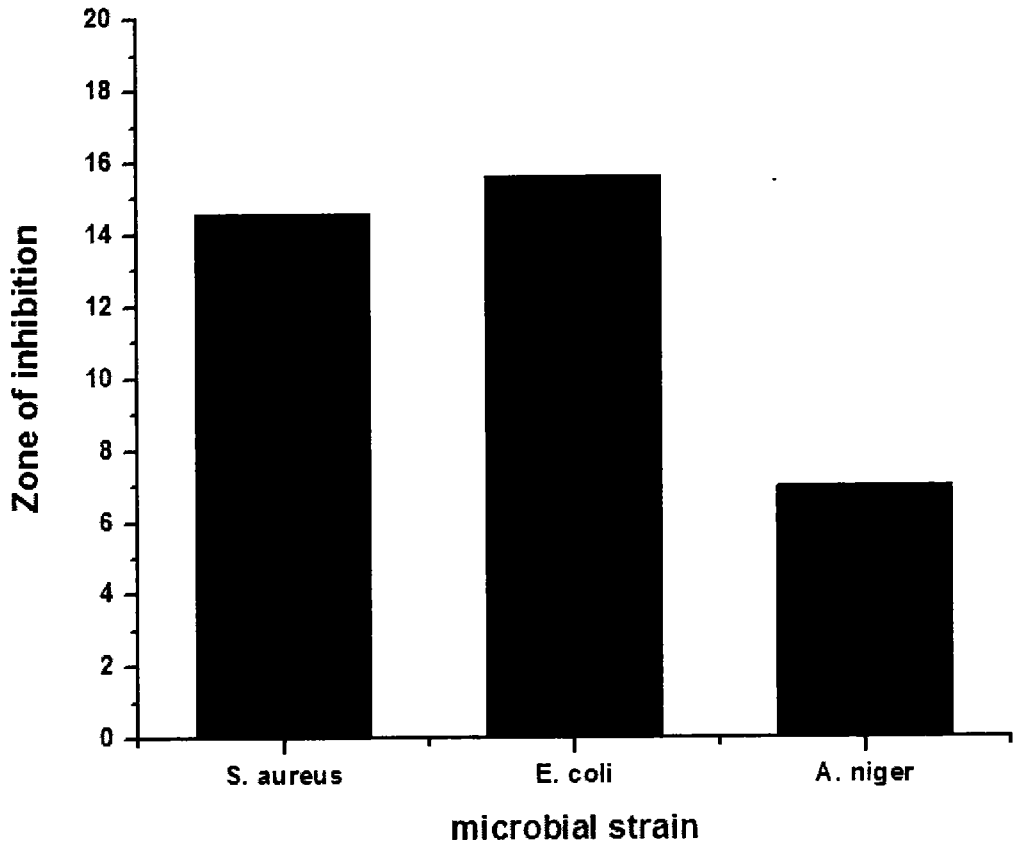


Figure 4.7 Antibacterial activity of Zr-S(80 µl) complex.

4.2 SEM

4.2.1 Zirconia nanostructures

Morphology of ZrO_2 was determined by JEOL SEM. SEM micrograph revealed the grain size of zirconia nanoparticles around 80 to 100 nm as shown in figure 4.8. Few spherical structures with irregular surface were formed. Stony appearance of particles is may be due to agglomeration of zirconia nanoparticles.

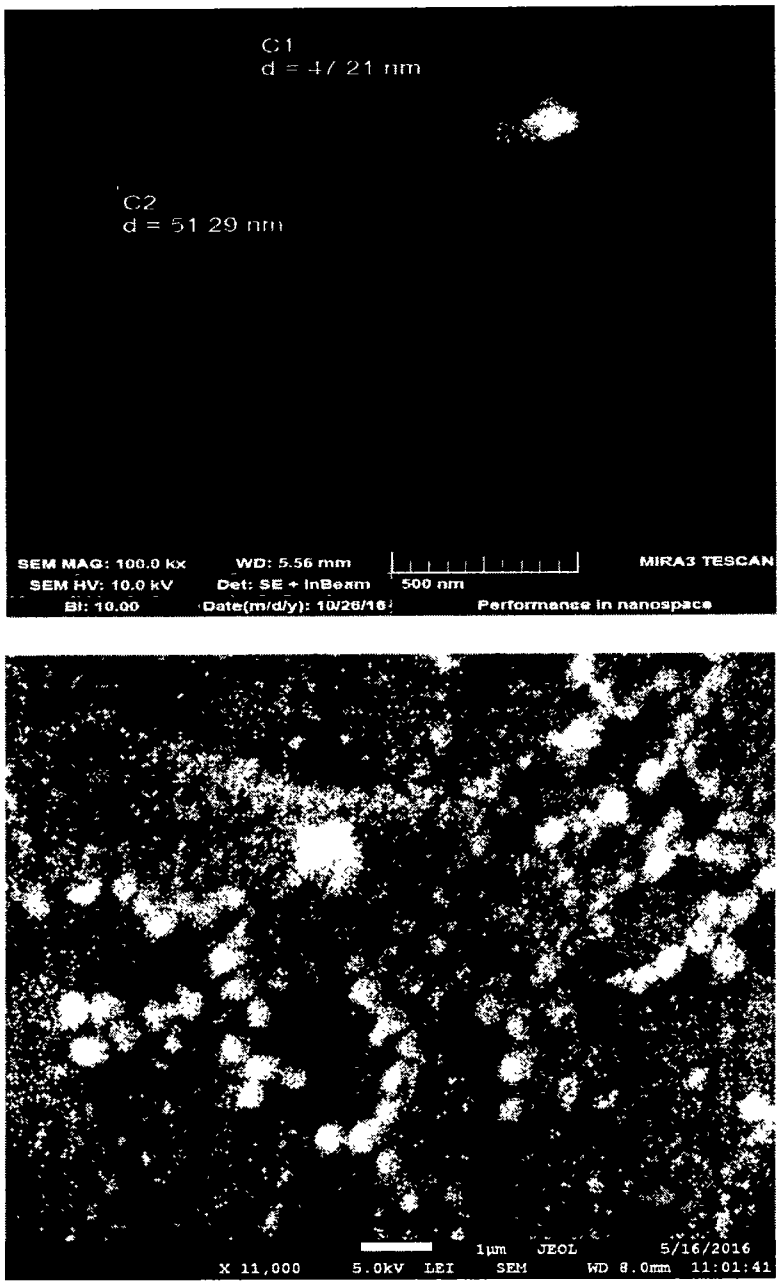


Figure 4.8 SEM images of ZrO_2 at different resolutions

4.2.2 Zirconium complexes

Morphology of zirconium complexes was determined by JEOL SEM. SEM micrographs of Zr(IV) complexes show stacked plates like elongated structures in case of Zr_S i.e., with serine as a secondary ligand as shown in figure 4.11, shows flowery irregular growths for Zr_A i.e., when alanine is used as a secondary ligand as shown in figure 4.9 and shows perfect mono dispersed spherical balls of for Zr_G as shown in figure 4.10 when glycine was used as a secondary ligands.

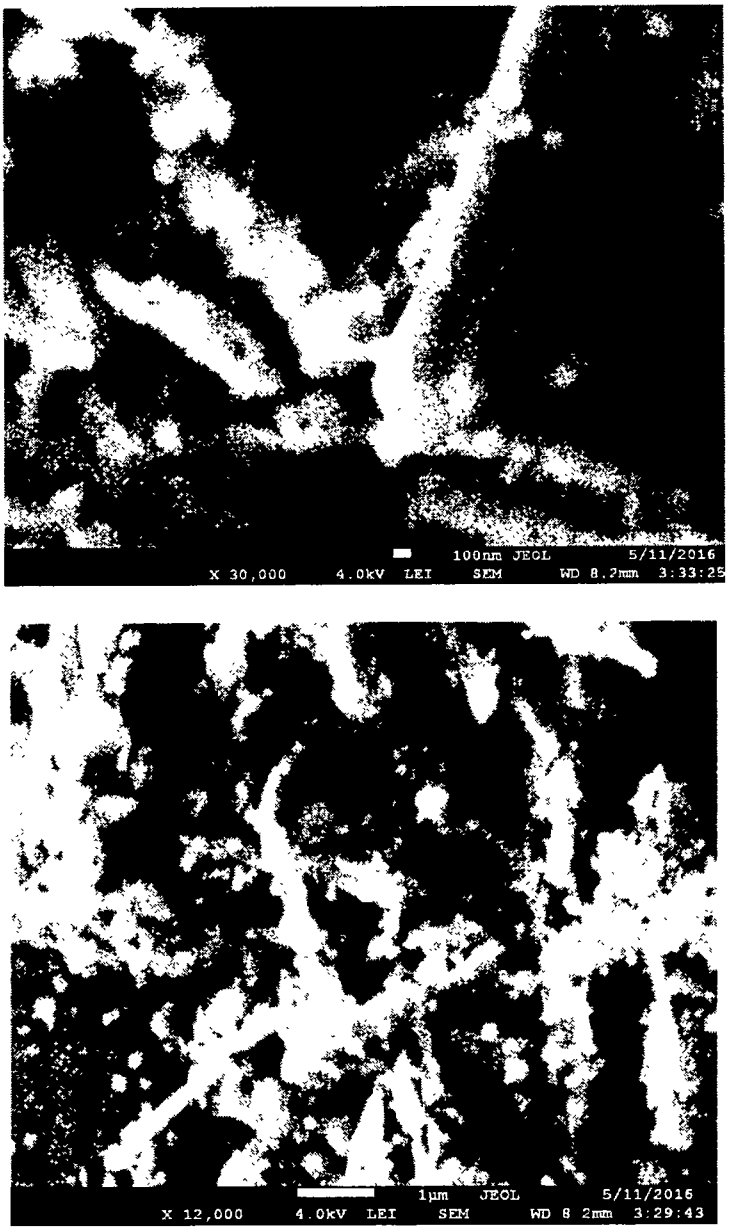
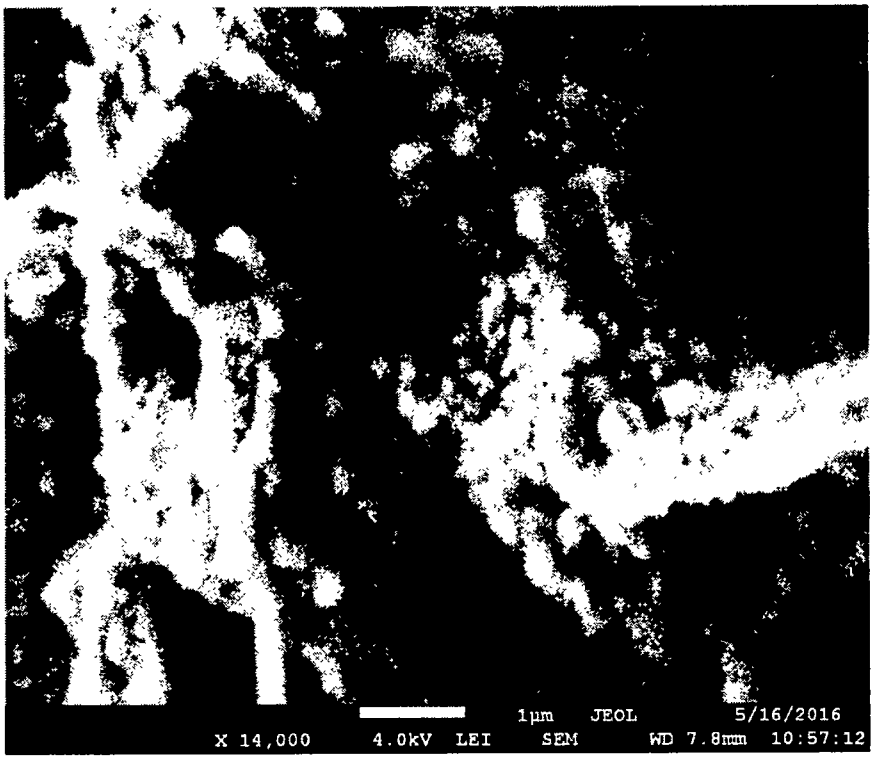


Figure 4.9 SEM images of Zr-A complex at different resolutions.



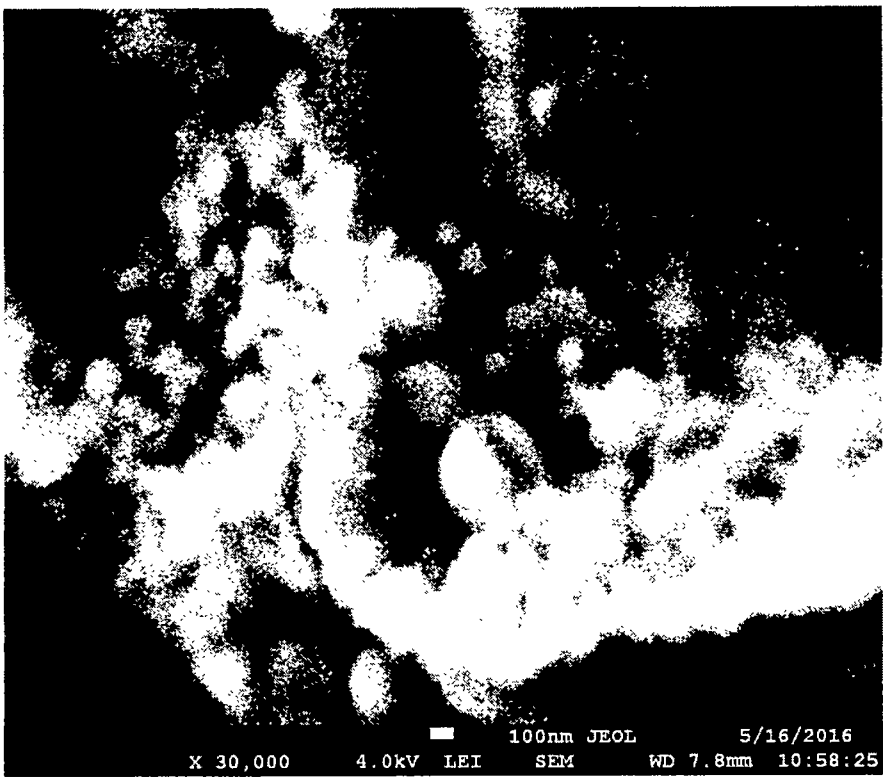


Figure 4.10 SEM images of Zr-G complex at different resolutions

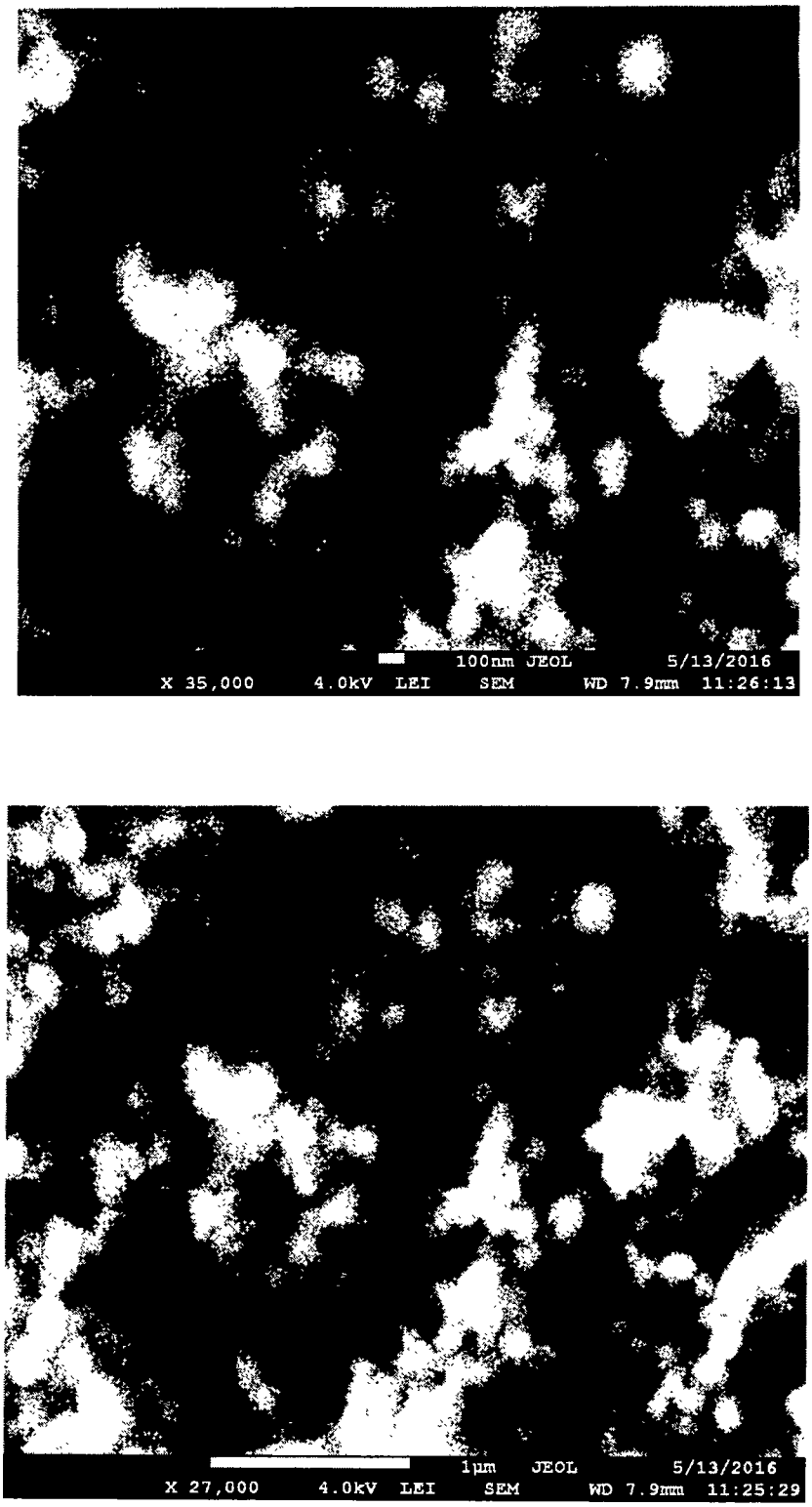


Figure 4.11 SEM images of Zr-S complex at different resolutions.

4.3 XRD

4.3.1 Zirconia nanostructures

XRD was performed on D8 Focus Bruker using copper K alpha rays on ZrO_2 sample. Sharp peaks were observed due to high temperature synthesis of the ZrO_2 and crystalline structure of zirconia is confirmed as shown in figure 4.12.

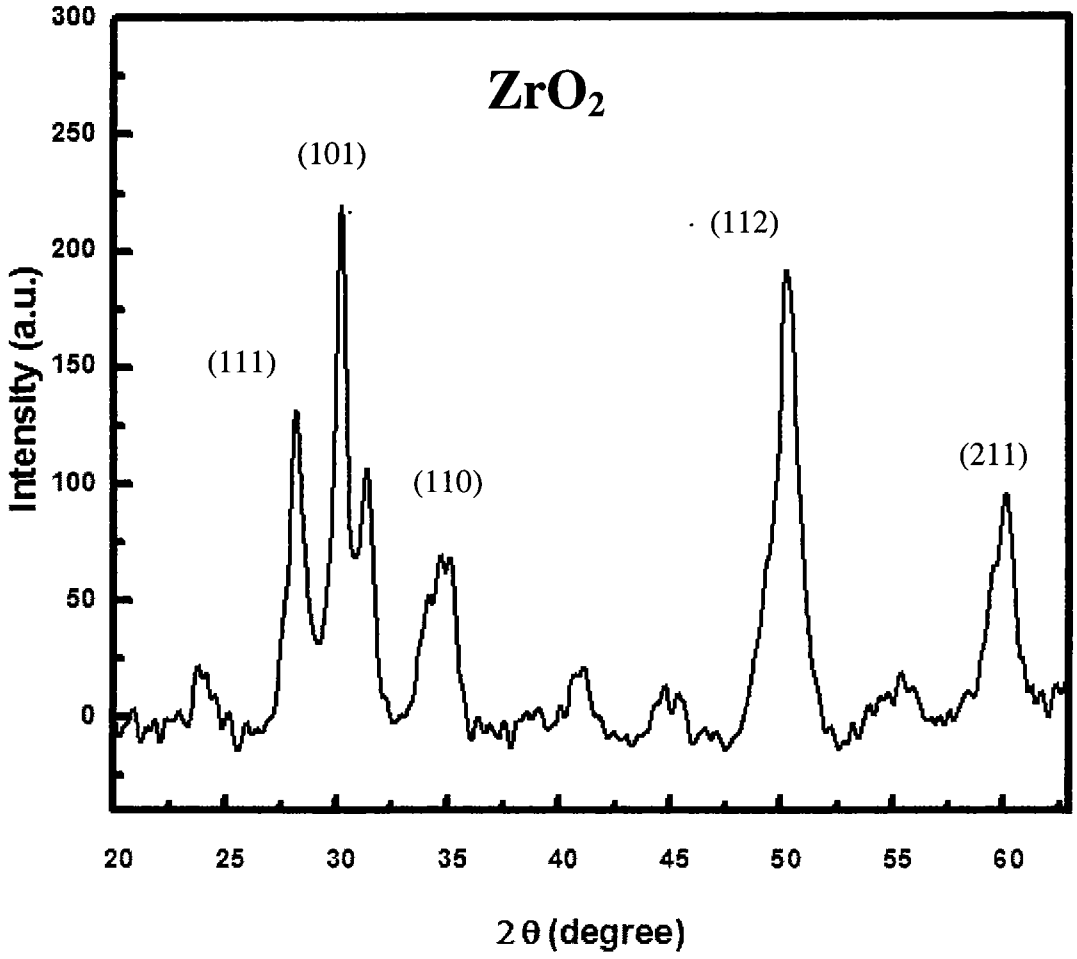


Figure 4.12. XRD pattern of ZrO_2

4.3.2 Zr(IV) Complexes

XRD of complexes was performed on D8 Focus Bruker using copper K alpha rays. XRD spectra of Zr(IV) complex synthesized using different ligands glysine,L-alanine, and L-serine) namely Zr_G(IV), Zr_A(IV) and Zr_S(IV) revealed that all complexes are in triclinic phase with few peaks.

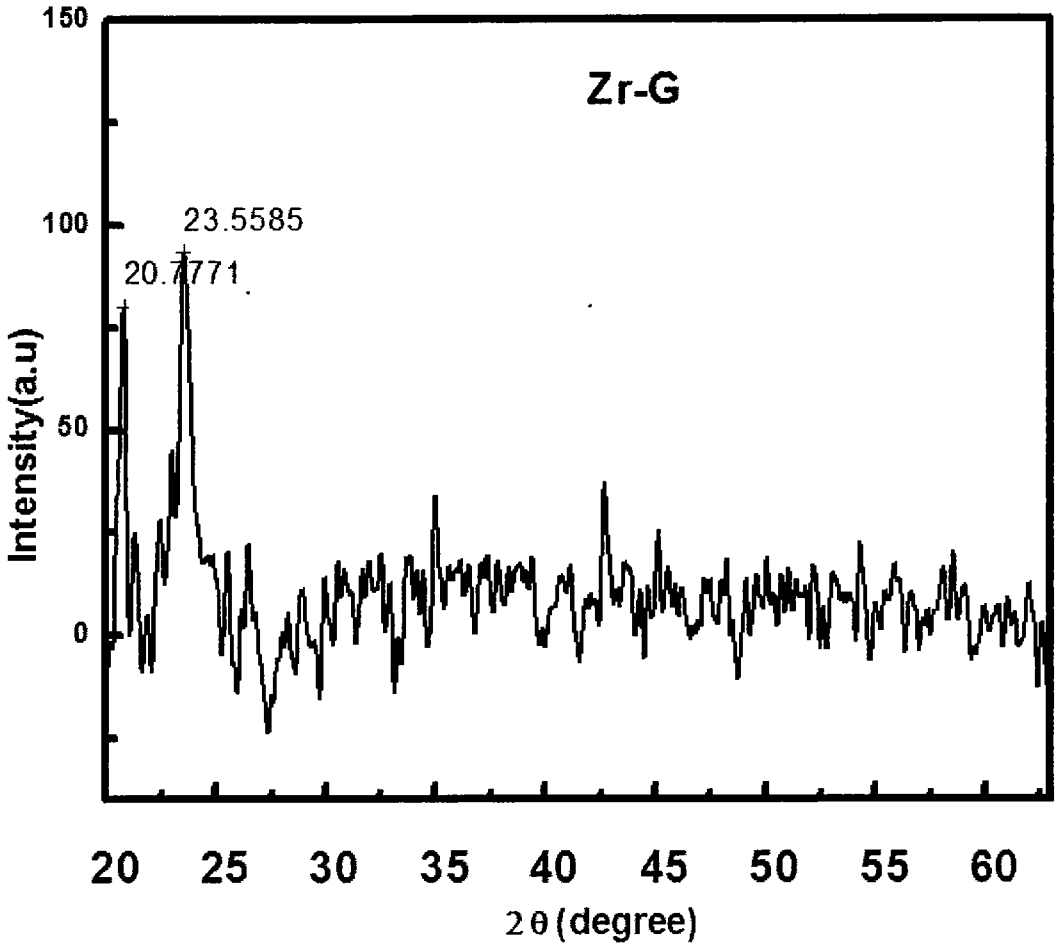


Figure 4.13 XRD pattern of Zr-G complex

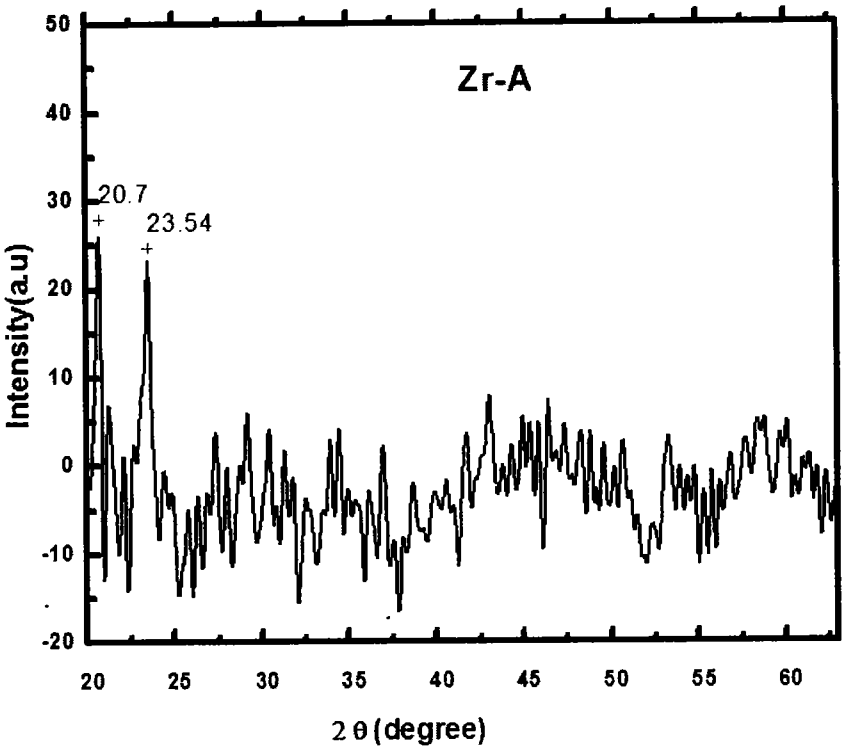


Figure 4.14 XRD pattern of Zr-A complex

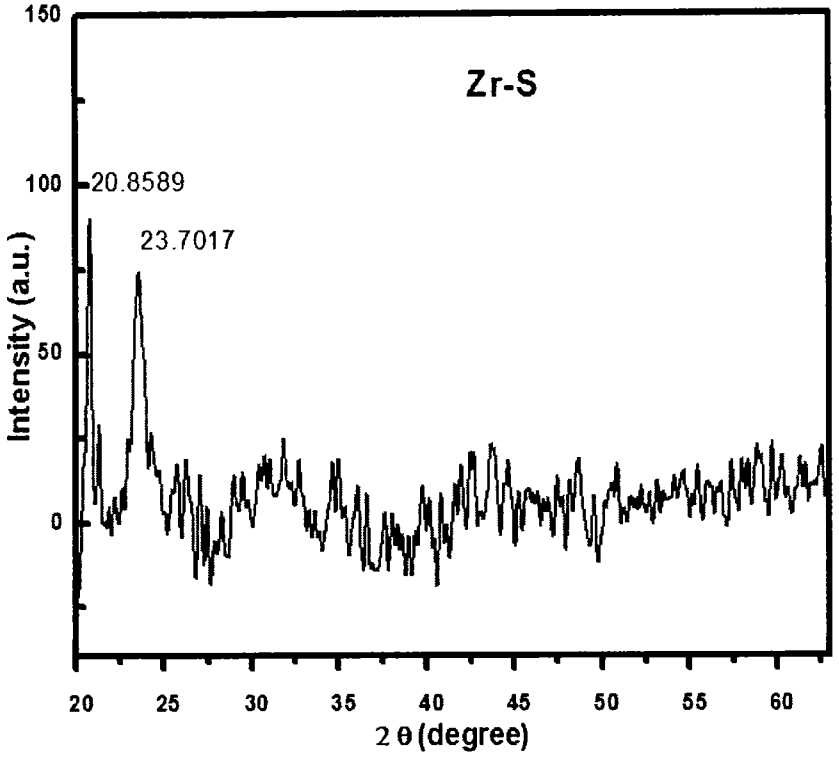


Figure 4.15 XRD pattern of Zr-S complex

DISCUSSION

5. DISCUSSION

The role of nanoparticle is vital in field of nano-medicine and life sciences especially for health care, biomedical, food and agriculture (Tachikawa *et al.*, 2011).

Nanoparticles synthesized from noble metals, such as oxides of silver, gold, zinc and zirconia are widely used in pharmaceutical and medical applications (Sahu, 2013)

Inorganic antimicrobial agents have gain much attention for the inhibition of microorganisms. To enhance the antimicrobial activity of nanoparticle, different chemicals and amino acids are used as primary and secondary ligands in the form of complexes with metals like Co(II) and Ni(II) complexes (Vitthal *et al.*, 2003)

Gowri *et al* ., 2014 reported that Zirconia nanoparticles of average size 50 nm can play a prominent role in textile field and is effective antimicrobial agent and an alternative to some traditional antimicrobial agents with detrimental effect. This kind of treated fabric can be used successfully to minimize the infections with pathogenic bacteria. Hence this work demonstrates that biological method may serve as a useful synthetic tool for producing biocompatible ZrO_2 nanoparticles.

Jangra *et al.*, 2014 have demonstrated that Zirconia and zirconium Zr(IV) mixed ligand complexes possesses significant antibacterial as well as antifungal activity.

They also found that the aqueous solution of zirconia nanoparticles has shown minor activity against *E coli* and no zones of inhibitions are formed in case of *S. aureus* and *A. niger.* In another study Gowri *et al.*, 2014 reported antimicrobial activity of zirconia nanoparticles against both bacterial and fungal strain the antimicrobial activity of methanolic solution of Zr-G complex which is combination of zirconia, primary ligand hydrooxyquinoline and secondary ligand glycine against strains of Bacteria (S. *aureus* and E. *coli*) and one strain of Fungi (A. *niger*) has been evaluated. It is also observed that complex was inhibitory to both bacterial and fungal growth. The highest antibacterial activities have been shown against (S. *aureus*). While significant antimicrobial activity was observed against *E. coli* and *A. niger* which is in accordance to Jangra *et al.*, 2012.

The antimicrobial activity of methanolic solution of Zr-A complex which was a combination of zirconia, primary ligand hydrooxyline and secondary ligand L-alanine was evaluated against *S. aureus* and *E. coli* and one strain of Fungi *A. niger.* It was observed that complex was excellent inhibitor to both bacterial and fungal growth.

Significant antimicrobial activity was also observed against all strains which is in accordance to Jangra *et al.,* 2012.

The antimicrobial activity of methanolic solution of Zr-S complex which was a combination of zirconia, primary ligand hydrooxyline and secondary ligand L-serine was evaluated against *S. aureus* and *E. coli* and one strain of Fungus *A. niger.* It was observed that complex was inhibitory to both bacterial and fungal growth. Significant antimicrobial activity was observed against all strains. The highest antibacterial activity was shown against *E. coli.*

SEM analysis was employed to visualize the size and shape of the calcined ZrO_2 nanoparticles.SEM micrographs of calcined ZrO_2 nanoparticles under different magnifications reveal that most of the particles are spherical in shape with smooth and fused surface. The particles are homogeneously distributed with some agglomeration and ensured the average size between 80 to100 nm. There as on for the unvarying size distribution of the particles may be attributed to the calcinations process that allows growth by aggregation of particles through their grain boundaries.

The SEM analysis of the complexes elucidates the morphological changes in the presence of different ligands. The formation of plates like flat structures of about 15–20 nm size for Zr-S(IV), spherical structures of about 5–7 nm for Zr-A(IV) and the oval structures of about 45–50 nm size for Zr-G(IV) were observed. Fig shows 4.2. XRD spectra which represent ZrO_2 samples prepared at 500 °C.

XRD shows diffraction peaks 28.22, 30.22, 34.7, 50.26 and 60.1at 2θ values corresponding to diffraction planes 111, 101, 110, 112 and 211 and are similar to the results of Gowri *et al.*, 2014. These diffraction planes are in good agreement with monoclinic (baddeleyite) phase of ZrO_2 (m-ZrO_2) with lattice constants a = 0.5313 nm, b = 0.5213 nm, c = 0.5147 nm and f_3 = 99.22*

The lattice spacing were measured from the high resolution micrographs and the values for the three complexes were found to be 0.254 nm, 0.242 nm and 0.351nm respectively, which correspond to the (–2 –1 2), (0 1 4) and (–101) planes of the triclinic phase of the complexes and is in good agreement with previous work as reported by Jangra *et al.*, 2012.

The XRD pattern of the calcined zirconia exhibit sharper and narrower diffraction peaks pointing out that calcination process has been known to change the phase from amorphous to crystalline state. There are no other crystal peaks related to organic impurities, zirconium metal and metal salts indicating the high purity as explained by Gowri *et al.*, 2014.

XRD spectra of Zr(IV) complex synthesized using different ligands (serine, alanine, and glycine) namely Zr-S(IV), Zr-A(IV) and Zr-G(IV) revealed that all complexes are in triclinic phase: a=8.1600(16), b=11.498(2), c=15.766(3) Å.

a=99.20(3) *, $f3$=103.07(3)*,y=107.94(3)* which is in accordance to Jangra *et al.*, 2012.

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6. REFERENCES

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APPENDICES

APPENDICES

Appendix I: List of chemicals used in experiments

S. No	Chemicals name	Amounts	Purpose
1.	Ethanol	100 ml	For preparation of complexes
2.	Methanol	100 ml	For preparation of solutions of complexes
3.	NaOH	1 molar	For pH control
4.	Zirconyl nitrate hydrate	5g	For preparation of zirconia nanoparticles and complexes
5.	8-hydroxyquinoline	2.318 g	Preparation of Zr(iv)complex
6.	L-serine	0.0021 g	Preparation of Zr(iv)complex
7.	Glysine	0.0015g	Preparation of Zr(iv)complex
8.	L-alanine	0.00089 g	Preparation of Zr(iv)complex
9.	Distilled water	500 ml	Washing of zirconia
10.	Acetone	40ml	Washing of zirconia
11.	Agar	15g/ 1liter	Bacterial and fungal media preparation
12.	Trypton	10g/ 1liter	Bacterial media preparation
13.	NaCl	10g/ 1liter	Bacterial media preparation
14.	Potato extract	100ml	Fungal media preparation
15.	Dextrose	2g/ 100ml	Fungal media preparation

Appendix II: List of Apparatus

S. No	Apparatus Name	Purpose
1.	Hot plate	Use to make uniform solutions.
2.	Incubator	Use to provide 37 °C temperature for the growth of microbes
3.	Dry oven	For drying of nanostructures
4.	Shaker	Used to shake extract
5.	Vacuum oven	For drying of complexes
6.	Mortar	Used to grind nanoparticles and complexes
7.	pH meter	For the maintenance of media pH
8.	Laminar hood	Used as control chamber for pouring media
9.	Auto clave	For the decontamination of media

Appendix III: Glassware and other Materials

Petri plats	Falcon tubes	Paper tape
spatula	Whatmann filter paper	Micropipettes
Conical Flasks	Cotton	Steel trays
Jars	Aluminum foils	Parafilm Tape
Conical flask	Cutter	Glass curve tube
Eppendrof tubes	Weighing balance	Tissue papers