INTRODUCTION

During the past few decades, the development of biomedical engineering has significantly contributed to different fields of research, particularly biomaterial and has improved on the characteristic features of biomedical devices, thereby allowing their commercial use. Hydroxyapatite (Hap), also called hydroxylapatitate, is one of the most extensively studied biomaterials due to its excellent biocompatibility, bioactivity, and osteoconductivity [1]. It is a bioceramic material that belongs to the family of apatites, having general formulae (Ca$_x$PO$_y$OH$_z$). Due to its chemical composition being similar to that of the bones and teeth, it is widely used in medical applications such as implants, coatings, and prostheses [2-4].

However, in recent years, the major concern regarding the health problem has been the development of resistance to antimicrobial agents by pathogenic bacteria. Therefore, in recent years, incorporation of certain antimicrobial agents (Ag+, Cu2+ and Zn2+) into the implant material has been suggested. Silver-containing materials, in particular, have been found to have a broad spectrum of antimicrobial properties and have received much attention. Their studies revealed that the silver release has a significant effect on the antibacterial efficiency [5].

Although the exact mechanism of antibacterial activity shown by silver ions is not yet understood, it is currently used to control bacterial growth in various applications such as dentistry and catheters. [6,7]. Different studies have shown that these ions have the ability to penetrate into the materials cell wall that causes DNA to transform into a condensed form. Further, these ions bind to the functional groups of proteins causing their denaturation [8]. Therefore, silver-doped ceramics have shown high antimicrobial activity and also chemical durability. The objective of this research work is to synthesize silver-doped bioceramic material so that we can evaluate the in vitro antimicrobial activity against Escherichia coli.

METHODS

Synthesis of silver-doped Hap

The synthesis of silver-doped Hap was done using the chemical precipitation method [9]. An appropriate amount of (Ca(NO$_3$)$_2$ $\cdot$ 4H$_2$O) (0.25 mol/L) was dissolved in 150 ml of distilled water and similarly, AgNO$_3$ (0.01 mol/L) was dissolved in 150 ml of distilled water in separate beakers. The two resulting solutions of calcium nitrate and silver nitrate were mixed together in a 500 ml beaker and stirrer continuously with magnetic stirrer for about 30 min. About 0.24 mol/L of diammonium hydrogen phosphate (NH$_4$)$_2$HPO$_4$ were taken in another beaker and dissolved in 300 ml of distilled water. The solution of diammonium hydrogen phosphate was added drop wise to a solution of beaker containing two mixture solution of calcium and silver nitrate solution, and resulting solution was heated up to 80°C on hot plate with continuous stirring by magnetic stirrer. For the variation of concentration of silver in silver-doped Hap, the synthesis of Hap was carried out with different concentration of silver as $X_{Ag}=0.3$, $X_{Ag}=0.4$, and $X_{Ag}=0.5$. The pH of the solution was maintained as 10.0 throughout the reaction with the help of ammonium hydroxide solution [1].

The resulting solution was allowed to stand undisturbed for the deposited mixtures to settle down and then washed 3-4 times with distilled water. The filtrate obtained was dried in an oven at 80°C for 24 h. The dried material was ground to obtain a fine powder of silver-doped Hap. The dried precipitate was processed to get fine powder with the help of a stove and pestle. Hap/Ag complexes were characterized using X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR) techniques. The powder was then given the shape of tablets.

Characterization of silver-doped Hap

Different techniques are used to characterize silver-doped Hap. Here, we have discussed some of the important techniques and their
principles that we have used to characterize silver-doped Hap in this project work. The techniques are FTIR and XRD.

FTIR analysis
FTIR is a sensitive technique which is used to identify organic and inorganic chemicals by checking the chemical bonds in a molecule by producing an infrared absorption spectrum. In this method, the infrared rays pass through the sample. Some of those IR are absorbed by the sample and some transmitted. No two unique molecular structures produce the same infrared spectrum. The functional groups present in the prepared powder were identified by FT-IR. For this, 1% of the powder were mixed and ground with 99% KBr. Pallet size of 10 mm diameter for FT-IR measurements was prepared by pressing the powder mixture at a load of 5 tons for 2 min, and the spectrum was taken in the range of 400-4000 cm$^{-1}$.

XRD analysis
XRD is currently a standard technique for the study of crystal structures and atomic spacing. The XRD measurements for the silver-doped Hap samples were recorded using a diffractometer with nickel-filtered CuKα ($\lambda=1.5418$ Å) radiation. The diffraction patterns were collected in the 2$\theta$ range of 0–90, with a step of 0.02.

Microbial strain
Microbial strain of *E. coli* was used to test the antimicrobial activity of silver-doped Hap.

Disc diffusion test
Disc diffusion is a common method to check the antibacterial activity of Hap with silver and without silver (control) evaluated against *E. coli* using the disc diffusion technique [10]. A disc diffusion test is performed to test whether the particular bacteria are susceptible to certain chemical or not. According to the diameter, a zone of inhibition of culture growth the microorganism can be observed sensitive or resistant. The experiments were performed in triplicates for each concentration of silver made in such a way that it contains nine plates containing solidified media and was incubated at 37°C for 24 h. The inhibition of bacterial growth was studied by measuring the zones formed around the tablets (cms).

RESULTS AND DISCUSSION

Synthesis of Hap
Silver-doped Hap was synthesized using chemical precipitation method as shown in Fig. 1. Although the preparation is simple, it requires a great care to make stable Hap. One of the most important parameters for synthesis is pH which was maintained at 10 throughout the reaction. Temperature too plays an important role which was maintained at 100°C.

**Formation of Hap powder to pallet**
Fig. 2 shows the formation of pallet of silver-doped Hap. The samples were dried in a hot air at 100°C for 48 h, and then, they were crushed in mortar and pestle. Silver-doped Hap tablets were prepared using the dye punch machine. The concentration of silver varies so as to check antimicrobial activity of silver-doped Hap against Gram-negative bacteria, *E. coli* by disc diffusion test at different concentrations, i.e., XAg-0.3, XAg-0.4, and XAg-0.5.

**FT-IR spectroscopy analysis**
To identify the functional groups present in the prepared Ag HAp powder by wet chemical precipitation method, FT-IR was done in Fig. 3. The results obtained clearly represent the presence of different vibrational modes that correspond to hydroxyl groups and phosphates. The spectrum can be divided into four main regions with the peaks having wave numbers as 3500 cm$^{-1}$, 1400 cm$^{-1}$, 1100 cm$^{-1}$, and 600 cm$^{-1}$. The presence of strong OH$^-$ peak could be observed from the spectrum. The peak which is observed at 3425 cm$^{-1}$ is because of the presence of –OH bond [11]. This peak is due to O-H stretching vibrations in Hap. The bands that are observed at 1600–1700 cm$^{-1}$ and 3200–3360 cm$^{-1}$ are due to H-O-H bands of water lattice [12]. Bands corresponding to phosphate and hydrogen phosphate groups observed in the spectrum include 565.16 cm$^{-1}$, 603.74 cm$^{-1}$, 632.67 cm$^{-1}$, 962.51 cm$^{-1}$, and 1000–1100 cm$^{-1}$ for the PO$_4^{3-}$ groups [12], and at 875.71 cm$^{-1}$ for the HPO$_4^{2-}$ ions [13]. Thus, from IR studies, the presence of PO$_4^{3-}$ group is confirmed.

Fig. 1: Silver-doped hydroxyapatite in powder form

Fig. 2: Silver-doped hydroxyapatite pallet

Fig. 3: Fourier transform infrared spectroscopy spectra from silver-doped hydroxyapatite
The zone of inhibition for X

Ag

in vitro analysis. The synthesized powder was pure white and crystalline precipitation method and was characterized using FTIR and XRD. Silver-doped Hap was synthesized successfully using the chemical to the bacterial cell [15]. In case of to the membrane protein of the bacteria cell and results in causing death to the growth of bacteria. The silver particles present in petri-plate binds to the zone of inhibition increases because of the effect of silver particles on understanding that as the concentration of silver in Hap was increased, the values were compared with amoxicillin (standard). It can be clearly shown in different concentrations of silver-doped Hap against E. coli.

Fig 4: X-ray diffraction spectra of synthesized silver-doped hydroxyapatite

XRD spectroscopy
XRD spectra of silver Hap show distinct diffraction patterns in Fig. 4. The maximum intensity of the peak was obtained at 2h=31.7684. The peak analysis matched very closely to the standard obtained from JCPDS File No-090432 [14].

Synthesis of silver-doped Hap using different concentrations of silver
Different concentrations of silver x=0.3, 0.4, and 0.5 were taken to synthesize silver-doped Hap. The precipitates formed by the procedure mentioned above in methodology were later used to check the antimicrobial activity of Hap with different concentrations of silver against E. coli.

Antibacterial activity test
Antibacterial test was done by measuring the zone of inhibition for the plates incubated with E. coli at different concentrations of silver-doped Hap using silver concentrations at silver x=0.3, 0.4, and 0.5 which is shown in Fig. 5. From Fig. 5, it is clear that XAg:Hap=0.5 showed good inhibition against E. coli. The zone of inhibition for X

Ag

=0.3 was 2.00±0.025 mm, X

Ag

=0.4 was 4.00±0.012 mm, and X

Ag

=0.5 was 5.00±0.016 mm. These values were compared with amoxicillin (standard). It can be clearly understood that as the concentration of silver in Hap was increased, the zone of inhibition increases because of the effect of silver particles on the growth of bacteria. The silver particles present in petri-plate binds to the membrane protein of the bacteria cell and results in causing death to the bacterial cell [15]. In case of E. coli, the effective concentration of silver was found at X

Ag

:Hap=0.5.

CONCLUSIONS
Silver-doped Hap was synthesized successfully using the chemical precipitation method and was characterized using FTIR and XRD analysis. The synthesized powder was pure white and crystalline in nature. The present study shows effect of bacterial growth on increasing concentrations of silver-doped Hap. In our study, effective concentration of silver was found at X

Ag

=0.5. Hence, it can be concluded that the proper concentration of silver required to inhibit the growth of bacteria in still to be explored at the high range of concentration of silver. It may be further modified or manipulated to synthesize silver-doped Hap at their optimum range of concentration of silver so that it can be substituted as dental implant device. The dental implant device may serve as antibacterial property and inhibit the growth of bacteria.

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