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Synthesis and Characterization of Silver Nanoparticle Incorporated Nanohydroxyapatite from Biomaterials and its Applications

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Abstract: ***Eco-friendly green synthesis is one of the promising branches of nano science for applications in different fields. It makes attractive potential option due to non toxic and very low cost of synthesis. In this work we propose a simple, cost-effective, and environmental friendly method for the synthesis of silver nanoparticle incorporated nanohydroxyapatite composite. Nanohydroxyapatite was isolated from eggshells and silver nanoparticles were prepared from the grape extract. The silver nanoparticles were incorporated with the prepared nanohydroxyapatite and the synthesis was confirmed by the characterizations using UV-Visible spectroscopy, FTIR, XRD, SEM and EDS. The synthesized nHA and nHA-AgNP were tested for photo catalytic degradation of Congo red and Methylene blue under visible light. Incorporation of AgNP significantly improved the photocatalytic activity of nHA under visible light. The samples also show good antibacterial activity with gram positive and gram negative strains. Therefore the prepared silver nanoparticle incorporated nanohydroxyapatite composite could be considered as good bioceramic material for use in tissue engineering and for treatment of effluents thereby protecting the environment from hazardous waste.***

Keywords: Green synthesis, biocomposite, nanohydroxyapatite, silver nanoparticle, photocatalytic activity

I. INTRODUCTION

Inorganic biomaterials based on calcium orthophosphate have their wide range of applications in medicine and bone tissue engineering [1]. Among them, synthetic hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is the most promising due to its composition and chemical structure similar to the mineral phase of bone [1,2].This bioactive ceramic has long been used for repairing and reconstructing bone fractures as well as for coating orthopaedic, maxillofacial and dental implants. However, one of the biggest current problems in the biomedical field is post-surgical infections arising from recent-implanted synthetic biomaterials, since these provide sites for potential bacterial adhesion. Such infections often lead to severe pain, bone tissue loss and eventually require implants removal, consequently raising morbidity. To overcome the limitations of antibiotics used in prevention and treatment of these infections that might lead to highly resistant bacteria, special attention is dedicated in controlling the release of alternative antimicrobial agents, such as copper, zinc and silver, all presenting wide activity and low bacterial resistance [3]. Several studies involving chemical synthesis reported the intentional inclusion of chemical elements into biomaterials in order to obtain antimicrobial effects, and silver has been the most common due to its strong and non selective antibacterial activity[3]-[5]. Furthermore, the reactivity of silver is even more efficient when used in nano meter-sized particles due to their high surface-to-volume ratio that allows better contact with microorganisms.

In recent years, much attention has been paid to the synthesis and characterization of nano composites because of their interesting properties. The aim of the present study is to synthesise silver nanoparticle incorporated nano hydroxyapatite composite from biomaterial in a fast, cost effective and eco-friendly way. Silver nanoparticle is prepared using *Vitis vinifera* (Grape) fruit extract and nano hydroxyapatite from eggshell. The removal and destruction of organic contaminants in ground water can be addressed through the impregnation of adsorbents with photoactive catalysts. The present study also focuses on the application of the as-synthesised nHA-AgNP composite for the industrial dye degradation and its antibacterial activity.

II. EXPERIMENTAL

A. Materials

Orthophosphoric acid was purchased from Nice Chemical Pvt. Ltd., Silver nitrate, Congo red, and Methylene blue were purchased from Thermo Fisher Scientific India Pvt. Ltd. All the reagents used were of analytical grade and were used as such without further purification.

B. Methods

1) *Synthesis of Nanohydroxyapatite from Eggshell* [7]: In the present study nano hydroxyapatite is synthesized using wet precipitation technique. Eggshells were collected, washed with distilled water, dried, then crushed and calcinated at 900°C for 2 hours. The decomposition of eggshell ($CaCO_3$) to CaO takes place. The freshly prepared CaO was mixed with the stoichometric amount of distilled water to prepare Ca $(OH)_2$. The 0.5M $Ca(OH)_2$ suspension was vigorously stirred at 70°C for 1 hour and the 0.3M H_3PO_4 solution was added at the end of the stirring time drop wise with a slow stirring rate. The pH of the solution was kept in the range 10.5-11. After complete addition of the H_3PO_4 solution, the contents were stirred for 1 hour at 70°C. The precipitate was washed with distilled water and dried in hot air oven at 105°C for 24hours. The formed powder was calcinated at 900°C for 2hours.
2) *Synthesis of Silver Nanoparticle from Grape Extract*: Grape fruits (*Vitis vinifera*) were used to make the aqueous extract. 25g of grape fruits were weighed, thoroughly washed in conductivity water, dried and were crushed into 100 ml conductivity water and filtered through WhatmanNo.1 filter paper. 10mM of 10 mL silver nitrate solution was prepared. 10 mL of grape extract was added into prepared silver nitrate solution for reduction into elemental Ag. The primary detection of synthesized silver nanoparticle was carried out in the reaction mixture by observing the colour change from colourless to dark brown. To get a concentrated solution of the nanoparticles free from organic contents of fruit juice, the solution was subjected to centrifugation at 10000rpm for 1 hour. Thereafter the supernatant was aspirated out from the centrifuge tube and the loose precipitate formed at the bottom was dried using a vacuum desiccator.
3) *Synthesis of Silver-Hydroxyapatite Nanocomposite* [5]: To 25 ml of deionised water, 0.50 g nano hydroxyapatite powder was added and dissolved completely under vigorous magnetic stirring at 60°C. Then 0.25g silver nanoparticles were added to it and allowed the reaction mixture to react overnight. The resulting sample was grey in colour. The sample was centrifuged at 9000 rpm to separate the silver incorporated hydroxyapatite nanocomposite precipitate.

C. Characterization

The synthesized nano hydroxyapatite (nHA), silver nanoparticles (AgNPs) and their composite (nHA-AgNP) were subjected to characterization by an array of techniques. UV-Visible spectroscopic analysis was carried out using JASCO V-750 UV/Vis Spectrophotometer. FT-IR spectra were recorded using a Bruker alpha-T ATR-FTIR spectrometer. The powder samples were made into KBr pellets using a hydraulic press and the IR spectra were recorded in the range of $400cm^{-1}$ to $4000cm^{-1}$. Powder XRD was obtained at room temperature using a Bruker D8-advance X-ray diffractometer (40kV, 30 mA). Cu-k_α (λ=1.5418A°) radiation was used as the X-ray source. The scan speed was 2°/min and measurement was carried out between the 2θ values of 5° to 90°. SEM images were obtained using a microscope JOEL Model JSM-6390LV. EDS analysis was carried out using JEOL JSM-6610LV SEM instrument equipped with a thermo EDX attachment

D. Study on Antibacterial Activity

The multidrug resistant human pathogen *Escherichia coli* (gram negative) and *Staphylococcus aureus* (gram positive) were used for the *in vitro* antibacterial studies. Stock cultures were maintained at 4°C on Nutrient agar Slant. Active cultures for experiments were prepared by transferring a loop full of culture from the stock cultures into the test tubes containing nutrient broth , that were incubated at 24hrs at 37ºC. Antibacterial of Sample was determined by disc diffusion method on Muller Hinton agar (MHA) medium. Muller Hinton Agar (MHA) medium is poured in to the petriplate. After the medium was solidified, the inoculums were spread on the solid plates with sterile swab moistened with the bacterial suspension. The disc were placed in MHA plates and add 20 µl of sample (Concentration: 500 µg) were placed in the disc .The plates were incubated at 37ºC for 24 hrs. Then the antimicrobial activity was determined by measuring the diameter of zone of inhibition.

E. Study on Photocatalytic Activity

The photolysis of Congo red (CR) and Methylene blue (MB) were carried out in the UV photo reactor. 100ml capacity Quartz tubes were used as reaction cells, the reaction solutions were illuminated by 8W mercury vapour lamp emitting 365nm wavelength. 10ppm of CR and 10ppm of MB each with 100mg of both nHA and nHA-AgNP respectively were studied. Aerator along with the connecting tubes, were introduced into the reaction system for effective mixing of the samples.

The aqueous solutions of CR and MB with nHA and nHA-AgNP were analysed in the dark for 30 minutes to ensure the adsorption equilibrium. The concentration of the dye in the reaction system at different reaction time was monitored spectrophotometrically by measuring the absorption intensity at a wavelength 400-800nm with a calibration curve. Sample of about 3 ml each was withdrawn

at given time interval of dark reaction and of illumination and absorbance of the resulting solution was measured. [9]- [10]. The Percentage of decolourization and percentage of decolourisation were calculated using the formula, % Adsorption = (($C_{blank} - C_{dark}$)/ C_{blank}) × 100, Where, C_{blank} =initial concentration, C_{dark}= concentration after adsorption equilibrium and % Decolourization= (($C_0 - C_t$)/C_0) ×100, Where, C_0=initial concentration after adsorption equilibrium and C_t= concentration at time (t)

III. RESULTS AND DISCUSSION

A. Characterization

1) *UV-Visible Analysis:* UV-Visible spectroscopy is an important technique for the analysis of nanoparticles. UV-Vis spectra of as-prepared samples is given in Fig.1. AgNP shows an absorption peak at about 405nm and a shoulder at about 248 nm. nHA shows λ max at 258 nm. Due to the small size, the synthesized nHA shows an absorption in the high energy UV region of the spectrum. The composite, nHA –AgNP shows two absorption peaks at 255nm and 468nm due to the presence of both AgNP and nHA. The peak appeared in the range between 350-620 nm for the composite and can be attributed to the Surface Plasmon Resonance (SPR) absorption of AgNP. The energy band gap of AgNP, nHA and nHA –AgNP are found to be 3.14eV, 4.38eV and 2.64eV respectively, which infers the synthesized samples, can function as semiconductor and this band gap could enable it to be a photocatalyst.

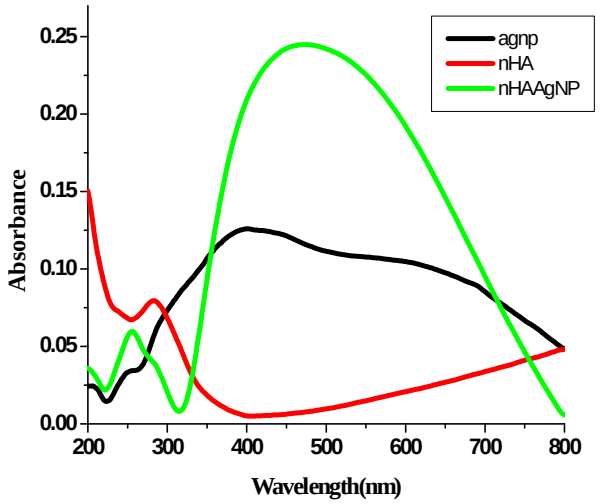

Fig. 1: UV-Vis Spectra of Samples of AgNP, nHA and nHA –AgNP Nanocomposite.

2) *FT-IR analysis:* FTIR spectroscopy was employed to characterize the different functional groups of the samples of AgNP, nHA and nHA-AgNP. The spectrum was recorded in the range of 4500-500cm-1. In the FTIR spectrum of synthesized AgNP (Fig. 2) the absorption peak at 3416cm-1 is assigned to O-H stretch of alcohols and phenolic compounds of the fruit extract. The band at 2893cm^{-1} is attributed to C-H stretching of vibrations of methyl, methylene, and methoxy groups. The absorption band at 1640cm^{-1} may be due to C=N bending in amide group or C=O stretching in carboxyl group. The absorption band at 1640cm^{-1} is close to that reported for native proteins, which suggests that proteins acted as reducing and capping agents for the biosynthesise of AgNP. The band at 1389cm-1 corresponds to C-O-C stretching mode of alcohols and carboxylic acids. The peak at 1056cm-1 was assigned to the stretch of the C-O bond.

Fig. 3 represents the FTIR spectra of nHA. The spectrum shows all characteristic absorption peak of nHA. The first indication for the formation of nHA is in the form of a strong broad FTIR band centered at about 1000-1110cm^{-1} due to asymmetric stretching mode of vibration for PO_4^{3-} .The band about 555-600cm^{-1} corresponds to symmetric P-O stretching vibration of the PO_4^{3-}. The crystalline powder generates two characteristic stretching modes of O-H bands at about 3559.6cm^{-1}. The broad band near 1983cm^{-1} and 3402cm^{-1} indicates adsorbed H_2O in the sample. The stretching bond corresponding to O-H is at 3559.6cm^{-1}, which is overlapping with the band at 3402cm^{-1} due to adsorbed water.

Fig.4 represents the FTIR spectra of nHA-AgNP. The absorption band at 3559.6cm^{-1} in nHA, characteristic of OH stretching was shifted to 3451cm^{-1} in nHA –AgNP and infers greater interaction between the AgNP and nHA. The absorption band at 1640cm^{-1} corresponds to the absorbed water and in evidence of the presence of absorbed water in the composite. The bands characteristics of

PO_4^{3-} in nanohydroxyapatite structure are clearly observed at 614-544cm^{-1} and 1044cm^{-1}. The small CO^{2-} band was presented in spectra at 1413cm^{-1}.

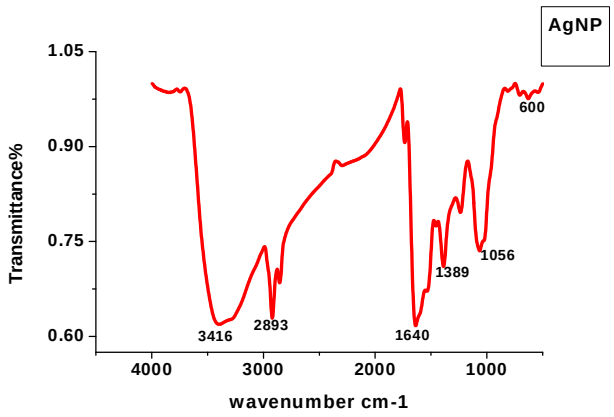

Fig. 2: FTIR of AgNP

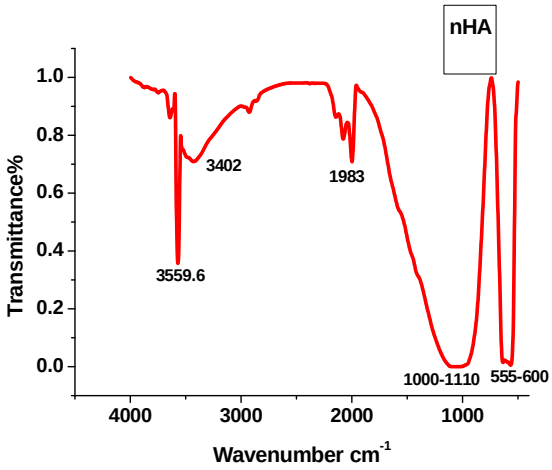

Fig. 3: FTIR of nHA

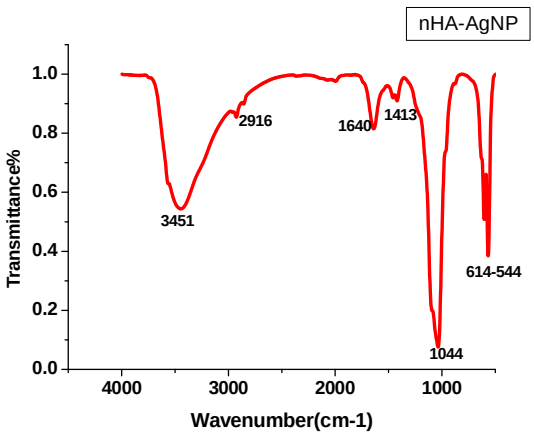

Fig. 4: FTIR of nHA –AgNP

3) *Powder X-ray Diffraction Studies:* The phase identification and crystallite size of the synthesized AgNPs, nHA and Ag-nHA nano composite were characterized by powder X-ray diffraction. Fig 5 shows the XRD pattern of AgNP. Four distinct diffraction peaks at 32.26°, 38.21°, 46.43°,55.02° were obtained and these were indexed with the planes (111), (200), (211), (220) respectively and these are matched with JCPDS card NO. 0065-2771. Other peaks were due to the presence of carbon in biomolecules. The well resolved and intense XRD pattern clearly showed that the AgNP formed by reduction of Ag+ ion using grape extract are crystalline in nature with face centred cubic structure.

Fig 6 shows the XRD pattern of nHA. Well resolved characteristic peak of high intensity for nHA was observed at 2θ value of 31.87°. The distinct diffraction peaks at 25.81°, 28.75°, 31.87°, 34.38°, 46.88° and 52.94° were obtained and these were indexed with the planes (002), (210), (211),(300), (222) and (004) respectively and it is matched well with the JCPDS card NO. 0009- 0432.

XRD pattern of nHA-AgNP is given in fig 7. The distinct diffraction peaks at 2θ values of 25.88°, 27.89°, 31.93°, 46.4° were obtained and these were indexed with the planes (111), (200), (210) and (300) respectively. The observed values are similar to those obtained in the XRD analysis of both AgNP and nHA.

The average crystallite size of the as- synthesized samples were calculated using Scherrer formula and found to be 43.7nm, 42nm and 60nm respectively.

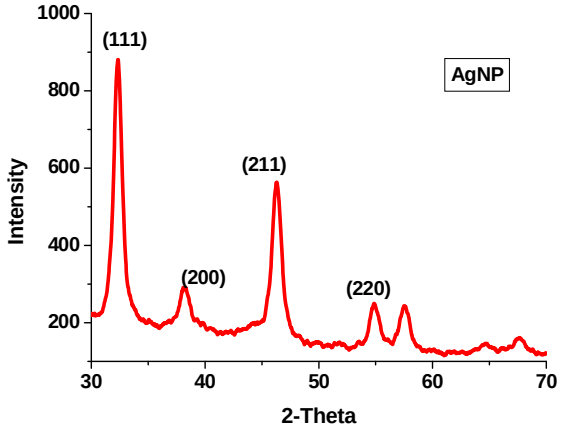

Fig .5: XRD of AgNP

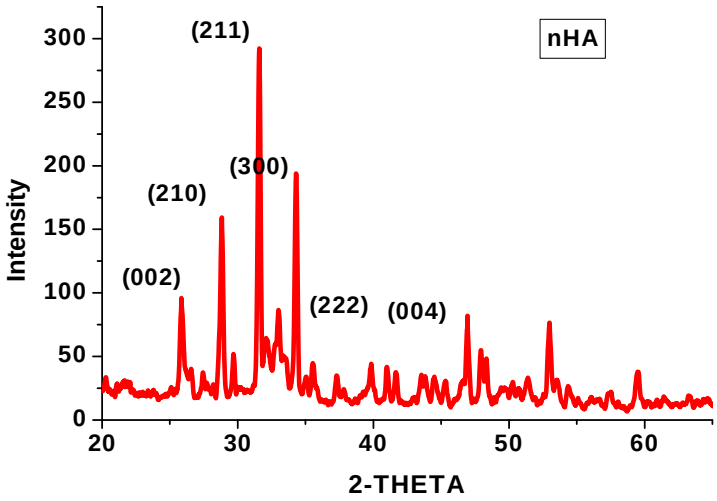

Fig .6:.XRD of nHA

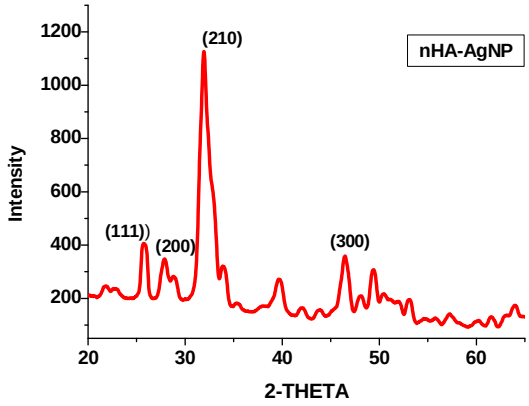

Fig. 7: XRD of nHA-AgNP nanocomposite

4) *SEM Analysis:* The morphology and size distribution of AgNP, nHA and nHA-AgNP were analysed using SEM analysis. The SEM image of silver nanoparticles (Fig 8) shows that the nanoparticles are spherical in shape and nHA (Fig 9) shows that the particles are rod shaped. The SEM image of nHA-AgNP nanocomposite (Fig 10) shows that the particles of nHA-AgNP having both spherical and rod shape with mild agglomeration confirming the biosynthesis of nHA-AgNP nanocomposite.

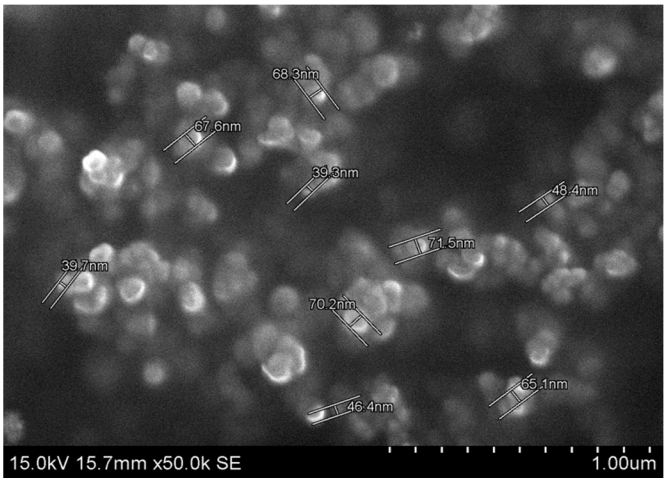

Fig .8: SEM image of AgNP

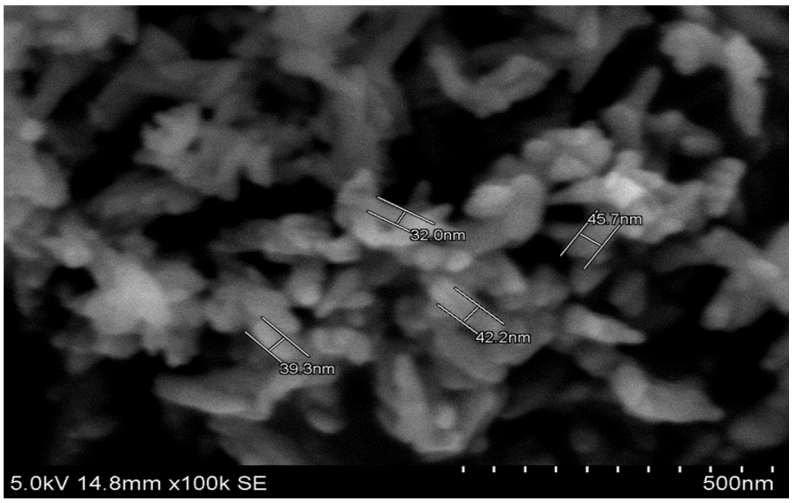

Fig. 9: SEM image of nHA

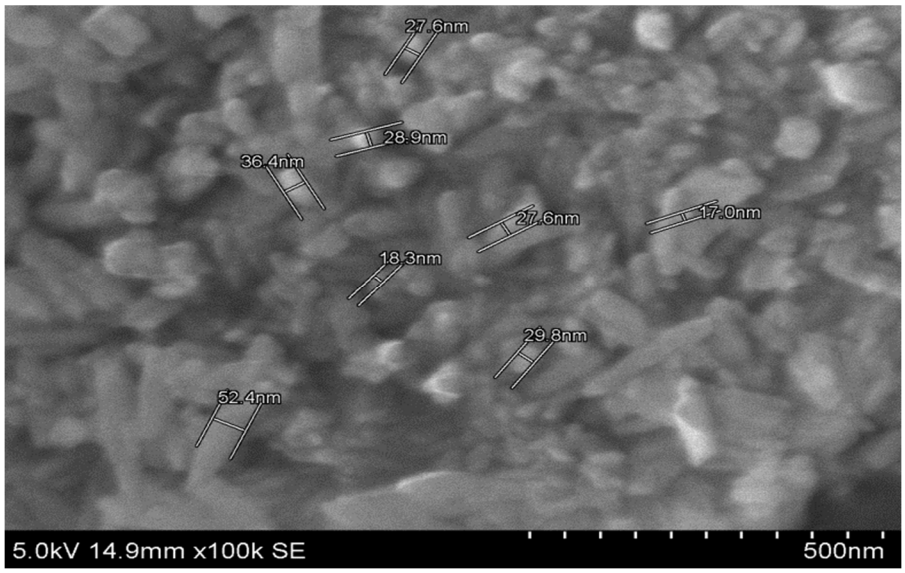

Fig. 10: SEM image of nHA-AgNP nanocomposite

5) *EDS Analysis:* The elemental composition of the synthesised nHA-AgNP composite is determined by EDS analysis.

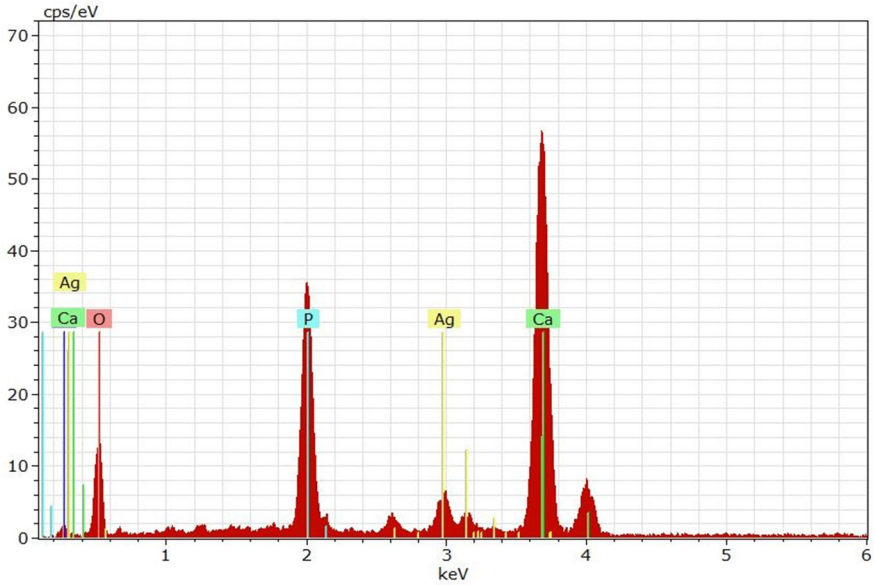

Fig .11: EDS analysis of nHA –AgNP nanocomposite

The EDS of nHA-AgNP shown in figure 11 shows that the elemental composition of the nanocomposite and it confirms the presence of Ca, P, Ag, O and C. The carbon observed may be due to the green synthesis of AgNP from Grapes (*Vitis vinifera*) extract. The EDS analysis of nHA-AgNP shows 28.08 weight% of Ca, 11.47 weight% of P and Ca/P weight ratio was found to be 2.448.

B. Photocatalytic Studies

1) *Preliminary Studies on Photocatalytic Activity in Sunlight:* Photo decolourisation of Congo red over a 10mg of nHA, AgNP, and nHA- AgNP and Methylene blue over a 10mg of nHA and nHA- AgNP were studied with 10ppm concentration of the dye in presence of sunlight. It was monitored extracting the dye solutions at different intervals. The preliminary studies shows that the nHA- AgNP nanocomposite is a better photocatalyst with reference to AgNP and nHA used separately (Fig. 12) and nHA-AgNP nanocomposite is better photocatalyst for CR than MB and leads to a more detailed study using photoreactor (Fig. 12,13)

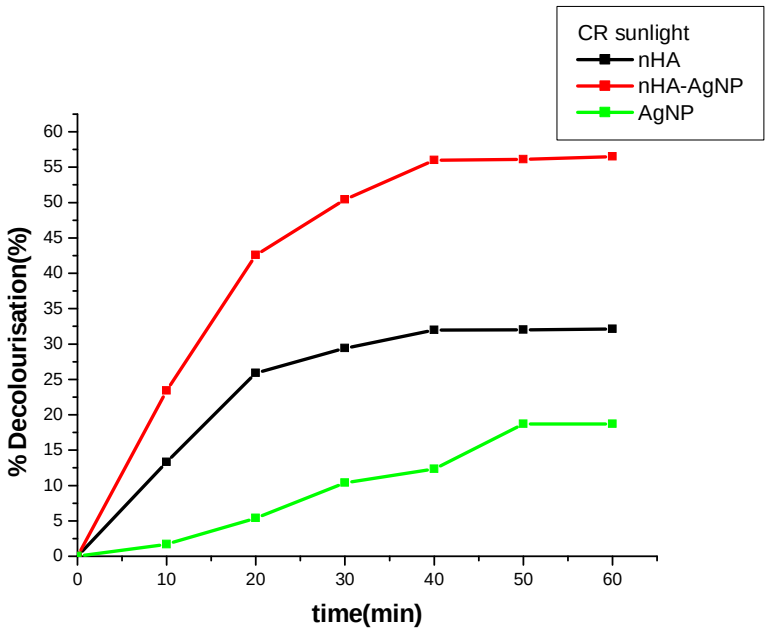

Fig .12: % Decolourization of CR over nHA, AgNP and nHA-AgNP nanocomposite.

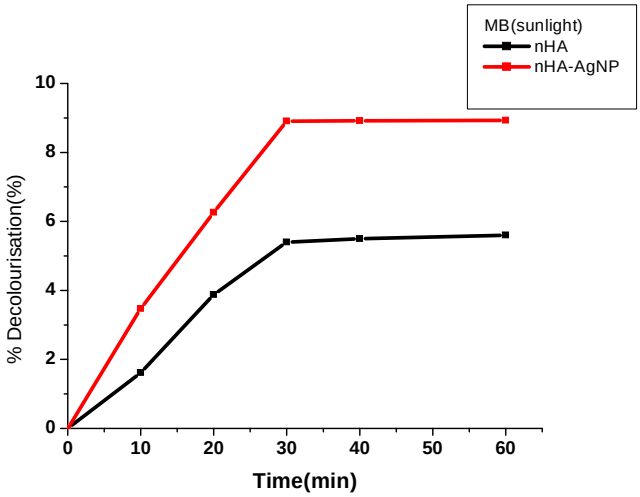

Fig .13: % Decolourization of MB over nHA and nHA-AgNP nanocomposite

2) *Photo Catalytic Studies of nHA and nHA- AgNP Nanocomposite on CR and MB using UV Photoreactor:* To find the %adsorption of CR and MB over the catalysts the dark reaction was carried out over the catalyst for 30 minutes. Samples were analysed using UV-Vis spectrophotometer. The % Adsorption of the dye over the catalyst was found using the equation ($C_{blank} - C_{dark}$)/C_{blank}) × 100. The % adsorption of CR over nHA and nHA-AgNP in 30 minutes (Dark) was 20.03 % and 23.02% while The % adsorption of MB over nHA and nHA-AgNP in 30 minutes (Dark) was 1.42% and 1.60% respectively.

Photo decolourisation of CR and MB over a 100mg of nHA and nHA-AgNP nanocomposite was studied with 10ppm concentration of dye and was monitored by extracting the dye solution at different time intervals. The % decolourisation of the dye over the catalyst was found using the equation ($C_0 - C_t$)/C_0) ×100 . The % decolourisation of CR over nHA and nHA-AgNP in 80 minutes (photoreactor- light) was 28% and 66% while the % decolourisation of MB over nHA and nHA-AgNP in 100 minutes (photoreactor- light) was 28.39% and 31.40% respectively.

The plot of % decolourisation with time (Fig. 14,15) shows that the photo decolourisation increases with time and attains equilibrium 80 minutes in the case of CR and 100 minutes in the case of MB. The time taken to attain equilibrium was 40 minutes in sunlight (Fig.12) and 80 minutes in photo reactor for CR (Fig. 14) and for MB, 30 minutes in sunlight (Fig.13) and 100 minutes in photoreactor (Fig.15). This could be due to the wide range of electromagnetic radiation in sunlight and the Mercury vapour lamp emitting specifically 365nm wavelength as the light source in the photoreactor.

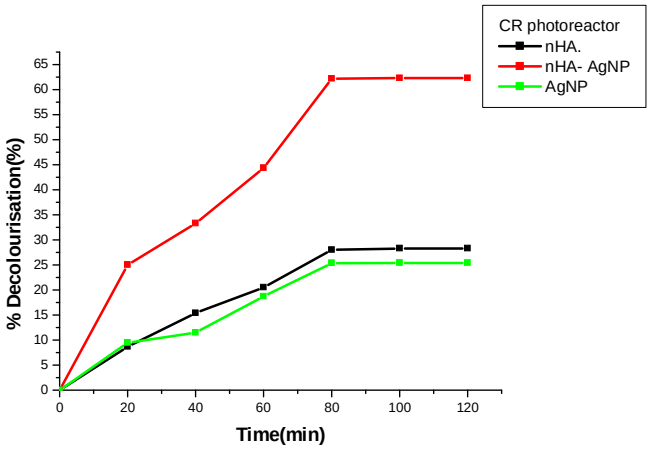

Fig .14: % Decolourization of CR over nHA, AgNP and nHA-AgNP nanocomposite.

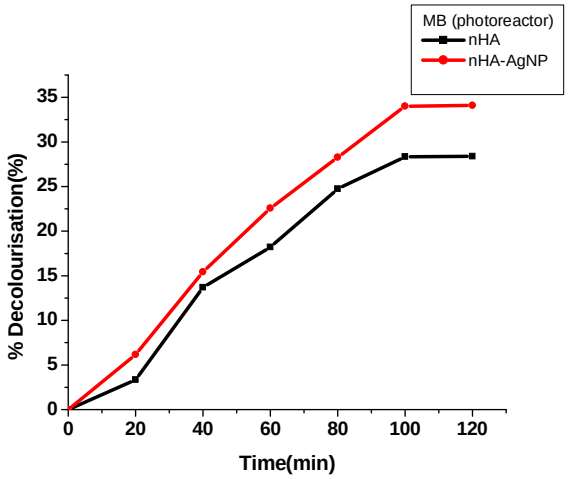

Fig. 15: % Decolourization of MB over nHA and nHA-AgNP nanocomposite.

From the above results it is clear that compared to nHA, the nanocomposite nHA-AgNP is a better photocatalyst. Among the organic dyes used in the study, CR was decolourised more effectively than MB.

High photocatalytic activity of nHA –AgNP nanocomposite system is due to absorption in the visible region, high surface area and its microstructure. The key factor that affects the activity of the photocatalyst is the abundant surface active sites which depends on the separation and transfer of the photo-generated electrons and holes. In metal nanoparticle like Ag, the conduction band and valence band lie very close to each other and through these electrons move freely. In literature it is reported that nHA is having good storage capacity of electrons. Thus, the photo-generated electrons could migrate to nHA leading to the separation of electron–hole pairs. The electrons accumulated on HA could adsorb the O_2 in solution to form $^{\bullet}O_2^-$, which could degrade the dyes. As a consequence, the nHA-AgNP nanocomposite acts as a photocatalyst and exhibited excellent photocatalytic performance and could be used for waste water treatment and enhanced protection of the environment [9,10].

C. Antibacterial Studies

The antibacterial activity was observed against *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram- negative) (Figure 16) with Ampicillin (20µl/disc) as the control.

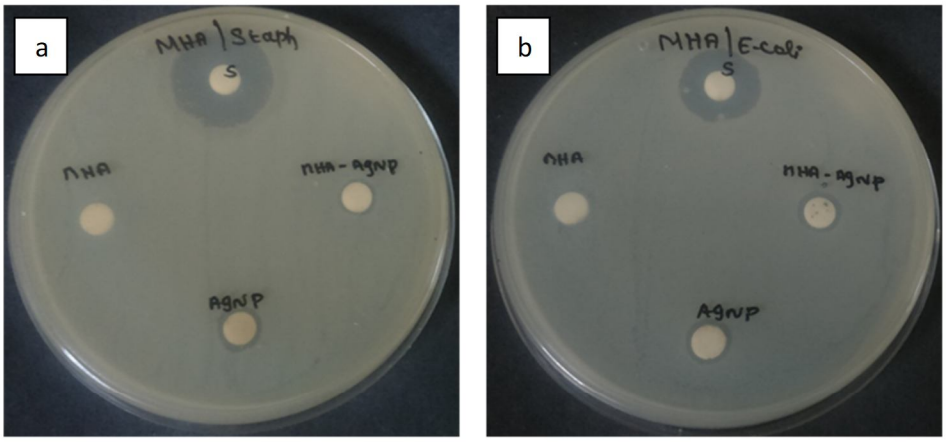

Fig. 16: Antibacterial activity of synthesized AgNP, nHA, nHA –AgNP against a) *Staphylococcus aureus*, b) *Escherichia coli*

All the three samples (AgNP, nHA, nHA–AgNP) showed antibacterial activity against the two strains. AgNP showed same activity against *Staphylococcus* aureus and *Escherichia coli* .In the case of nHA the zone of inhibition is more for Escherichia *coli* and it is clear that incorporation of AgNP to the nHA enhances the activity against both *Staphylococcus aureus* and *Escherichia coli.* Thus the as-synthesised nHA-AgNP nanocomposite exhibited enhanced antibacterial activity and could be utilised for orthopaedic and dental implants.

IV. CONCLUSIONS

Nanohydroxyapatite is a calcium phosphate bioceramic with versatile applications in the field of bone tissue engineering, dentistry, photocatalyst, coating, drug delivery etc. In this present report, silver nanoparticle incorporated nanohydroxyapatite composite prepared from biomaterial was isolated from waste material viz., eggshells and the green synthesis of silver nanoparticles were prepared using the grape extract. The green syntheses were confirmed by UV-Vis spectroscopy, FTIR, XRD, SEM and EDS studies. The UV-Vis spectra for the as- synthesised AgNP confirmed by exhibiting λ max 430nm characteristic of the capping and reducing agent from fruit extract for the synthesis of AgNP and the energy band gap was found to be 3.14eV, 4.38eV and 2.64eV for AgNP, nHA, nHA-AgNP respectively. Thus, the nHA-AgNP nanocomposite could act as a semiconductor. The FTIR analysis indicated the presence of different functional groups in the as- prepared samples. XRD results showed that the AgNP, nHA, nHA -AgNP were successfully synthesized and the average grain size was found to be 43.7nm, 42nm and 60nm respectively, using Scherrer formula. The SEM images showed that the AgNP synthesized from Grape extract are spherical in shape, nanohydroxyapatite prepared from eggshells are rod shaped and the composite nHA-AgNP contains both spherical and rod shaped particles. The EDS analysis of the nHA-AgNP revealed the presence of elements Ca, P, Ag, O and C confirmed the synthesis of nanocomposite. The as-prepared samples could be used as photocatalyst at a cheap cost for environmental applications which includes treatment of water waste from industrial effluent. The biosynthesized nHA –AgNP nanocomposite via a cost effective green synthesis route is a promising candidate in removal of organics in waste water and enhance environmental protection. The antibacterial study infers that the nHA – AgNP nanocomposite was effective against both *Staphylococcus aureus* and *Escherichia coli* and could be used for orthopaedic and dental implants.

V. ACKNOWLEDGEMENT

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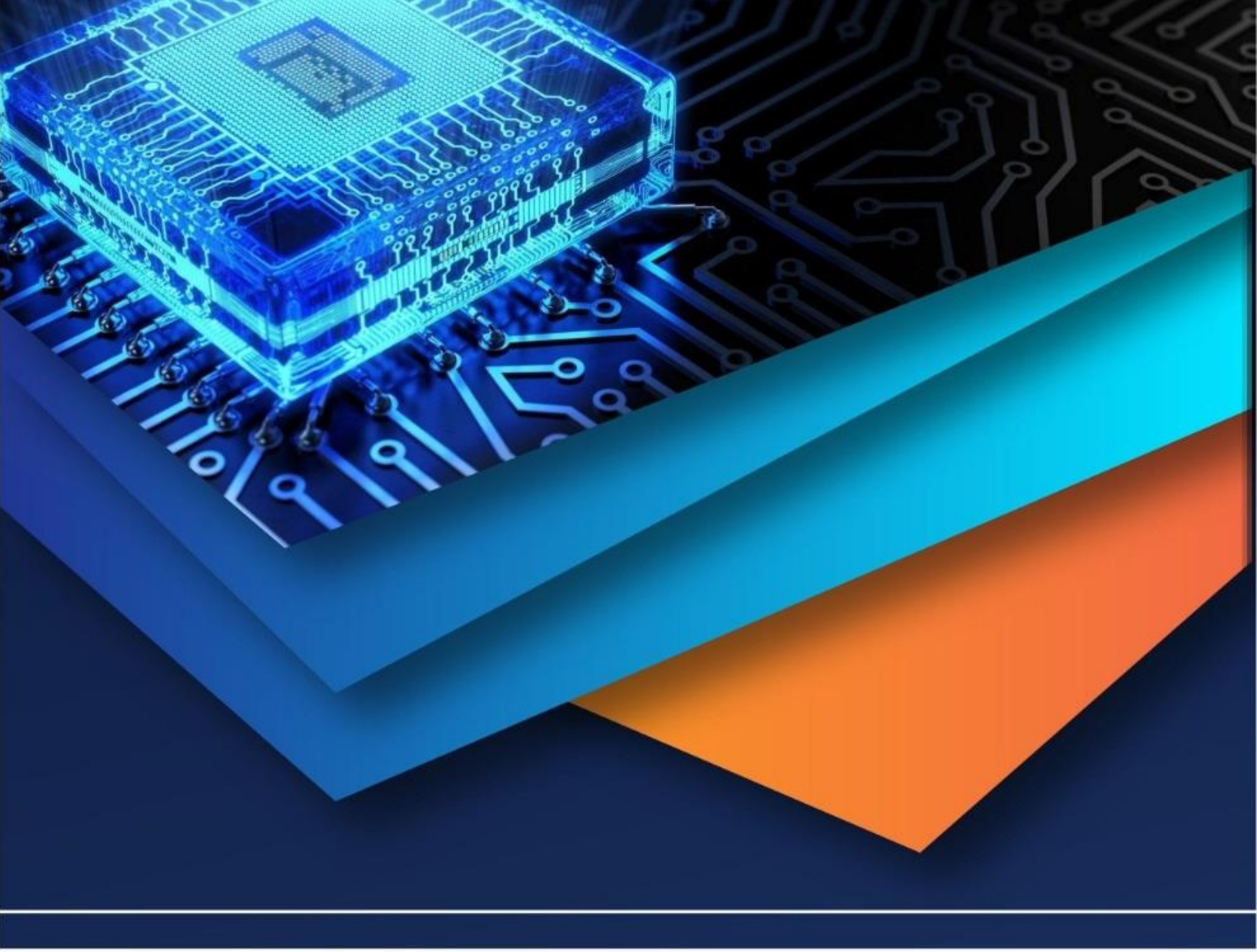

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