Carbohydrate-directed Synthesis of Silver and Copper Nanoparticles, and their Antibacterial Property

A Dissertation

Submitted in the partial fulfillment of the requirement for the award of degree of

Master of Science in Biotechnology

Submitted by

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July 2015

CANIDATE'S DECLARATION

I hereby declare that the research work on the topic of **'Carbohydrate-directed synthesis of Silver and Copper nanoparticles and their applications'** embodied in this thesis has been carried out by me at School of Chemistry and Biochemistry and Department of Biotechnology under supervision of Dr. Niranjan Das, Professor, Dept. of Biotechnology and Dr. Bonamali Pal, Head and Professor, SCBC, Thapar University, Patiala. I also affirm that this work is original and has not been submitted in part or full, for any degree or diploma to this or any other University or Institution.

Patiala

Date - 15-7-2015.

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CERTIFICATE

This is to certify that the entitled "**'Carbohydrate-directed synthesis of Silver and Copper nanoparticles and their applications'**" submitted for the award of the degree of Master of Science in the Department of Biotechnology by Miss. Ramandeep Deep Sarwara is the record of bonafide research work carried out by the candidate during the period from Jan-2015 to June-2015 under my guidance and supervision, and this work has not formed the basis for the award of any degree, diploma, associate ship, fellowship, or other titles in this University or any other University or Institution of higher learning.

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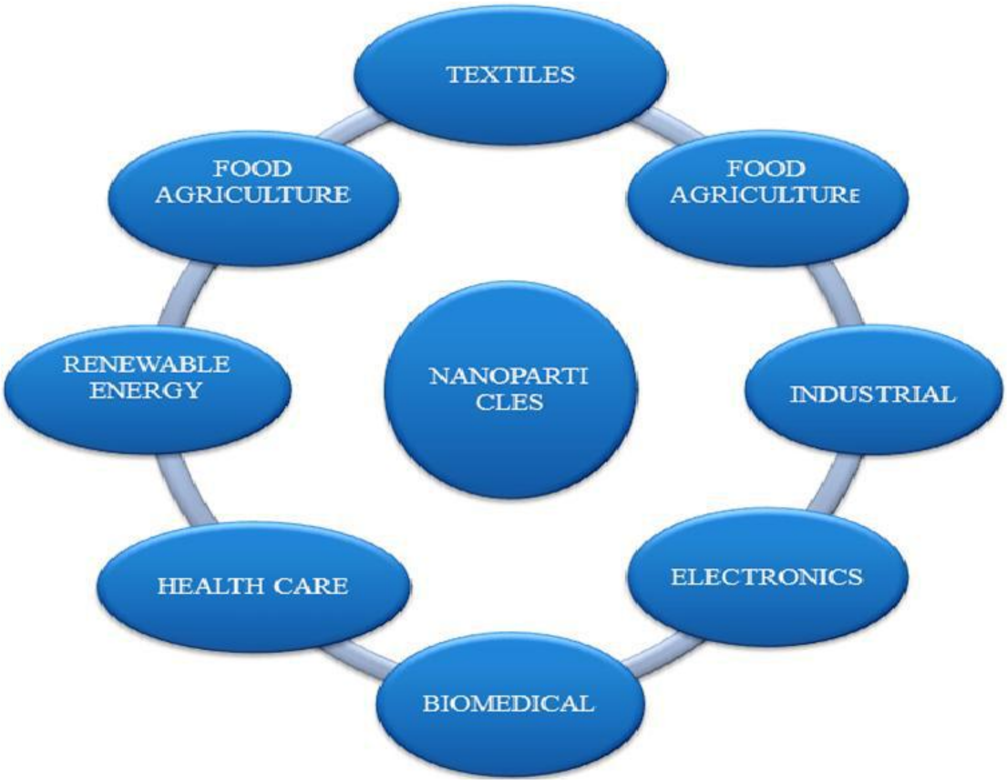
ABSTRACT

This dissertation work mainly deals with an in-depth study pertaining to the synthesis and characterization of copper and silver nanoparticles based on different carbohydrates, and assessing their antibacterial activity by using agar-well diffusion method. The existing physical and chemical methods of nanoparticle synthesis are expensive and sometimes toxic to environment and human health. Therefore, biological or green synthesis becomes more preferred option currently. Different carbohydrates such as glucose, sucrose, fructose and starch were used successfully for the synthesis of respective nanoparticles having well-defined sizes. The individual reactions went to completion in less than 20 min at room temperature. This method was easy and simple to synthesize high purity of Ag NPs and Cu NPs with different carbohydrates as mentioned above. Gelatin was used to control the agglomeration of the nanoparticles, and acted as a stabilizing agent. UV-Visible spectroscopy had shown a shift of the SPR peak depending upon the different average particle size of Ag NPs and Cu NPs synthesize by different carbohydrates. Visible photoluminescence maximum emission of Ag NPs was observed at 328-345 nm and Cu NPs at 700 nm. The structural and morphological characterization of the samples was performed using Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM). The carbohydrate-based nanoparticles of this study were found to be effective in growth inhibition of Gram-Negative bacteria namely *E. coli*; hence showed their antibacterial activity. Starch-based Ag NPs exhibited strong inhibitory effect as compared with other carbohydrate-based Ag NPs and Cu NPs. The diameters of the individual zone of inhibition were noted and compared in this report. Strikingly, starch-based copper NPs showed practically no inhibitory effect on the growth of *E. coli*. The data clearly suggested that carbohydrate-based silver nanoparticles appear to be more effective in terms of bactericidal activities as compared to carbohydrate-based copper nanoparticles (except fructose-based Cu NP). It is apparent that overall size and shape of the carbohydrate-based nanoparticles are crucial in terms of their inhibitory effect on the microbes. However, the precise molecular mechanisms of antibacterial activities of the individual carbohydrate-based nanoparticles need to be further understood.

CHAPTER 1: INTRODUCTION

Nanotechnology and Nano science are new emerging fields which have shown potential impact on many scientific areas such as energy, medicine, pharmaceutical industries, electronics, and space industries [1-5]. Nanoparticles are small-sized materials of size range between 1-100 nm. Nanoparticles are more active and exhibit unexpected properties because of high surface to volume ratio and quantum size effect [6-7]. Nano size particles (NP's) of metals like Ag, and Cu are found to exhibit enhanced optical and catalytic activity due to the Quantum Size Effect. They have distinct physical, chemical, electronic, electrical, mechanical, magnetic, thermal, dielectric, optical and biological properties. Metal NP's are considered as building blocks for the next generation optoelectronics, electronics, and various chemical and biochemical sensors as, they have advantages over bulk materials. Their enhanced effect on different properties because of their surface Plasmon resonance (SPR) enhanced Rayleigh scattering and surface enhanced Raman scattering (SERS) [8-12], makes them more reliable as compared with bulk metals. Metal nanoparticles are diverse on the basis of their outstanding application in different fields. The main characteristics of NPs are large surface-area-to-volume ratio, large surface energies, Plasmon excitation, quantum confinement, short range ordering, and increased number of kinks. The large surface area to volume ratio of nanoparticles provides differing magnetic properties. Bulk gold and platinum are non-magnetic but at the Nano size they show magnetic properties. [6-10]. As the system length scale is reduced, the changes which occur in electronic properties are related mainly to the increasing influence of the wave-like property of the electrons (quantum mechanical effects) and the scarcity of scattering centers. As the size of the system becomes comparable with the de Broglie wavelength of the electrons, the discrete nature of the energy states becomes apparent. This phenomenon can be utilized to produce radically different types of components for electronic, optoelectronic and information processing applications, such as resonant tunneling transistors and single-electron transistors. The colloidal solution of coinage nanoparticles show intense color and display characteristic surface Plasmon resonance (SPR) band as a result of the coherent oscillations of the conduction band electrons induced by the interacting electromagnetic field [10-12].

Nanoparticles are non-toxic, safe antibacterial agents being used for centuries and are capable of killing wide range of microbes that causes diseases. They possess a wide range of biological applications such as antibacterial agents for antibiotic resistant bacteria, healing wounds, anti-inflammatory and preventing infections (Taylor et al., 2005). Silver and copper nanoparticles are highly toxic to microorganisms, exhibits strong bactericidal effect on many species of bacteria but have low toxicity towards animal cells [10-17]. Currents nanotechnology is turning as central science for all relevant fields as shown in scheme 1.



Scheme 1 Applications of nanoparticles

1.1 Coinage Metal Nanoparticles

Among the transition metals, coinage metals have attracted a great deal of interest from both scientific and technological viewpoints. Owing to their brilliant luster and metallic properties and stability, these metals were used to make ornaments, utensils, currency coins, colored window glazes, etc. during prehistoric times [18-19]. But with the advent of Nano science, coinage metal nanoparticles like Ag and Cu became the subject of intense research due to their fascinating physical, optical, and electronic properties. The study of coinage metal nanoparticles is advantageous because these noble metals, copper, silver, and gold, can be easily synthesized, show an intense color and exhibit a strong tunable absorption band in the visible region, which is absent in the bulk material as well as for the individual atoms. Many strategies have been employed to synthesize well-controlled morphologies of coinage metal nanostructures like spheres, cubes, rods, boxes, cages, wires, etc., to tune their optic-electronic and catalytic properties. Very little information is available regarding Ag and Cu catalysis despite of their low-cost and suitable work functions. The main drawback of Ag and Cu NPs is their severe susceptibility to oxidation, which makes their optical and catalytic reactions and properties non-reproducible. Therefore, these NPs are stabilized through adsorption or by covalent attachment of organic compounds (namely, carboxylic acids, polymers, polysaccharides, amino acids) on the NP surface which provides electrostatic or electrostatic repulsive forces between particles, allowing them to resist from oxidation and aggregation [18-23].

1.1.1 Silver and Copper Nanoparticles

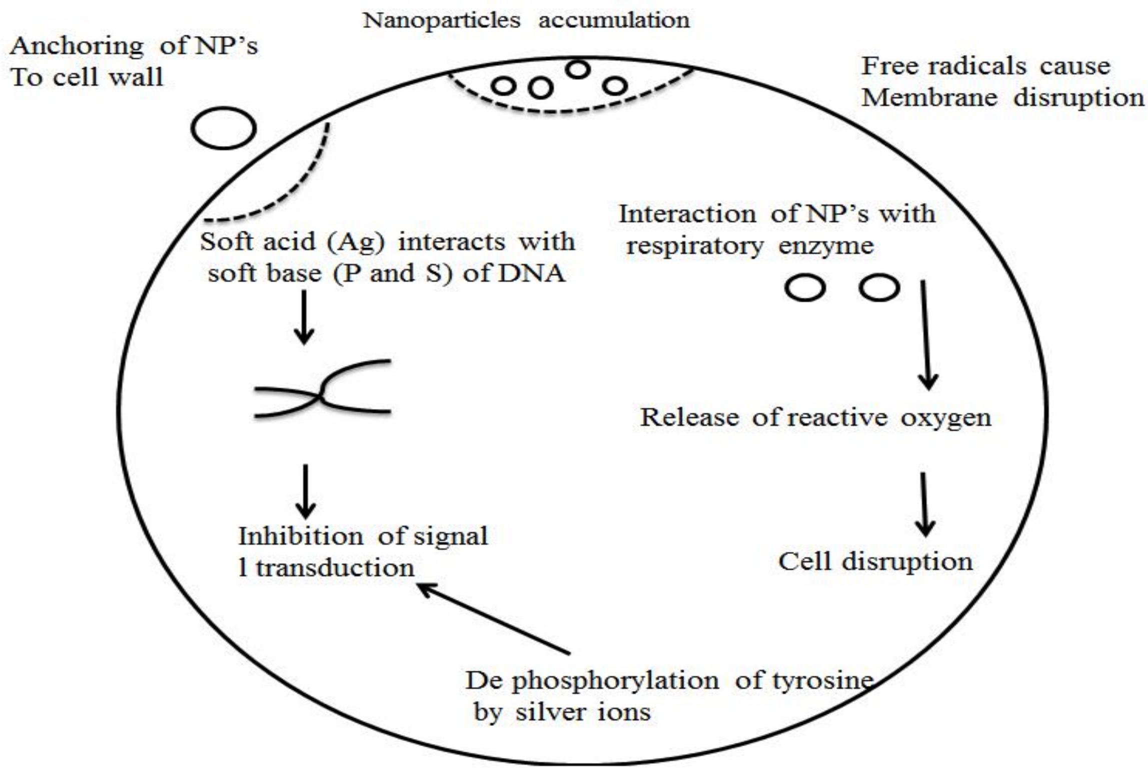
Silver and copper nanoparticles are one of the most promising products in the field of nanotechnology. Development of consistent processes for the synthesis nanoparticles is an important aspect of current nanotechnology. These nanoparticles can be synthesized by several chemical, physical and biological methods [23-26]. One of the promising processes is green synthesis process because it avoids toxicity and, is harmless and cost effective and increases the nanoparticle quality.

1.1.2 Applications of Coinage Metal Nanoparticles (Silver and Copper Nanoparticles)

Cu and Ag nanoparticles are most commonly used in emerging interdisciplinary field of Nano biotechnology and in biomedical technology. Silver and copper nanoparticles have their extensive applications in various fields. They are used for purification and quality management of air, bio sensing, imaging, drug delivery system [27-30]. Antimicrobial properties of silver and copper nanoparticles caused the use of these Nano-metals in different fields of medicine, various industries, animal husbandry, packaging, accessories, cosmetics, health and military. Copper is a ductile metal with very high thermal and electrical conductivity. It is combined with other metals to form alloys as brass because it found to be too soft for some applications [30-31]. Though silver nanoparticles are cytotoxic but they have tremendous applications in the field of biomedical detection and diagnostics, therapeutics, and microelectronics [28-31]. Many consumer goods manufacturers already are producing household items that utilize the antibacterial properties of silver and copper nanoparticles. These products are – silver lined refrigerators, washing machines and air conditioners. They both have some potential applications like diagnostic biomedical optical imaging, biological implants (like heart valves) and various medical applications (wound dressing, surgical instruments, contraceptive and bone prostheses) [26-32].

1.1.3 Silver and copper nanoparticles as antimicrobial agents

Ag NP's and Cu NP's are both exhibits highly antimicrobial activities for several species of bacteria. Like common kitchen microbe *E. coli.* The exact mechanism of silver and copper nanoparticles which cause antimicrobial effect is not clearly known. According to the mechanism reported silver nanoparticles have the ability to adhere to the bacterial cell wall and penetrate it, thereby causing many structural changes and leads to the death of bacteria. As shown in scheme 2, formation of 'pits' on the cell surface of bacteria there is accumulation of the nanoparticles on the cell surface [33], Formation of free radicals by silver nanoparticles has ability to damage the cell membrane which can leads to cell death [34-35]. Generation of reactive oxygen species (ROS) which are produced possibly through the inhibition of a respiratory enzyme by silver ions, attack the cell itself. Silver is a soft acid and there is a natural tendency of an acid to react with a base [36]. Sulfur and phosphorus group are the major components of DNA, nanoparticles act on these and leads to failure in DNA replication and destroy the DNA which leads to death of bacteria [37].

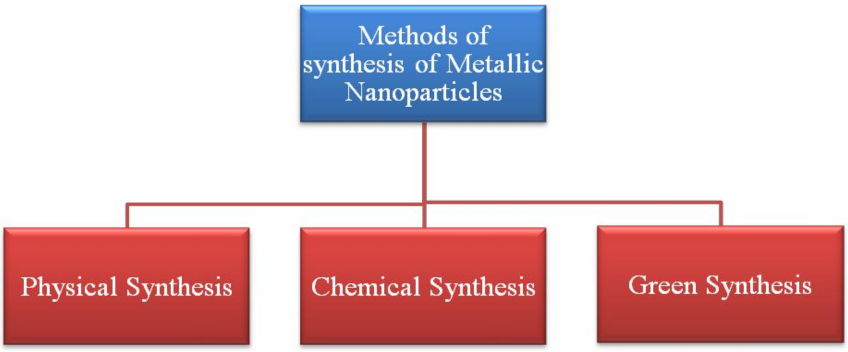


Scheme 2 Possible mechanisms regarding antimicrobial effects of nanoparticles

In the new era of pharmaceutical and medical industries, silver is the metal of choice as it holds the promise to kill the microbes effectively. Silver and copper nanoparticles have been recently known to be promising antimicrobial agents that effects on a broad range of target site both extracellular as well as intracellular. They both shows very strong bactericidal activity against gram positive and gram negative bacteria including multi resistant strains (Shrivastava et al.,2007). Hence there is a huge scientific progress in the study of biological applications and antimicrobial applications of silver and copper nanoparticles and other metallic nanoparticles [38-39].

1.2 Various Methods of Metallic Nanoparticle Synthesis

Different methods for synthesis of NP's are outlined in the following scheme:



Scheme 3 Different Method of Nanoparticles Synthesis

1.2.1 Green Synthesis of Metallic Nanoparticles

Today, the "green" synthesis of metallic nanoparticles is receiving substantial attention due to the development of eco-friendly technologies in materials science. Well-dispersed and ultrafine metal nanoparticles, especially transition metals, are of great interest due to their distinctive physicochemical and thermodynamic properties, which have made them suitable for use in various fields, such as catalysis, optics, and medicine [40]. The green synthesis of MNPs relies mainly on the following:

- The selection of a biocompatible and nontoxic solvent medium
- The selection of environmentally benign reducing agents
- The selection of non-toxic substances for stabilization of the nanoparticles.

Green nanotechnology, thus, aims to the application of green chemistry principles in designing Nano scale products, and the development of nanomaterial production methods with reduced hazardous waste generation and safer applications [40-41].

1.2.2 Polysaccharide Method for Synthesis of Metallic Nanoparticles

In this process, Ag-NPs are synthesized in water as an eco-friendly benign solvent with polysaccharides (Carbohydrates) as the stabilizer agent. In some cases, polysaccharides are used as both a reducing agent and a stabilizer. For example, preparation of Ag-NPs using soluble starch was carried out as a stabilizer with β-D-glucose as a reducing agent in low temperatures. Ag-NPs are prepared by the reduction of silver cations inside the starch templates. The extensive network of hydrogen bonds in the templates can be passivated by the surface nanoparticles and/or can be protected from aggregation. This method must normally be done with a gentle heat system because the binding interactions between polysaccharides and metallic nanoparticles are weak and can be reversible with high heat systems, allowing separation from the obtained particles. Another synthesis of Ag-NPs can be performed with gelatin which act as as a reducing as well as capping agent. It is advantageous as being able to use renewable materials like gelatin and glucose [42-43].

Carbohydrates: A carbohydrate is a biological molecule consisting of carbon (C), hydrogen (H) and oxygen (O) atoms, having empirical formula $C_m(H_2O)_n$ (where m could be different from n). Carbohydrates are technically hydrates of carbon. Structurally it is more accurate to view them as polyhydroxy aldehydes and ketones**.** The use of carbohydrates such as (glucose, sucrose, maltose, fructose, starch) has recently become important in green synthesis methods of nanoparticles, because these biopolymers acts as an effective surfactant agent and is environmentally friendly. According to Bozanic et al., it is possible to obtain silver and copper NPs of spherical shape by using sago starch as coating agent. Others studies found that stable silver NPs could been synthesized by using soluble starch both as reducing and stabilizing agents, with sizes in the range 10–34 nm. Nersisyan et al. developed an effective way for preparation of Nano sized colloidal dispersions of Ag (with NP size of 10–50 nm) using glucose as a reducing agent. Polymers have also been used as matrices in nanocomposites or as stabilizers to provide stability or the metal nanoparticles against oxidation, agglomeration and precipitation.

Natural polymers, such as natural rubber, polysaccharides, cellulose, chitosan and starch have been used as matrices or stabilizers for the preparation of metallic nanoparticles because of their non-toxicity and biocompatibility. Gelatin is a natural biopolymer, extracted from the partial hydrolysis of collagen and which has good biocompatibility and biodegradability. Currently, it has been widely used in wound dressings and as a drug carrier and tissue scaffold [44-45].

In respect of the excellent antimicrobial properties of coinage metal nanoparticles and foremost need of greener technology herein we have synthesized the coinage metal nanoparticles by a green synthesis method (Sugar reducing and stabilized by gelatin) and evaluated there antimicrobial activities.

CHAPTER 2: LITERATURE REVIEW

Many chemical and physical methods have been applied for the synthesis of metallic nanoparticles (NPs). However, various problems are related with these methods which include use of harmful and expensive chemical reagents, production of hazardous commodities, etc. In this regard, there is a need to develop reliable, nontoxic, clean, eco-friendly and green experimental protocols for the synthesis of NPs. The following sections mostly deal with the green synthesis methods for various nanoparticles as reported earlier.

Various methods of nanoparticle synthesis: Many biological systems, including plants, fungi, actinomycetes, bacteria, yeast and carbohydrates have the ability to transform inorganic metal ions into metal nanoparticles via the reductive capacities of the proteins and metabolites present in these organisms and sugars. Use of plants in synthesis of nanoparticles has drawn more interest of workers as it provides single step process and rapid synthesis of nanoparticles than chemical methods Makarov *et al.,* (2014). Plants tender a superior option for synthesis of nanoparticles, as the protocols involving plant sources are free from toxicants; moreover natural capping agents are readily supplied by plants [46]. Arunachalam *et al*., (2013) produced silver and gold nanoparticles by treating silver and gold ions with an extract of *Memecylon umbellatum* leaf. The reaction process was simple and easy to handle, and was monitored using ultraviolet-visible spectroscopy [47].

Synthesis of silver nanoparticles was reported by Song *et al.,* (2009).They treated aqueous solution of $AgNO_3$ with five different plant leave extracts as reducing agent for Ag^+ to Ag^0. It was found that *Magnolia* leaf broth was the best reducing agent in terms of synthesis rate and conversion of silver nanoparticles took only 11 min for 90% conversion at reaction temperature of 95 ºC [41]. Vigneshwaran et al., (2007) demonstrated a simple route of synthesis of silver protein nanoparticles using spent mushroom substrate. It was observed that the substrate exhibited an organic surface that effectively reduced silver. Barrasa et al., (2010) synthesized silver nanoparticles using the Lee-Meisel and the Creighton method using $AgNO_3$ instead of $HAuCl_4$ as metallic precursor and sodium citrate as the reducing agent. The obtained nanoparticles displayed a broad size distribution. The Creighton method is a popular method for generating Ag NPs showing a narrow size distribution [27]. Panacek et al., (2006) synthesized silver colloidal nanoparticles with controllable size. Reduction of $[Ag(NH_3)_2]^+$ complex cations by four saccharides was performed i.e., glucose, galactose, maltose and lactose. The synthesis was carried at various ammonia concentrations (0.005- 0.20 mol L^{-1}) and pH conditions (11.5-13.0) produced a wide range of particle sizes (25-450 nm) with a narrow distributions, especially at the lowest ammonia concentrations. Antibacterial activity of silver nanoparticles was found to be dependent on the size of silver particles. A very low concentration of silver (as low as 1.69 μg/mL Ag) gave good antibacterial performance [48]. Dhas et al., (1998) synthesized Nano scale particles of metallic copper by two methods, namely the thermal reduction and sonochemical reduction of copper hydrazine carboxylate ($Cu\text{-}(N_2H_3COO)_2.2H_2O$) complex in an aqueous medium. Reduction processes occurred under an argon atmosphere for a period of 2-3 h [16].

Pettegrew et al., (2014) synthesized Ag NPs using monosaccharaides and their growth inhibitory activity against gram- negative and gram- positive bacteria. Using various monosaccharaides as reluctant, they synthesized Ag NPs containing colloidal solution showed distinctive colors with varying λ max. The size of the NPs formed varied significantly from 10 to 35 nm in good agreement with the localized Plasmon resonance ranged from ~300 to ~600 nm. The antimicrobial properties of these NPs were compared in Gram-negative and positive bacteria in liquid culture. Gram positive bacteria were highly susceptible compared to Gram-negative microbes the additional lipopolysaccharide layer covering the peptidoglycan cell wall in the latter somewhat lessens the effect. The results indicated that larger NPs produced by glucose inhibited bacterial growth better than the smallest NPs produced by ribose. This may be attributed to the higher aggregation rate for larger NPs on cell wall [43]. Das et al., (2009) synthesized uniform and stable silver nanoparticles by reduction of silver ions using ethanol. It is a simple process for obtaining silver nanoparticles. XRD and TEM of the samples revealed the nano nature of the particles [2]. Vasudev D. Kulkarni et al., (2013) reported the synthesis of ecofriendly copper nanoparticles using *Ocimum sanctum* leaf extract was by. They observed that

Ocimum sanctum leaf extract can reduce copper ions into copper nanoparticles in within 8-10 minutes. Easily available starting materials and no use of toxic reagents provide evidence that this method can be used for rapid green biosynthesis of stable copper nanoparticles [49].

Abdelmonema et al., (2014) synthesized nontoxic, ecofriendly metallic nanoparticles using *pomegranate peel* (PPE). It was observed that the bioactive constitutes and the potential antioxidant capacity of pomegranate *(Punica granatum L)* extract play a role in the production of metallic nanoparticles. High mono disperse character of the metallic nanoparticles was reported. Biosynthesis of metallic nanoparticles using PPE gives high yield of nanoparticles which can be a better alternative to traditional, physical, and chemical and even the microbial synthesis [50]. Khalil et al., (2013) synthesized silver nanoparticles (Ag-NP) using hot water *Olive* leaf extract (OLE) as reducing and stabilizing agent and were evaluated for their antibacterial activity against drug resistant bacterial isolates. They observed that the aqueous OLE had no effect at concentrations used for synthesis of Ag-NPs and thus these Ag-NPs showed broad spectrum antibacterial activity at low concentrations of OLE [51]. Rajendran et al., (2015) reported the green synthesis of silver and copper nanoparticles using *origanum heracleoticum* L. leaf extract. It was observed that *O. heracleoticum* L. plant extract could be used as a proficient green reducing agent for the synthesis of Ag-NPs. Antibacterial activity of synthesized Ag-NPs showed effective inhibition against pathogenic microbes. These green synthesized nanoparticles showed potential cytotoxic effect on MCF7 cell line in dose dependent manner [52].

Mubayi et al., (2012) synthesized Ag-NPs using aqueous extract of *Moringa oleifera* leaf extract having high medicinal value. This technique gave a simple and efficient way for the synthesis of nanoparticles because of tuneable optical properties governed by particles size. Antimicrobial activity of these synthesized Ag-NPs was examined in both gram positive and gram negative bacteria [26]. Pantidos et al., (2014) reviewed the biological synthesis of metallic nanoparticles using bacteria, plants, and fungi. This review highlights the potential importance of these environment friendly methods of biological metallic nanoparticle synthesis in assessing nanoparticle risk to both health and the environment [53]. Gopinath et al., (2014) synthesized copper nanoparticles (Cu NPs) using *Nerium oleander* leaf extract as the reducing agent. Antibacterial activity of these Cu NPs was studied against *E.coli*, K. pneumonia, S.aureus, B. subtilis and S. typhi using gentamycin as the control [54]. Banerjee et al., (2012) investigated an efficient and sustainable route for Ag NP preparation using leaf extracts of *Musa balbisiana* (banana), *Azadirachta indica* (neem) and *Ocimum tenuiflorum* (black tulsi). Efficient capping and stabilization properties of these Ag NPs were reported [55]. Krishnaraj et al., (2010) using leaf extract of *Acalypha indica* synthesized silver nanoparticles. Formation of nanoparticles observed within 30 min and checks its activity on water born bacterial pathogens [56].

Veerasamy et al., (2011) synthesized silver nanoparticles using *Garcinia mangostana* leaf extract. It acts as reducing agent. Silver ions when exposed to *leaf* extract were reduced and silver nanoparticles produced. They are highly effective against different multi drug resistant human pathogens [57].

Namratha et al., (2013) synthesized silver nanoparticles using *Azadirachta indica* (Neem) extract and usage in water purification. One such application is the use of these nanoparticles as adsorbents or catalysts to remove toxic and harmful substances from air and water [58]. Rao et al., (2014) synthesize plant mediated metallic nanoparticles and their applications in the field of biomedical, catalysis optoelectronics, chemical sensing, bio sensing, environmental engineering and in biotechnology [28]. Shobha et al., (2014) green synthesis of copper nanoparticles and their applications in agriculture, industrial and technology field. Copper nanoparticles find wide applications in the agricultural field and has much effort has been made in recent years to ascertain the necessity of certain minor elements in the economy of plants [59].

2.1 ORIGIN OF THE PROBLEM

The antibacterial effect of metallic nanoparticles has been a topic of great interest for many years. The effects of various materials/ nanoparticles have been widely studied. They affect the cell membranes, cause inactivation of enzymes. Moreover, the nanoparticles adversely affect DNA replication, and cause DNA degradation as found in many bacteria. Nanoparticles rapidly interact with the proteins present in various microorganisms. However, to date, few studies have been conducted focusing on the antibacterial effect of copper and silver nanoparticles synthesized by using some of the carbohydrates. Therefore, many more carbohydrates could be used for the green synthesis of various metallic nanoparticles. The green synthesis and green chemistry usually involve the selection of biocompatible, ecofriendly, and nontoxic solvents which do not cause any harmful effects. In other words, the green synthesis of useful molecules or products avoids hazardous solvents and toxic catalysts.

Apart from synthesis and studying the structural attributes/physicochemical properties of various carbohydrate-based metallic nanoparticles, assessment of their relative efficacies with regard to bactericidal activities is also a focus area of research in the field of nanobiotechnology. Keeping the aforesaid points in view, the following objectives were framed in the study;

OBJECTIVES

- Preparation of Ag and Cu nanoparticles of different sizes using different carbohydrates namely glucose, fructose, sucrose and starch, and their Morphological studies.
- Effect of molecular structure of carbohydrates on the morphology of Ag and Cu nanoparticles
- Optimization of optical properties, electro-kinetic parameters (zeta potential and conductance) and particle size.
- Studying antibacterial activity of the nanoparticles

CHAPTER 3: MATERIALS AND METHODS

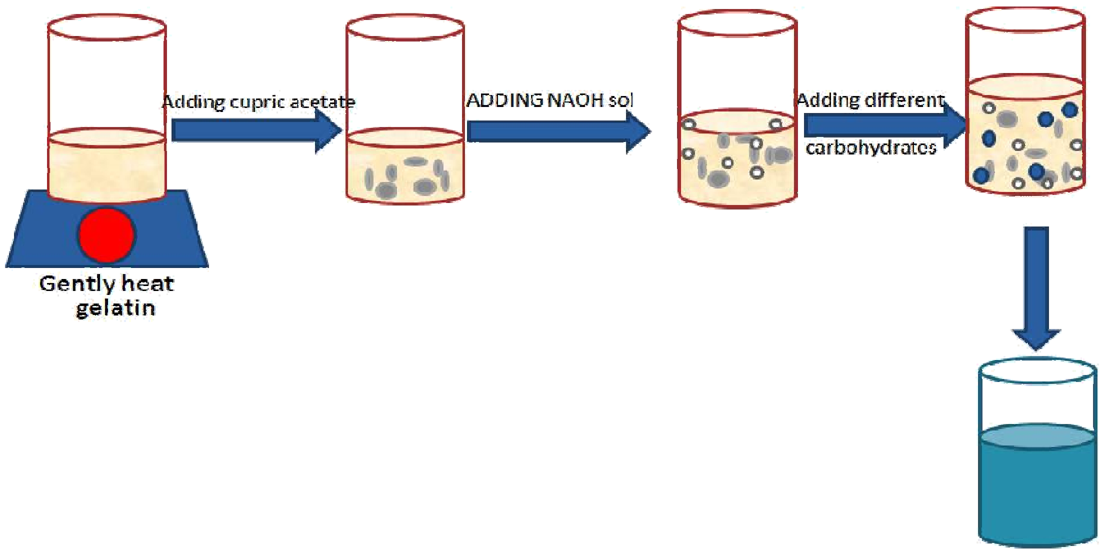
3.1 Chemicals and Reagents

All sugars and chemicals Glucose ($C_6H_{12}O_6$), Fructose ($C_6H_{12}O_6$), Sucrose ($C_{12}H_{22}O_{11}$), Starch ($C_6H_{10}O_5$), Agar, and Luria broth were purchased from Hi media. Silver nitrate ($AgNO_3$), Sodium hydroxide (NaOH) and Cupric acetate $(CH_3COO)_2.Cu.H_2O$ were purchased from loba chemie , s-d fine chem ltd and Qualigen respectively. Gelatin and *Escherichia coli* strain for antibacterial activity were obtained from CDH (central drug house). All the chemicals were used without any further purification. De-ionized water used was obtained from ultra-filtration system (Milli Q, Millipore) with a measured conductivity above 35 mho cm1 at 25 1C.

3.2 Synthesis of Noble Metal (Cu and Ag) Nanoparticles (NP's)

3.2.1 Cu Nanoparticles

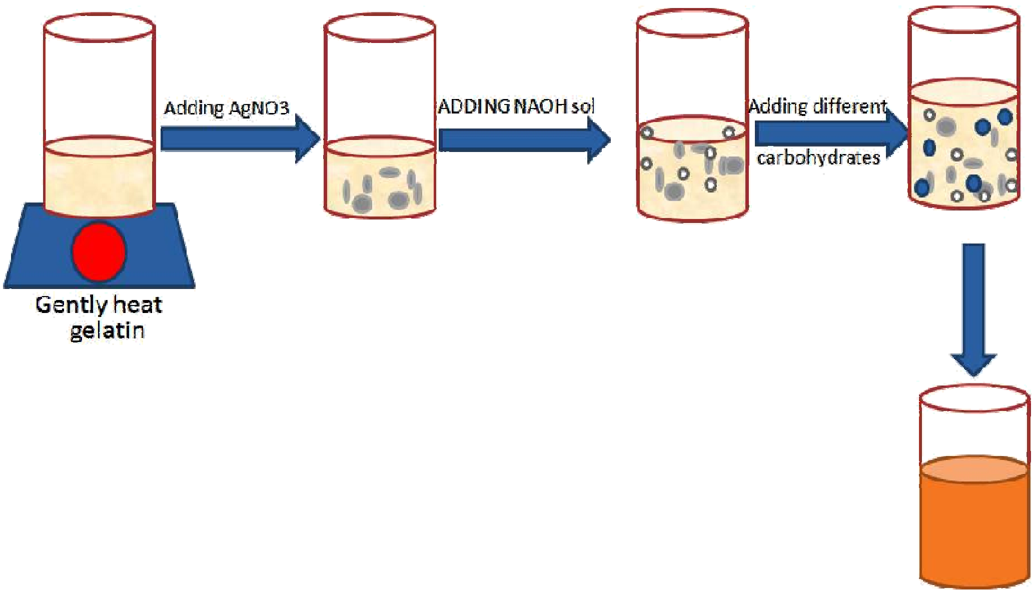
Copper nanoparticles (Cu NP's) were synthesized by gelatin- Sugar based reduction method. For the synthesis of Cu-NPs(0.5g), of gelatin was added per 10mL of distilled water in a 100 ml Flask and was stirred till a clear solution was obtained. 5 ml salt solution of cupric acetate (0.2M) and 5 ml NaOH (0.5M) was added to above solution drop wise with continuous stirring to obtain a light blue color solution. For the reduction 5ml of 1M solution of different sugars (Glucose, fructose, sucrose and starch) were added to above solution and further sonicated until a Sol of fast blue color was obtained. The synthesized NP's were washed under several cycles of Centrifugation (REMI RPM=1000) with distilled water and 70 percent ethanol Synthesis NP's were kept in dark to avoid any photochemical reaction.



Scheme 4 Schematic pathway for synthesis of Cu nanoparticles by reducing sugars

3.2.2 Silver Nanoparticles

Silver nanoparticles (Ag NP's) were synthesized by gelatin- Sugar based reduction method according to Ramakrishna V. et al. The procedure of synthesis is systematically shown in scheme 4. For the synthesis of Ag-NPs, 0.5g of gelatin was added per 10mL of distilled water in a 100 ml flask and was stirred till a clear solution was obtained. 5 ml salt solution of $AgNO_3$ (0.2M) and 5 ml NaOH (0.5M) was added to above solution drop wise with continuous stirring to obtain a brown color solution. Now for reduction purpose 5ml of 1M solution of different sugars (Glucose, fructose, sucrose and starch) were added to above solution and further sonicated until a sol of reddish brown color was obtained. These solutions were kept in dark to avoid photochemical oxidation or reaction.



Scheme 5 Schematic pathway for synthesis of Cu nanoparticles by reducing sugars

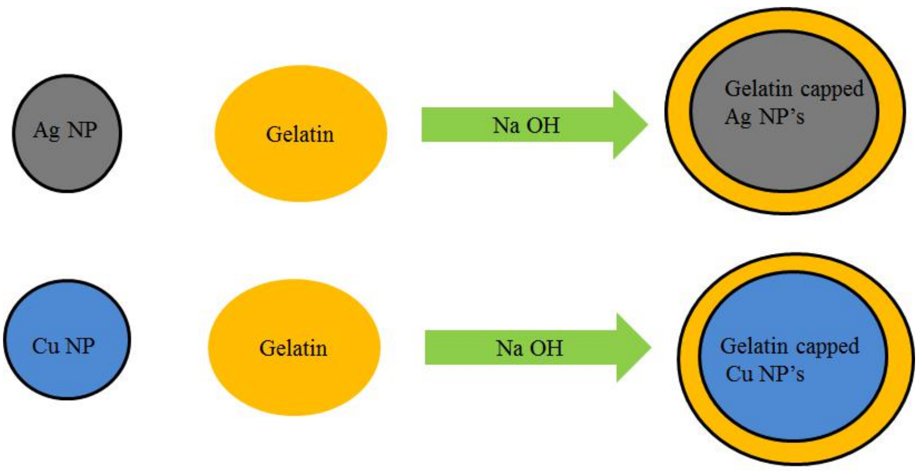


Fig.6 Capping of gelatin on silver and copper nanoparticles

3.3 Antibacterial Activity Assay

3.3.1 Media

Luria Bertani medium (LB) was used for the testing of anti-bacterial activities. The medium was prepared by dissolving Yeast extract – 0.5% (w/v)**,** Tryptone – 1.0% (w/v)**,** NaCl – 1.0% (w/v) and Agar – 1.5% (w/v) in 250 ml in distilled water. The flask was properly covered and autoclaved for 15 min at 121 psi.

3.3.2 Bacterial strains, culture conditions

The antibacterial properties of NP's were tested against bacterial Gram negative strain

Escherichia coli DH5 α. The strain *E.coli* DH5α was grown in Luria broth medium for overnight at 37^0C with 120 rpm shaking in an incubator. 100µl of *E.coli* culture from overnight grown culture was then inoculated in 50ml Luria broth, at 37^0C and 120 rpm for 4-5 hours. *E.coli* cells were taken from log phase of growth when the O.D.is 0.5 at 590 nm.

3.3.3 Preparation of LA plates

Luria Agar (LA) plates were prepared in glass petri plates under continuous laminar air flow and were kept there for solidification. After solidification the plates were wrapped with clin film and kept in incubator (37^0C) for 24 hrs.

3.3.4 Antibacterial activity by agar-well diffusion method

100 µl of *E.coli* strain was spread on LA plates with the help of sterile glass spreader under laminar air flow to avoid contamination. After spreading the plates were left for 1 hour for proper absorption or drying of *E.coli* culture. Then, in each plate 5 to 6 wells of 15 µl capacity was made with the autoclaved tips. For antibacterial activity 15 µl nanoparticles dispersion was added to the wells in each plate without overflow and was kept for 20 minutes. The plates were wrapped with Para film and incubated at 37^0C for overnight under proper culture conditions for growth of *E.coli* cells. The antibacterial assay was recorded after 24 hours and results clearly indicate that the given concentration of nanoparticle dispersion inhibit the bacterial growth, as zone of inhibition was observed.

3.4 Characterization of Nanoparticles

3.4.1 UV- Visible Spectroscopy

UV spectroscopy was carried out using double beam Analytikejena (Secord 205) spectrophotometer. 2ml solution of nanoparticles were taken in a cuvette (3.5ml quartz).The scan range was set between 190-1100nm and background was minimized using water.

UV-visible spectroscopy measures the intensity of light passing through a sample (*I)*, and compares it to the intensity of light before it passes through the sample (I_0).

The ratio I/I_0 is called the transmittance, and is usually expressed as a percentage (%T).The absorbance, *A* is based on the transmittance:

$$A = -\log (\%T/100)$$

3.4.2 Dynamic Light Scattering (DLS)

The hydrodynamic size distribution of as prepared NP;s dispersed in water was determined by using Brookhaven 90 plus particle size analyzer at 25^{o}C. It contains a 15 mW solid state laser operating at 635 nm and an avalanche photodiode detector. The stirred light was detected at an angle of 90^{0}.

3.4.3 Fluorescence

The fluorescence property of Cu and Ag NP’s were analyzed by Perkin-Elmer spectrofluorometer using xenon lamp as an excitation source. 2mL of sample in a cuvette was analyzed. Ag NPs excited at 225nm and Emission of Ag NPs at 328-345 and Cu NPs excited at 500nm and emission at 700 nm.

3.4.4 Transmission Electron Microscopy (TEM)

TEM technique was used to visualize the morphology of the nanoparticles and determination of the size, shape and arrangement of particles. TEM images were taken on the Hitachi 7500 model with resolution 2 A^{0} operating at voltage 120 kV.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Optical Properties

The optical properties of Cu and Ag NPs arising due to the SPR (surface Plasmon resonance) have been received noteworthy research attention in recent years because of their potential applications in diverse areas such as, biological sensors, photochromic, photoluminescence, bio-imaging, antimicrobial agents etc. [4-10]. SPR is the optical feature of metal NPs and can be defined as the resonance energy produced when the frequency of photons of the incident light matches with the frequency of the coherent oscillation of conduction electrons confined at the surface of the metal (Cu and Ag) NPs. Large particles the Plasmon band width increases with increasing size as the wavelength λ of the interacting light becomes comparable to the dimension of the nanoparticle [60-61].

Herein the absorption studies of as synthesized Ag and Cu NP's revealed an intense surface

Plasmon resonance(SPR) band at maximum wavelength ranging from 400-641nm. As illustrated in Fig.7 sugar reduced silver nanoparticles showed the characteristic absorption at 434nm, 423nm, 412nm, 400nm corresponding to sucrose, fructose, glucose and starch reduced silver nanoparticles with different colors (color panel). It was noted that Ag NP's synthesized via sucrose and fructose reduction method showed their absorption bands at higher wavelength than that of starch and glucose reduced NP's.

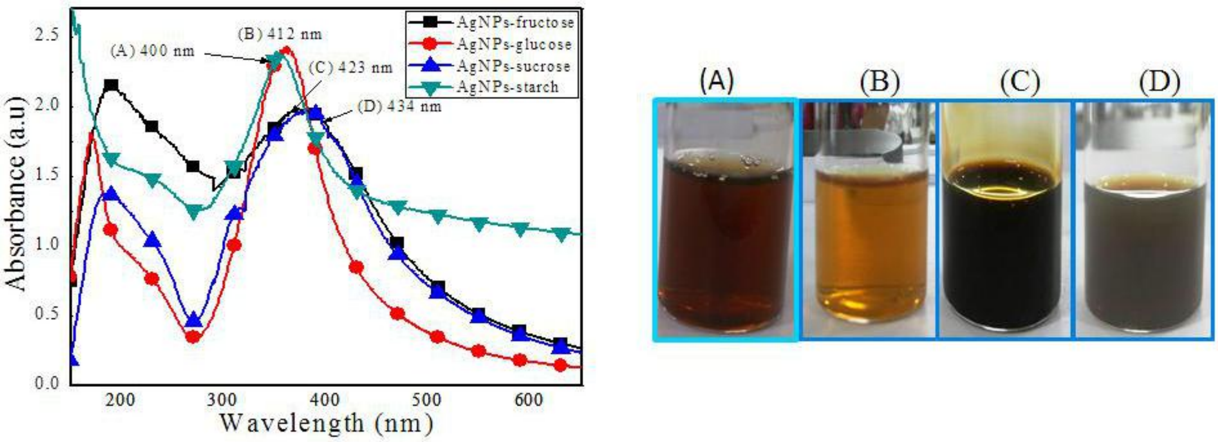


Fig.7 Sugar reduction mediated synthesis of Ag Nanoparticles

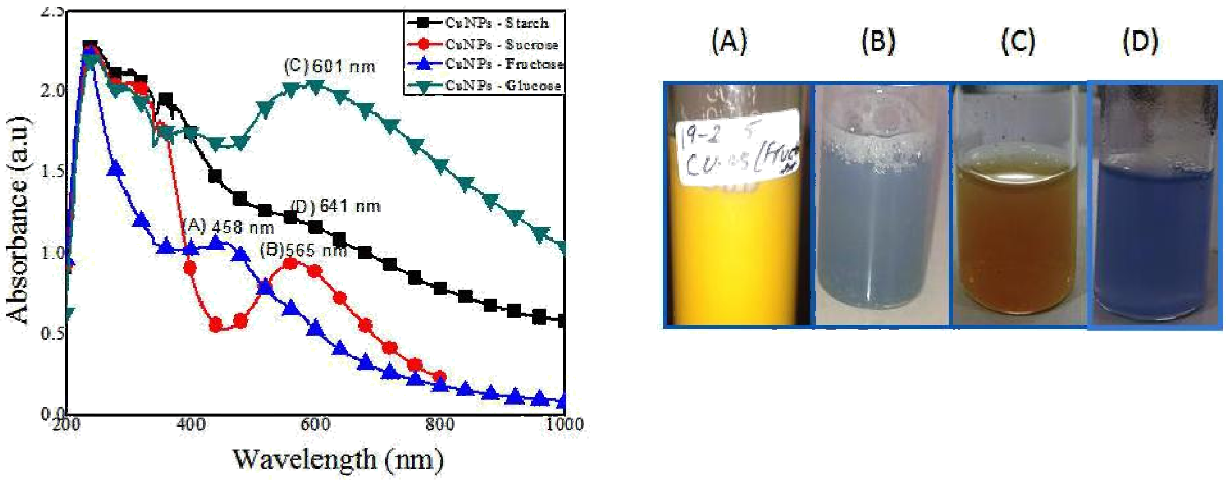


Fig.8 Sugar reduction mediated synthesis of Cu Nanoparticles

Similarly a characteristic SPR absorption bands were observed for Cu NP's. As shown in Fig. 8, with maximum wavelength 641nm, 601nm, 565nm, 458nm corresponding starch, glucose, Sucrose and fructose reduced copper nanoparticles. In case of synthesized copper nanoparticles the fructose reduced Cu NP's showed the absorption at 458 nm while a red shift was observed when the particles were synthesized by glucose (601 nm) as reducing agent. As it is clear from the color panel the nanoparticles of different size have been synthesized which is supported by TEM analysis discussed later in this chapter.

4.1.1 Effect of reducing sugars on SPR absorption of Cu and Ag NP's

The absorption maximum or SPR band of Cu and NP nanoparticles was found to be dependent on type of reducing sugar. As shown in Fig. 9 Cu NP's synthesized by glucose reduction showed absorption maximum at 601 nm while as in case of sucrose, fructose and starch the absorption maximum was 560, 458 and 458 nm respectively indicated that shift of SPR band depends on type of reducing sugar. Similarly in case of Ag NP's the reducing sugar alters the absorption maximum this might be because of formation nanoparticles of different size.

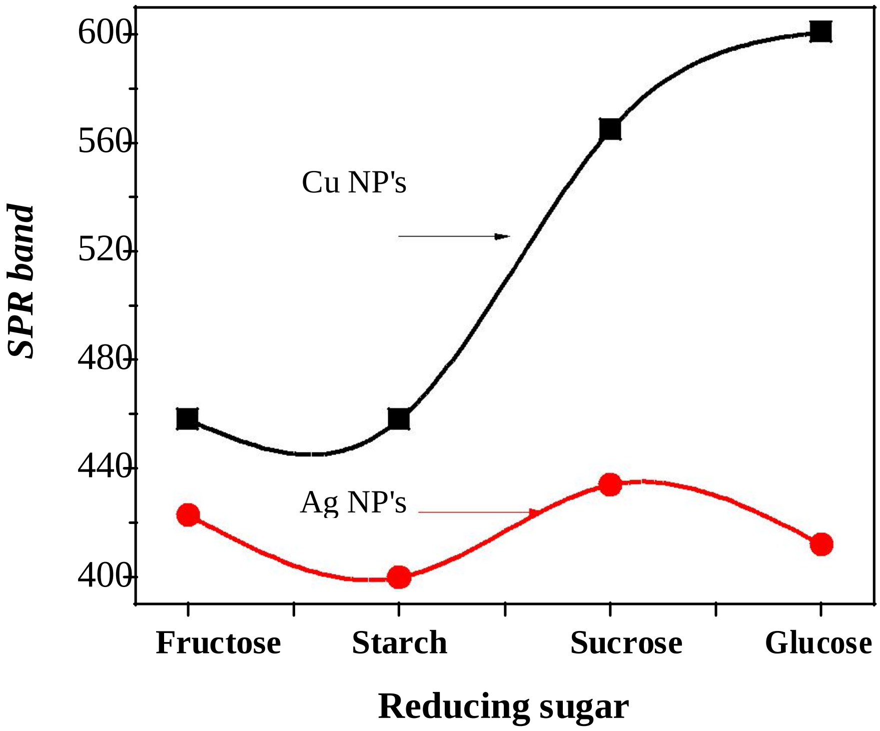


Fig.9 Effect of reducing sugars on SPR absorption of Cu and Ag NP’s

4.2 Fluorescence

The change in the photo-luminescent behavior of synthesized silver and copper nanoparticles on its surface was studied by measuring their fluorescence emission spectra. The emission maximum of silver nanoparticle was observed at 328-345 nm on excitation at 225nm. The emission maximum of copper nanoparticles was observed at 700 nm on excitation at 225 nm as shown in the Fig. 10. Strong emission was seen in case of Cu NP's (fructose) and Ag NP's (Glucose) while as weak fluorescence emission was observed in Cu NP's (sucrose) may be because of radiation less recombination of charge carriers or quenching effect.

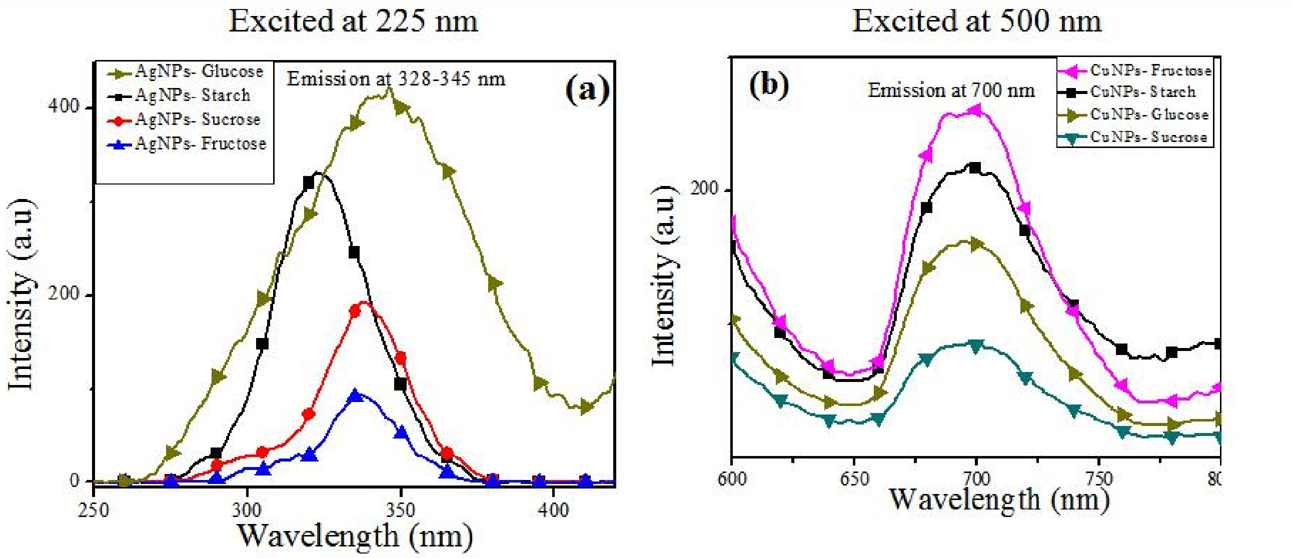


Fig.10 Fluorescence emission spectra of Ag and Cu NP's

4.3 Dynamic Light Scattering (DLS)

Also known as photon correlation spectroscopy is a technique that can be used to determine the size distribution of nanoparticles in solution or suspension. The observed shifts in absorption bands shown in (Fig. 7-8) are attributed to different size and distribution of nanoparticles. As shown in Fig. 11. starch reducing nanoparticles shows highest particle size starting from 43 nm to 76 nm similarly DLS studies glucose reducing nanoparticles shows particle size starting from 20 nm to 38 nm, sucrose reducing nanoparticles shows particle size starting from 20 nm to 39 nm and fructose reducing nanoparticles particle size starting from 10 nm to 20 nm.

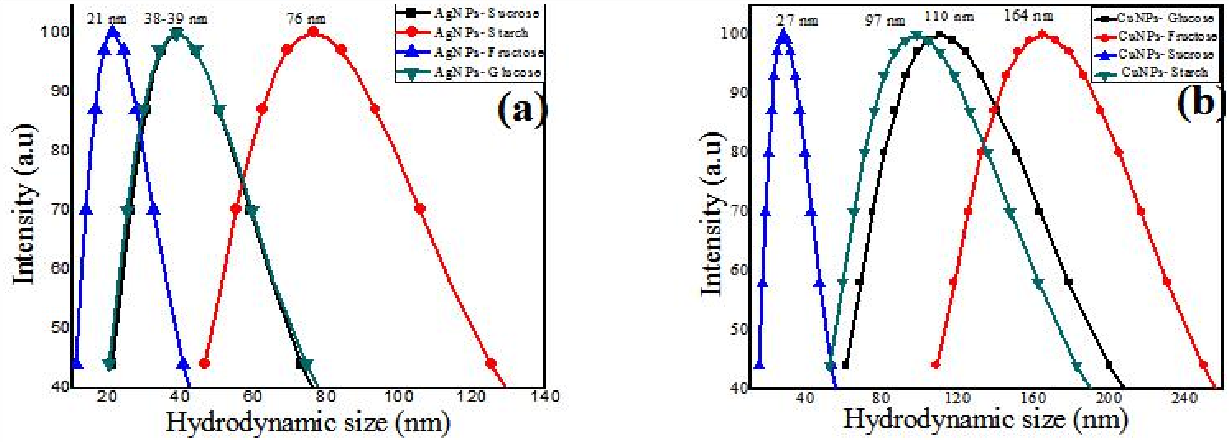


Fig. 11A Hydrodynamic size of Ag and Cu Nanoparticles

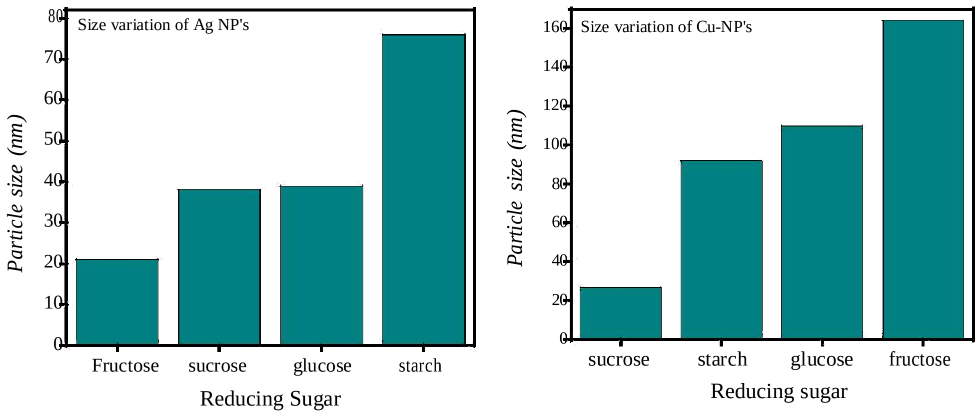


Fig. 11B Particle size distribution

4.4 Morphological Studies

Using gelatin as the reaction stabilizer the reduction of metal salts of silver and copper resulted in the formation of spherical nanoparticles as shown in Fig. 12(a, b and c). The morphological characteristics like shape, size and arrangement of Ag and Cu nanoparticles were studied by transmission electron microscopy (TEM). TEM results revealed the well dispersed silver and copper nanoparticles over gelatin coating. As shown in Fig. 12a Ag nanoparticles produced by fructose shows spherical shape but a little aggregation was seen. Average size of these nanoparticles was determined by simple particle distribution method and for fructose reduction method the average size was of 6.52nm.

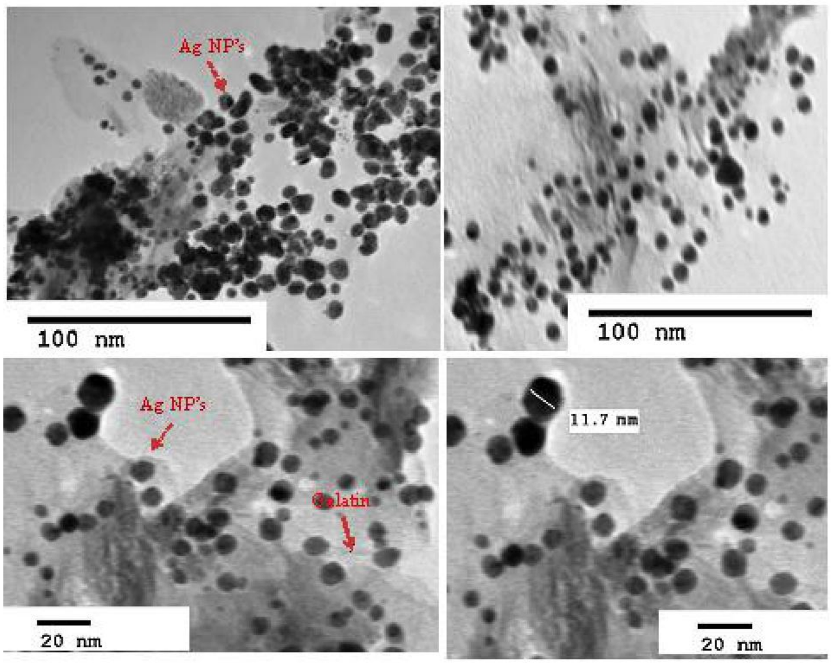
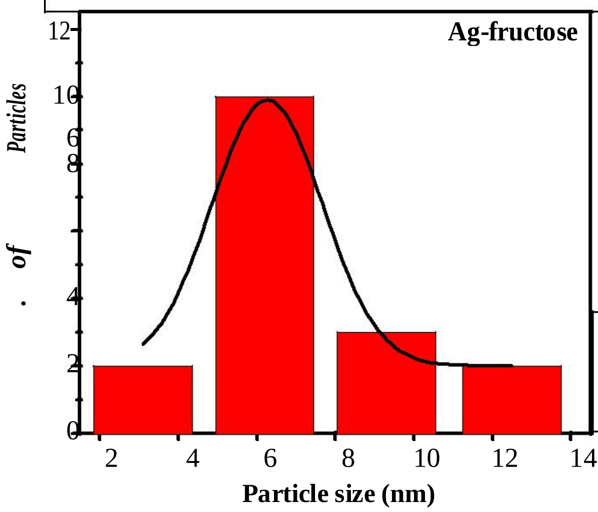


Figure 12a TEM Images of silver nanoparticles



Particle size distribution of Fructose-based Ag NP's

In case of Cu NP’s TEM studies reveal uniform and well dispersed particles (Fig. 12b and 12c) average size of Cu NP’s with glucose reduction method is 41nm. Cu NP’s glucose has smaller average size of nanoparticles then Ag NP’s fructose and Cu NP’s sucrose (65nm). It was also observed that no correlation was found between both size and shape of the nanoparticles produced with the functional groups (aldehyde or ketone) present in the monosaccharaides or disaccharides or with the C length of the sugars.

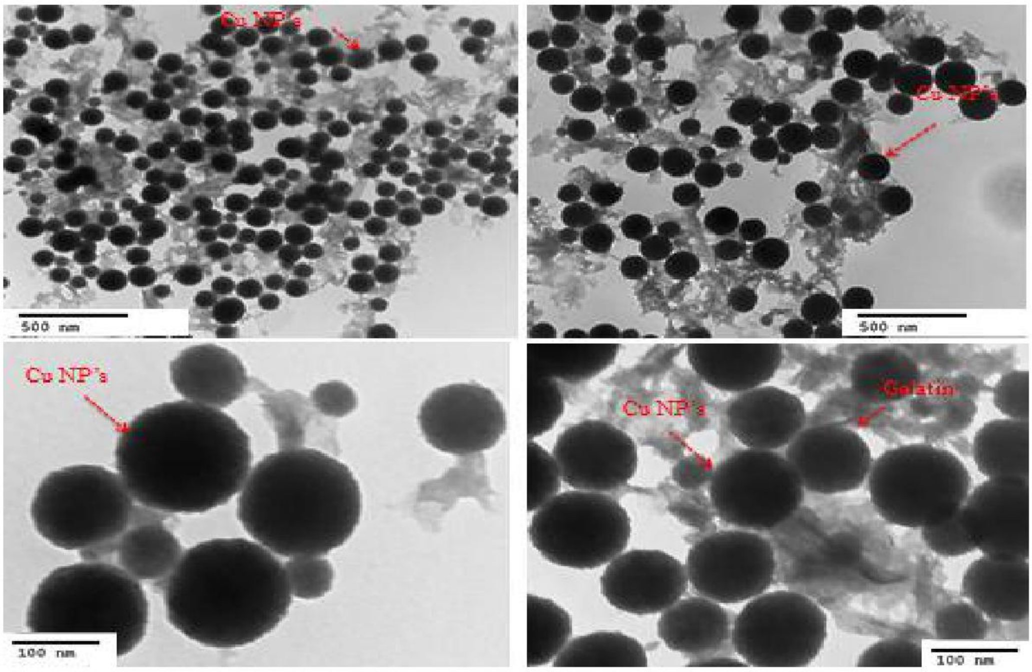
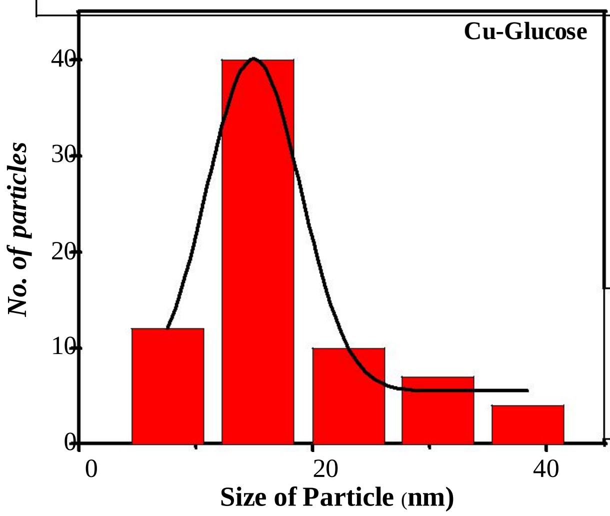


Fig. 12b TEM Images of Cu Nanoparticles (Glucose)



Particle size distribution of

Glucose-based Cu NP’s

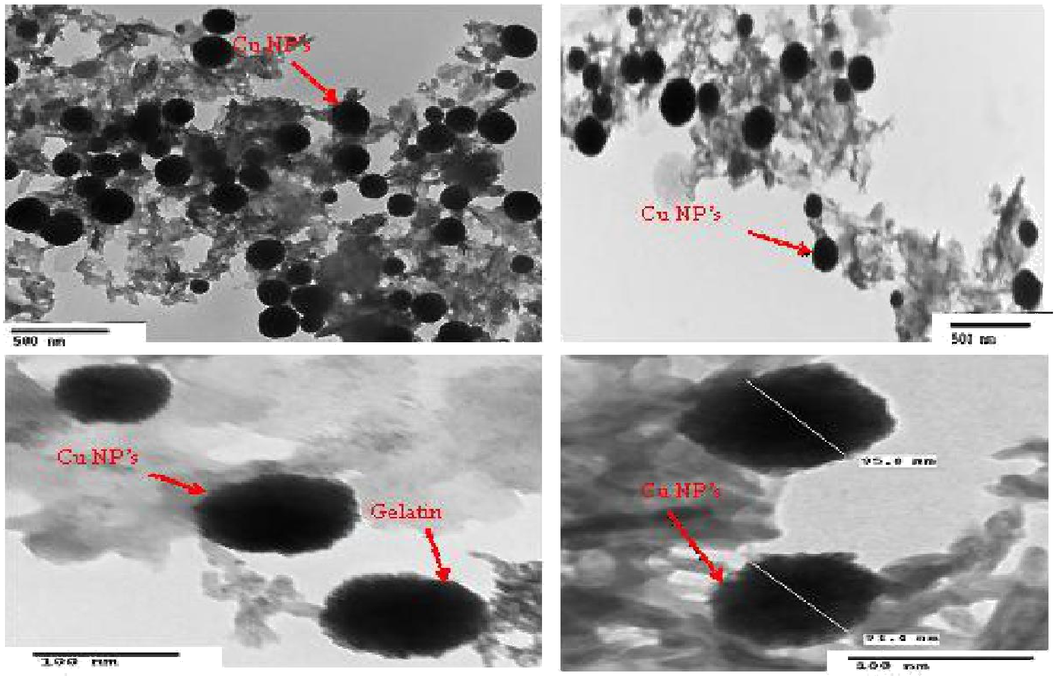
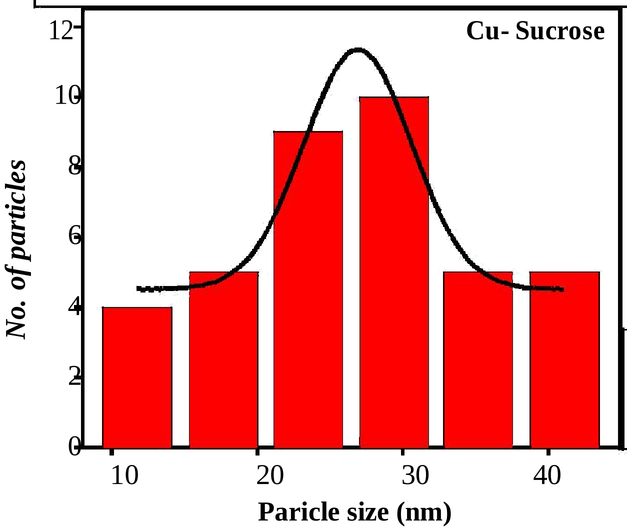


Fig. 12c TEM Images of Cu Nanoparticles (Sucrose)



Particle size distribution of Sucrose-based Cu NP’s

4.5 ANTIMICROBIAL EFFECT

During last few decades' extensive research has been done on metallic nanoparticles such as silver, gold, copper, copper oxide to study their applications in all trades of science [34]. Due to higher bacterial infections there is a necessity to develop powerful antibacterial agents using various metallic nanoparticles [62]. Antibacterial metallic nanoparticles are being applied in the areas of food preservation, medical [29-30], cosmetics, and diagnostic devices, burn dressing and waste water treatments [31]. Antibacterial effect of metallic nanoparticles depends upon its size, shape, morphology, size distribution, functionalization of surface and stability [45].

From last few decades copper and silver nanoparticles are used as antibacterial agents. They have revealed a strong antibacterial activity which is able to decrease the microorganism's concentration by 99.9% [33]. Copper and silver nanoparticles are known to exhibit a wide range of antibacterial activities against different strains of gram negative and gram positive bacteria.

The US Environmental Protection Agency [EPA] has approved registration of copper as antimicrobial agent that is able to reduce specific harmful bacteria linked to potentially deadly microbial infections [63].

In this study antibacterial property of silver and copper nanoparticles was checked against gram negative bacteria *E. coli* (DH5α) on Luria agar (LA) plates. Silver ions show antibacterial activity by interacting with thiol group of vital enzymes and inactivating them. Once the bacteria are treated with silver ions, DNA loses its replication ability (Morones et al., 2005) which leads to death of bacteria [64]. We removed silver ions as much as possible by washing Ag NP's extensively with double distilled water and with 70 percent ethanol to avoid antimicrobial effects resulting from silver ions.

Copper nanoparticles have high potency for bacterial cell filamentatation (formation of filaments) and cell killing. A result demonstrates that nanoparticles mediated dissipation of cell membrane potential was the probable reason for the formation of cell filaments. Copper nanoparticles cause DNA degradation in *E. coli* cells [64]. Gelatin plays an important role in the rupturing of the cell wall of bacteria. Gelatin is act as a stabilizing molecule which was expected to be advantageous for interaction of the particles with cell membranes and their subsequent entry into the cell cytosol [65].

4.5.1 Antibacterial Activity of the Carbohydrate-based Nanoparticles

The effects of silver and copper nanoparticles as synthesized by using same concentration of different carbohydrates were studied on *E. coli*. For each metallic nanoparticle, 15 µl solutions were used in the agar-well diffusion method.

Starch-based Ag NPs: Starch nanoparticle showed clear and relatively larger zone of inhibition as compared with nanoparticles synthesized by using glucose, fructose and sucrose. In this case, average diameter of the zone of inhibition was found to be ~ 1.82 cm as shown in the Fig. 13A.

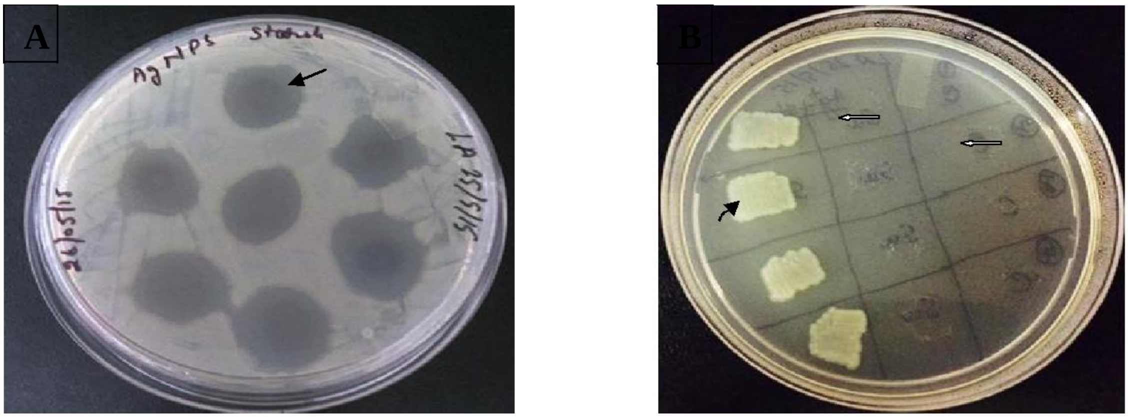
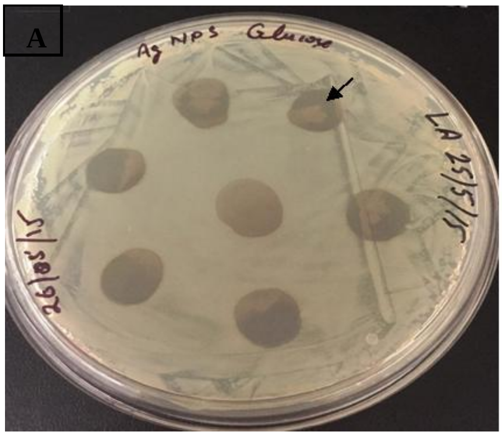


Fig. 13 Antibacterial property of starch- based Ag NPs

A Formation of zone of inhibition (indicated by →); **B** Streaking from different parts of the inhibition zones (indicated by⟹) and the surrounding region as indicated by curved arrow to assess the viability of *E. coli* cells

In order to assess the viability *of E. coli* cells**,** streaking was done from the different parts of the inhibition zones and the surrounding region. No growth was observed in the cases of streaking from both the inhibition zone and centre of the inhibition zone; whereas proper growth was noted if streaked from the surrounding region as expected (Fig. 13B). The result clearly showed the killing effect of the starch based nanoparticles.

Glucose-based Ag NPs: in case of glucose-based silver nanoparticle showed clear zone of inhibition. In this case, average diameter of the zone of inhibition was found to be ~ 1.35 cm as shown in the Fig. 14A.



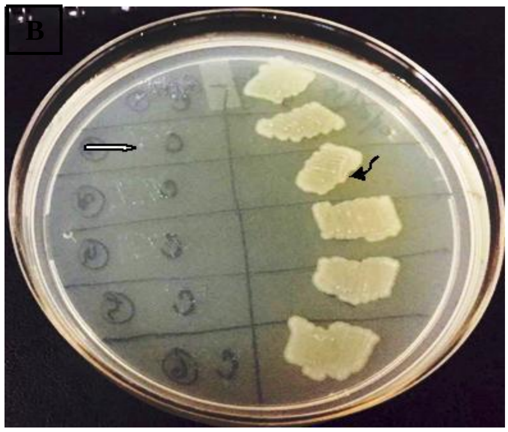


Fig.14 Antibacterial property of glucose-based Ag NP's

A Formation of zone of inhibition (indicated by →); B Streaking from the inhibition zone (Indicated by ⇨), and the surrounding region as indicated by curved arrow to assess the viability of *E. coli* cells

In order to assess the viability *of E. coli* cells**,** streaking was done from the inhibition zone and the surrounding region. No growth was observed in the cases of streaking from the inhibition zone and centre of the inhibition zone; whereas proper growth was noted if streaked from the surrounding region as expected (Fig. 14B). The result clearly showed the killing effect of the glucose-based nanoparticles.

Fructose-based Ag NPs: In the Ag NP's fructose plates, the zone of inhibition was clearly observed. In this case, average diameter of the zone of inhibition was found to be ~ 1.26 cm as shown in the Fig. 15A.

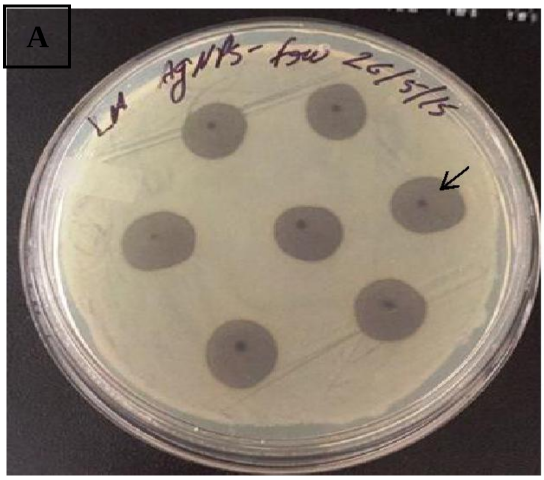


Fig. 15 Antibacterial property of fructose-based Ag NP's

A Formation of zone of inhibition (indicated by→); B Streaking from the inhibition zone (Indicated by ⟹), and the surrounding region as indicated by curved arrow to assess the viability of *E. coli* cells

In order to check the viability of *E. coli* cells**,** streaking was done from the inhibition zone and the surrounding region. No growth was observed in the cases of streaking from the inhibition zone; whereas proper growth was noted if streaked from the surrounding region as expected (Fig. 15B). The result clearly showed the killing effect of the fructose-based nanoparticles.

Sucrose-based Ag NPs: sucrose-based silver nanoparticle showed clear zone of inhibition. In this case, average diameter of the zone of inhibition was found to be ~ 1.21 cm as shown in the Fig. 16A.

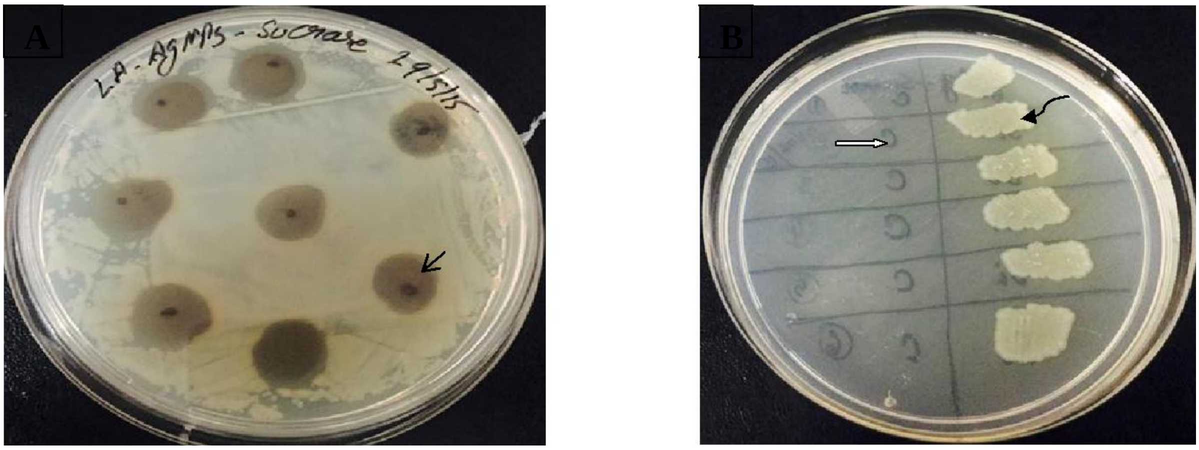


Fig. 16 Antibacterial property of sucrose-based Ag NP’s

A Formation of zone of inhibition (indicated by →); B Streaking from the inhibition zone (Indicated by ⇒), and the surrounding region as indicated by curved arrow to assess the viability of *E. coli* cells

Further, In order to check the viability of *E. coli* cells**,** streaking was done from the inhibition zone and the surrounding region. No growth was observed in the cases of streaking from the inhibition zone; whereas proper growth was noted if streaked from the surrounding region as expected (Fig. 16B). The result clearly showed the killing effect of the sucrose-based nanoparticles.

Glucose-based Cu NPs: Similarly, glucose-based copper nanoparticles showed clear and relatively larger zone of inhibition as compared with Cu NPs synthesized by using fructose, sucrose and starch. In this case, average diameter of the zone of inhibition was found to be ~ 1.2 cm as shown in the Fig. 17A.

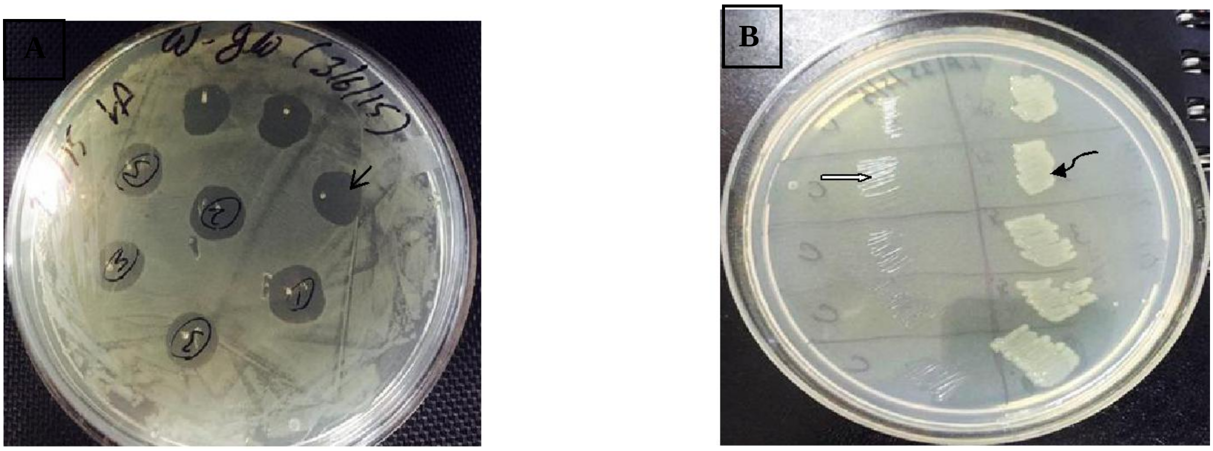


Fig. 17 Antibacterial property of glucose-based Ag NP's

A Formation of zone of inhibition (indicated by →); B Streaking from the inhibition zone (Indicated by ⟹), and the surrounding region as indicated by curved arrow to assess the viability of *E. coli* cells

In order to assess the viability *of E. coli* cells**,** streaking was done from the inhibition zone and the surrounding region. No growth was observed in the cases of streaking from the inhibition zone and; whereas proper growth was noted if streaked from the surrounding region as expected (Fig.17B). The result clearly showed the killing effect of the glucose-based copper nanoparticles.

Fructose-based Cu NPs: in case of fructose-based copper nanoparticle showed clear zone of inhibition. In this case, average diameter of the zone of inhibition was found to be ~ 1.19 cm as shown in the Fig. 18A.

Fig.18 Antibacterial property of fructose-based Ag NP's

A Formation of zone of inhibition (indicated by →); B Streaking from the inhibition zone (Indicated by ⟹), and the surrounding region as indicated by curved arrow to assess the viability of *E. coli* cells

In order to assess the viability *of E. coli* cells**,** streaking was done from the inhibition zone and the surrounding region. No growth was observed in the cases of streaking from the inhibition zone and; whereas proper growth was noted if streaked from the surrounding region as expected (Fig.18B). The result clearly showed the killing effect of the fructose-based copper nanoparticles.

Sucrose-based Cu NPs: in case of sucrose-based copper nanoparticle some growth of cells were observed in clear zone of inhibition. In this case, average diameter of the zone of inhibition was found to be ~ 0.76 cm as shown in the Fig. 19A.

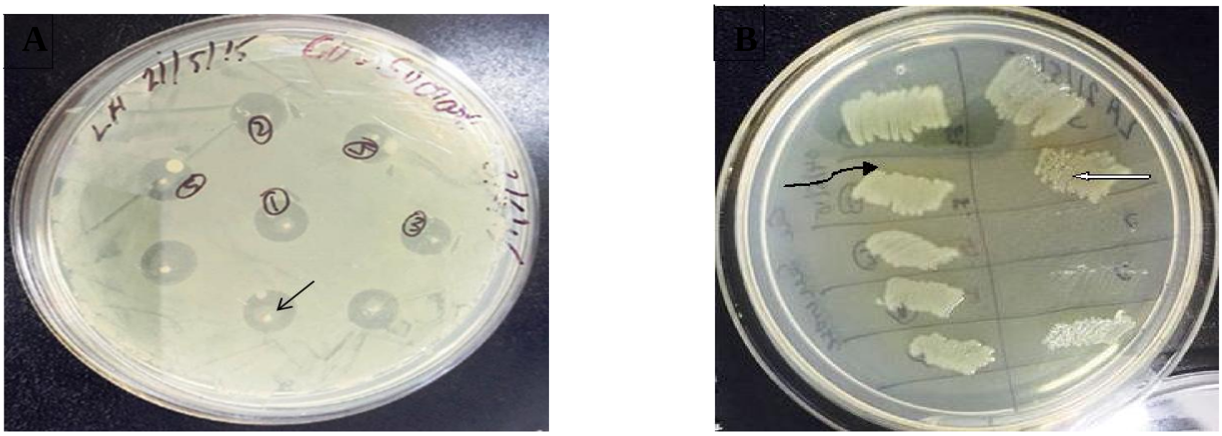


Fig.19 Antibacterial property of sucrose-based Ag NP's

A Formation of zone of inhibition (indicated by →); B Streaking from the inhibition zone (Indicated by ⇒), and the surrounding region as indicated by curved arrow to assess the viability of *E. coli* cells

In order to assess the viability *of E. coli* cells**,** streaking was done from the inhibition zone and the surrounding region. Growth was observed in the cases of streaking from the inhibition zone and; whereas proper growth was noted if streaked from the surrounding region as expected as shown in the (Fig. 19B). The result clearly showed that fructose-based copper nanoparticles are not capable to show any antibacterial property.

Starch-based Cu NPs: In the Cu NP's starch plate no zone of inhibition was observed. This indicates the Cu NP's sucrose has no antibacterial property as compared with other Ag NP's and Cu NP's as shown in the Fig.20.

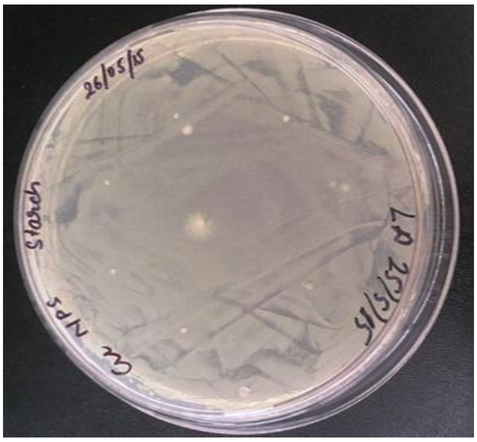


Fig.20 Cu NP's doesn't show any antibacterial property

Table 1 Sizes of the zone of inhibition using different carbohydrate-based silver nanoparticles

Carbohydrates used in silver nanoparticles	Zone of inhibition (diameter in cm)
Starch	1.82 cm
Glucose	1.35 cm
Fructose	1.26 cm
Sucrose	1.21 cm

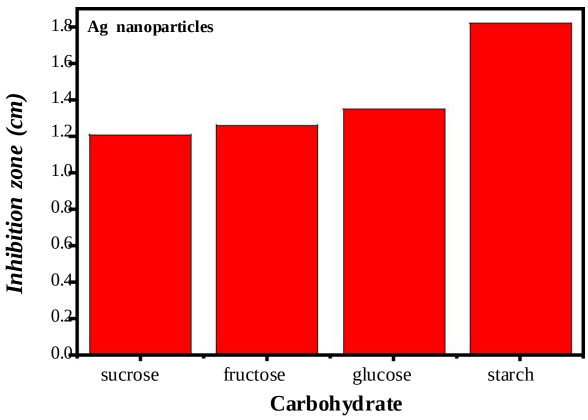


Fig.21 lowest to highest zone of inhibition (Diameter in cm) of Ag NPs

Table 2 Sizes of the zone of inhibition using different carbohydrate-based copper nanoparticles

Carbohydrates used in copper nanoparticles	Zone of inhibition (diameter in cm)
Fructose	1.19 cm
Glucose	1.2 cm
Sucrose	0.76 cm
Starch	Noclearzone formation.

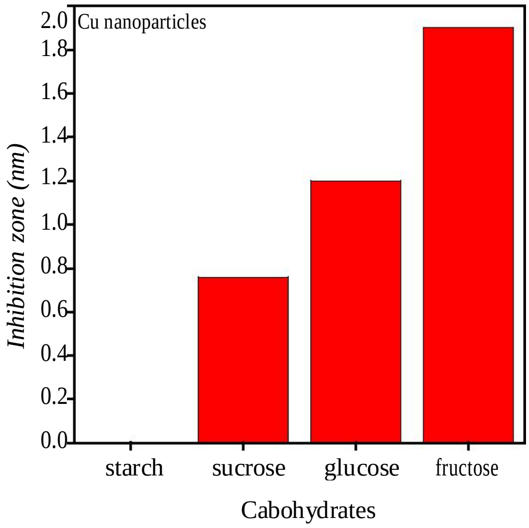


Fig.22 lowest to highest zone of inhibition (Diameter in cm) of Cu NPs

Table 3 Comparison between Ag NP's and Cu NP's in terms of their bactericidal activity

	Ag NP'	***Cu NP's***
Glucose	1.35 cm	1.2 cm
Sucrose	1.21 cm	0.76 cm
Fructose	1.26 cm	1.19 cm
Starch	1.82 cm	______

CONCLUSION

In the present investigation, we conducted an in-depth study on the synthesis and characterization of copper and silver nanoparticles synthesized by different carbohydrates, and checked their antibacterial effect by using agar-well diffusion method. There are many ways involved in the synthesis of nanoparticles. Physical and chemical methods of nanoparticle synthesis were being followed over the decades but they are expensive and sometimes toxic to environment and human health. Currently, biological or green synthesis is more preferred option.

Here, silver and copper nanoparticles were synthesized using green synthesis method. Different carbohydrates such as glucose, sucrose, fructose and starch were used successfully for the synthesis of respective nanoparticles having well-defined sizes. The individual reactions went to completion in less than 20 min at room temperature. The advantages of the procedure are:

(1) fast reaction time; (2) mild conditions; (3) cost effective; (4) aqueous solution; (5) good size control; (6) no harsh/ harmful chemicals involved. Synthesis in neutral aqueous solution at room temperature is attractive for biological applications. These carbohydrates are biological molecules and available in large quantities in nature. This method was easy and simple to synthesize high purity of Ag NPs and Cu NPs with different carbohydrates (glucose, sucrose, fructose and starch). In the reduction of aqueous $AgNO_3$, NaOH was used as a reaction accelerator. Gelatin controls the agglomeration of the nanoparticles and act as a stabilizing agent. It is a biopolymer which is biocompatible and biodegradable. Gelatin capping may help their easy attachment with biological systems.

UV-visible spectroscopy had shown a shift of the SPR peak depending upon the different average particle size of Ag NPs and Cu NPs synthesize by different carbohydrates. Visible photoluminescence maximum emission of Ag NPs was observed at 328-345 nm and Cu NPs at 700 nm. The structural and morphological characterization of the samples was performed using zeta DLS and TEM. Silver and Copper nanoparticles has always been an excellent antimicrobial property and used for the purpose for ages. There are many mechanisms attributed to the antimicrobial activity shown by silver and copper nanoparticles, the actual and most reliable mechanism is not fully understood.

The carbohydrate-based nanoparticles as prepared in this study were found to be effective in growth inhibition of Gram-Negative bacteria namely *E. coli*; hence showed their antibacterial activity. Starch-based Ag NPs exhibited strong inhibitory effect as compared with other carbohydrate-based Ag NPs and Cu NPs. The diameters of the individual zone of inhibition were noted and compared in this report. Strikingly, starch-based copper NPs showed practically no inhibitory effect on the growth of *E. coli*. The data clearly suggested that carbohydrate-based silver nanoparticles appear to be more effective in terms of bactericidal activities as compared to carbohydrate-based copper nanoparticles (except fructose-based Cu NP). It is very likely that overall size and shape of the carbohydrate-based nanoparticles are crucial in terms of their inhibitory effect on the microbes. Underlying molecular mechanism of antibacterial activities of the individual carbohydrate-based nanoparticles need to be further understood.

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