

Biosynthesis, Characterization, Antimicrobial Activity Of Copper Nanoparticles Using Fresh Aqueous Ananas Comosus L. (Pineapple) Extract

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Abstract: The synthesis of metal nanoparticles is a growing area for research due to its potentiality in the application and development of advanced technologies. In general, nanoparticles are synthesized by using chemical methods which are not eco-friendly. In this study we used the fast, convenient, eco-friendly method for synthesis of copper nano particles by using the fresh aqueous pineapple extract.

The fresh aqueous pineapple extract was mixed with copper sulphate solution by heating to a temperature of 60-80°C and under optimum condition at pH 11. The reduction reaction was studied by observing the color change from blue to reddish brown. Water soluble antioxidant constituents present in the fresh aqueous pineapple extract were mainly responsible for the reduction of copper ions to nano sized Cu particles.

The resulting copper nanoparticles were characterized by UV Visible Absorption Spectrometer, powder X-Ray Diffraction (PXRD), Fourier Transform Infrared (FTIR), Scanning Electron Microscopy (SEM) and EDAX analysis. UV-Vis spectral analysis showed Copper nanoparticles exhibit an absorption peak at around 576 nm. X-ray diffraction analysis shows that the crystalline morphology of nanoparticles are FCC in nature. The FTIR spectrum analysis has confirmed the presence of functional groups and active proteins in the biomass before and after reduction. SEM results display the formation of copper nanoparticles with an average size of 30-50 nm. The presence of elemental copper was characterized by EDS. The antibacterial and antifungal activity of these nanoparticles was studied against E-Coli, Staphylococcus aureus, Aspergillus niger and Trichoderma viride. The biosynthesized nanoparticles are quite stable in aqueous solution for a month without any sign of precipitation.

Keywords: Eco-friendly, copper nanoparticles, fresh aqueous pineapple extract, UV-Vis, FT-IR, EDS, SEM, Antimicrobial activity, Antioxidant.

Introduction

The term **Nanotechnology** comes from the combination of two words: the Greek numerical prefix nano referring to a billionth and the word technology. As an outcome, Nanotechnology or Nanoscaled Technology is generally considered to be at a size below 0.1 μ m or 100 nm (a nanometer is one billionth of a meter, 10⁻⁹ m). Nanoscale science (or nanoscience) studies the phenomena, properties, and responses of materials at atomic, molecular, and macromolecular scales, and in general at sizes between 1 and 100 nm. In this scale, and especially below 5 nm, the properties of matter differ significantly (i.e., quantum-scale effects play an important role) from that at a larger particulate scale. Nanotechnology is considered an emerging technology due to the possibility to advance well-established products and to create new products with totally new characteristics and functions with enormous potential in a wide range of applications. In addition to various industrial uses, great

innovations are foreseen in information and communication technology, in biology and biotechnology, in medicine and medical technology, in metrology, etc. Significant applications of nanosciences and nanoengineering lie in the fields of pharmaceuticals, cosmetics, sports, therapy, automotives, environmental monitoring, fuel cells and energy devices, water purification, food and beverage industry, chemical engineering, high-performance materials, electronics, precision mechanics, optics, and environmental sciences.

Among the various nanoparticles, metal nanoparticles assume special importance because they are easier and cheaper to synthesis and are most promising in application. Synthesis of copper nanoparticles is of great interest because of many advantages. Copper is highly conductive and also less expensive than silver and gold. Copper nanoparticles have wide applications as heat transfer system, antimicrobial materials, super strong materials, sensors and catalysts. Copper nanoparticles are very reactive because of their high surface-to-volume ratio and can easily interact with other particles and increase their antimicrobial efficiency.

However, aggregation appears immediately due to surface oxidation are the main problems concerned with copper nanoparticles. But, we can overcome these problems by selecting suitable stabilizer for capping of copper nanoparticles [1].

Copper is most widely used material in the world due to their electrical, optical, catalytic, biomedical and antifungal/antibacterial applications among various metal particles such as gold, silver, iron, palladium, zinc and quantum dots.

Copper can give more yields and reaction rate in mild reaction conditions when compared to other traditional catalysts [2]. Green synthesis of colloidal copper nanoparticles is used for treating the rubber tree which is affected by pink disease [3].

The need for biosynthesis of nanoparticles rises because the physical and chemical processes are costly, hazardous, longer time, tedious process to isolate nanoparticles. This green synthesis method have several advantages over other methods namely cost effectiveness, simplicity, use of less temperature, the usage of less toxic materials, moreover it is compatible for medical and food applications. Therefore, in the search for cheaper pathways for Nano particle synthesis, scientists used microorganisms and then plant extracts for synthesis.

Many researchers used green synthesis methods for different metal nanoparticles due to their growing need of eco-friendly properties. Green synthesis method was found to be the best method when compared to the other method such as chemical reduction, photochemical reduction, electrochemical reduction, heat evaporation etc., In this method, the plant extract has been used as capping and reducing agent for the synthesis of copper nanoparticles due to their reducing properties present in the leaf and fruit extract.

Ananas comosus L. belongs to Bromeliaceae family, a large, diverse family of about 2000 species and is a subtropical fruit native to Thailand, Phillipines, China, Brazil and India.

Pineapple has several beneficial properties including antioxidant activity. It contains mixture of protease enzymes which acts as a nutritional supplement to “promote digestive health” and used as an anti-inflammatory medication.

Phytochemicals, especially phenolics, in fruits and vegetables are suggested to be the major bioactive compounds for the health benefits [4]. Pineapple contains phytochemicals which include antioxidant substances that fight against free radical cell damage and have been commonly used in traditional Chinese medicine [5]. Polyphenols possess outstanding antioxidant and free radical scavenging properties, suggesting a possible protective role in humans [6,7]. Their antioxidant potential is closely related to the number of hydroxyls, the higher the number, the more potent the chain breaking antioxidant action of the compound. Plant phenolics are the largest class of plant secondary metabolites, which serve in plant defense mechanism to counteract reactive oxygen species (ROS) in order to survive and prevent molecular damage.

Antimicrobial Applications

Copper as an antimicrobial agent which is able to reduce specific harmful bacteria linked to potentially deadly microbial infections (European Copper Institute, 2008). In addition, no research has discovered any bacteria are able to develop immunity to copper as they often do with antibiotics. Copper nanoparticles act as antimicrobial agent in various fields. The copper is highly toxic to microorganism such as bacteria (E-Coli, Staphylococcus Aureus, Pseudomonas aeruginosa) and non-toxic to animal cells, due to these phenomena it is considered to be an effective bactericidal metal. It is consider safe for human beings in food package and in water treatment applications. CuNps with bactericidal properties can be immobilized and coated on to the surface find application in water treatment and in medical instrument and devices as sterilizing agent respectively.

Keeping this in view the present work is focused for the first time to carry out the biosynthesis of copper nanoparticle using fresh aqueous pineapple extract and to evaluate the antifungal and antibacterial activities against human pathogens.

Materials and Methods

Pineapple, the sample for the biosynthesis of the copper nanoparticles was procured from the local supermarket (Fig.1). The bacterial and fungal strains employed in this work were procured from Microbial Type Culture Collection Centre (MTCCC) located at the institute of microbial technology, Chandigarh, India.



Fig.1: Photograph of fresh pineapple fruit

Scientific classification of pineapple

Table-1:- Scientific Classification of pineapple

| | |
|---------------|---------------------------------------|
| Kingdom | Plantae – Plants |
| Subkingdom | Tracheobionta – Vascular plants |
| Superdivision | Spermatophyta – Seed plants |
| Division | Magnoliophyta – Flowering plants |
| Class | Liliopsida – Monocotyledons |
| Subclass | Zingiberidae |
| Order | Bromeliales |
| Family | Bromeliaceae – Bromeliad family |
| Genus | Ananas Mill. – pineapple |
| Species | Ananas comosus (L.) Merr. – pineapple |

Preparation of sample extract

50g of pineapple fruit was accurately weighed, thoroughly washed under running tap water followed by washing it with double deionized water to remove surface impurities. They were crushed using a blender and finely macerated. After homogenization 100ml of double deionized water was added and heated over a water bath maintained at 80 degree Celsius for 15 minutes. The extract obtained was filtered through muslin cloth and then through whatmann no: 1 filter paper and used immediately for the biosynthesis of copper nanoparticles.

Biosynthesis of copper nanoparticles

The four step preparation scheme for copper nanoparticles starts with dissolving copper sulphate (0.02M) in deionised water to obtain a blue solution. Next, poly ethylene glycol 6000 (PEG 6000) 0.1 M was dissolved in water and added to the aqueous solution containing the copper salt with vigorous stirring. In this step, solution colour changes from blue to white. In the third step, fresh pineapple extract was added to the CuSO₄ solution containing PEG 6000. The colour change occurred in the aqueous phase from white to blue. Finally 0.1 M sodium hydroxide were added to the solution under continuous rapid stirring and instant colour change started to occur in the aqueous phase from blue to green in cold condition. The appearance of this colour indicates that the reduction has started. The formation of copper nanoparticle is confirmed by the colour change from green to reddish brown when it is kept on the waterbath at 80°C. The formation of copper nanoparticles is strongly inferred by visual observation followed by UV-Visible spectrum, FTIR, SEM, XRD and EDAX studies.

Fixation of parameters for biosynthesis of copper nanoparticles

❖ Biosynthesis of copper nanoparticles using different composition

(volume ratio of CuSO₄ : Extract):-

The biosynthesis of copper nanoparticles was carried out at different volume ratio of copper sulphate and extract (1:1, 1:2, 1:3, 1:4) and vice versa at pH 11. The time taken for the colour change in the reaction mixture as well as the formation of nanoparticle was monitored by visual inspection and also by UV-Visible spectrometer.

❖ Biosynthesis of copper nanoparticles at different temperatures:-

The biosynthesis of copper nanoparticles was done at different temperatures namely; room temperature and at 80°C over water bath and the time taken for visual colour change from green to reddish brown was recorded followed by recording the UV-Visible spectrum.

❖ Biosynthesis of copper nanoparticles at different pH:-

The biosynthesis of copper nanoparticles was carried out at different pH viz 8, 9, 10, 11, 12. The time taken for colour changes as well as the UV-Visible spectrum for the reaction mixture was monitored.

❖ Biosynthesis of copper nanoparticles at different intervals of time:-

The synthesis was carried out at pH 11 in the ratio 1:4 (CuSO₄: Extract) and the time taken for the formation was noted at the interval of every 30 minutes. The completion of the reaction was monitored by the colour change as well as recording the UV-Visible spectrum.

❖ Biosynthesis of copper nanoparticles in presence of peg 6000:-

The biosynthesis of copper nanoparticles was carried at pH 11 in the ratio 1:4 with and without PEG 6000 and completion of the reaction was monitored by the colour change as well as the UV-Visible spectrum.

❖ Biosynthesis of copper nanoparticles in the presence of starch:-

The biosynthesis of copper nanoparticles was carried out at pH 11 in the ratio 1:4 using three different concentration of freshly prepared starch viz (1 %, 2%, 3%). Completion of the reaction was monitored by the colour change as well as the UV-Visible spectrum.

❖ **Stability of copper nanoparticles:-**

The stability of the colloidal solution of copper nanoparticles was determined at room temperature at the interval of 24 hrs for 15 days.

Characterization of biosynthesized copper nanoparticles:-

❖ **Visual inspection:-**

The bio reduction of the copper sulphate using aqueous pineapple extract was monitored and the appearance of reddish brown colour indicates the formation of copper nanoparticles. Photograph of the copper nanoparticles are shown in results and discussion.

❖ **pH Analysis:-**

The pH was determined by using Digital pH meter. The pH of the extract, precursor, PEG 6000 and the resulting mixture after adding NaOH was determined.

❖ **UV - Visible spectroscopy:-**

The reduction of copper sulphate to copper was monitored by measuring UV-Visible spectrum of the reaction medium after diluting a small aliquot of the sample with deionised water. The measurements are recorded on Shimadzu Dual Beam Spectrometer (Model UV-1650 PC) operated at resolution of 1nm.

❖ **FT - IR Analysis of biomass before and after bioreduction:-**

FT-IR measurements was carried out for both the extract and copper nanoparticles to identify the possible bioactive molecules responsible for the reduction of the copper ions and the capping ability of the bioreduced copper nanoparticles by the pineapple extract using KBr pellets and the spectra was recorded in the wavelength interval 4000 to 400cm⁻¹. The FTIR spectrum was also recorded for the solid copper nanoparticle isolated after centrifugation.

❖ **X – Ray Diffraction studies:-**

X-ray diffraction (XRD) measurements of the pineapple reduced copper nanoparticles was carried out using powder x-ray diffractometer instrument (SEIFERT JSO DEBYE FLEX-2002) in the angle range of 20°-80° operated at a voltage of 40KV and current of 30mA with Cu K α radiation in a θ -2 θ configuration. The crystallite domain size was calculated by using Debye-Scherrer formula.

❖ **Scanning electron microscopy:-**

The sample was prepared by placing a drop of colloidal solution of copper nanoparticles on carbon coated copper grid and subsequently drying in air., before transferring it to the microscope operated at an accelerated voltage of 130 KV (Hitachi-s 3400N)

❖ **Energy dispersive X-ray spectroscopy:-**

The presence of elemental copper was confirmed through EDS. Energy dispersive analysis x-ray spectrometer takes advantage of the photon nature of the light. In the x-ray range the energy of a single photon is just sufficient to produce a measurable pulse X-ray. A semiconductor material is used to detect the x-ray along with processing electronics to analysis the spectrum. The EDS observations were carried out by instrument coupled with SEM.

Pharmacognostic evaluation of biosynthesized copper nanoparticles:-

❖ **Determination of antibacterial activity:-**

Antibacterial activity of copper nanoparticles synthesized using fresh aqueous pineapple extract was determined by Agar disc diffusion method on Muller & Hinton agar medium (Hi-Media Pvt. Ltd. Mumbai) [8]. 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 μ l of sample (Concentration: 1000 μ g, 750 μ g and 500 μ g) were placed

in the disc .The plates were incubated at 37°C for 24 hrs. Then the antimicrobial activity was determined by measuring the diameter of zone of inhibition.

❖ Determination of antifungal activity:-

Antifungal activity of copper nanoparticles synthesized using fresh aqueous pineapple of extracts was determined by disc diffusion method on Sabouraud Dextrose agar (SDA) medium [8]. SDA 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 fungal suspension. The disc were placed in SDA plates and add 20 µl of sample (Concentration: 1000µg, 750µg and 500 µg) were placed in the disc .The plates were incubated at 37°C for 24 hrs. Then the antifungal activity was determined by measuring the diameter of zone of inhibition.

Results and Discussion

Qualitative Pharmacognostic evaluation of aqueous extract:-

The results of the phytochemical analysis of aqueous pineapple extract are shown in (table-2), which indicate the presence of secondary metabolites such as carbohydrates, proteins, tannins, flavanoids, phenols, terpenoids and steroids etc.,

Table-2: Qualitative phytochemical screening of fresh pineapple extract

| No. | Phytochemicals | Result |
|-----|----------------|--------|
| 1. | Carbohydrates | + |
| 2. | Tannins | + |
| 3. | Phenols | + |
| 4. | Flavanoids | — |
| 5. | Saponins | + |
| 6. | Proteins | + |
| 7. | Steroids | — |
| 8. | Alkaloids | + |
| 9. | Anthroquinones | — |
| 10. | Quinones | — |

Indication of sign (+) present and (-) absent.

The presence of phenolic compounds indicated in (table-2) constitute a major group of compounds that act as primary antioxidant which are mainly responsible for the reducing property of a fresh aqueous pineapple extract.

Phenolic compounds are very important phytoconstituents because of the scavenging ability of their —OH groups. The antioxidant property of phenolic compounds is mainly due to the redox property which allows them to act as reducing agents. The presence of ascorbic acid as one of the phyto constituents present along with polyphenols and other phytonutrients present in aqueous pineapple extract are reported in the literature [9] might be responsible for the reduction process.

Determination of total reducing power:-

The higher absorbance at 700nm shown in (table-3) indicates high reducing power of the aqueous pineapple extract.

Table-3:- Determination of total reducing power

| S.No | Sample | Absorbance at 700 nm |
|------|---------------------------|----------------------|
| 1. | Aqueous pineapple extract | 0.40 |

Determination of total antioxidant capacity:-

Pineapple contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components. Pineapple contains a number of essential nutrients, including vitamin C, manganese, and fiber. It also contains beneficial phytochemicals (Ferulic acid and chlorogenic acid) which have antioxidant and anti-cancer activities. These antioxidative compounds delay or inhibit the oxidation of molecules by inhibiting the initiation or propagation of oxidative chain reaction. The antioxidative activity of phenolic compounds is mainly due to their redox property, which plays an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [10].

Another phenol, with high radical scavenging capacity present in the pineapple extract is the chlorogenic acid. Hydrocinnamic acids, caefferic acids, rosmarinic acid and chlorogenic acids are more efficient scavengers of free radicals than benzoic acid derivatives [11]. Thus the different types of antioxidants presented in the pineapple extract synergistically reduce the Cu metal ions as each antioxidant is unique in terms of its structure and antioxidant function of trapping the different free radicals. In addition different antioxidants react with one another to regenerate each other forming an antioxidant network.

Therefore it is necessary to determine the free radical scavenging activity by DPPH method. The DPPH activity of fresh aqueous pineapple extract was found to be 82.8% shown in (table-4) indicates that fresh aqueous pineapple extract has potent antioxidant property.

Table-4:- Determination of DPPH activity (%)

| S.No | Sample | DPPH activity (%) |
|------|-------------------|-------------------|
| 1. | Pineapple extract | 82.8 |
| 2. | Standard-BHT | 99.9 |

Visual characterization:-

The preparation of copper nanoparticles using aqueous pineapple extract involves a four stage process. When the pH of the solution was increased by the addition of 0.1M NaOH the colour of the solution changed from blue to green and finally to reddish brown colour on heating over water bath. The appearance of reddish brown colour indicates the reduction of CuSO_4 and formation of copper nanoparticles as shown in (Fig.2).



Fig.2: Photograph indicating the formation of copper nanoparticles

Different parameters were optimized viz, ratio of CuSO_4 and extract, different temperature, different time intervals, different pH, in the presence of capping agent PEG 6000 and in the presence of starch. These parameters have been identified as factors affecting the rate of formation of copper nanoparticles using fresh aqueous pineapple extract and aqueous copper sulphate solution.

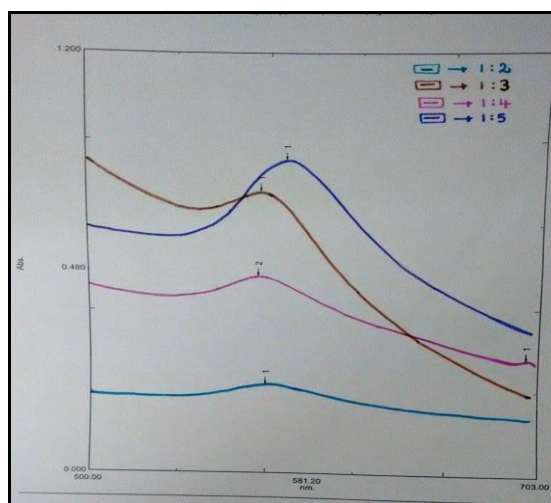
Ratio of volume of CuSO_4 : extract:-

The time taken for the formation of CuNps using different ratio of volume of CuSO_4 solution to extract ratio are given in (table-5).

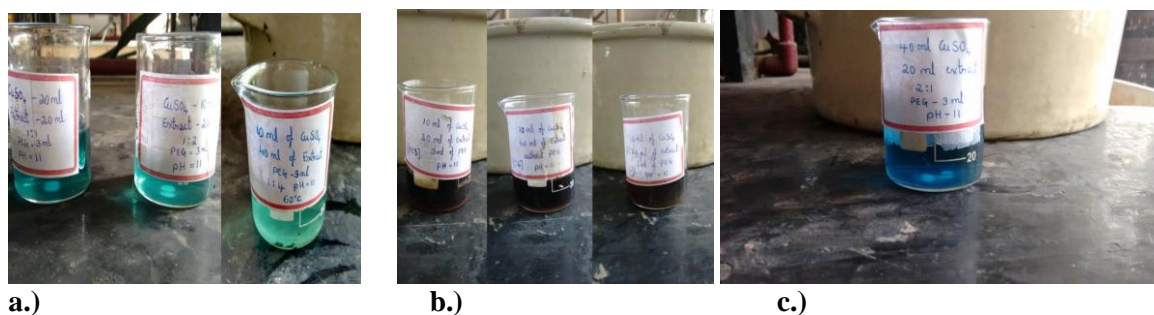
Table-5 : Rate of formation of copper nanoparticles using different ratio of volume of fresh aqueous pineapple extract and aqueous 0.02 M CuSO₄ at 80°C in PEG 6000 at pH 11.

| S.No | Ratio of (CuSO ₄ : extract) | Time taken for the formation of nanoparticles in (minutes) | Δ_{\max} |
|------|---|---|-----------------|
| 1. | 1:1 | No colour formation | - |
| 2. | 1:2 | 150 | 580.60 |
| 3. | 1:3 | 120 | 577.00 |
| 4. | 1:4 | 60 | 576.20 |
| 5. | 1:5 | 45 | 588.60 |

The UV-Visible Spectral lines recorded for the shift in SPR peaks position with variation in the amount of extract to precursor salt are shown in (fig.3).

**Fig.3: UV – Visible spectra of Copper Nanoparticles at different composition.**

A blue shift in the wavelength from 580 to 576 nm was observed with the increase in amount of extract. This shift can be explained on the basis of increased nucleation rate due to greater amount of Cu²⁺ ions reduced and generation of smaller nanoparticles in the solution. However further increase in the ratio of extract to precursor ion from 1:4 to 1:5, the red shift was observed in SPR from 576.2 to 588.6 nm. This may be due to collision between smaller nanoparticles due to agglomeration which leads to particle growth. This is in accordance with the literature reported [12]. The inset digital photograph in (fig.4a and 4b) clearly shows the formation of copper nanoparticles. Reddish Brown colour was noted for the optimum amount of extract and precursor producing greatest number of copper nanoparticles in aqueous medium.

**Fig.4 : (a,b,c) Photograph of formation of copper nanoparticles at different composition and vice versa**

From (Fig.4) it is seen that the time taken for the formation of CuNPs was found to be less for 10 ml of 0.02M CuSO₄ and 40 ml of extract solution. When the biosynthesis was carried out keeping the ratio vice versa, 2:1 composition (20ml of 0.02M CuSO₄ and 10ml of extract solution) remains blue in colour as seen in (fig.4c) indicating that this condition is not at all ideal or suitable for the formation of copper nanoparticles due to the less percentage amount of antioxidant and other secondary metabolites present in the aqueous pineapple

extract. Hence it needed a large amount of aqueous pineapple extract to reduce a Cu^{2+} ions. It is also inferred that the amount of antioxidant and other secondary metabolites present in the aqueous pineapple extract plays an important role in the reducing reaction as indicated by the formation of nanoparticles.

Effect of temperature on biosynthesis of copper nanoparticles:-

The effect of temperature on the rate of formation of CuNPs was studied for the composition of 10 ml of CuSO_4 and 40 ml of extract 1:4. The copper nanoparticles were formed within 60 minutes at 80°C . However, at room temperature and 60°C the formation of copper nanoparticles were formed only after 24 hours respectively and above 80°C under boiling condition the solution level decreases and becomes charred and no particles formation is seen. The rate of formation of CuNPs are indicated in the (fig.5) and (table-6).



Fig.5: Photograph of formation of CuNPs at different temperature

Table-6:- Rate of formation of CuNPs under different temperatures for 1:4 ratio (10ml 0.002M CuSO_4 and 40ml of aqueous pineapple extract) in PEG 6000 at pH 11

| S.No | Temperature | Absorbance |
|------|--------------------|------------|
| 1. | Room temperature | 436.40 |
| 2. | 80°C | 572 |

The UV-visible absorption spectra of copper nanoparticles synthesized under room temperature and 80°C are shown in the (fig.6). Hence, the reaction at 80°C favours the biosynthesis of copper nanoparticles using aqueous pineapple extract.

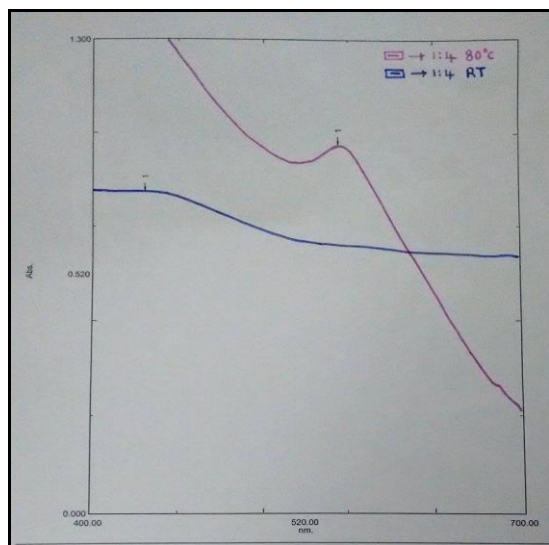


Fig.6:- UV – Visible absorption spectra of Copper Nanoparticles at different temperatures Effect of PEG 6000:-

PEG is frequently used as the surfactant to prepare nanomaterials and as stabilizing agent for metal colloids, because of its availability, low cost and non toxicity. It has already been shown [13] that the size and shape of nanomaterials strongly depends on the solution concentration of PEG. Once nuclei are formed, they tend to aggregate in order to decrease the total surface energy. This aggregation, which can be a consequence of

attractive Van der Waals forces between crystals, should be inhibited or limited to restrict the final particle size at the nanometric scale. One way to prevent nanoparticles from aggregation is the use of substances that lead to steric repulsion between individuals. PEG inhibits this type of growth and aggregation among biosynthesized nanoparticles.

In the present work PEG 6000 was used, which act as size controller and polymeric capping agent, as it hinders the nuclei from aggregation through the polar groups which are strongly adsorbed at the surface of the copper nanoparticles with the co-ordination bonds. The UV-Visible absorption spectra of copper nanoparticles synthesized under identical condition in presence and absence of PEG 6000 are shown in (fig.7). The spectrum shows a blue shift in the position of SPR from 577 to 572nm in the presence of PEG 6000. This indicates that the PEG 6000 molecules are adsorbed on the copper nanoparticles surface preventing them from further growth and leading to the generation of smaller nanoparticles [14]. Formation of copper nanoparticles are shown in the (fig.8a and 8b).

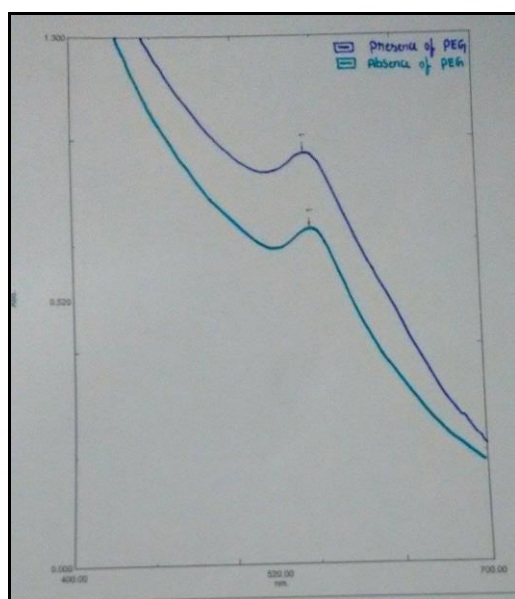


Fig.7: UV – Visible absorption spectra of Copper nanoparticles in presence and absence of PEG 6000.



a.)

b.)

Fig.8: (a,b) Photograph of formation of CuNP's in absence and presence of PEG 6000.

Effect of starch:-

Starch is a unique polymeric material used as capping agent. The biosynthesis of copper nanoparticles were also carried out using different concentration of starch as capping agent for 1:4 ratio under 80°C at pH 11. The UV-Visible absorption spectra of Copper nanoparticles at different concentration of starch are shown in (fig.9) which indicates that the starch might act as capping agent only at the higher concentration. The deep

investigation with regard to starch will be carried out in future. As far as the stability is concerned, it is stable only for 4 days where as biosynthesized copper nanoparticle using PEG6000 was stable for more than a week.

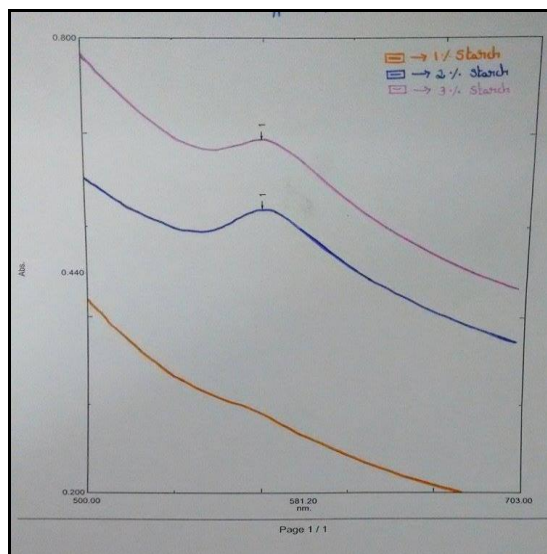


Fig. 9:- UV – Visible Absorption spectra of formation of Copper Nanoparticles at different concentration of starch.

Effect of pH:-

The work reported by Thi My Dung Dang et al [15] showed that the pH of the solution has an influence on the progress of copper sulphate reduction reaction. The pH of the pineapple extract, copper sulphate and PEG 6000 on mixing was found to be 5. The probable kinetic enhancement could be conducive to a reduction in crystallite size because of the enhancement of the nucleation rate. The antioxidant present in the extract induces a reduction in the solution pH which was adjusted back in the range from 7 to 12 with addition of 0.1 M NaOH solution and it also accelerates the reduction reaction in water. The UV-Visible absorption spectra for the pH ranging from 7 to 12 are shown in (fig.10). The Surface Plasmon absorbance of copper solution nanoparticles was obtained for all pH except at pH 7. This probably indicates the formation of very small particles at such low pH. The Plasmon Resonance is clearly visible for pH 9 to 11. At pH 12 the peak is still detectable but shows red shift when compared with other pH values.

The maximum blue shift in SPR peak position at pH 11 could be attributed due to the decrease in particle size [16]. The exact position of the Plasmon absorption may depend on several factors (including particle size, shape, solvent type and capping agent) and in this case, there might be some variation in the arrangement of capping molecules around the copper nanoparticles as a consequence of variation in pH. The pH 11 is found to be ideal due to the appearance of the reddish brown colour within 60 minutes because biosynthesized copper nanoparticles showed maximum absorption at 576.20 nm which is in agreement with the reported values in literature [17]. The formation of copper nanoparticle at different pH are shown in (fig.11). The rate of formation of CuNPs at different pH for ratio 1:4 (CuSO₄: Extract) are given in table-7.

Table-7: Rate of formation of copper nanoparticles at different pH

| S.N o | pH | Time Taken For The Formation Of Copper Nanoparticles in (minutes) | Absorbance |
|----------|----|---|------------|
| 1. | 7 | 360 | - |
| 2. | 8 | 240 | 593.00 |
| 3. | 9 | 120 | 580.60 |
| 4. | 10 | 90 | 577.00 |
| 5. | 11 | 60 | 576.20 |
| 6. | 12 | 45 | 588.60 |

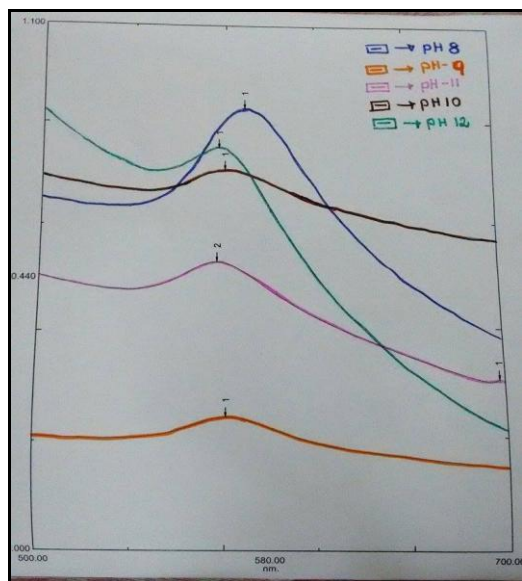


Fig.10: UV – Visible Absorption spectra of formation of Copper Nanoparticles at different pH.



Fig.11: Photograph of formation of copper nanoparticles at different pH.

Effect of reaction time:-

Time is a very important parameter in nanoparticle synthesis. As an empirical rule the availability of a larger number of nuclei at a given time induces a decrease in the nanoparticle size because smaller metal nuclei grow and consume metal ions at the same time. Copper nanoparticle synthesis was carried out under optimum condition. In order to optimize the reaction process the UV-Visible absorption spectra was recorded for every 20 minutes. The UV-Visible spectra taken at different time intervals are shown in (fig.12). Initially there was no characteristic absorption peak. The formation of CuNps at different intervals of time for ratio 1:4(CuSO_4 : Extract) at pH 11 are given in table-8.

Table-8:- Rate of formation of copper nanoparticles at different intervals of time.

| S.No | Reaction time of solution (minutes) | Colour | Absorbance (nm) |
|------|--|-----------------|-----------------|
| 1 | Immediately after the addition of NaOH | Green | - |
| 2 | 20 | Yellowish green | - |
| 3 | 45 | Dark red | 586 |
| 4 | 60 | Reddish brown | 576 |
| 5 | 100 | Dark brown | 583,590 |
| 6 | 130 | Dark brown | 687 |

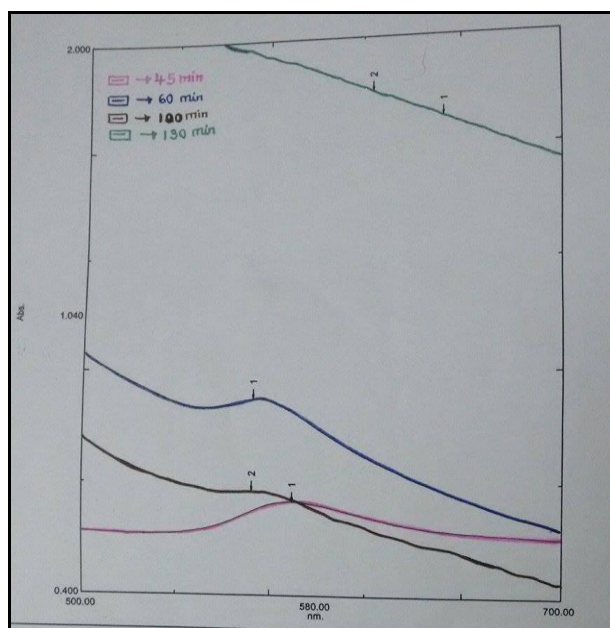


Fig.12: UV-Visible Absorption spectra of CuNPs at different intervals of time

From (table-7) and the (fig.12) it is seen that the CuNPs were formed within 60 minutes exhibited Plasmon Resonance at 576nm. It suggests a homogenization mechanism, which provides a larger number of nuclei with time. For 100 and 130 minutes no clear Plasmon Resonance was observed at higher range of wavelength 590, 687nm. This could indicate an formation of copper oxide on heating for longer time interval. At this moment the mechanism associated with this phenomenon is not clearly understood. Ascorbic acid present in the pineapple extract as reported in the literature [18] is well known to scavenge free radicals. This provides the right condition for subsequent rapid reduction by phytonutrients, polyphenols along with ascorbic acid and hence copper nanoparticles formation [14].

Stability:-

The stability of nanoparticles dispersion is a key factor in their application. In order to prevent agglomeration of nanoparticles, several capping agent is added in this media. In this present work, antioxidant present in the pineapple extract acts as both reducing and capping agent along with the protecting agent viz PEG 6000. The antioxidant and PEG 6000 stabilized copper nanoparticles dispersion was centrifuged at 8000 rpm for 15 minutes. It is seen that the residue gets settled at the bottom of the centrifuge tube. The supernant liquid was decanted under ambient conditions and no sign of sedimentation was observed even after 10 days of storage. The photos of dispersion before and after the storage are shown in (fig.13a and 13b).

The UV-Visible spectrum for the biosynthesized of CuNPs using 10ml of 0.02M CuSO_4 and 40ml of extract solution under optimum condition was recorded over a period of time for 10 days is shown in (fig. 14).



a.)

b.)

Fig 13: (a and b) Photograph of dispersion of copper nanoparticles taken before and after storage.

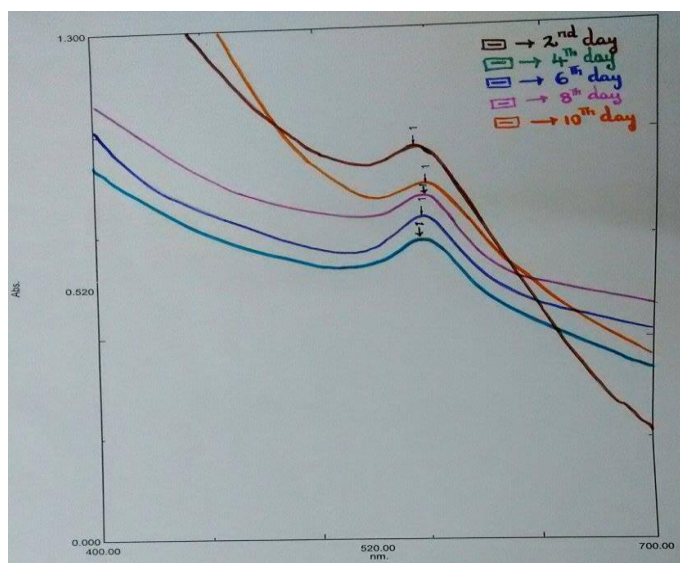


Fig.14: UV – Visible spectra indicating the Stability of copper nanoparticles.

There is no change in the SPR absorbance and the λ_{max} was found to be at 576nm without any change. This indicates that the antioxidant, PEG 6000 stabilized copper nanoparticles are highly stable due to the extreme capping effect of both antioxidant, PEG 6000 and the dispersion effect of the polyhydroxyl structure.

This stability against oxidation is likely attributable to the presence of antioxidant and PEG 6000 which forms a capping layer at the surface of the particle. Based on the UV-Vis spectral line taken at alternate days for 10 days (fig.14) and photograph shown in (fig.13a and 13b) indicates that the copper nanoparticles are stable for a period of 10 days. Finally the solution was turned into dark green colour on the 12th day, indicating the formation of cuprous oxide as reported in the literature [19]. It is understood that antioxidant plays a key dual role as reducing and capping agent. During nano particle synthesis Copper ions can coordinately bond with carbon and oxygen present in PEG 6000, and forms a protective layer over a biosynthesized copper nanoparticles.

Characterisation of biosynthesized copper nanoparticle:-

UV – Visible spectroscopy:-

Nanosized particles exhibit unique optical properties having an exponential-decay Mie scattering profile with decreasing photon energy. UV-Visible absorbance spectroscopy has proved to be a very useful technique for studying metal nanoparticles because the peak positions and shapes are sensitive to particle size. The surface Plasmon peak of CuNPs has been reported to appear around 570nm. In our present work, the UV-Vis absorption spectrum of CuNPs as observed in (fig.15) showed a single SPR peak at 576 nm, which confirms the formation of CuNPs. However, a broad SPR band was generated by the same sample within 45 minutes of reaction time with maximum absorption around 586nm, resulting in dark red colour solution. A reddish brown colour appeared after 60 minutes together with a highly sharp SPR peak having maximum absorption at 576 nm. These experimental investigations were found to be in good agreement with the results already presented in the literature [17]. The band was observed only after the addition of sodium hydroxide. The time evolution of the UV-Visible absorption spectra as shown in (fig.16) indicates initially there is no characteristic visible peak, which suggests that NPs were formed only after the addition of NaOH and after 60 minutes of reaction time. Generally, CuNPs are known to show an SPR band in the 573–600 nm range [20]. Moreover, UV-Visible spectral analysis was carried out for a number of samples prepared in the aqueous medium to optimize parameters such as volume ratio of copper sulphate to aqueous fresh pineapple extract, effect of pH, effect of temperature, reaction time, effect of starch and effect of PEG 6000 as depicted in (figure 4, 7, 8, 10, 11, 13). It is also evident that the small increase in absorbance (550-600 nm) turn yellow colour to red, which indicates the formation of nano particles of Zero valent Cu under the influence of reduction reaction [21].

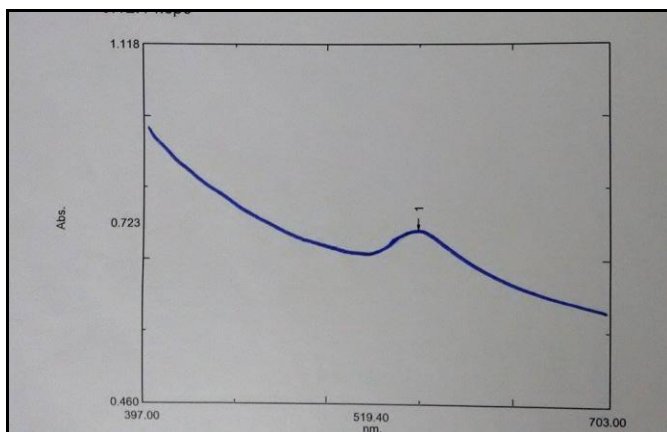


Fig.15: UV – Visible Absorbion of spectrum of copper nanoparticles taken at optimum conditions.

FT - IR Analysis:-

FTIR measurements of both the aqueous fresh pineapple extract and the synthesized dried CuNPs were carried out to identify the possible biomolecules responsible for the reduction, capping and efficient stabilization of the bio-reduced CuNPs. The FTIR spectra of the aqueous fresh pineapple extract and the synthesized CuNPs are shown in (Fig.17a and 17b). The pineapple extract displays a number of adsorption peaks, reflecting its complex nature. The spectrum was recorded in the wavelength region between 400cm^{-1} to 4000cm^{-1} . The spectrum of aqueous fresh pineapple extract (Fig.16a), shows the peaks at wave numbers 3555cm^{-1} , 673cm^{-1} and 1643cm^{-1} , 2362cm^{-1} . The peak at 1643cm^{-1} was due to presence of C=O stretching [22]. The peak at 3555cm^{-1} showed broad O-H stretching of phenolic compound. The N-H stretching of primary amine were obtained from the peak at 673cm^{-1} . The alkyne stretching frequency were obtained at 2362cm^{-1} .

After the reduction process, the absorption peaks at 3500 , 1637 , 667 , 2360cm^{-1} was observed shown in (fig.16b). Peak at 1637 corresponds to C=O stretching of amides and 3500 corresponds to O-H stretching of phenolic compound. The other peak obtained at 667 and 2360cm^{-1} are due to N-H stretching of primary amine and alkyne. Based on the peaks obtained for CuNPs it is inferred that biosynthesized copper nanoparticles might be surrounded by the any one of these organic molecules such as polyphenols, alkaloids and terpenoids which are in accordance with the facts already reported in the literature [23].

The IR spectrum of the CuNPs indicates the disappearances of $\nu_{\text{O-H}}$ bending vibration of the phenolic compound at 1397cm^{-1} and peaks of lower intensity confirms the reduction and the stabilization of copper nanoparticles proceed via these groups which confirm that water soluble compounds such as terpenoids and phenols are present in extract. The phyto constituents present in pineapple extract such as Flavonoids, alkaloids and terpenoids might be responsible for the reduction of copper ions to copper nanoparticles due to their capping and reducing capacity.

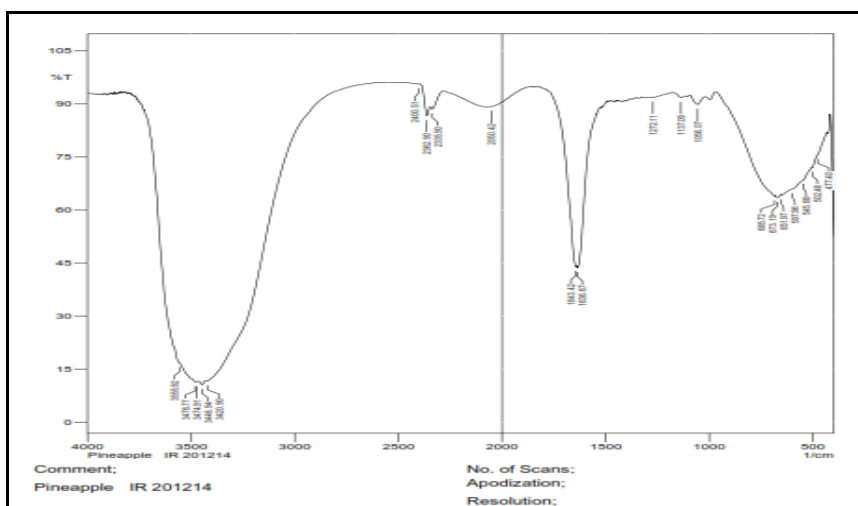


Fig.16 (a):- FTIR Spectra of aqueous pineapple extract.

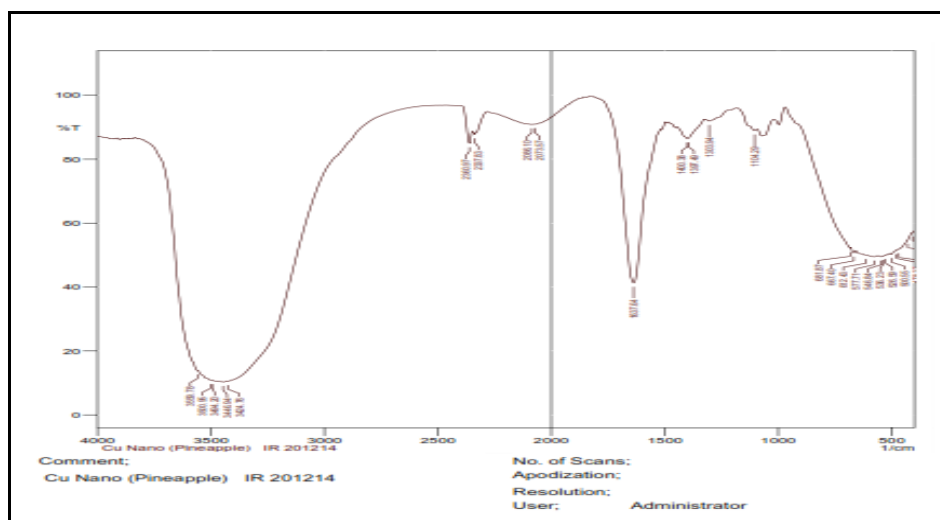


Fig.16. (b):- FTIR Spectra of Biosynthesized Copper Nanoparticles.

PXRD Analysis:-

PXRD was performed on the biosynthesized copper nanoparticles, using powder X-ray diffractometer instrument (SEIFERT JSO DEBYEFLEX – 2002) in the angle range between of 10° - 75° of the CuNPs at 2θ , scan axis :2:1 sym is shown in (fig.18). Three main characteristic diffraction peaks for Cu were observed at around $2\theta = 43^\circ, 50^\circ, 74^\circ$ correspond to (111), (200), (220) crystallographic planes of face-centred cubic (fcc) Cu crystals. The peak matches with Joint Committee of Powder Diffraction Standards, which further proves the formation of crystalline CuNPs [24]. XRD pattern of the biosynthesized copper nanoparticles at optimum conditions at pH 11 in presence of PEG 6000 is indicated in (fig.17). The average diameter of the copper nanoparticles is calculated and found to be in range 30 to 45 nm by Scherrer formula using FWHM obtained from the diffraction peaks.

$$D = K \lambda / \beta \cos \theta$$

Where, D is the crystalline size of NPs, (FWHM) K is the Scherer constant with a value from 0.9 to 1 λ is the wavelength of the X-ray source (0.1541 nm) used in XRD, β is the full width at half maximum of the diffraction peak and θ is the Bragg's angle.

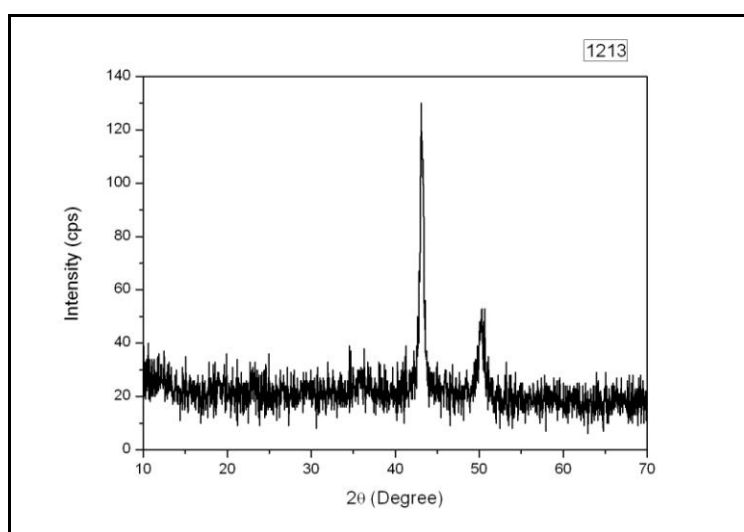
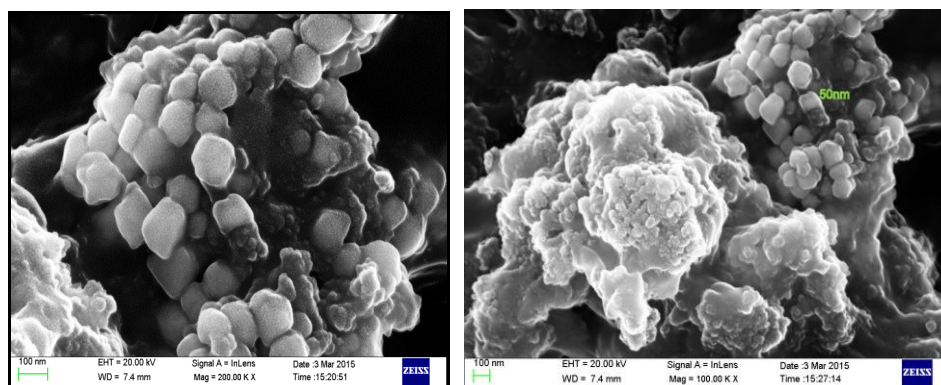


Fig.17: XRD pattern of Biosynthesized CuNPs at 2θ in presence of PEG 6000

Scanning electron microscopy:-

Scanning electron microscopy provided further insight into the morphology and size details of the copper nanoparticles. Experimental results showed that the diameter of biosynthesized copper nanoparticles

were about 40-50 nm and the shape was cubic in nature as shown in (fig.18b). A similar phenomenon has been reported [25].



(a) (b)
Fig.18(a, b) : SEM Micrograph of Biosynthesized Copper Nanoparticles at different magnifications.

EDAX:-

The EDX pattern also clearly shows that copper nanoparticles are crystalline in nature. The EDX spectrum of the solution containing copper nanoparticles observed at 1KeV, which is typical for the absorption of metallic CuNPs. The EDAX analysis confirmed the weight percentage of copper nanoparticles are 8.41% since the measurements were done one month after the powder preparation, an aerial oxidation might have taken place which led the formation of copper oxide. Weak signals depicting the presence of sulphur, calcium, phosphorous and potassium as seen in (fig.19) indicates that these phytonutrients, which are present in fresh aqueous pineapple extract along with secondary metabolites might be involved in reduction process.

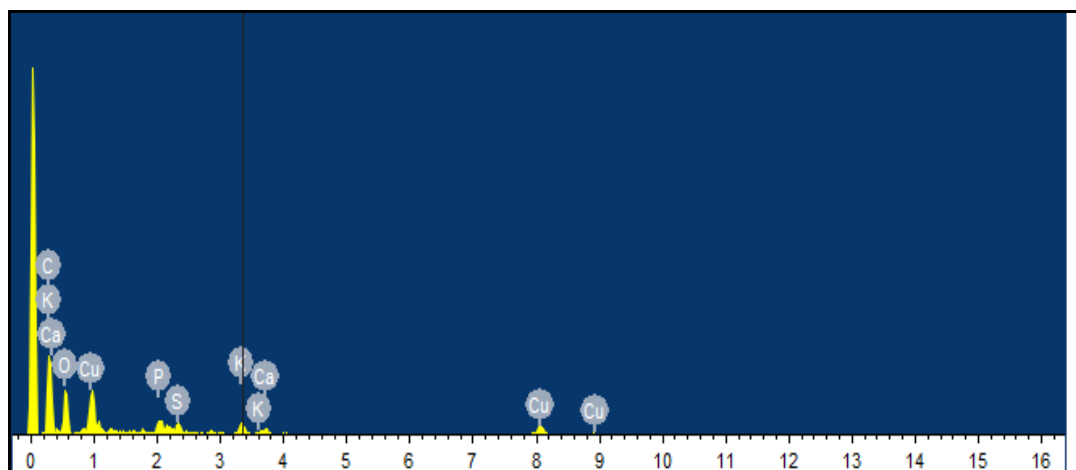


Figure 19: EDS Spectra of Biosynthesized copper nanoparticles.

Antimicrobial and antifungal activity of biosynthesized copper nanoparticles

Antimicrobial Activity

The emergence of nanoscience and nanotechnology in the last decade presents opportunities for exploring the bactericidal effect of metal nanoparticles. The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio, which allows them to interact closely with microbial membranes and is not merely due to the release of metal ions in solution. A cell wall is present around the outside the bacterial cell membrane and it is essential to the survival of bacteria. It is made from polysaccharides and peptides named peptidoglycon. There are two different types of cell wall in bacteria, called gram-positive and gram-negative. The names originate from the reaction of cells to the gram stain, a test long-employed for the classification of bacterial species. Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan. In contrast, gram-negative bacteria have a relatively thin cell wall consisting of a

few layers of peptidoglycan. Surfaces of copper nanoparticles affect / interact directly with the bacterial outer membrane, causing the membrane to rupture and killing bacteria.

The antimicrobial activity of biosynthesized copper nanoparticles was carried out on two human pathogens such as, *Escheria Coli* and *Staphylococcus aureus* by Agar disc diffusion method at different concentration as shown in (fig.20a and 20b). *E.Coli* are gram –ve bacteria and *Staphylococcus aureus* are gram +ve bacteria. Ampicillin 20µg/ml was used as +ve control. Biosynthesized CuNPs showed clear zone of inhibition as indicated in (Table-9) against *E.coli* and *Staphylococcus aureus*.

As the concentration increases, the zone of inhibition increases. At the lower concentration of 500 µg/ml *Staphylococcus aureus* was found to be resistant and at higher concentration CuNPs showed the antimicrobial activity against *Staphylococcus aureus*. The presence of an inhibition zone clearly indicates the mechanism of the biocidal action of nanoparticles involves, disrupting the membrane. Extent of inhibition depends on the concentration of nanoparticles as well as on the initial bacterial concentration. The reason could be that the smaller size of the particles which gets tightly adsorbed on the surface of the bacterial cells so as to disrupt the membrane, thereby leading to the leakage of intracellular components, thus killing the bacterial cells. Another proposed mechanism involves the association of copper with oxygen and its reaction with sulfhydryl (-S-H) groups on the cell wall to form R-S-S-R bonds, thereby blocking respiration and causing cell death.

From the result of zone of inhibition values indicated in (table-9) proves that biosynthesized copper nanoparticles exhibits high antibacterial activity again gram –ve bacterium. This observation is in agreement with the earlier studies [26]. The interaction between Gram-positive bacteria and nanoparticles was stronger than that of Gram-negative bacteria because of the difference in cell walls between Grampositive and Gram-negative bacteria.

Table-9 Antimicrobial activity of biosynthesized copper nanoparticles against two human pathogens at different concentrations

| Organisms | Zone of Inhibition (mm) | | | Antibiotic (mg/ml) |
|-----------|----------------------------|-------|------|-----------------------|
| | Concentration(µg/ml) | | | |
| | 1000 | 750 | 500 | |
| E.coli | 14 mm | 9 mm | 6 mm | 15 mm |
| S. aureus | 12 mm | 11 mm | - | 14 mm |



(a) *E. coli*



(b) *S. aureus*

Fig.20: Plates showing diameter of zones of inhibition of a) *E.coli* and b) *Staphylococcus aureus*.

Antifungal activity

In the present study, the antifungal activity was determined using the agar well diffusion assay method for different concentration of CuNPs against *Aspergillus niger* and *Trichoderma viride* is shown in (fig.21a and 21b). CuNPs exhibited a potent antifungal activity against fungal strains tested, similar to the antifungal activity in the MIC values of Amphotericin B which was used as a positive control. These results indicate that CuNPs have remarkable potential antifungal activity in treating fungal infectious diseases.

Biosynthesized CuNPs showed clear zone of inhibition as indicated in the (Table-10) against *Aspergillus niger* and *Trichoderma viride*.

Based on these result, it might be concluded that synthesized copper nanoparticles had significant antifungal action on both organisms used. In particular CuNPs shows efficient zone of inhibition with increase in concentration for *Aspergillus niger*, whereas *Trichoderma viride* was found to be resistant towards lower concentration (500 µg/ml)

Fungal cells by having ergosterol in the membrane and by making various gradients between cytoplasmic membranes, can keep their membrane potential ability. As a result of using antifungal drugs and CuNPs, these gradients and membrane conformity might have been destroyed and causes the cell death.

At present the exact mechanism behind this is not clearly known. This needs to be studied further.

Table-10 Antifungal activity of biosynthesized copper nanoparticles at different concentrations.

| Organisms | Zone of Inhibition (mm) | | | Antibiotic (mg/ml) |
|--------------------|-------------------------|------|------|--------------------|
| | Concentration(µg/ml) | | | |
| | 1000 | 750 | 500 | |
| Trichoderma viride | 11 mm | 8 mm | - | 13 mm |
| Aspergillus niger | 10 mm | 7 mm | 6 mm | 14 mm |



(a) *Trichoderma viride* (b) *Aspergillus niger*

Fig.21 Plates showing diameter of zones of inhibition of a) *Trichoderma viride* and (b) *Aspergillus niger*

Conclusion

In the present study we found that the aqueous pineapple extract proved to be a efficient natural source for the biosynthesis of copper nanoparticles. The green chemistry approach is simple, fast and cost effective technique and does not involve any harmful and environmentally toxic chemicals used in conventional chemical reduction method. The CuNPs are crystalline in nature and the size of copper nanoparticles is around 50 nm. Biomolecules responsible for the reduction and stabilization of CuNPs are phenols. The phenolic group present in fresh aqueous pineapple extract exhibit excellent antioxidant activity and these phenols can react with a free radical to form the phenoxyl radicals. Therefore, the use of natural anti-oxidant for the synthesis of CuNPs seems to be a effective root for the green synthesis. The biologically synthesized CuNps shows excellent antibacterial activity. The CuNPs also exhibited potent antifungal effects on fungi tested, probably through destruction of membrane integrity, therefore it may be concluded that CuNPs has considerable antifungal activity, deserving further investigation for various biomedical application such as catheters, topical antimicrobial gel formulation, food packaging materials, food processing equipments and so on. The fruit material responsible for the reduction and stabilization of NP's needs further study including extraction and identification of the compounds present in the extract.

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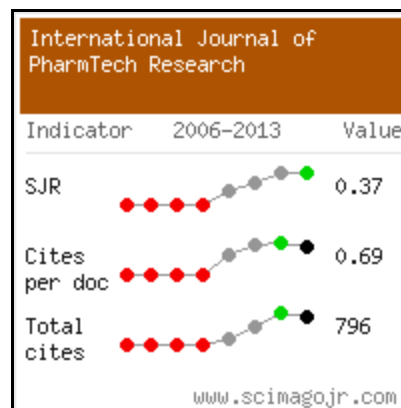
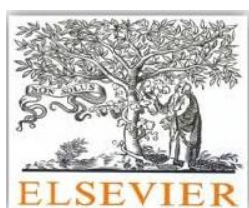
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