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Biosynthesis of Copper Nanoparticles Using Aqueous Phyllanthus Embilica (Gooseberry) Extract- Characterisation and Study of Antimicrobial Effects

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Abstract: Vegetable mediated synthesis of nanoparticles is a green chemistry approach that connects nanotechnology and biotechnology. In the present investigation we have used a fast, convenient and environment friendly method for the synthesis of copper nanoparticles by biologically reducing $CuSO_4$ with aqueous extract of Goose Berry (Phyllanthus Embilica) under optimum conditions (pH-6-10). The formation of copper nanoparticles was indicated by the colour change from colourless to brown. Biosynthesized nanoparticles were characterized by UV-VIS, FT-IR, XRD, SEM, and EDAX analysis. These biologically synthesised Cu nanoparticles were tested for antimicrobial activity against four human pathogens viz. Staphylococcus Aureus and Escheria Coli. The reduction process was simple and convenient to handle and monitored by UV-Vis spectroscopy which showed surface plasmon resonance at 294 nm. The presence of active proteins and phenolic groups present in biomass before and after reduction was identified by FT-IR. The crystalline morphology and size of the nanoparticles were determined by SEM and X-ray diffraction studies, which showed the average particle size of Cu nanoparticles was in the range 15-30nm as well as revealed their FCC structure. Presence of elemental copper was revealed by EDAX analysis. These biologically synthesised Cu nanoparticles were found to be effective in controlling growth of human pathogens viz. Staphylococcus Aureus and Escheria Coli. The reducing property of aqueous extract is due to the presence of antioxidant viz. ascorbic acid, polyphenols which is confirmed by quantitative assay.

Keywords: Phyllanthus Embilica, SEM, XRD, EDAX, FT-IR, Scherrer formula,UV-Vis, Staphylococcus Aureus, and Escheria Coli.

1 Introduction

Nanoparticles have been extensively studied over the last decade due to its characteristic: physical, chemical, electronic, electrical, mechanical, magnetic, thermal, dielectric, optical and biological properties [1]. This is primarily based on their smaller size and their high surface to volume ratio Nanoparticles are considered as building blocks for the next generation of technology with applications in many industrial sectors. In particular metal and metal oxide nanoparticles are receiving increasing attention in a large variety of applications. The oxides of transition metals are an important class of semiconductors, which have applications in magnetic storage media, solar energy transformation, electronics, gas sensors and catalysis [2].

Although various physical and chemical methods have been extensively used to produce nanosized copper particle such as micro-emulsion method [3], arc submerged nanoparticle synthesis system [4], flame based aerosol methods [5], sonochemical [6], hydrothermal [7] and solid state techniques [8] the use of toxic chemicals are subjects of paramount concern. The use of toxic chemicals for the synthesis of nanoparticle limits their applications in clinical fields. Therefore, development of clean, biocompatible, nontoxic and eco-friendly methods for nanoparticles synthesis deserves merit. The interest in this field has shifted toward 'green' chemistry and bio-processor approach. These approaches focus on utilization of environmental-friendly, cost-effective and biocompatible reducing agents for synthesis of copper nanoparticles.

Various plant extracts have been utilized in the synthesis of nanoparticles. Amla is rich in vitamin C, aminoacids, polyphenols and other antioxidants. Ascorbic acid present in the gooseberry extract is a good reducing and capping agent and aids in the biosynthesis of copper nanoparticles [9].

Here, in this work we report vegetable assisted synthesis of Copper nanoparticles, reducing the copper ions by the aqueous extract of Goose Berry fruit, characterized by UV-Vis spectroscopy, FT-IR, XRD, SEM and EDAX. Further, these biologically synthesized nanoparticles were found to be toxic against multidrug resistant human pathogens. The investigation is also focused for the first time to outline the

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potential use of Goose berry extract as reducing, stabilising and capping agent in the reaction.

2 Materials and Method

2.1 Materials

Gooseberry, the sample for the biosynthesis of the copper nanoparticles was procured from the local supermarket. Copper sulphate, sodium hydroxide, PEG 6000 and other reagents used in the study were of analytical grade. The bacterial strains employed in this work were procured from microbial type culture collection centre (MTCCC) located at the institute of microbial technology, Chandigarh, India. (E-coli and Staphylococcus aures).

2.2 Preparation of Sample Extract

50gm of gooseberry fruit (phyllanthus emblica) was accurately weighed, thoroughly washed under running tap water followed by washing it with double deionised water to remove surface impurities. They were crushed using a blender and finely macerated. After homogenization 100ml of double deionised water was added and heated over a water bath maintained at 80^0C for 15 minutes. The extract obtained was filtered through muslin cloth and then through Whatmann no: 1 Filter paper (pore size 25µm) and used immediately for the biosynthesis of copper nanoparticles.

2.3 Phytochemical Screening-Qualitative Analysis

Fresh extract of the fruit of gooseberry was used for phytochemical screening- Qualitative analysis. Preliminary phytochemical screening was carried out by standard phytochemical methods [10].

2.3.1 Test for carbohydrates

To 2ml of extract, 1ml of Molish's reagent and few drops of concentrated sulphuric acid were added. Presence of purple or reddish color indicates the presence of carbohydrates.

2.3.2 Test for tannis

To 1ml of extract, 2ml of 5% ferric chloride was added. Formation of dark blue or greenish black colour indicates the presence of tannis.

2.3.3 Test for saponins

To 2ml of extract, 2ml of distilled water was added and shaken in a graduated cylinder for 15 minutes lengthwise. Formation of 1cm layer of foam indicates the presence of saponins.

2.3.4 Test for flavonoids

To 2ml of extract, 1ml of 2N sodium hydroxide was added. Presence of yellow color indicates the presence of flavonoids.

2.3.5 Test for alkaloids

To 2ml of extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.

2.3.6 Test for anthraquinone

To 1ml of exact few drops 10% ammonia solution was added. Appearance of pink color precipitate indicates the presence of anthraquinone.

2.3.7 Test for anthocyanosides

1ml of the filtrate was mixed with 5ml of dilute Hcl, a pale pink color indicates the positive test.

2.4 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, polyethylene glycol 6000 (0.01M) was dissolved in water and added to the aqueous solution containing the copper salt with vigorous stirring. In this step, the solution color changed from blue to white. In the third step, gooseberry extract was added to the copper sulphate solution containing PEG 6000. The color of the aqueous phase remains the same. Finally, 0.1M sodium hydroxide was added in drops to the solution under continuous rapid stirring.

The instant color change started to occur in the aqueous phase from white to yellowish green. The appearance of this color indicates that the reduction has started. The formation of copper nanoparticles is confirmed by the color change from yellowish green to brown when it is kept on a water bath at 80^0C. The formation of copper nanoparticles is inferred by visual observation followed by recording UV-Visible spectrum. The biosynthesized copper nanoparticles are characterized by FTIR, SEM, XRD and EDAX studies [11].

2.5 Fixation of parameters for biosynthesis of copper nanoparticles

2.5.1 Biosynthesis of copper nanoparticles using different ratios (volume ratio of extract: CuSO4)

The biosynthesis of copper nanoparticles was carried out at different volume ratio of extract and copper sulphate (1:1, 1:2, 1:3, 1:4, 1:5) at pH 10. Time taken for the color change in the reaction mixture as well as the formation of nanoparticles was monitored by visual inspection and also by its UV-Visible spectrum.

2.5.2 Biosynthesis of copper nanoparticles at different temperatures

The biosynthesis of copper nanoparticles for the fixed composition was done at different temperatures namely; room temperature and by heating in the water bath at 60^0C and 80^0C. The time taken for visual color change from yellow to brown was recorded followed by recording UV-Visible spectrum.

2.5.3 Biosynthesis of Copper Nanoparticles at Different pH

The biosynthesis of copper nanoparticles for 1:3 ratios of extract and copper sulphate was carried out at different pH viz. 6, 8, 10. The time taken for color change as well as the UV- Visible spectrum for the reaction mixture was monitored.

2.5.4 Biosynthesis of copper nanoparticles at different intervals of time

The synthesis was carried out at pH 10 in the ratio 1:3(extract:$CuSO_4$) and the time taken for the formation was noted at an interval of every 10 minutes and completion of the reaction was monitored by the color change as well as the UV- Visible spectrum.

2.5.5 Biosynthesis of Copper Nanoparticles in the Presence of PEG

Biosynthesis of copper nanoparticles was carried at pH 10 in the ratio 1:3 with and without PEG 6000 and completion of the reaction was monitored by the color change as well as by recording the UV-Visible spectrum.

2.5.6 Stability of Copper Nanoparticles

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

2.6 Characterization of Biosynthesized Copper Nanoparticle

2.6.1 Visual Inspection

The bio-reduction of the aqueous solution of copper sulphate using gooseberry extract was monitored and the appearance of brown color indicates the formation of copper nanoparticles. Photograph of the copper nanoparticles at different parameters.

2.6.2 pH analysis

The pH of the extract, precursor as well as the resulting mixture after addition of PEG 6000 and NaOH was determined using digital pH meter.

2.6.3 UV-Visible Spectroscopy

The reduction of copper sulphate to copper was monitored by recording UV-Visible spectrum of the reaction mixture after diluting a small aliquot of the sample with deionised water. The measurements are recorded on Shimadzu dual beam spectrometer (model uv-1650pc) operated at resolution of 1nm.

2.6.4 FT-IR Analysis of Bio-Mass before and after Bio-Reduction

FT-IR measurement 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 of the copper nanoparticles by the gooseberry extract using KBr pellet and the spectrum was recorded in the wavelength interval 4000 to 400cm^{-1}. The FT-IR spectrum was also recorded for the solid copper nanoparticles isolated after centrifugation.

2.6.5 X-Ray Diffraction Studies

X-ray diffraction (XRD) measurement of the gooseberry reduced copper nanoparticles was carried out using powder x-ray diffractometer instrument (SEIFERT JSO DEBYEFLEX-2002) in the angle range of 10^0-70^0 operated at a voltage of 40Kv and a current of 30mA with CuKα radiation in a θ-2θ configuration. The crystallite domain size was calculated by using Debye- Scherrer formula. XRD pattern was also taken for nanoparticle prepared in the absence of PEG.

2.6.6 Scanning Electron Microscopy (SEM)

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

2.6.7 Energy Dispersive X-ray Spectroscopy (EDAX)

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.

2.7 Pharmocognostic evaluation of biosynthesized copper nanoparticle

Determination of antibacterial activity: Antibacterial activity of the extract was determined on Muller and Hinton Agar (Hi-Media Pvt. Ltd .Mumbai) using Kirby-Bauer disk diffusion method [12]. Test pathogens were spread on the test plates- Muller Hinton Agar (MHA) for bacterial using sterile swabs. Sterile wells are made with the help of a sterile cork borer at aseptic conditions. Samples (1500µg and 2000µg) were added to the wells at aseptic conditions. Stock solutions of the extract were prepared using DMSO. The test plates were incubated for 24hrs. The zone of inhibition (in mm diameter) were read and taken as the activity of the extract against the test organisms.

3 Results and Discussion

3.1 Qualitative Pharmocognostic Evaluation of Extract

The results of qualitative phytochemical analysis of the gooseberry extract are shown in table-1 which indicate the presence of secondary metabolites such as carbohydrates, tannis, saponins, flavonoids, etc.,

Table 1

Test	Result
Carbohydrate	+
Tannin	+
Saponin	+
Flavonoid	+
Alkaloid	+
Quinone	+
Glycoside	-
Cardioglycoside	-
Terpenoid	-
coumarine	-
Anthraquinone	+
anthocyanosides	+
Phenols	+

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

The presence of ascorbic acid, polyphenols and other phytonutrients in aqueous gooseberry extract is mainly responsible for the bio-reduction process [13]. From the literature it has been found that the amount of ascorbic acid (natural vitamin C) present in gooseberry extract was found to be 445mg of ascorbic acid/100gm of fruit. Polyphenolic compounds are very important plant constituents because of the scavenging ability of their –OH groups.

The antioxidant property of polyphenolic compounds is mainly due to its redox property which allows them to act as reducing agents [9].

3.2 Visual characterization

The preparation of copper nanoparticles from gooseberry extract involves a four stage process. When the pH of the solution was increased to 10 by the addition of 0.1M sodium hydroxide, the color of the solution changed from colorless to greenish yellow and finally brown on heating in a water bath. The color change to brown indicates the reduction of copper sulphate and formation of copper nanoparticles. Fig 1 indicates the formation of nanoparticles.

Figure 1: Formation of copper nanoparticles.

Different parameters were optimized for the biosynthesis of copper nanoparticles viz,

- Volume ratio of extract and copper sulphate
- Temperature
- In the presence and absence of PEG 6000 (capping agent)
- Different pH
- Effect of time

3.2.1 Ratio of volume of extract: $CuSO_4$

The time taken for the formation of CuNp depends on the ratio of volume of extract to $CuSO_4$ solution as given in (Table-2).

Table-2 Time taken for the formation of copper nanoparticles using different ratio of volume of fresh aqueous gooseberry extract and aqueous 0.02M $CuSO_4$ at 80^0C in the presence of PEG at pH 10.

S.No	Ratio (Extract:$CuSO_4$)	Time taken for the formation of CuNp	Λmax
1.	1:1	2 hours	324
2.	1:2	1 hour	268
3.	1:3	30 mins	294

The UV-Visible spectrum was recorded for the shift in SPR peaks position with variation in the amount of precursor salt to extract as shown in fig 2.

A blue shift in the wavelength from 324 to 268nm was observed with the increase in amount of precursor salt. This

shift can be explained on the basis of increased nucleation rate due to greater amount of $cu2^{+}$ ions and generation of smaller nanoparticle in the solution. However with further increase in the precursor ion from 1:2 to 1:3, a red shift was observed in SPR from 268 to 294nm. This may be due to collision between smaller nanoparticles which leads to particle growth [14]. The inset digital photograph in fig 1 clearly shows the formation of copper nanoparticle. Brown color was noted for the optimum amount of precursor and extract producing greatest number of copper nanoparticle in aqueous medium.

From the table it is also seen that the time taken for the formation of CuNp was found to be less for 25ml of the extract and 75ml of 0.02M $CuSO_4$ solution (1:3). This ratio was found to be ideal as the biosynthesized nanoparticles showed maximum absorption at 294nm which is in agreement with the values reported in the literature.

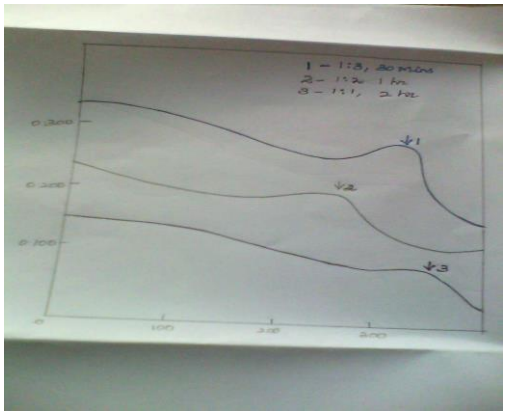


Figure 2: UV Visible spectrum of biosynthesized nanoparticle at different ratios

3.2.2 Effect of temperature on biosynthesis CuNp's

The effect of temperature on the rate of formation of CuNp was studied for the 1:3 composition of the extract and $CuSO_4$ solution. The CuNp were formed within 30 mins at 80^0C. However, at room temperature and 60^0C the formation of CuNp were formed after 1 day and 2 hours respectively and above 80^0C under boiling condition the solution becomes charred and no particle formation is seen. Hence, the reaction at 80^0C favours the biosynthesis of CuNp using aqueous gooseberry extract.

3.2.3 Effect of PEG

An important feature in the production of the metal nanoparticles is to prevent agglomeration and oxidation processes. The stabilization is commonly achieved by using surfactants which avoid the aggregration by binding to the nanoparticle surface. PEG 6000 is frequently used as the stabilizer or capping agent for metal colloids because of its availability, low cost and non-toxicity. The stabilization of metal colloids and the shape of nano material depend strongly on PEG 6000. In the present work PEG 6000 was used which works as size controller and polymeric capping agent because it hinders the nuclei from aggregation through the polar group which are strongly adsorbed at the surface of the CuNp with the co-ordination bonds. Fig- 4 shows UV-Visible absorbance spectra of CuNp synthesized under identical condition in absence and presence of PEG 6000. The spectrum shows a blue shift in the position of SPR from 326-294nm in the presence of PEG. This indicates that the PEG molecule was adsorbed on the CuNp surface keeping them from excessive growth and leading to the generation of smaller nanoparticles [15]. Formation of CuNp in the presence and absence of PEG 6000 is shown in the fig 3.

Figure 3: Formation of copper nanoparticle a) presence of PEG 6000 and b) absence of PEG 6000 for the composition (1:3) (extract: $CuSO_4$)

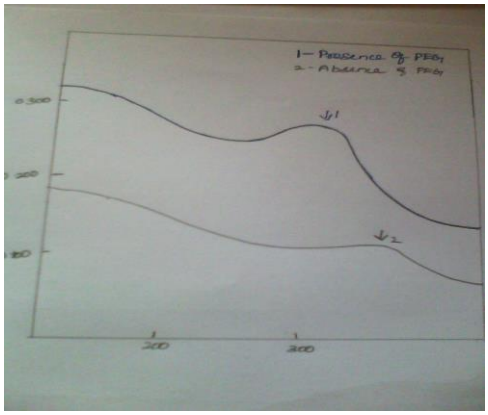


Figure 4: Biosynthesized copper nanoparticle in the presence and absence of PEG.

3.2.4 Effect of pH

The present work shows that the pH of the solution has an influence on the progress of bio-reduction of copper sulphate solution. The pH of the gooseberry extract, $CuSO_4$ and PEG on mixing was found to be 5.4. The probable kinetic enhancement could also be conducive to a reduction in crystallite size because of the enhancement of the nucleation rate [15]. The ascorbic acid present in the extract induces a reduction in the solution pH which was adjusted back in the range from 6 to 12 with addition of 0.1M NaOH solution.

Fig-6 shows UV-Visible absorption spectra for the pH ranging from 6 to 12.

The surface plasmon absorbance of copper colloids was obtained for all pH except at PH 6. This probably indicates very small particles at such low pH. The Plasmon resonance is clearly visible for pH 8 to 10 at 308 and 294nm respectively. At pH 12, the peak is still detectable but much weaker when compared with other pH's.

The maximum blue shift in SPR peak around the maximum value at pH 10 could be attribute to the decrease in the particle size [16], but 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 the capping molecules around the copper particles as a consequence of the variation in pH. Thus pH 10 is found to be ideal due to the appearance of brown color within 30 mins because biosynthesized CuNp showed maximum absorption at 294nm which is in agreement with reported values in the literature [14]. Fig-5 shows the formation of CuNp at different pH.

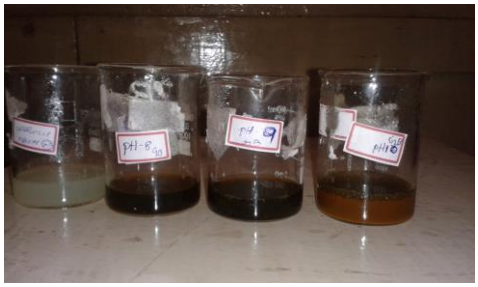


Figure 5: Formation of copper nanoparticles at different pH for the ratio (1:3) (extract: $CuSO_4$).

Table-3 Time taken for the formation of CuNp at different pH for the ratio 1:3(extract: $CuSO_4$) in the presence of PEG 6000.

S.No	pH	Time taken for the formation CuNp	λmax(nm)
1.	6	1 day	350
2.	8	4 hours	308
3.	10	30 mins	294
4.	12	45 mins	272

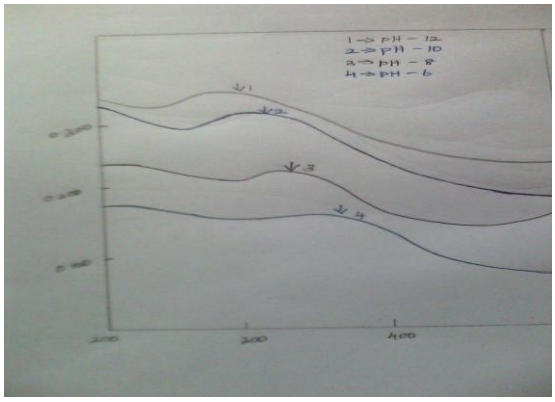


Figure 6: UV-Visible spectra for the biosynthesized copper nanoparticle at different pH for 1:3 (extract: $CuSO_4$).

3.2.5 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 clarify the reaction process the UV-Visible absorption spectra was recorded for every 10 mins.

The evaluation of the UV-Visible spectrum is shown in fig 7. Initially there was no characteristic absorption peak.

Table- 4 Formation of copper nanoparticle at different intervals of time

S.NO	Reaction time of solution (mins)	Color	Λmax (nm)
1.	Immediately after the addition of NaOH	Greenish yellow	-
2.	10	Dark green	-
3.	20	Pale brown	350
4.	30	Brown	294
5.	40	Brown	250
6.	50	Brown	240
7.	60	Brown	230

From the table-4 and fig-9 it is seen that the CuNp were formed within 30 mins exhibited plasmon resonance at 294nm. For 50 and 60 mins no clear plasmon resonance was obtained despite a clear absorption in the lower range of wavelength. It suggests a homogenization mechanism, which provides a larger number of nuclei with time. This could indicate an even smaller particle size.

At this moment the mechanism associate with this phenomenon is not clearly understood. Ascorbic acid present in gooseberry extract is well known to scavenge free radicals thus provides anti-oxidant action during copper nuclei formation. This provides the right condition for subsequent rapid reduction by phytonutrients, polyphenols along with ascorbic acid and hence copper nanoparticle formation [15].

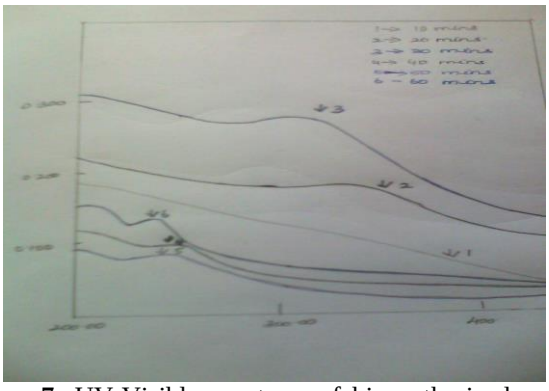


Figure 7: UV-Visible spectrum of biosynthesized copper nanoparticles at different intervals of time.

3.3 Stability

The stability of nanoparticle dispersion is a key factor in its applications. In order to prevent agglomeration of nanoparticles, several capping agent is added in this media. In this work, ascorbic acid present in the gooseberry extract is used as both reducing and capping agent along with the protecting agent viz PEG 6000. The ascorbic acid and PEG stabilized copper nanoparticles dispersion was centrifuged at 8000 rpm for 15 mins. It is seen the residue get 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 15 days of storage. The UV-Visible spectrum for the biosynthesized CuNp using 25ml of the extract and 75ml of $CuSO_4$ solution was recorded under optimum condition over a period of time for 15 days. There was no change in UV spectrum.

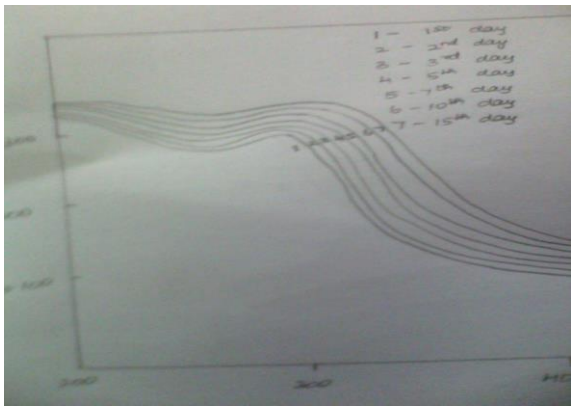


Figure 8: UV-Visible spectra showing stability of biosynthesized copper nanoparticles.

There is no change in the SPR and the λmax was found at 294nm without any change. This indicates that the ascorbic acid, PEG 6000 stabilized copper nanoparticle are highly stable due to the extreme capping effect of both the ascorbic acid, PEG 6000.

This stability against oxidation is likely attributable to the presence of ascorbic acid and PEG which forms a capping layer at the surface of the particle. From the observation (fig 8), it is understood that ascorbic acid plays a key dual role as reducing agent and capping agent. During particle synthesis Cu ions can co-ordinatively bond with carbon and oxygen present in PEG, so that the synthesized CuNp are covered by an adsorbed layer of PEG [16].

3.4 Characterization of bio-synthesized copper nanoparticle by spectral methods

3.4.1 UV-Visible studies on copper nanoparticles

Nanosized particles exhibit unique optical properties having an exponential-decay Mie scattering profile with deceasing photon energy [17]. 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 effect of ascorbic concentration in the extract on the UV-Visible absorbance spectroscopy of the synthesized CuNp showed single peak at around 294nm. The surface plasmon peak of CuNp has been reported to appear at around 570nm. However, when the particle size is less than 4nm, the distinctive Plasmon peak is known to be broadened and replaced by a featureless absorbance, which increases monotonically towards higher energies [18-20].

In our work, the resulting Cu dispersion did not show a plasmon peak at around 570nm, but displayed a broadened peak at a short wavelength, indicating the presence of very small separated CuNp. Fig 9 shows the UV-Visible spectrum for CuNp for the ratio 1:3 under optimum conditions [16].

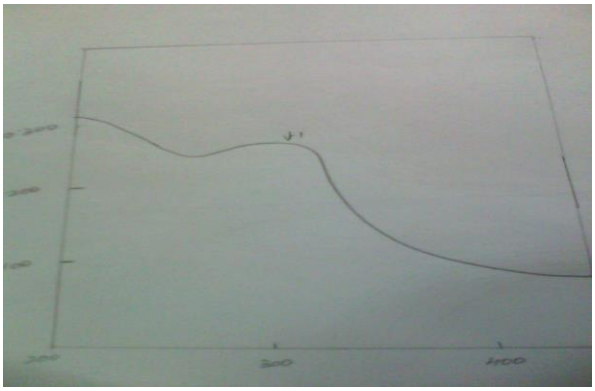


Figure 9: UV–Visible spectrum for the formation of CuNp under optimum conditions.

3.4.2 Fourier Transform-InfraRed (FT-IR) Characterisation

FT-IR spectroscopy was used to investigate the interactions between different species and changes in chemical compositions of the mixtures during bio-synthesis. FT-IR measurements of both the aqueous gooseberry extract and

the synthesized dried copper nanoparticles were carried out to identify the possible bio-molecules responsible for the reduction, capping and efficient stabilization of the bio-reduced CuNp.

The FT-IR spectra of the gooseberry extract and the synthesized CuNp are shown in fig 10a and 10b. The gooseberry extract displays a number of adsorption peaks, reflecting its complex nature. Terpenoids from the phyllanthus embilica (gooseberry) can be identified by the strongest peaks of hydroxyl group at 3419 cm^{-1}, α, β-unsaturated ketone band at 1710 cm^{-1}, olefinic band at 1612 cm^{-1}, primary and secondary alcohols functionalities bands at 1043 cm^{-1} as well as the peaks around 3000 and 1400 cm^{-1} can be attributed to aliphatic C-H stretching and bending modes [21].

By comparing the spectrum of copper nanoparticles with that of the gooseberry extract, we can conclude that the two spectra are similar in their spectral features. There is no question, therefore, that the compound on the surface of copper nanoparticles has a very close chemical composition to the gooseberry. It was found that many peaks obtained by the gooseberry extract have been repeated in the FT-IR spectrum of copper nanoparticles with changes in the position as well as the intensity of absorption.

The absorption peaks at 3419, 1604, 1107 and 1053 cm^{-1} corresponding to OH, C=C and C-O observed in the plants extract get narrower and shifted to higher frequently regions, while those at around 3000 and 1400 cm^{-1} attributable to aliphatic C-H stretching and bending modes deceased in intensity and shifted to low frequency regions. In addition the disappearances of γC=O stretching vibration of α, β-unsaturated ketone at 1710 cm^{-1} confirm that the reduction and the stabilization of copper nanoparticles proceed via these groups. This also confirms that water soluble compounds such as terpenoids which are present in gooseberry extract have the ability to perform dual functions of reduction and stabilization of copper nanoparticles. A similar observation has been reported by several works [22].

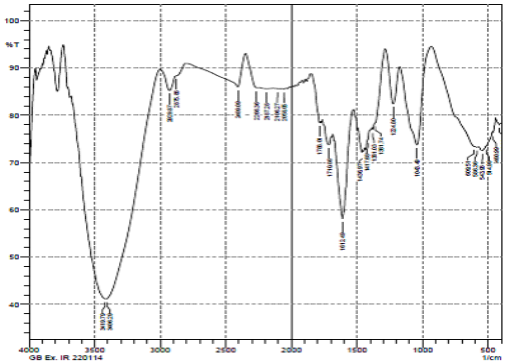


Figure 10(a): IR spectrum of aqueous gooseberry extract.

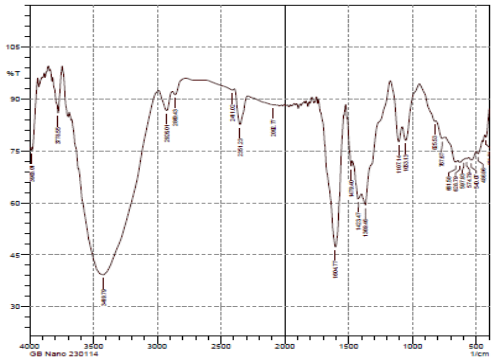


Figure 10(b): IR spectrum of copper nanoparticles.

3.4.3 XRD

XRD patterns taken using powder X-ray diffractometer instrument (SEIFERT JSO DEBYEFLEX 2002) in the angle range 10-80^0 of the copper nanoparticles at 2θ, scan axis 2:1 sym is shown in fig 11. A number of Bragg reflections corresponding to (111), (200) and (220) sets of lattice planes are observed, which can be indexed to face-centered cubic copper [23]. The peaks match with the Joint Committee of powder Diffraction Standards (File No. 089-2838), which further proves the formation of crystals of copper nanoparticles. Furthermore, the average diameter of the copper nanoparticles is calculated in the range 15-30nm by Scherrer's formula using FWHM obtained from the diffraction peaks:

$$\mathbf{D= 0.89\lambda/\beta\cos\theta}$$

Where D is the mean grain size, λ is the wavelength of copper target, β is the FWHM of the diffraction peaks and θ is the diffraction angle. Thus XRD is commonly used to determine the chemical composition and crystal structure of a material.

XRD pattern was taken for both copper nanoparticle in the presence and the absence of PEG 6000 (capping or protecting agent). The fig 11a and 11b shows copper nanoparticle formation in the presence and absence of PEG 6000. In the absence of PEG 6000 XRD showed that the Cu_2O was formed whereas in the presence of capping or protecting agent i.e, PEG 6000 it showed that Cu has formed. This clearly proves that PEG 6000 plays vital role in the formation of copper nanoparticles and protects them from oxidation.

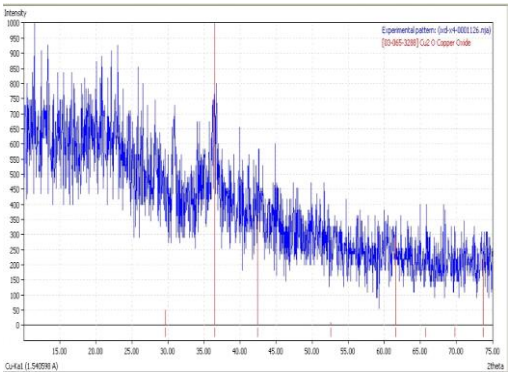


Figure 11 (a): XRD spectrum of biosynthesized copper nanoparticle in the presence of PEG 6000.

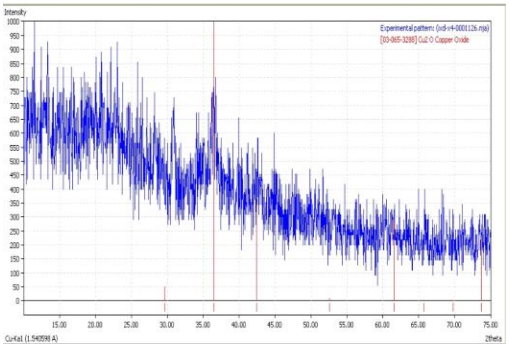
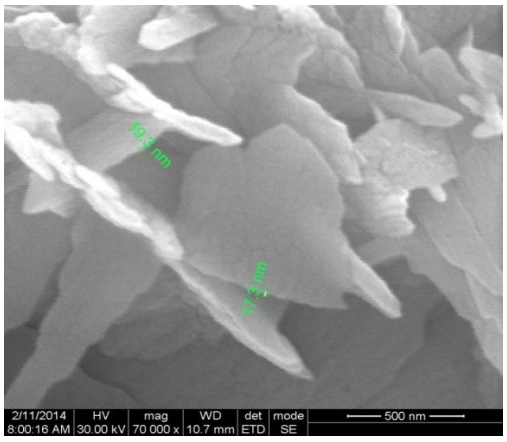


Figure 11 (b): XRD spectrum of Cu_2O in the absence of PEG 6000.

3.4.4 SEM (Scanning Electron Microscopy)

Scanning Electron Microscopy provided further insight into the morphology and size details of the copper nanoparticles. The experimental result showed that the diameter of the prepared nanoparticle was about 15-30nm and the shape is found to be flakes as shown in the fig 12a and 12b. Similar phenomenon was reported [24].



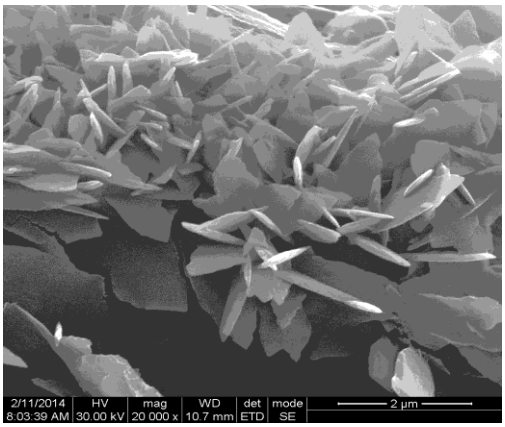


Figure 12(a) and **12(b)** SEM picture of biosynthesized nanoparticles.

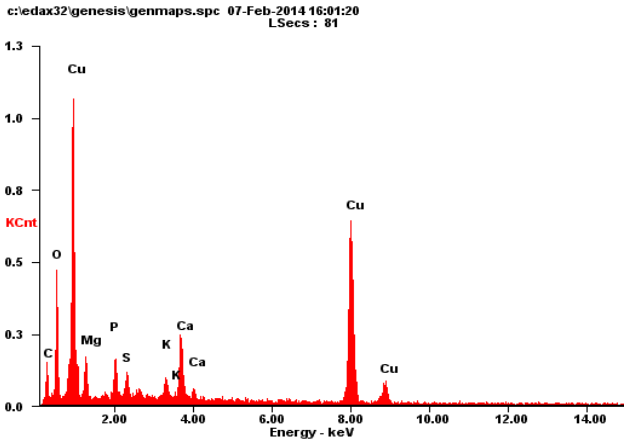


Figure 13: EDS spectrum of biosynthesized copper nanoparticle.

3.4.5 EDAX

The EDAX pattern clearly shows that copper nanoparticle formed by the reduction of copper ions using fresh aqueous gooseberry extract are crystalline in nature (fig 13). The EDS spectrum was recorded in the spot-profile mode. The optical absorption peak is observed at 1Kev, which is typical for the absorption of metallic copper nanoparticles. Strong signals from the copper atoms are observed, while weaker signals for Ca, P, Mg and K atoms were also recorded. From the EDS signals, it is clear that copper nanoparticles reduced by aqueous gooseberry extract have the weight percentage of elemental copper as 71%.

3.5 Antibacterial activity of copper nanoparticles

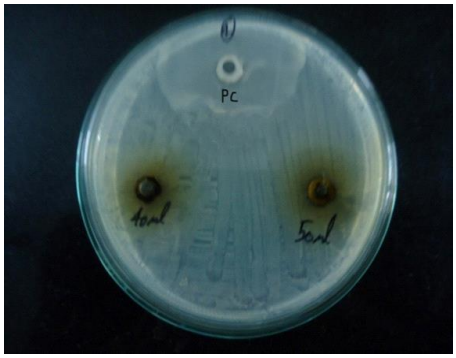
The emergence of nanoscience and nanotechnology in the last decade presents opportunities for exploring the bactericidal effect of metal nanoparticles. The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio, which allows them to interact 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 of the bacterial cell membrane and it is essential to the survival of bacteria. It is made from polysaccharides and peptides named peptidoglycon. There are broadly speaking 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 strain, a test long employed for the classification of bacterial species. Gram positive bacteria possess a thick cell wall containing many layers of peptidoglycon. In contrast, gram negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycon. 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 E-Coli and Staphylococcus aureus by standard disc diffusion method (fig 14a and b). E-Coli are gram –ve bacteria and Staphylococcus aureus are gram +ve bacteria. Biosynthesized CuNps showed clear zone of inhibition as indicated in the table-5 against E-Coli and Staphylococcus aureus was found to be resistant. 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 nanoparticle as well as on the initial bacterial concentration. The reason could be that the smaller size of the particles which leads to tightly adsorbed on the surface of the bacterial cells so as to disrupting the membrane which would lead to the leakage of intracellular component, thus killing the bacterial cells [25]. 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. Ciprofloxacin 25µg/ml was used as +ve control.

Table-5 showing zone of inhibition against bacterial pathogens.

Culture name	CuNp (1500µg)	CuNp (2000µg)	Pc
E. coli	10	14	24
Staphylococcus aureus	12	14	22



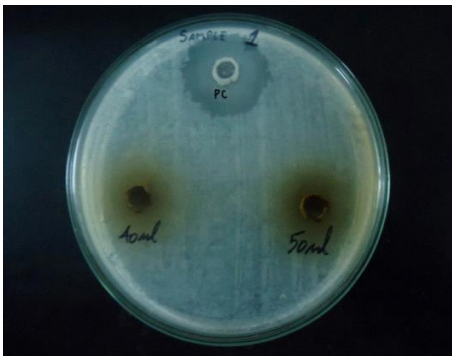


Figure 14: Zone of inhibition of biosynthesized copper nanoparticle against bacterial pathogens. (a) E-Coli & (b) Staphylococcus aureus.

4 Conclusion

Here we have reported a simple reproducible and low cost approach for the preparation of stable copper nanoparticles by using aqueous extract of the Gooseberry as the reducing, stabilizing and capping agent. The biosynthesized nanoparticles have been characterized by SEM, EDS, FT-IR, XRD and UV-VIS spectroscopy. The copper nanoparticles have antibacterial activity effects. The biosynthesized copper nanoparticles proved to be potential candidates for medical applications where antioxidant and antimicrobial activities are highly essential. Hence the biosynthesized copper nanoparticles are more effective in the drug delivery process. As the biosynthesized copper nanoparticles showed excellent antimicrobial activity, the green synthesized metal nanoparticles could replace some antibiotic medicines used to combat human pathogenic microorganism (bacteria) and cost effective in the pharmaceutical industry. The present work can be further extended to characterize the biomolecules involved in the bioreduction of CuNp.

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