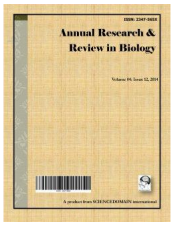

Annual Research & Review in Biology

23(1): 1-10, 2018; Article no.ARRB.38894
ISSN: 2347-565X, NLM ID: 101632869

Studies on Green Synthesis of Copper Nanoparticles Using *Punica granatum*

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2018/38894
Editor(s):
(1) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Alessandro Buccolieri, University of Salento, Italy.
(2) Kiran R. Halkai, Rajiv Gandhi University of Health Sciences, India.
(3) Merlis P. Alvarez-Berríos, Inter American University of Puerto Rico, Puerto Rico.
(4) Claudia Antonetti, University of Pisa, Italy.
Complete Peer review History: http://www.sciencedomain.org/review-history/22925

Original Research Article

Received 19th November 2017
Accepted 24th January 2018
Published 30th January 2018

ABSTRACT

Aim: The present study aimed at green synthesis of copper nanoparticles using various plant extracts as reducing and stabilizing agents. It would also study the antibacterial effect of the synthesized copper nanoparticles.
Place and Duration of Study: Department of Microbiology, Bhavan's Vivekananda college of Science, Humanities and Commerce, Hyderabad, India. The duration of the study is for six months between May 2017 to October 2017.
Methodology: The aqueous solutions of different plant extracts were mixed with $CuSO_4$ solution and incubated for green synthesis of stable copper nanoparticles. These were tested by UV-Visible spectroscopy and SEM analysis. Antibacterial tests of the biosynthesized nanoparticles were carried out on Gram-positive Bacteria *Staphylococcus aureus* by Agar well assay.
Results: The aqueous solutions of different plant extracts yielded stable copper nanoparticles as indicated by the O.D values tested using UV-Visible spectroscopy. The best plant extract that yielded higher amount of copper nanoparticles was fruit rind extract of *Punica granatum*. The synthesized nanoparticles were found to be 56-59 nm, characterized by Scanning Electron

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Microscopy (SEM). The synthesized copper nanoparticles exhibited a strong antibacterial activity against *Staphylococcus aureus*.
Conclusion: The copper nanoparticles can be green synthesized using fruit rind extract of *Punica granatum* and these can be used as efficient antimicrobial agents against *Staphylococcus aureus* and the study is significant currently as drug resistant infections of *Staphylococcus aureus* are gaining much prevalence and prominence.

Keywords: $CuSO_4$ solution; copper nanoparticles; leaf extract; Punica granatum; antimicrobial agent.

1. INTRODUCTION

The field of nanotechnology is one of the most active areas of research in modern materials science. Nanoparticles usually referred to as particles with a size approximately extending from 1 nm up to 100 nm in length in at least one dimension [1], exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. The application of nanoscale materials and structures is an emerging area of nanoscience and nanotechnology. Nanoparticles provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine, and water-treatment [2]. Particularly copper nanoparticles have assumed a great deal of importance as they often display unique and considerably modified physical, chemical and biological properties as compared to their counterparts of the macro scale [3,4].

Copper nanoparticles, due to their unique physical and chemical properties and low cost preparation, have been of great interest recently. Copper nanoparticles have diverse applications as heat transfer systems [5], as super strong materials [6], as sensors [7] and as catalysts [8]. Their other properties like antimicrobial activity, disinfecting property and stability as matrix bound particles can be further exploited for use in wall paints and plasters to coat hospital equipment [9,10].

The synthesis of copper nanoparticles is an active area of academic and, more importantly, application research in nanotechnology. A variety of chemical and physical procedures such as chemical reduction [11], electrochemical reduction [12], chemical vapor deposition [13], thermal decomposition [14] and solvo thermal reduction [15] have been reported for synthesis of metallic nanoparticles. However, these methods have many problems including use of toxic solvents, generation of hazardous by-products, high energy consumption and are non eco friendly. Taking this aspect into consideration there is an essential need to develop clean, reliable, biocompatible, cost-effective, environmentally friendly and sustainable procedures for synthesis of nanoparticles [16,17].

Considering the vast potentiality of plants as sources for the green synthesis of different nanoparticles, and especially copper nanoparticles researchers worked with plant extracts, some specific plant parts or whole plant for the green synthesis [18]. Many of them reported that extracts from plants like *Nerium oleander* [19], *Punica granatum* [20], *Aegle marmelos & Ocimum sanctum* [21,22], *Zingiber officinale* [23] efficiently yielded copper nanoparticles on green synthesis and so were used for the same. The present study also concentrated on green synthesis of copper nanoparticles using concentrated and wet leaf extracts of *Ricinus communis, Punica granatum, Psidium guajava, Eucalyptus globules, Ocimum tenuiflorum, Tagetes sp., and Phyllanthus emblica* as both reducing and stabilizing agent. The antimicrobial activity of synthesized copper nanoparticles was studied against Gram positive bacteria like *Staphylococcus aureus* and it was found to be very potent. This is significant currently when drug resistant infections of *Staphylococcus aureus* are becoming more prevalent.

2. MATERIALS AND METHODS

2.1 Collection of Plant Leaf Samples

The young leaves of *Ricinus communis, Punica granatum, Psidium guajava, Eucalyptus globules, Ocimum tenuiflorum, Tagetes sp*, *and Phyllanthus emblica* were collected from the college campus, local garden and nursery.

2.2 Preparation of Plant Extracts

Fresh plant samples selected for the study were thoroughly washed with tap water first and then with distilled water to remove debris and other contaminants. These thoroughly washed samples were air dried and used. For the preparation of extract 15 gm of respective samples were boiled in 100 mL of sterile water for 20 min and filtered through Whattman filter paper No.1 after cooling. The filtered extracts were stored at 4°C and used for further studies [24].

2.3 Biosynthesis of Copper Nanoparticles

Different aqueous leaf extracts in a fixed volume 10 mL were added to 100 mL of 1mM of aqueous copper sulphate solution in a 250 mL Erlenmeyer flask. The color of the solution changed from blue to pale yellow [25] when both the solutions of leaf extract and copper sulphate were mixed in equimolecular ratio and stirred thoroughly for 30-60 min to form homogeneous mixture. The flask was kept at room temperature overnight and the copper nanoparticles formed separated out and settled at the bottom of the flask.

2.4 Biosynthesis of Copper Nanoparticles Using Different Molar Solutions and Concentrations of Copper Sulphate Solution

Copper sulphate solutions in variable molarities namely 1 mM, 5 mM 10 mM and 20 mM were prepared by adding copper sulphate to double distilled water separately. Then these copper sulphate solutions were mixed in various concentrations such as 2.5%, 5% and 7.5% with the selected plant extract (50% concentrated). The solution was converted to the pale yellow color, indicating the formation of copper nanoparticles [26].

2.5 Characterization of Biosynthesized Copper Nanoparticles

The green synthesized copper nanoparticles were characterized by UV-vis spectroscopy and SEM analysis.

2.5.1 UV-Vis spectroscopy

The reduction of pure copper ions was monitored by measuring the UV-Vis spectrum of the reaction. UV-vis absorption spectrum of the samples (copper nanoparticles synthesized from various plant extracts) was done in an Electronics India-371 UV-Vis spectrophotometer in the wavelength range from 300 to 600 nm to determine maximum absorption. The measurement of copper nanoparticles synthesized under different conditions like variation of concentration of plant extracts (fruit rind & leaf extract of *Punica granatum*), different molarity of copper sulphate solutions and incubation time were taken at the particular wavelength that gave maximum absorption.

2.5.2 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was used to observe the size, shape of the synthesized nanoparticles copper nanoparticles (prepared from 10 mM copper sulphate solution with 5% concentrated fruit rind extract).

2.6 Assessment of Antimicrobial Activity

The antimicrobial activity was carried out using Agar well diffusion method [27,28]. For this preseeded agar plates were prepared by adding 5% inoculum of the test organism *Staphylococcus aureus* in broth (having an O.D of 0.5 and containing 10^5 cfu/mL) to nutrient agar and then pouring the plates (Pour plate method).Wells were bored in the agar layer of each preseeded plate using sterile cork borer of 6 mm diameter. Concentrated, wet and different dilutions of leaf extracts of various samples (50 µL) were introduced into the wells using micropipettes and allowed to diffuse at room temperature for 2 h. The plates were incubated at 37°C [29]. After the incubation period, the mean diameters of the zones of inhibition around the wells were recorded. The experiments were conducted in triplicates along with control and the average values were recorded for antimicrobial activity.

3. RESULTS

When copper nanoparticles were synthesized using plant extracts like *Ricinus communis, Punica granatum, Psidium guajava, Eucalyptus globules, Ocimum tenuiflorum, Tagetes sp.*, *Phyllanthus emblica* as reducing and stabilizing agent, the aqueous copper sulphate solution turned to yellow color within 30 min. The absorption maxima for the synthesized copper nanoparticles was determined and reported graphically. The characteristic absorption maxima was 450 nm [Fig. 1]. As observed from the intensity of the surface Plasmon absorption peak at around 450 nm as a function of time, the rate of reduction of copper ions using different leaf extracts are in the order of: *Tagetes sp.*< *Ricinus communis* < *Eucalyptus globules*<

Psidium guajava < *Ocimum tenuiflorum* < *Phyllanthus emblica* < *Punica granatum* [Fig. 2].

Further absorbance of copper nanoparticles synthesized from different molarity of copper sulphate solution with selected leaf, fruit rind, seed, flower extracts of *Punica granatum* was observed at 450 nm (Fig. 3 and Fig. 4). The results indicated that as concentration of copper sulphate increases optical density of synthesized nanoparticles from concentrated fruit rind extract increased up to 10 mM then decreased (Fig. 5, Fig. 6 and Fig. 7). The size and morphology of green synthesized copper nanoparticles (prepared from 10 mM with 5% copper sulphate and fruit rind extract of *Punica granatum)* was determined by scanning electron microscopy (Fig. 8). SEM images indicate that the nanoparticles prepared from 10 mM copper sulphate with 5% fruit rind extract of *Punica granatum* were spherical in shape with 56-59 nm diameter.

The antibacterial activities of synthesized copper nanoparticles using various extracts of *Punica granatum* were tested against pathogenic bacteria like *Staphylococcus aureus* using the Agar Well Assay (Fig. 9 and Fig. 10). The results indicate that copper nanoparticles synthesized via green route are promising antibacterial agents against the multidrug resistant *Staphylococcus aureus* strains and so these have*,* great potential in biomedical applications.

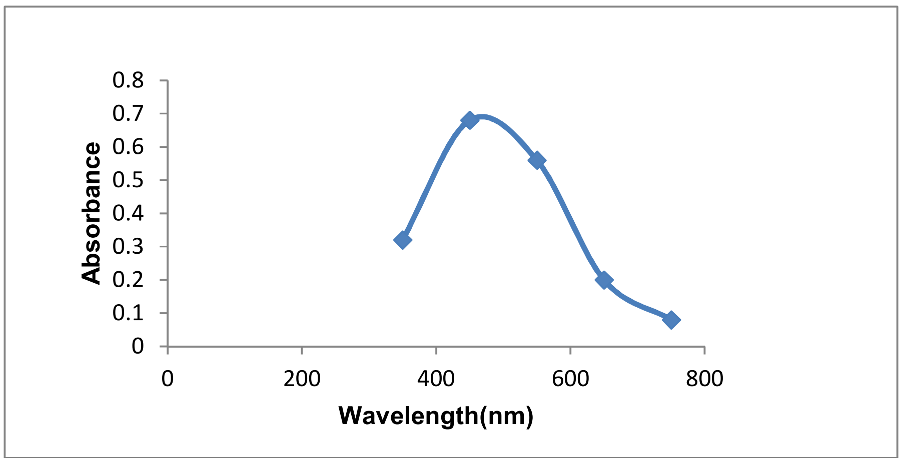

Fig. 1. UV/Vis absorption spectra of copper nanoparticles

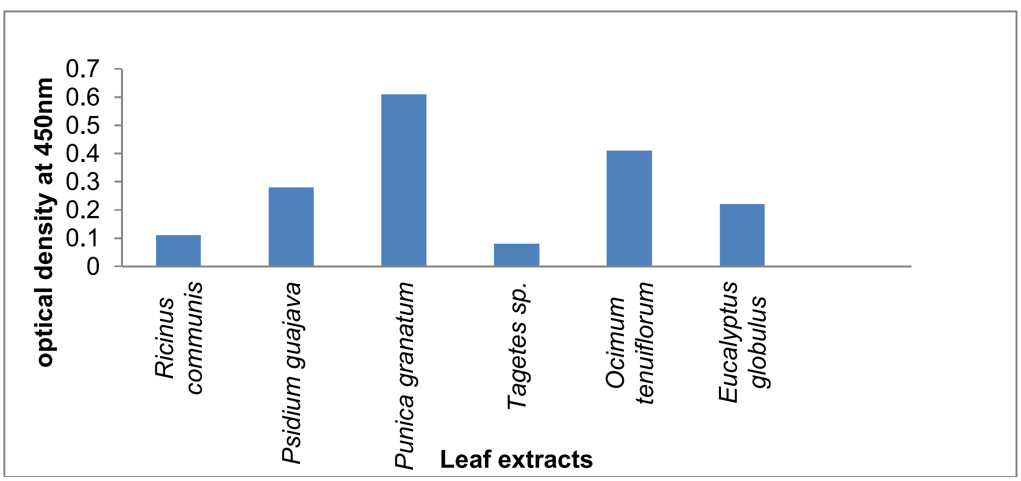

Fig. 2. Synthesis of copper nanoparticles from different plant extracts

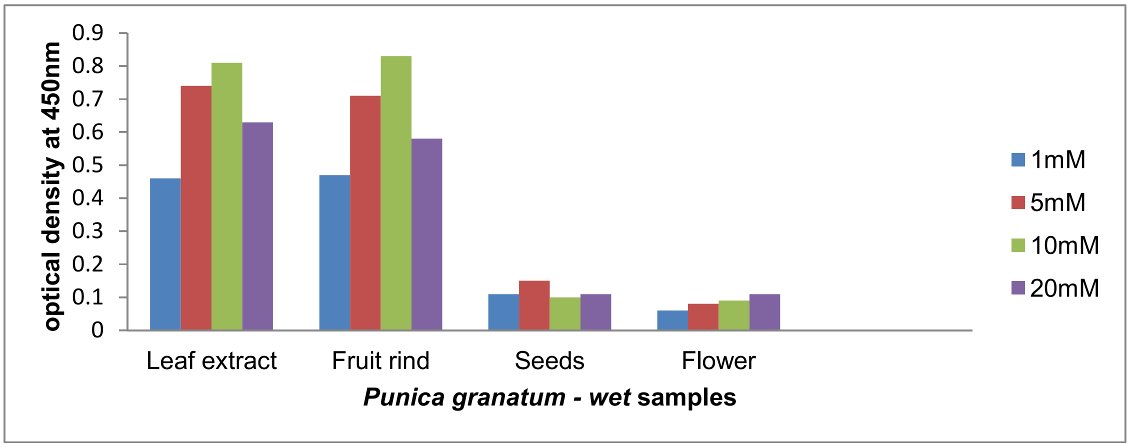

Fig. 3. Effect of different molar solutions of copper sulphate on copper nanoparticles synthesis using wet samples

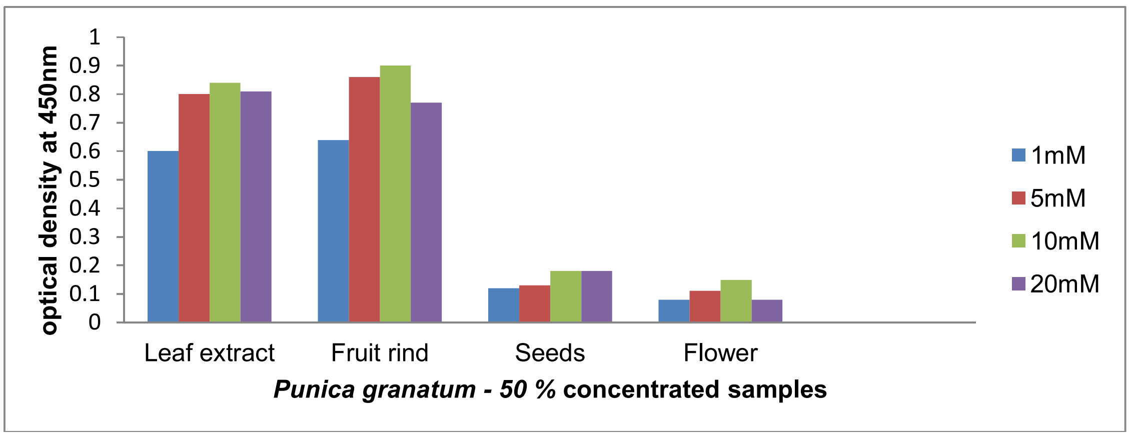

Fig. 4. Effect of different molar solutions of copper sulphate on copper nanoparticles synthesis using 50 % concentrated samples

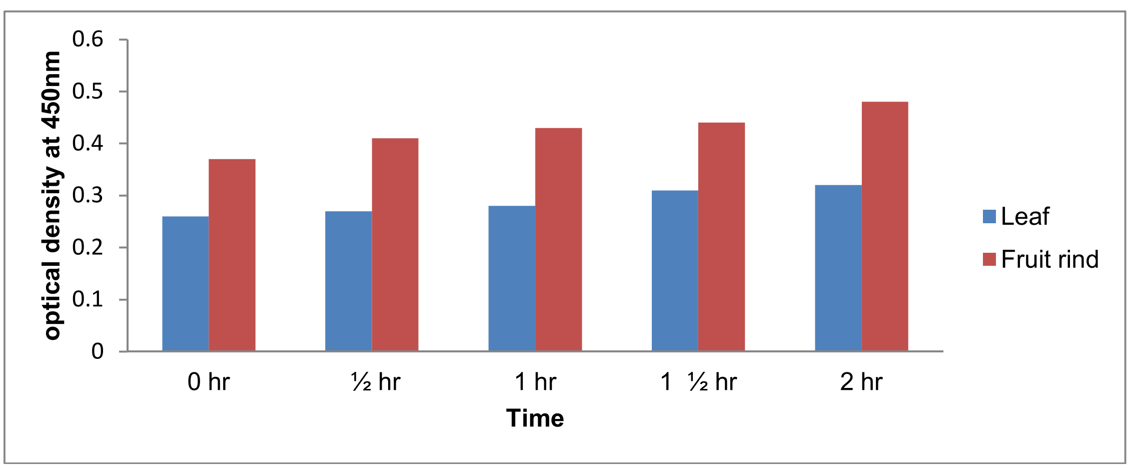

Fig. 5. Effect of 2.5% of fruit rind & leaf extract using 10 mM of copper sulphate on synthesis of copper nanoparticles

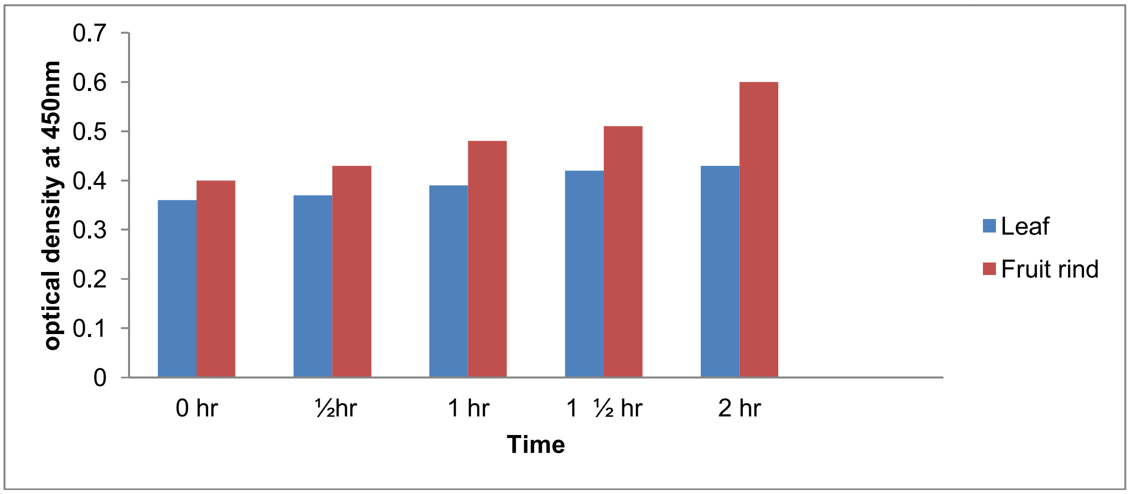

Fig. 6. Effect of 5% of fruit rind & leaf extract using 10 mM of copper sulphate on synthesis of copper nanoparticles

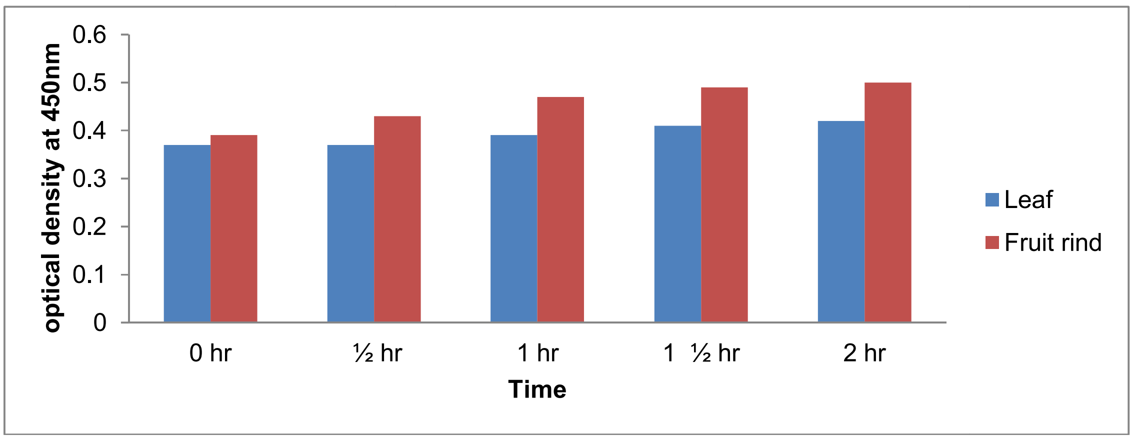

Fig. 7. Effect of 7.5% of fruit rind & leaf extract using 10 mM of copper sulphate on synthesis of copper nanoparticles

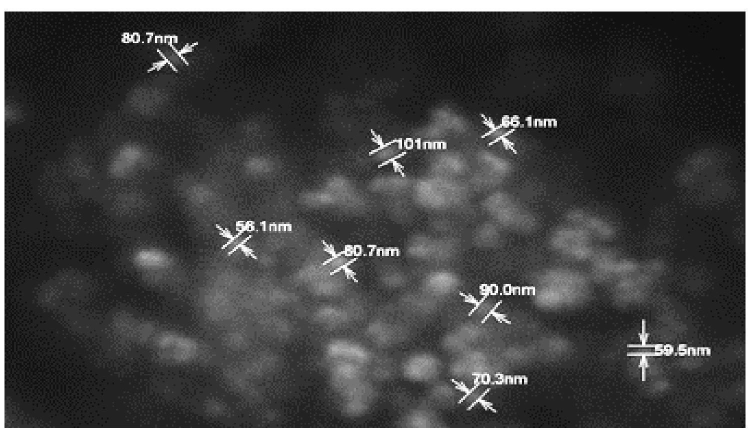

Fig. 8. SEM image of copper nanoparticles synthesized from 10 mM copper sulphate solution with 5% *Punica granatum*-fruit rind extract after 2 hours of incubation

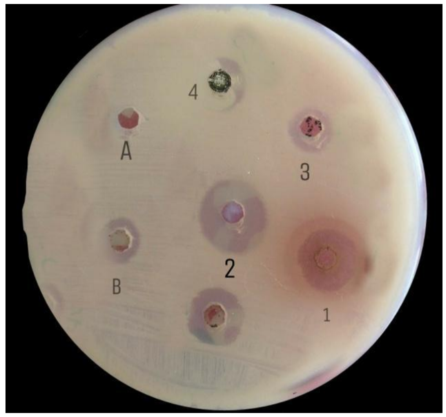

Fig. 9. Antibacterial activity of copper nanoparticles assayed by the agar well method. In the plate, the cavity A- sterilized water, cavity-B copper sulphate solution, cavity-1 leaf extract, cavity-2 fruit rind extract, cavity-3 seeds extract,cavity-4 flower extract. The clear zone of the central well indicates growth restriction by diffused nanoparticles and copper sulphate solution against *Staphylococcus aureus*

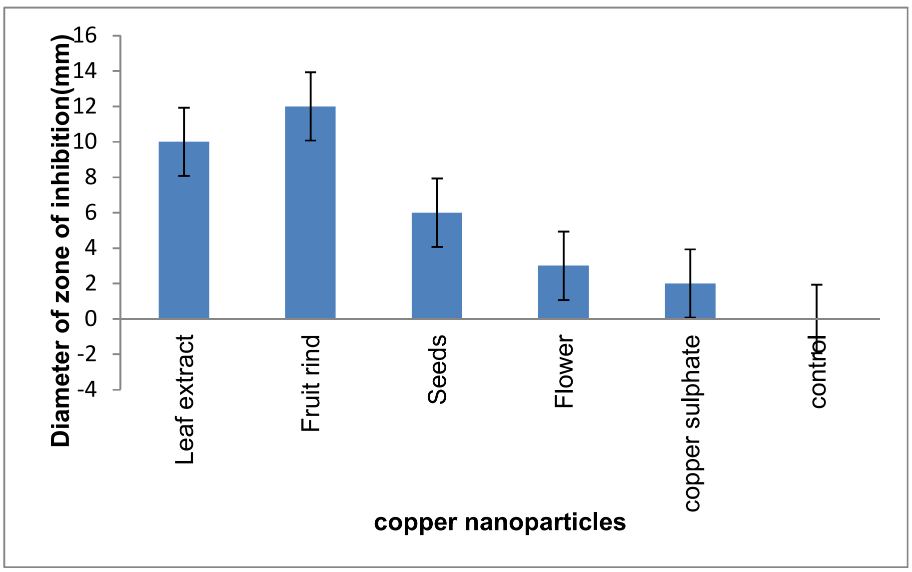

Fig. 10. Antibacterial effect indicated by zone of inhibition of synthesized copper nanoparticles against *Staphylococcus aureus*

4. DISCUSSION

It is known that copper (II) sulphate salt when dissolved in water, dissociates into Cu^{2+} and SO_4^{2-}. These Cu^{2+} ions thus formed are unstable and so are reduced to Cu° by reducing action of components present in plant extracts, forming metallic copper nuclei, that can further be reduced in size to form nanoparticles. Thus copper sulphate favors formation of copper nanoparticles. Also the growing nanoparticles are stabilized by several molecules of plant extracts distributed around them. These agglomerates grow to a certain size and eventually get precipitated. Green synthesis of copper nanoparticles can be observed by the color change of the solution from blue to yellow and intensity of the yellow color increased in direct proportion to the reaction time. It may be due to the excitation of surface Plasmon resonance (SPR) effect and reduction of copper ions.

The synthesis and utilization of novel antimicrobial metal nanoparticles has increased due to the gradual increase of drug resistance among microorganisms. Copper has been recognized as a hygienic material since the beginning of civilization and so copper compounds have been employed as antimicrobial agents. Copper especially in its nanoscale, has significant antibacterial activity and also synthesis of copper nanoparticles is very cost effective [30]. The mode of action of copper nanoparticles is not clear but it is known that copper ions disrupt biochemical processes inside bacterial cells. In addition, no research has discovered to date that indicated development of resistant bacteria to copper as seen in case of antibiotics.

Pomegranate (*Punica granatum*) is a polyphenol-rich fruit. Polyphenols have received a great deal of attention due to their biological functions. There are reports on anti bacterial effect of *Punica granatum* on multidrug-resistant *Staphylococcus epidermidis* strains. Tannins found in abundance in Pomegranate, are also toxic to microorganisms [31] as they interact with the bacterial membrane causing instability of the membrane, thus affecting the transport of substrates into the cell [32]. Likewise, Naz et al. [33] suggested phenolic toxicity through reactions with sulfhydryl groups or through more non-specific interactions with proteins leading to loss of function [34,35]. Copper ions released may also interact with DNA molecules and intercalate with nucleic acid strands. This encouraged more and more researchers to exploit biological systems as possible eco-friendly nano-factories leading to green synthesis of nanoparticles.

5. CONCLUSION

In conclusion, the green synthesis of copper nanoparticles using plant material as reducing and capping agent has many advantages like ease of synthesis, economic viability, eco friendly nature along with it being renewable and also giving good scope for scale up. Green synthesis of copper nanoparticles with 10 mM solution of copper sulphate using 50% concentrated *Punica granatum* fruit rind extract gave good yield of nanoparticles of medium size within 1½ to 2 hours. These copper nanoparticles showed good anti-bacterial effect against *Staphylococcus aureus.* Thus these green synthesized eco friendly copper nanoparticles with bactericidal effect against wound causing *Staphylococcus aureus* could be used for development of nano bandages.

ACKNOWLEDGEMENTS

The authors (Thanveer Banu, Chaitanya and Naga Padma) are grateful to the management of BVB Bhavan's Vivekananda College for their encouragement to carry out this work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/22925