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(RESEARCH ARTICLE)

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Green synthesis of copper nanoparticles by using aqueous extract of *Ocimum sanctum* leaves and its antibacterial activity

Jivan Munja Dhotare [1], Mukundraj Govindrao Rathod [2], Praneeta Dattatraya Barkhude [1], Gautam Tanaji Kamble [3], Namrata Vinodkumar Dongrajkar [3], Shidheshwar Narayanrao Shivankar [3], Umesh Pravin Dhuldhaj [3] and Anupama Prabhakarrao Pathak [3,*]

[1] *College of Agricultural Biotechnology (affiliated to Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani), Hatta 431705, Taluka Basmat, Dist. Hingoli, Maharashtra state, India.*
[2] *Department of Biotechnology & Bioinformatics (U.G. & P.G.), Yeshwant College of Information Technology, Parbhani 431401(affiliated to Swami Ramanand Teerth Marathwada University, Nanded, Maharashtra, India.*
[3] *School of Life Sciences (DST-FIST phase-I and UGC-SAP DRS-II sponsored school), Swami Ramanand Teerth Marathwada University, Nanded-431606, Maharashtra, India.*

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Abstract

Nanoparticles are described as substances with at least one exterior dimension between 1 and 100 nm. Nanoparticles can be produced by physical, chemical, or biological processes. Nanoparticle production techniques include chemical reduction, electrochemistry, photochemistry, and physical vapour condensation. However, some of these methods are very expensive and risky. The biological method for creating nanoparticles is simple, quick, inexpensive, dependable, and risk-free in comparison to these technologies. Our current study documented the green synthesis of copper nanoparticles using *Ocimum sanctum* (Tulsi) leaves extract and their antibacterial activity. We have used UV-vis spectroscopy to characterise copper nanoparticles. *Ocimum sanctum* leaves extract contained alkaloids, flavonoids, tannins, and saponins that might serve as capping and stabilising agents for the green synthesis of *Ocimum sanctum*-copper nanoparticles. In the form of a zone of growth inhibition, the copper nanoparticles demonstrated promising effects against all of the chosen bacteria. It was discovered that copper nanoparticle formation increased along with the reaction time of *Ocimum sanctum* leaves extract and copper sulphate. The inhibitory action of copper nanoparticles was found to increase along with the concentration of copper sulphate in solution. The effectiveness of different volumes of *Ocimum sanctum* leaves extract in reducing copper ions was assessed, and its effectiveness against *Escherichia coli* was confirmed.

Keywords: Antimicrobial; Leaf extract; Nanoparticles; *Ocimum sanctum*

1. Introduction

Materials with at least one exterior dimension between 1 and 100 nm are referred to as nanoparticles [1]. Any material's intrinsic qualities may alter if it is shrunk to the nanoscale. Numerous varieties of nanoparticles, including magnetic, non-magnetic, and metallic ones, are employed in a wide range of applications such as water purification, electronics, catalysis, solar energy conversion, and optics [2,3,4]. The majority of applications for gold, silver, and copper metal nanoparticles include surface-enhanced Raman scattering detection, photonics, bioengineering, medicine, agriculture, and catalysis [5,6,7]. Additionally, some functionalized, biocompatible, and inert nanoparticles may be used in the detection and treatment of cancer. Although there are many different kinds of nanomaterials and their uses are already

* Corresponding author: Anupama Prabhakarrao Pathak; Email: anupama.micro@rediffmail.com

understood, the discipline of nanoscience and nanotechnology is still developing since there are several uses for nanomaterials that have yet to be discovered [8,9,10,11].

It is possible to create nanoparticles through physical, chemical, or biological processes [12]. Chemical reduction, electrochemistry, photochemistry, and physical vapour condensation are a few well-researched ways to make nanoparticles. However, some of these techniques are very pricy and dangerous. In contrast to these technologies, the biological method for synthesizing nanoparticles is straightforward, quick, affordable, dependable, and risk-free [13]. Furthermore, it is noteworthy that nanoparticles produced through biological means exhibit outstanding yield, solubility, and durability. The biological method incorporates environmentally friendly techniques that allow for the optimum production of chemical products like nanoparticles [14]. Metal salts are combined with vitamins, sugars (carbohydrates), plant extracts, proteins, or microbes to create nanoparticles in a green manner. *Aloe vera*, *Buddleja globosa*, *Carica papaya*, *Cassia auriculata*, *Allophylus cobbe*, *Typha angustifolia*, *Jatropha curcas*, *Ocimum sanctum* (Tulsi), and *Azadirachta indica* (neem) are a few examples of plants whose extracts might function as a reducing agent in the reaction with metal salts [15,16]. Among the microorganisms utilised for the synthesis of nanoparticles are *Pseudomonas stutzeri* AG259, *Lactobacillus* sp., *Bacillus licheniformis*, *Escherichia coli*, *Brevibacterium casei*, *Bacillus amyloliquifaciens*, *Bacillus flexus*, *Staphylococcus aureus*, *Fusarium oxysporum*, and *Ganoderma neo-japonicum* [17]. Starch (biodegradable polymer), fibrinolytic enzymes, and amino acids are among the biomolecules used in the green chemistry approach to nanoparticle synthesis [18]. Some plants' stems, roots, shoots, flowers, barks, and seeds have been successfully involved in nanoparticle synthesis [19].

Our current study documented the green synthesis of copper nanoparticles using *Ocimum sanctum* (Tulsi) leaves extract and their antibacterial activity.

2. Material and methods

2.1. Preparation of leaves extract and green synthesis of nanoparticles

20 g fresh *Ocimum sanctum* leaves were collected and thoroughly washed with distilled water to remove adhered dirt particles. Finely chopped leaves were added to 100 mL of distilled water and stirred for 1 hour at 60 ^{o}C. The mixture was cooled after boiling and filtered through Whatman filter paper No. 1. For the bioreduction process, 5 mL of filtrate (leaves extract) was added to 45 mL of 1 mM $CuSO_4.5H_2O$ solution and stirred continuously for 24 hours at 30 ^{o}C [3,4,9]. The resulting copper nanoparticle solution was purified by centrifugation at 12,000 revolutions per minute for 15 minutes. The copper nanoparticles were dried in a hot air oven at 80 °C for three hours. UV visible spectroscopy techniques were used to characterise the bio synthesised copper nanoparticles. The absorbance of the samples measured in wavelength ranged from 400 to 600 nm [20].

2.2. Phytochemical analysis

The principal phytochemicals, including alkaloid, flavonoid, tannin and saponins, were identified using the established techniques as described by Panchal and Parved for qualitative phytochemical analysis of *Ocimum sanctum* leaves extract [21,22].

2.3. Cultivating active cultures of test organisms to determine copper nanoparticles' antibacterial effectiveness

A loop full of culture was inoculated into 10 ml of nutrient broth from a pure culture of the relevant bacteria, namely *Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhi* that had previously been cultured on nutrient agar slants. The broth suspension was then incubated for the following 24 hours at 37 °C. After incubation, the growth that occurred was used as an active culture for the antibacterial activity [23,24,25,26,27,28,29,30,31].

2.4. Copper nanoparticles' antimicrobial properties as evaluated by the agar well diffusion method

The antimicrobial activity of copper nanoparticles prepared using *Ocimum sanctum* leaves extract was investigated using the agar well diffusion method. In this method, first of all, the freshly prepared and autoclaved nutrient agar media was seeded individually with 3 mL active culture of the respective organism, i.e. *Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhi*. For optimal mixing of microbial cells in molten nutritional agar, each flask was vigorously agitated for 2-3 minutes. Petri plates were filled with seeded nutrient agar, and the plates were left to set. The cork borer was disinfected in ethanol by the surface sterilization method before being used to pierce the wells in the agar medium. Each well was filled with 0.1 mL of copper nanoparticle solution using a micropipette. Then, to allow for diffusion, these nutrient agar plates were put in the freezer for ten minutes. Plates were then kept at 37 °C for a further 24 hours. After incubation, the plates were examined to see if growth inhibition zones had developed around the wells. The size of the

inhibitory zone was measured. The same procedure was performed for the samples of leaves extract of *Ocimum sanctum*, copper sulphate solution, and distilled water [5,6,9,32].

2.5. Effect of reaction time period

5 mL of *Ocimum sanctum* leaves extract was mixed thoroughly with 20 mL of 1 mM $CuSO_4.5H_2O$. The copper nanoparticles that had developed in the tube were extracted out after every hour, up to a maximum of 8 hours. Sterilized filter paper discs of 6 mm diameter were immersed in copper nanoparticles solution extracted at different time interval and kept on nutrient agar plates seeded with *Escherichia coli*. Then plates were kept in the freezer for better diffusion for 10 minutes. Then, the plates were incubated at 37 °C for 24 hours. The plates were observed for zone of growth inhibition [33,34].

2.6. Effect of metal ion concentration

20 mL of copper sulphate solution of different concentrations (0.25 mM, 0.50 mM, 1 mM, 1.5 mM, 2 mM, and 2.5 mM) were prepared in various screw cap tubes. 5 ml of *Ocimum sanctum* leaves extract was added to each screw-cap tube and thoroughly mixed. For the synthesis of copper nanoparticles, all tubes were incubated for 24 hours, and optical densities were measured. Following incubation, 0.1 ml of each tube's solution was added with a micropipette into the corresponding wells of nutrient agar plates that had been seeded with *Escherichia coli*. For proper diffusion, plates were then placed in the freezer for 10 minutes. All the plates were then incubated for 24 hours at 37° C. The plates were observed for zone of growth inhibition. To ensure the reliability and repeatability of the findings, each experiment was carried out three times [2,3,9,35].

2.7. Effect of reducing precursor

Five screw cap tubes holding 4 mL of a 1 mM coper sulphate solution were used, and 1, 2, 3, 4, and 5 ml of *Ocimum sanctum* leaf extract were added to each tube and thoroughly mixed. For 24 hours, these tubes were kept for incubation. Then, the nutrient agar plates seeded with *Escherichia coli* were made. After solidifying, sterile cork borer was used to create the wells. A solution from the appropriate screw cap tube containing 0.1 mL was added to each well. For better diffusion, the plates were placed in the freezer for 10 minutes. Each plate was then kept for 24 hours at 37 °C of incubation. Zones of inhibition were looked for on the plates [5,8,10,36].

3. Results and discussion

3.1. Green synthesis of nanoparticles

Using an extract of *Ocimum sanctum*'s leaves as a reducing agent, green synthesis of copper nanoparticles was accomplished in aqueous solution. Within 24 hours of combining this extract with copper sulphate solution, the colour of the aqueous solution had changed to a green hue, indicating the emergence of copper nanoparticles (Fig. 1). The UV visible spectrophotometer was used to characterise the synthesised copper nanoparticles, and the maximum absorbance (1.21) was found at 298 nm. The electronic spectra of the copper nanoparticles arise due to the excitation of the surface plasmon resonance (SPR) phenomenon.

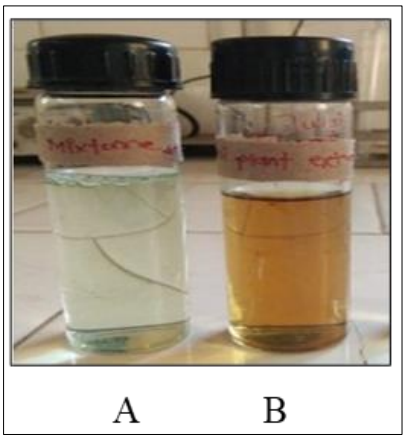

Figure 1 A: Mixture of leaves extract of *Ocimum sanctum* and $CuSO_4$ solution B: Leaves extract of *Ocimum sanctum*

3.2. Phytochemical analysis

Alkaloid, flavonoid, tannin, and saponin were found in leaves extract of *Ocimum sanctum* (Table 1). These phytochemicals could act as capping and stabilizing agents for the green synthesised *Ocimum sanctum*-copper nanoparticles, and they may also be responsible for the reduction of Cu^{+} to Cu^{0}.

Table 1 Qualitative analysis of principal phytochemicals in *Ocimum sanctum* leaves extract

Phytochemical	Test	Inference
Alkaloid	Mayer's test	Present
	Wagner's test	Present
Flavonoid	Sodium hydroxide test	Present
	Ferric chloride test	Present
Tannin	Gelatin test	Present
Saponin	Foam test	Present

3.3. Copper nanoparticles' antimicrobial properties as evaluated by the agar well diffusion method

The antibacterial activity of copper nanoparticles against *Bacillus subtilis*, *Escherichia coli*, and *Salmonella typhi* was observed. The copper nanoparticles showed promising effects against all selected bacteria in the form of zone of growth inhibition. Copper nanoparticles showed greater effect than the cooper sulphate and leaves extract of *Ocimum sanctum*. The copper nanoparticles showed maximum effect on *Escherichia coli* (12 mm), as compared to *Bacillus subtilis* (11 mm), and *Salmonella typhi* (09 mm) (Table 2). In this study we found that the copper nanoparticles are able to inhibit the growth of all selected bacteria.

Table 2 Measurement of diameter of zone as revealed by the selected samples

Test culture	Copper nanoparticles solution	Copper sulphate solution	Leaves extract of *Ocimum sanctum*	Distilled water
Escherichia coli	12	10	8	0
Bacillus subtilis	11	10	6	0
Salmonella typhi	09	07	6	0

(Size of zone is given in mm).

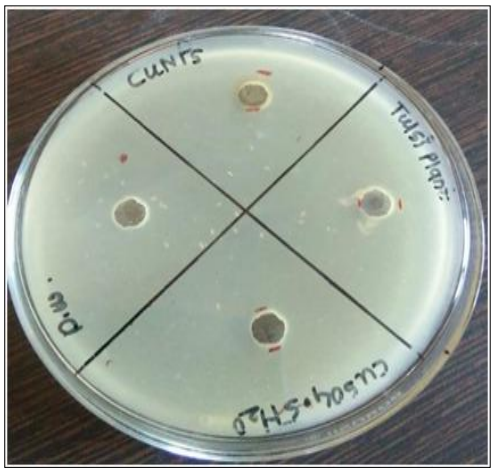

Figure 2 Antibacterial activity of copper nanoparticles (Cu-NPs) against *Bacillus subtilis* as evaluated by the agar well diffusion method

3.4. Effect of reaction time period

It was observed that as the reaction time of leaves extract of *Ocimum sanctum* and copper sulphate increased, the formation of copper nanoparticles also increased, which was confirmed by seeing the diameter of zone of growth inhibition on seeded plates of *Escherichia coli* (Figure 3).

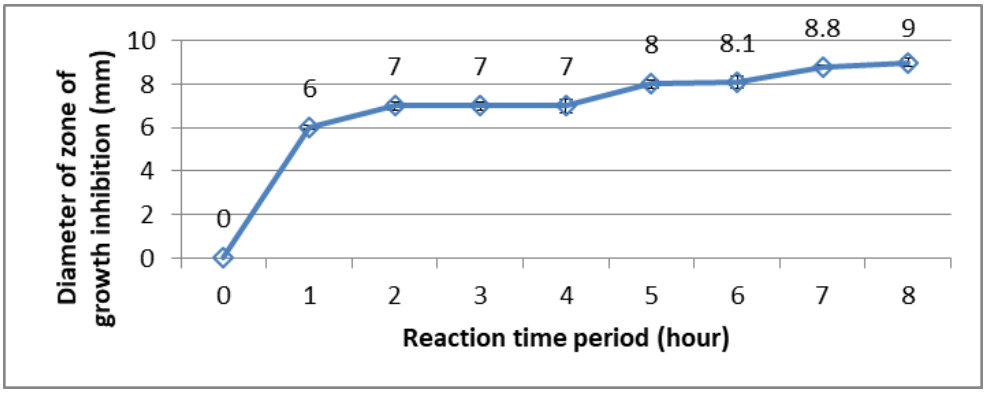

Figure 3 Effect of reaction time period (0-8 hours) on antibacterial activity of copper nanoparticles against *Escherichia coli*

3.5. Effect of metal ion concentration

The inhibitory action of copper nanoparticles was found to increase along with the concentration of metal ions (Figure 4). The absorbance peak at 298 nm was shown by using leaves extract of *Ocimum sanctum* as a reducing agent on the UV visible spectrophotometer.

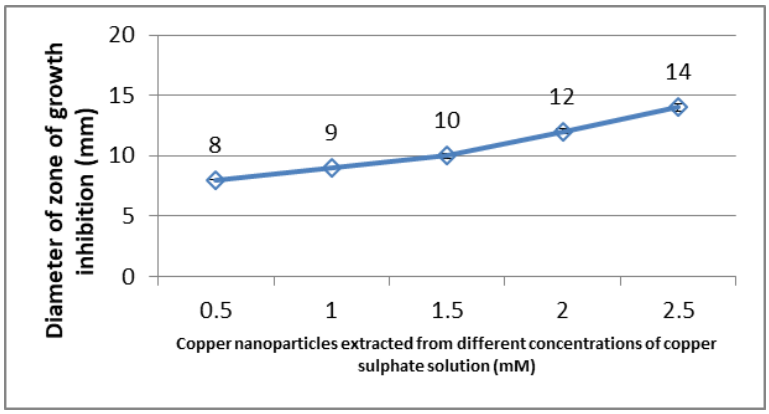

Figure 4 The inhibitory action of copper nanoparticles extracted from different concentrations of copper sulphate solution (mM), against *Escherichia coli*

3.6. Effect of reducing precursor

From figs. 5 and 6, it was observed that even 1 mL of plant extract has ability to reduce 4 mL of a 1 mM coper sulphate solution. Diameter of zone of growth inhibition has shown in Fig. 5.

In the current study, we have only used UV-vis spectroscopy to characterise copper nanoparticles. However, additional characterization is essential in order to evaluate the created particles' functional attributes. The samples are usually characterised using a variety of analytical techniques, including atomic force microscopy, scanning electron microscopy, Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy, and dynamic light scattering [2]. In addition to being used to monitor the synthesis and stability of copper nanoparticles, UV-vis spectroscopy is a very helpful and trustworthy technique for the initial characterization of synthesised nanoparticles [37]. Due to their distinctive optical characteristics, copper nanoparticles strongly interact with particular light wavelengths [2]. Additionally, UV-vis spectroscopy can characterise the particles in colloidal suspensions quickly, easily, simply, sensitively, and selectively for different types of nanoparticles [2, 38].

The copper nanoparticles described in this study can be used to test the effectiveness of various nano-biotechnological applications.

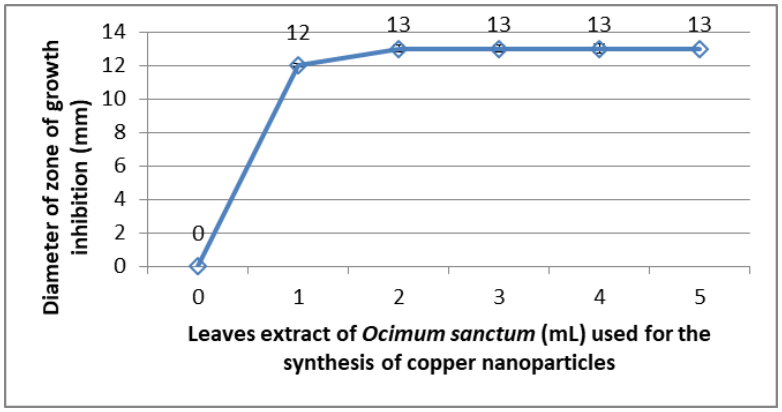

Figure 5 Effect of volume of Leaves extract of *Ocimum sanctum* used for the synthesis of copper nanoparticles

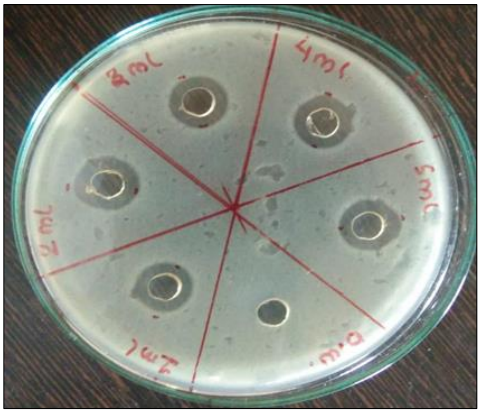

Figure 6 Effect of copper nanoparticle solutions made with various amounts of reducing precursors (1–5 mL) on growth of *Escherichia coli*. (Extract from the leaves of *Ocimum sanctum* functioned as reducing precursors.)

4. Conclusion

In summary, green synthesis of copper nanoparticles was accomplished in aqueous extract of *Ocimum sanctum* leaves by a simple green chemistry approach. Alkaloid, flavonoid, tannin, and saponin were found in leaves extract of *Ocimum sanctum* which could act as capping and stabilizing agents for the green synthesis of *Ocimum sanctum*-copper nanoparticles. The copper nanoparticles showed promising effects against all selected bacteria in the form of zone of growth inhibition. It was found that as the reaction time of leaves extract of *Ocimum sanctum* and copper sulphate increased, the formation of copper nanoparticles also increased. The inhibitory action of copper nanoparticles was found to increase along with the concentration of metal ions. In conclusion, for a variety of nano-biotechnological applications, these copper nanoparticles can be used to screen their efficacy.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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Author's short Biography

	**Mr. Jivan Munja Dhotare (B.Sc. Agri., M.Sc.)**: Mr. J.M. Dhotare serves as an assistant professor at the College of Agricultural Biotechnology, Hatta. He has mastered the art of instructing students in microbial genetics, r-DNA technology, and related topics. He has taken part in numerous extracurricular and co-curricular activities and published more than 5 full-length research papers. He works as a programme officer for the National Service Scheme (NSS) in his college. Additionally, he attended the NSS State Adventure Camp in Chikhaldara, Amravati. He coordinates community service projects through NSS, such as blood donation, tree plantings, AIDS awareness campaigns, and awareness campaigns for intoxicants.
	**Dr. Mukundraj Govindrao Rathod** (**Ph.D. & MH-SET**): At Yeshwant College of Information Technology in Parbhani, Maharashtra, India, Dr. Mukundraj G. Rathod is the in-charge Principal. In addition, he heads this college's Biotechnology and Bioinformatics department. More than 77 research papers, including review articles in various peer-reviewed international and national journals and proceedings of conferences in various research fields, haveindeed been published by him. He has 300 citations in Google Scholar, an i10 index of 10, and a H index of 10, which is impressive. He has an amazing H index of 4, with 31 Scopus citations. He had given oral talks and posters at numerous conferences and seminars. He has also reviewed publications for a number of reputable scientific publishers. He had donated seven crucial industrial cultures to the National Center for Cell Science's Microbial Culture Collection in Pune, Maharashtra, India, for use by the general public. He is currently the lead researcher on a study supported by Swami Ramanand Teerth Marathwada University in Nanded through the Rajiv Gandhi Science and Technology Commission's application scheme for science and technology (Government of Maharashtra). According to the AD Scientific Index 2023, he recently held the 17th rank among the top 20 scientists at Swami Ramanand Teerth Marathwada University, Nanded, and its jurisdiction.
	**Prof. Dr. (Mrs.) Anupama Prabhakarrao Pathak (Ph.D. & MH-SET)**: Dr. Anupama P. Pathak was previously serving as the School of Life Sciences' director and currently serving as the microbiology department's head at Swami Ramanand Teerth Marathwada University, Nanded. More than 200 research papers, including review articles, in various peer-reviewed international, national journals and conference proceedings were published by her. She has 774 citations in Google Scholar, an i10 index of 28, and a H index of 15, which is really impressive. She has an amazing H index of 8, with 239 Scopus citations. More than 110 16S rRNA gene sequences and numerous industrially significant bacterial cultures are among her contributions. She had finished two research projects in microbiology on extremophiles that were funded by the University Grants Commission of New Delhi. She is a member of the university's Microbiology Board of Studies. She is a renowned scientist who, according to the AD Scientific Index 2023, is ranking currently in ninth place of the top 20 scientists at Swami Ramanand Teerth Marathwada University, Nanded, and its jurisdiction.