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Original Article

Development of Herbal-Metal Nanocomposite using Hemigraphis colorata and Copper nanoparticles: Investigating the Antimicrobial Activity against Bacterial and Fungal Pathogens

Ms. Poorna Shri M[1], T. H. Sukirtha[2]

[1]Research Scholar, Department of Microbiology, Rathnavel Subramaniam College of Arts and Science, Sulur, Coimbatore
[2]Associate Professor, Department of Microbiology, Rathnavel Subramaniam College of Arts and Science, Sulur, Coimbatore

Corresponding Author:

Ms. Poorna Shri M
Research Scholar, Department of Microbiology, Rathnavel Subramaniam College of Arts and Science, Sulur, Coimbatore
Email: poornakam04@gmail.com

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ABSTRACT

This study explores the biological synthesis of copper nanoparticles (CuNPs) using the leaf extract of Hemigraphis colorata as both a reducing and stabilizing agent. The synthesis was carried out by treating aqueous solutions of copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) with the leaf extract, resulting in the formation of stable copper nanoparticles. Antibacterial tests were conducted by impregnating the synthesized CuNPs onto paper discs and evaluating their efficacy against bacterial and fungal strains: After 24 hours of incubation, zones of inhibition were measured, demonstrating that the copper nanoparticles exhibited significant antimicrobial activity against both bacterial and fungal strains. The findings from this study contribute to the growing body of research on plant-based nanocomposites and their applications in both biomedical and environmental fields, offering a novel, sustainable alternative for future material development.

Keywords: *Copper nanoparticles, Plant-mediated synthesis, Hemigraphis colorata, Antibacterial activity, Gram-positive bacteria, Gram-negative bacteria.*

INTRODUCTION

The field of nanotechnology is one of the most active areas of research in modern materials science. Nanoparticles, generally defined as particles with sizes ranging from 1 nm to 100 nm in at least one dimension (Hutchison, 2008), exhibit new or significantly improved properties based on specific characteristics such as size, distribution, and morphology. The application of nanoscale materials and structures is an emerging area in nanoscience and nanotechnology, offering solutions to various technological and environmental challenges, including those in solar energy conversion, catalysis, medicine, and water treatment (Sangiliyandi et al., 2009).

Nanomaterials, particularly metallic nanoparticles, have become increasingly important due to their unique and modified physical, chemical, and biological properties compared to their macroscopic counterparts (Fayaz et al., 2011). Copper nanoparticles, for example, can be easily mixed with polymers and are relatively stable in both their chemical and physical properties. They are particularly valuable as antimicrobial agents because they can be prepared with high surface areas and unusual crystal morphologies (Nezhad et al., 2014). Copper ions and copper nanoparticles have demonstrated strong antibacterial activity against a wide range of bacteria, including Salmonella enterica, Campylobacter jejuni, E. coli, Listeria monocytogenes, and S. aureus. Additionally, copper is an essential metal for human health and is considered to have low toxicity, making it safe for use in medical applications, such as intrauterine devices (Pham and Lee, 2014).

Over the past decade, nanomaterials have attracted significant attention due to their excellent physical and chemical properties, which differ from those of their bulk counterparts (Eustis and El-Sayed, 2006). These unique properties are often determined by factors such as size, shape, composition, crystallinity, and structure. As a result, controlling the synthesis of nanomaterials with defined morphology and uniform size is crucial for achieving desired properties. Metal

nanoparticles with bactericidal activity can be immobilized and coated onto surfaces, offering potential applications in various fields. Their antimicrobial effects are attributed to their small size and high surface area to volume ratio, which allow them to interact closely with microbial membranes (Morones, 2005). While only a few studies have been reported on the antibacterial properties of copper nanoparticles, these studies indicate that copper nanoparticles hold significant promise as bactericidal agents. Yoon et al. (2007) reported that copper nanoparticles demonstrated superior antibacterial activity compared to silver nanoparticles against representative strains of *E. coli* and *Bacillus subtilis*., where the copper nanoparticles demonstrated superior antibacterial activity compared to the silver nanoparticles.

REVIWE OF LITERATURE

2.1 Hemigraphis colorata

Hemigraphis colorata is an important plant adapted to the Indian climate. It is a versatile, low-growing, creeping perennial herb that typically reaches a height of 15–30 cm (Subramoniam et al., 2001; Anitha et al., 2012; Silja et al., 2008). This plant is known by several names, including Aluminium Plant, Cemetery Plant, Metal Leaf, Red Flame Ivy, Waffle Plant, and Java Ivy. In Kerala, it is popularly referred to as 'murikootti' or 'murianpacha' due to its incredible potency in healing wounds (Priya, 2012; Subramoniam et al., 2001).

The leaves of Hemigraphis colorata exhibit a metallic purple luster on the upper surface and a solid dark purple color on the underside. In traditional medicine, the juice extracted from the leaves is directly applied to fresh wounds to stop bleeding (Silja, 2008). The plant contains several phytoconstituents such as phenols, saponins, flavonoids, terpenoids (Sheu et al., 2012), coumarins, carbohydrates, carboxylic acids, xanthoproteins, tannins, proteins, alkaloids, steroids, and sterols (Saravanan et al., 2010). These phytochemicals contribute to the plant's curative properties. The crude leaf paste is known for its efficacy in excision wound healing (Bhargavi et al., 2011; Pawar and Toppo, 2012).

Hemigraphis colorata is widely grown as an ornamental indoor and outdoor plant due to its attractive and vivid foliage. In folk medicine, the leaves are ground into a paste and applied to fresh wounds to promote healing and are also used to treat anemia. Although traditional knowledge about the usage of this plant is rich and varied, scientific studies supporting these claims are relatively limited (Devi Priya, 2013).

2.1.1 Characteristic Features of *Hemigraphis colorata*

The plant *Hemigraphis colorata* is a versatile tropical, low-creeping perennial herb. It is a prostrate-growing plant with spreading, rooting stems. The leaves are opposite, ovate to cordate, bearing well-defined veins. They are slender, lance-shaped, and have toothed, scalloped margins. The upper surface is green stained with red-purple, while the underside is a darker shade of purple. The plant bears small, five-lobed, bell-shaped white flowers and are held in spikes on the stem tip. Seed are small ,flat and white in colour.

2.1.2 Medicinal Properties

Hemigraphis colorata possesses a variety of medicinal properties and is widely used in traditional medicine, particularly in the southern parts of India, for wound healing. The leaf paste of *H. colorata* has been shown to exhibit anti-inflammatory effects in the carrageenan-induced paw edema model (Subramaniam et al., 2001). Additionally, leaf extracts have demonstrated antibacterial properties (Anitha et al., 2012).

Traditionally, the leaves are ground into a paste and applied to fresh wounds. This paste is also used to promote urination, control hemorrhage, stop dysentery, treat venereal diseases, and heal hemorrhoids. In folk practices, the entire plant is ground with water to make a paste that is believed to have immense healing power for fresh wounds, cuts, ulcers, inflammation, and various skin complaints.

2.2 Ethnobotany

The traditional healthcare system of India relies heavily on indigenous medicinal practices based on plants and plant extracts. Initially, Hemigraphis colorata was used primarily for decorative purposes, such as in aquariums and goldfish bowls. However, over time, its medicinal potential has gained recognition. The plant is known to pacify vitiated pitta in Ayurvedic terms and is used to treat fresh wounds, cuts, ulcers, inflammation, and various skin conditions.

In folklore, the leaf juice is applied directly to open wounds to stop bleeding (Silja V. P. et al., 2008). In folk medicine, the plant is also used internally to treat anemia (Gayathri V. et al., 2012). Traditionally, the leaves are consumed to address gallstones, control excessive menstruation, and even serve as a natural contraceptive. In Vanuatu, the sap of young leaf buds is squeezed into water and consumed at dawn for four days as a contraceptive and to induce sterility (Bourdy G. et al., 1992). In Java, the leaves are used to treat bloody dysentery and hemorrhoids. The plant is also credited with diuretic properties.

2.3 Phytochemistry and Pharmacology

Phytochemicals have long been utilized as medicines, dyes, and nutritional supplements. These bioactive compounds, which are secondary metabolites, contribute significantly to the therapeutic potential of medicinal plants. The phytochemical constituents present in Hemigraphis colorata include phenols, saponins, flavonoids, terpenoids (Sheu J. et

al., 2012), coumarins, carbohydrates, carboxylic acids, xanthoproteins, tannins, proteins, alkaloids, steroids, and sterols (Saravanan J. et al., 2010).

Different parts of the plant contain specific phytochemicals. The leaves are rich in flavonoids, polyphenols, tannins, and also have a high potassium and low sodium: stem contains saponins and tannins ,while the root contain flavonoids and polyphenols. These phytochemicals provide curative properties.

2.3.1 Anti-Bacterial Activity

The benzene extract of Hemigraphis colorata leaves has shown significant antibacterial activity, particularly against Acinetobacter species and Streptococcus aureus (Anitha V. T. et al., 2010). This antibacterial property is attributed to the presence of phenolic compounds in the extract, which are known for their antimicrobial potential.

2.3.2 Anti-Diabetic Activity

Studies evaluating the hypoglycemic and anti-diabetic effects of H. colorata revealed that the n-hexane extract, and to a lesser extent the ethanol extract, of the whole plant significantly reduced blood glucose levels in glucose-fed rats. The presence of steroids and coumarins in these extracts is believed to contribute to the anti-diabetic activity (Gayathri V. et al., 2012).

2.3.3 Wound Healing Activity

The crude leaf paste of Hemigraphis colorata has demonstrated effective wound healing properties. In experimental studies, application of the leaf paste on mice resulted in faster wound contraction and enhanced epithelialization. However, oral administration of the paste was found to be ineffective (Bhargavi C. H. S. et al., 2011). Further studies using excision and incision wound models indicated that the methanolic extract of the plant showed wound healing effects comparable to the standard reference drug, Vokadine (Saravanan J. et al., 2012). The herbal scaffold made from chitosan has been found to be highly haemostatic and can be effectively applied to treat infectious wounds, making it a valuable material in wound care (Annapoorna M. et al., 2013).

2.3.4 Anti-Oxidant Activity

Phenolic compounds present in Hemigraphis colorata are effective hydrogen donors, which contributes to their strong antioxidant properties. Phenolic acids such as chlorogenate, cinnamate, coumarate, gallate, and ferulate found in the plant act as pro-oxidants and exhibit free radical scavenging activity, helping to protect cells from oxidative stress (Deepak R. P. et al., 2017).

2.3.5 Miscellaneous Activity

Volatile indoor pollutants, often released from paints, cleaning agents, and odorants, can cause a variety of health issues when people are exposed to them regularly. Hemigraphis colorata has been recently evaluated as one of the highest-rated ornamental plants capable of removing harmful volatile organic compounds (VOCs), thereby improving indoor air quality (Yang D. S. et al., 2009).

2.3. Nanotechnology

Nanoscience involves the study of materials at the nanoscale level, typically between approximately 1 and 100 nanometers in length in at least one dimension (Hutchison, 2008). It also encompasses the study of methods to control the formation of two- and three-dimensional assemblies of molecular-scale building blocks into well-defined nanostructures or nanomaterials (Rosi and Mirkin, 2005).

Nanotechnology refers to the application of science and technology to control matter at the molecular level. It involves the design, production, characterization, and application of structures, devices, and systems by manipulating shape and size at the nanometer scale (Uskokovic, 2008). Emerging from the physical, chemical, biological, and engineering sciences, nanotechnology uses novel techniques to probe and manipulate individual atoms and molecules.

This technology has advanced significantly due to developments in material science, particularly the ability to fabricate nanoscale materials in a uniform and reliable manner, at a practical scale and cost. These advancements have turned many scientific dreams into reality, enabling the construction of micro- and nanodevices.

Nanomaterials possess broad applications across various fields due to their unique and size-dependent optical, magnetic, electronic, and chemical properties (Burda et al., 2005). Nanoparticles, in particular, are noted for their extremely large surface area-to-volume ratio, with their properties largely influenced by surface behavior (Hodes, 2007). These particles find well-established applications in cosmetics, pharmaceuticals, coatings, electronics, polishing, semiconductors, and catalysis.

The design and preparation of novel nanomaterials with tunable physical and chemical properties continues to be a growing area.

Nanobiotechnology is an emerging area of opportunity that seeks to fuse nano/micro fabrications and biosystem to the benefit of both. Increasing awareness towards green chemistry and other biological processes has led to the development of simple and ecofriendly approaches towards the synthesis of nanomaterial (Hsiao et al.,2006). There has been considerable amount of recent interest in using biotechnological approaches to achieve scaleable ,cost effective bioproduction option.

2.3.1 Copper Nanoparticles

Copper (Cu) is a transition metal with a distinct red-orange color and metallic luster, possessing an atomic number of 29 and an atomic mass of 63.546. It is one of the more abundant metallic elements in the Earth's crust (ranking 8th) and is known for its high electrical and thermal conductivity, strong corrosion resistance, good ductility, malleability, and reasonable tensile strength. These properties make copper an essential element for various societal functions and have led to its use for thousands of years.

Copper's excellent conductivity and ease of joining to itself, combined with its corrosion resistance and cost-effectiveness, make it the number one material used in modern household water piping, plumbing, and a preferred choice for vehicle radiators and air conditioners.

Copper has been used as a biocide for centuries. In ancient Egypt (around 2000 BC), it was used to sterilize water and wounds. The ancient Greeks (around 400 BC) prescribed copper for pulmonary diseases and for purifying drinking water. During the Roman Empire, copper cookware was employed to prevent the spread of diseases.

Copper is also essential to human health. It is a component of many enzymes involved in numerous bodily functions and contributes to the composition of hair and elastic tissues found in skin, bone, and other organs (Goyer, 1997). Recent studies have shown the potential of copper to reduce bacterial loads on surfaces, suggesting its application in healthcare and the food industry as an alternative to stainless steel (Airey and Verran, 2007; Mehtar et al., 2008).

Among noble metals, gold, silver, and copper are most commonly used in the synthesis of stable nanoparticle dispersions, which have applications in photography, catalysis, biological labeling, photonics, optoelectronics, and surface-enhanced Raman scattering (SERS) detection (Smith et al., 2006). In recent years, copper nanoparticles have garnered increasing attention as potential alternatives to silver and gold nanoparticles due to their promising anti-microbial, antibiotic, and anti-fungal properties. They also hold potential in electrical, dielectric, magnetic, optical, imaging, catalytic, biomedical, and bioscience applications (Theivasanthi and Alagar, 2011a).

Although copper is one of the most widely used materials across various applications, synthesizing it at the nanoscale is challenging due to its high tendency for oxidation. Unlike gold and silver, copper is extremely sensitive to air, and its oxide phases are thermodynamically more stable (Jeong et al., 2008). As a result, the formation of a surface oxide layer on copper nanoparticles is inevitable. However, the presence of copper oxides on the surface is undesirable in many industries, such as electronics, where copper is considered a cost-effective alternative to more expensive metals. The electrical conductivity of copper nanoparticles decreases significantly if they become impure with oxide phases. It is rare to find a method in the literature that produces pure copper nanoparticles unless the entire procedure is conducted under an inert atmosphere (Mott et al., 2007). Khanna et al. (2007) reported success in synthesizing pure copper nanoparticles by reducing copper salts in the presence of a surfactant.

2.3.3 Applications of Copper Nanoparticles

Copper nanoparticles have garnered considerable attention recently due to their unique physical and chemical properties, along with the low cost of preparation. These nanoparticles have wide-ranging applications in heat transfer systems (Eastman et al., 2001), as antimicrobial materials (Esteban-Cubillo et al., 2006), in the development of super-strong materials (Guduru et al., 2007), sensors (Kang et al., 2007), and catalysts (Kantam et al., 2007). However, copper nanoparticles easily oxidize to form copper oxide. For applications where oxidation is a concern, the particles are often encapsulated in organic or inorganic materials such as carbon or silica (Moya et al., 2006).

Copper nanoparticles, due to their high surface-to-volume ratio, are highly reactive and can effectively interact with other particles, enhancing their antimicrobial efficiency. Colloidal copper has been used as an antimicrobial agent for decades. Monodispersed copper nanoparticles (2–5 nm) embedded in a polysilicate called sepiolite [$Mg_8Si_{12}O_{30}(OH)_4(H_2O)_4 \cdot 8H_2O$] have demonstrated strong antibacterial activity, reducing microorganism concentrations by 99.9% (Esteban-Cubillo et al., 2006). Similarly, copper nanoparticles measuring approximately 6 nm, when embedded in polyvinylmethylketone films, have shown significant inhibitory effects on the growth of microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* (Cioffi et al., 2005).

Due to the stability of copper nanoparticles when supported on a matrix and their potent disinfecting properties, they can be incorporated into paint or plaster to serve as bactericidal agents for coating hospital equipment. Metallic nanoparticles, including copper, can also be utilized in heat transfer systems to enhance efficiency. Fluids containing metallic nanoparticles with thermal conductivity approximately three times that of pure fluids can significantly improve heat

transfer rates. For example, adding only 0.3% by volume of copper nanoparticles, with an average diameter of less than 10 nm, to ethylene glycol has been reported to increase its thermal conductivity by up to 40% (Eastman et al., 2001).

A major challenge in fuel-cell technologies is the formation of high levels of carbon monoxide (CO), which is generated during hydrogen production. One effective method to eliminate this CO byproduct is to react it with water to produce hydrogen gas and carbon dioxide (CO_2) in a process known as the water-gas shift reaction:
$CO + H_2O \rightarrow H_2 + CO_2$

With the aid of appropriate catalysts, the water-gas shift reaction can convert a large portion of carbon monoxide into carbon dioxide. For this purpose, nanoparticles (2–4 nm) of either gold or copper supported on metal oxides such as zinc oxide (ZnO) and cerium oxide (CeO_2) have been employed. Although gold nanoparticles exhibit the highest catalytic activity in the water-gas shift reaction, copper is nearly as reactive and is significantly more cost-effective (Rodriguez et al., 2007).

2.3.4 Mode of Action of Copper Nanoparticles
Das et al. (2010) investigated the antibacterial activity of copper nanoparticles against three bacterial strains: Staphylococcus aureus, Bacillus subtilis, and Escherichia coli using the Kirby–Bauer diffusion method. They found that copper nanoparticles were effective growth inhibitors against all three bacteria.

In another study by Ramyadevi et al. (2012), copper nanoparticles demonstrated promising antibacterial activity against Micrococcus luteus, S. aureus, E. coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa. Among these, E. coli was the most susceptible, followed by S. aureus, M. luteus, and K. pneumoniae, while P. aeruginosa exhibited resistance to copper nanoparticles.

Yoon et al. (2007) examined the susceptibility of *E. coli* and *B. subtilis* to silver and copper nanoparticles. Their study reported that bacterial survival rates decreased with increasing nanoparticle concentrations. Complete inhibition of E. coli and B. subtilis occurred at concentrations above 70 µg/mL for silver and 60 µg/mL for copper nanoparticles, indicating the superior antibacterial efficacy of copper nanoparticles compared to silver.

Furthermore, copper oxides are gaining recognition as valuable antimicrobial agents because they can be prepared with extremely high surface areas and unusual crystal morphologies.

However in time-kill experiment ,the gram negative strains showed a greater susceptibility to copper nanoparticles (Xu et al.1999)

It has been reviewed that a reduced amount of negatively charged peptidoglycans makes Gram-negative bacteria such as *Pseudomonas aeruginosa* and Proteus species less susceptible to positively charged antimicrobials (Ren et al., 2009).

Biologically synthesized copper oxide nanoparticles also exhibit antimicrobial activity. Usha et al. (2010) demonstrated the biosynthesis of copper oxide nanoparticles using a Streptomyces species isolated from the Pichavaram mangrove. The activity of copper oxide nanoparticles (100–150 nm) coated onto fabric showed 100% reduction of E. coli, S. aureus, and *Aspergillus niger* after 48 hours of incubation. These results highlight the potential of such nanoparticles when applied as coatings to the surface of protective clothing, significantly reducing the risk of transmission of infectious agents.

Gopalakrishnan et al. (2012) also reviewed the antibacterial nature of biologically synthesized cuprous oxide from Tridax procumbens leaf extract against E. coli. All these studies confirm the antibacterial activity of copper nanoparticles against common pathogens.

Similarly, there are several other reports on the effectiveness of copper nanoparticles against different microbes. These include activity against:

E. coli and *S. aureus* (Esteban-Cubillo et al., 2006) ,*E. coli, B. subtilis, and S. aureus* (Ruparelia et al., 2008) ,*E. coli*, *M. luteus*, and Issatchenkia orientalis (Esteban-Tejeda et al., 2009),E. coli and Bacillus megatherium (Theivasanthi and Alagar, 2011),E. coli (Raffi et al., 2010; Lee et al., 2013) and Copper alone and in combination with lactic acid against E. coli (Gyawali et al., 2011)

Like silver nanoparticles, copper nanoparticles have also demonstrated size-dependent antibacterial activity (Duran et al., 2010; Prabhu et al., 2010). From these findings, it can be concluded that to achieve maximum antibacterial efficacy, there is a significant need to develop methods for the synthesis of monodisperse copper nanoparticles with small particle sizes.

2.4 Hemigraphis colorata
Hemigraphis colorata (Blume) (= H. alternata (Burm. f.)) is a plant in the family Acanthaceae, native to the eastern Malesia region. Various extracts of H. colorata leaves have recently been shown to possess antibacterial activity against certain

pathogens (Anitha et al., 2012). Additionally, leaf paste of H. colorata has been observed to promote wound healing in mice (Subramoniam et al., 2001).

The leaves of *H. colorata* are green on the adaxial (upper) side and purple on the abaxial (lower) side. The permanent red to purple coloration of the abaxial leaf surface is commonly seen in deeply shaded understory plants, particularly in tropical regions. However, the functional significance of this abaxial anthocyanin coloration—especially its potential role in photosynthetic adaptation—remains unclear (Hughes et al., 2008).

Hemigraphis colorata is an herbal plant traditionally used to treat cuts and bleeding. It is considered a tribal medicinal plant in Kerala and is known to possess various bioactive compounds and secondary metabolites. The known properties of *H. colorata* include antimicrobial, antidiabetic, and wound-healing activities, as well as functioning as a natural dye for solar energy conversion. Species of Hemigraphis are also used as antibiotics in the treatment of urinary tract infections (UTIs).

Due to its numerous medicinal properties and easy perennial growth, Hemigraphis colorata presents promising potential for future pharmacological and phytochemical research.

2.4.1 Classification

Domain	:	EukaryotaEukaryot
Kingdom	:	Plantae
Phylum	:	Spermatophyta
Subphylum	:	Angiospermae
Class	:	Dicotyledonae
Order	:	Scrophulariales
Family	:	Acanthaceae
Genus	:	Hemigraphis
Species	:	Hemigraphiscolorata

Figure 1. *Hemigraphis colorata*

Hemigraphis is a genus of plants belonging to the Acanthus family (Acanthaceae), consisting of about 30 species native to tropical Asia. In some classifications, Hemigraphis is included within the genus Strobilanthes.

The genus is characterized by gray-green leaves that are stained with red-purple on the upper surface and a darker purple on the underside. Hemigraphis species are typically prostrate plants with spreading, rooting stems, allowing them to form dense ground cover.

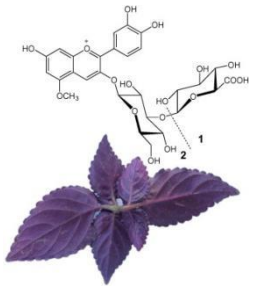


Fig. 2. Structures of 5-O-methylcyanidin 3-O-(3″-(β-glucuronopyranosyl)-β-glucopyranoside) (1) and 5-O-methylcyanidin 3-O-β-glucopyranoside (2), isolated from the leaves of Hemigraphis colorata with purple abaxial surfaces (Andersen et al., 2010).

2.4.2 Wound Healing Activity:

Hemigraphis colorata is considered to be a medicinal plant known for its ability to heal bleeding wounds and cuts. The juice extracted from the leaves is applied to cut wounds, which helps in curing them (V.P. Silja et al., 2008). Studies have been conducted on the wound healing properties of this plant through the formulation of hydrogels using the polymer Carbopol 934 (A. Subramoniam et al., 2001). This activity was shown to be effective in promoting wound healing.

2.4.3 Anti-inflammatory Activity:
The oral administration of leaf suspension and topical application of Hemigraphis colorata were found to be devoid of anti-inflammatory effects in mice (Subramoniam et al., 2001). However, the methanolic extract of H. colorata showed significant anti-inflammatory activity in rats (Bhagyalakshmi Chengattu Prakashbabu et al., 2017). The ethyl acetate extract of H. colorata also exhibited anti-inflammatory activity by inhibiting 5-LOX, COX-1, and COX-2 enzymes responsible for inflammation, in a dose-dependent manner in human keratinocyte cell lines (HaCaT cells) (Arun K. Kashyap et al., 2013). In an in vitro experiment, acetone extract showed 43% and 48% inhibition of inflammatory activity (Shahid Adangapurath and Sudeesh Sudhakaran, 2018).

2.4.4 Antibacterial and Antimicrobial Activity:
The aqueous extract of H. colorata showed antibacterial and antimicrobial activity against Staphylococcus aureus and Pseudomonas species (Dhinesh et al., 2016). Cotton fibers treated with extracts of H. colorata and three other herbal plants exhibited antimicrobial activity against Escherichia coli and Staphylococcus aureus (Shabrin Farhana G. and Palaniswamy N.K., 2018).

2.4.5 Antidiabetic Activity:
The antidiabetic activity of H. colorata was studied in rats. The study showed that n-hexane and ethanol extracts of the whole plant significantly lowered blood glucose levels in rats. The presence of coumarins and steroids in the plant extract is believed to be responsible for its antidiabetic properties (Gayathri V. et al., 2012).

2.4.6 Cytotoxicity Screening:
The in vitro short-term cytotoxicity of the ethanolic extract of H. colorata was assessed using DLA (Dalton's Lymphoma Ascites) tumor cell lines by the Trypan blue exclusion method. The cell lines were obtained from Amala Cancer Research Institute, Thrissur, Kerala, India.

Cell viability was determined by the Trypan blue exclusion method. A viable cell suspension (1×10^6 cells in 0.1 ml) was added to tubes containing various concentrations of the ethanolic extracts of H. colorata dissolved in DMSO. The volume was made up to 1 ml using phosphate-buffered saline (PBS). A control tube contained only the cell suspension. These assay mixtures were incubated for 3 hours at 37°C. Further, the cell suspension was mixed with 0.1 ml of 0.4% Trypan blue and kept for 2–3 minutes before being loaded on a hemocytometer. Dead cells took up the blue color of Trypan blue, while live cells did not. The number of stained (dead) and unstained (live) cells was counted separately.

2.4.7 Implications of Nanoparticles to Human Health and the Environment:
Nanoparticles are considered special and interesting because their chemical and physical properties differ significantly from their macro counterparts. Most of their properties are attributed to their small size, and they are gaining attention for their potential applications in achieving specific processes and selectivity, especially in biological and pharmaceutical fields (Theivasanthi and Alagar, 2011a). However, it is also recognized that nanoparticles may pose undesirable and unforeseen effects on the environment and ecosystems (Long et al., 2006).

Some metallic nanoparticles show increased toxicity, even if the same material is relatively inert in its bulk form (e.g., silver, gold, and copper). Nanoparticles can also interact with proteins and enzymes within mammalian cells and may interfere with antioxidant defense mechanisms, leading to the generation of reactive oxygen species (ROS). As nanoparticle discharge into the environment continues to grow—due to expanding industrial use—their fate and environmental impact must be studied further. This is crucial because of known toxicities, existing knowledge gaps, and challenges in risk assessment and management (Handy et al., 2008). Therefore, there is a pressing need to develop economical, commercially viable, and environmentally benign routes for the synthesis of metal nanoparticles.

AIM AND OBJECTIVES

3.1 Aim
Synthesis of Herbal-Metal Nanocomposite Using Hemigraphis colorata and Copper Nanoparticles: Investigating the Antibacterial Activity Against Bacterial and Fungal Pathogens

3.2 Objectives:
1. Collection and Processing:
Collection and processing of the medicinal herb Hemigraphis colorata and procurement of standard antibiotic drugs (e.g., Gentamicin).
2. Extraction:
Soxhlet extraction of Hemigraphis colorata using solvents such as acetone or methanol.
3. Synthesis of Copper Nanoparticles:
Preparation of copper nanoparticles using the chemical reduction method.
4. Development of Herbal-Metal Nanocomposite (HMNC):
Formation of the Herbal-Metal Nanocomposite using a standard magnetic stirrer method
5. Determination of Minimal Inhibitory Concentration (MIC):

Evaluation of the MIC of the synthesized HMNC against bacterial and fungal pathogens.

6. Qualitative Antibacterial Activity:

Qualitative antibacterial activity of HMNC against bacterial pathogens.

7. Qualitative Antifungal Activity:

Qualitative antifungal activity of HMNC against fungal pathogens

MATERIALS AND METHODS

4.1 Materials

4.1.1 Plant Used

Hemigraphis colorata (commonly known as "Moorikooti")

The plant were taken from a nursery garden in Coimbatore, Tamil Nadu, India.

4.1.2 Solvents Used for Extraction

Acetone,1% Dimethyl Sulphoxide (DMSO)

4.1.3 Test Organisms Used

Isolates of the following organisms were used for antimicrobial testing:

Staphylococcus aureus, Escherichia coli ,Candida albicans, Candida tropicalis

4.1.4 Media Used

Nutrient Agar,Nutrient Broth

4.2 Methodology

Screening of Medicinal Plant for Antimicrobial Activity

4.2.1 Collection and Processing of Medicinal Herb – Hemigraphis colorata

The leaves of Hemigraphis colorata (Moorikooti) were collected from a nursery garden in Coimbatore, Tamil Nadu, India. The collected leaves were pre-washed using deionized water to remove any dust or impurities. They were then dried using a dry towel and cut into smaller pieces for further processing.

To ensure the removal of enzymes and to retain phytochemicals, the leaves underwent a blanching pre-treatment. The blanching process involved immersing the leaves in hot water at 96–98°C for 90 seconds.

Figure 3 Blanched leaves

4.2.2.a Sample preparation - Drying and milling of leaves (Lin et al., 2012)

The blanched leaves were separated and arranged in aluminium trays in each drying method. The leaves were dried using oven drying (40 °C for 7h, 50 °C for 6h, 60 "C for 4h and 120°C for 15min) using oven dryer until both the blanched leaves reached a moisture content of below 10%. The moisture content of 10% and below is the recommended value for drying of leaves and for powder production. The dried leaves were grounded to powder and the powder was then sieved manually by using sieve with size 250mm. Sieved particles were stored at room temperature prior testing.

Figure 4 Dried leaves were grounded to powder

4.2.2.b Soxhlet extraction of *Hemigraphis colorata* leaves using solvent (Saohin et al., 2007)

Extraction is the separation of medicinally active portions of plant using selective solvents through standard procedures. The purpose of all extraction is to separate the soluble plant metabolites, leaving behind the insoluble cellular residue. The initial crude extracts contain complex mixture of many plant metabolites, such as alkaloids, glycosides, phenolics,

terpenoids and flavonoids. The initial stage in studying medicinal plants is the preparation of plant samples to preserve the biomolecules in the plants prior to extraction. Plants samples such as leaves, barks, leaves, fruits and flowers can be extracted from fresh or dried plants material. Other pre-preparation of plant materials such as grinding and drying also influences the preservation of phytochemicals in the final extracts. Some of the initially obtained extracts could be used as medicinal agents however most of plant extracts need further processing.

Figure 5 Soxhlet extraction **Figure 6 Leaf extracts of *Hemigraphis colorata***

For the present study, soxhlet method which follows the principle of infusion method was chosen to extract the content from the given herbs (Banana leaves). In the Soxhlet extraction method, finely ground sample - *Hemigraphis colorata* leaf herbal powder was placed in a porous bag or "thimble" made from a strong filter paper or cellulose, which is placed, is in thimble chamber of the Soxhlet apparatus. Extraction solvent (acetone) is heated in the bottom flask, vaporizes into the sample thimble, and condenses in the condenser and drip back. When the liquid content reaches the siphon arm, the liquid contents is emptied into the bottom flask again and the process is continued. For the study, infusion method of Soxhlet Extraction had been adopted. The powdered herbs of *Hemigraphis colorata* leaves were filled in the thimble and placed in the soxhlet extractor. The extractor had been filled with solvent solution of ethanol and the temperature of 60ºC was set and left for 6hours. Slowly and steadily the temperature was increased upto 100 degree C. The extract from the thimble was collected in the round botton flask kept in the heating mantle below by passing through a side arm tube. Thus collected extract was taken in a separating funnel and stored at room temperature prior to testing.

4.3 Synthesis of Copper nanoparticles

4.3.1: Synthesis of Copper nanoparticles using chemical reduction method (Shende et al., 2016)

For the synthesis of copper nanoparticles, 50 ml of *Hemigraphis colorata* leaf extract was mixed with 50ml aqueous solution of 1mM copper sulphate (1:1 ratio of plant extract and copper solution) and stirred continuously for 2min at 30^0C. Reduction takes place rapidly which is indicated by the change in colour of the solution. The mixture was incubated at room temperature overnight. Mixture was centrifuged at 3500rpm for 10min to get copper nanoparticles. Green synthesis of copper nanoparticles was achieved in aqueous solution using plant extract as reducing agent. When plant extract was mixed with copper sulphate solution, the colour of aqueous solution was changed immediately within 10 min, which turns dark brown within 24 hours (Fig) indicated the formation of copper nanoparticles. The nanoparticles were washed and dried at room temperature.

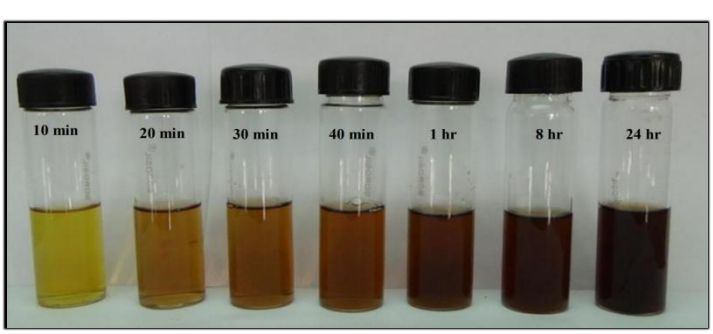


Figure 7 Copper nanoparticles at different incubation period and (Dried, powdered and sieved Copper nanoparticles)

4.3.2: Development of Herbal-Metal nanocomposite (HM_{NC}) using standard stirrer method

Using the herbal extracts and copper nanoparticles, Herbal-Metal nanocomposite (HM_{NC}) was developed. Briefly, the herbal extracts was kept under stirring conditions using a magnetic stirrer (110 rpm, 40ºC) in a beaker. Followed by copper nanoparticle solution was added drop wise onto the herbal extract at the rate of 20ml per minute. The magnetic stirring condition was kept constant for 2 to 3h till complete development of Herbal-Metal nanocomposite. Developed nanocomposites were stored in brown amber bottle at refrigeration temperature prior to antibacterial activity and other tests.

Figure 8 Synthesized Herbal-Copper nanocomposite

4.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING – MINIMUM INHIBITORY CONCENTRATION (Tripathi, 2013)

4.4.1 Principle

Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC is used to determine the susceptibility of an organism to antimicrobials**.**

4.4.2 Culture Medium [NAPH (pH 7.0 ± 0.2)]

- Peptone: 5 g
- Yeast extract: 5 g
- Beef extract: 3 g
- Sodium chloride: 5 g
- Agar agar: 2 g

4.4.3 Preparation of Bacterial and Fungal Inoculums

The following strains were used to study the antimicrobial activity:

- *Staphylococcus aureus,Escherichia coli,Candida albicans,Candida tropicalis*
- All the test cultures were inoculated in sterile Nutrient Agar (Composition per liter: Peptone: 5 g; Yeast extract: 5 g; Beef extract: 3 g; Sodium chloride: 5 g; Agar agar: 2 g; Final pH 7.0 ± 0.2) and allowed to grow for 24 to 48 hours.
- The Minimum Inhibitory Concentration (MIC) of the Herbal-Metal Nanocomposite (HMNC) was determined by the standard agar well diffusion method. Nanocomposite was dissolved in 5% dimethyl sulfoxide (DMSO) prior to experiments**.**

4.4.4 Well Diffusion Method

To determine the MIC of the nanocomposite, a set of Nutrient Agar (NA) plates were prepared under sterile conditions. About 20 µl of the nanocomposite at different concentrations (100µg/ml, 200 µg/ml, 300 µg/ml, and 400 µg/ml) was added to their respective wells on plates pre-seeded with Staphylococcus aureus, Escherichia coli, Candida albicans, and Candida tropicalis. All the inoculated plates were incubated at 37°C ± 0.2°C for 24 to 48 hours for bacterial strains and at 28°C ± 0.2°C for 48 hours for fungal strains. The antimicrobial drug ciprofloxacin + tinidazole (100 µg/ml) was used as the positive control.

After the incubation period, the plates were observed for zones of inhibition around the wells. The zone surrounding the lowest concentration of the nanocomposite that showed visible inhibition was considered as the Minimum Inhibitory Concentration (MIC)

Qualitative Antibacterial activity of HM_{NC} against bacterial pathogens

The antibacterial activity of Herbal-Metal nanocomposite (HM_{NC}) as a novel composite was evaluated against the test organisms by well diffusion method. Based on the obtained MICs, three different volumes (1X – 10ul, 2X – 20ul and 3X – 30ul) of selected concentrations were evaluated for effective antibacterial activity. Sterile Nutrient Agar (Composition g/L: Peptone: 5g; Yeast extract: 5g, Beef extract: 3g, Sodium chloride: 5g, Agar 15 g; Final pH (7.0 ± 0.2) plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of the test organism (*Staphylococcus aureus* and *Escherichia coli*) were swabbed uniformly over the agar surface separately. Under sterile conditions, 6mm wells were cut on the agar surface of each NA plates. About 20µl of herbal-metal nanocomposite fractions were loaded from each selected volumes (1X – 10ul, 2X – 20ul and 3X – 30ul) into the well and the plates were incubated at 37ºC for 24h. The antibacterial

activity was evaluated in terms of zone of inhibition around the wells in all the inoculated NA plates. The inhibition clear zones were measured and recorded in millimeter.

Qualitative Antifungal activity of HM_{NC} against fungal pathogens

The antifungal activity of Herbal-Metal nanocomposite (HM_{NC}) as a novel composite was evaluated against the test organisms by well diffusion method. Sterile Nutrient Agar (Composition g/L: Peptone: 5g; Yeast extract: 5g, Beef extract: 3g, Sodium chloride: 5g, Agar 15 g; Final pH (7.0 ± 0.2) plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of the test organism (*Candida albicans* and *Candida tropicalis*) were swabbed uniformly over the agar surface separately. Under sterile conditions, 6mm wells were cut on the agar surface of each NA plates. About 20µl of herbal-metal nanocomposite fractions were loaded into the well and the plates were incubated at 28ºC for 48h. The antifungal activity was evaluated in terms of zone of inhibition around the wells in all the inoculated NA plates. The inhibition clear zones were measured and recorded in millimeter.

RESULT AND DISCUSSION

5.1 Preliminary Screening of Hemigraphis colorata Plant Extracts and Their Nanoparticles for Antimicrobial Assessment

5.1.1 Antimicrobial Susceptibility Testing – Agar Well Diffusion Method

In the present study, crude extracts of Hemigraphis colorata were prepared using acetone. After evaporation, the extract was mixed with 5% DMSO, and its efficacy in inhibiting the growth of Staphylococcus aureus, Escherichia coli, Candida albicans, and Candida tropicalis was studied using the agar well diffusion method.

The same method was also applied to test copper nanoparticles synthesized from the methanolic extract of *Hemigraphis colorata*. These copper nanoparticles exhibited maximum inhibition against *Staphylococcus aureus* and *Escherichia coli.* A concentration of 400 µg/ml of the nanocomposite showed the highest inhibition zones:

- 16 mm against Escherichia coli,14 mm against Staphylococcus aureus .No zones of inhibition were observed at 100 µg/ml and 200 µg/ml concentrations of the herbal-metal nanocomposite against *Staphylococcus aureus* and *Escherichia coli*.
- Similarly, the nanocomposite exhibited maximum inhibition of 16 mm against *Candida albicans 15 mm* against *Candida tropicalis*
- Again, no zones of inhibition were observed at 100 µg/ml and 200 µg/ml concentrations against *Candida albicans* and *Candida tropicalis.*

The antibacterial efficiency of metal nanoparticles was investigated by introducing the particles into media containing bacteria. The antibacterial studies were performed both in solutions and on Petri dishes. Copper has been used for its antibacterial properties for centuries. However, copper nanoparticles have demonstrated significantly greater antibacterial activity than copper in its bulk form. This is primarily due to their unique properties, especially a high surface area-to-volume ratio. The large surface area in nanocrystals leads to an increased number of reactive sites, enhancing their overall reactivity. Copper nanoparticles can directly interact with bacterial outer membranes, leading to membrane rupture and ultimately killing the bacteria. Ivasanthi and Alagar (2011a) reported the synthesis of copper nanoparticles using electrolysis and chemical reduction methods. In both methods, copper nanoparticles showed higher antibacterial activity, particularly against *Escherichia coli.*

Although limited, some studies have explored the mode of action of copper and copper oxide nanoparticles on bacteria (Gopalakrishnan et al., 2012). Researchers have proposed that the mechanism of metallic copper nanoparticles may be similar to that of silver and copper oxide nanoparticles (Ruparelia et al., 2008). Das et al. (2010) suggested that linoleic acid-capped copper nanoparticles penetrate bacterial cells and inactivate enzymes by generating hydrogen peroxide, which causes bacterial cell death.

In another study, Schrand et al. (2010) hypothesized that copper nanoparticles act as effective antibacterial agents against a wide range of bacterial species through interactions with –SH (sulfydryl) groups, leading to protein denaturation. These nanoparticles also affect bacterial cell membranes due to their affinity toward amine and carboxyl groups on the cell surface of organisms like *Bacillus subtilis* (Beveridge and Murray, 1980; Ren et al., 2009).

Once inside the bacterial cell, copper nanoparticles may bind with DNA molecules, disrupting the helical structure by cross-linking within and between nucleic acid strands. Additionally, copper ions can interfere with internal biochemical processes (Kim et al., 2000; Stohs and Bagchi, 1995). Gopalakrishnan et al. (2012) also proposed a possible mechanism for the mode of action of copper oxide nanoparticles against *E. coli.*

According to studies, copper oxide nanoparticles adsorb onto the bacterial cell surface, interact with the cell wall, and subsequently damage the cell membrane. This damage increases membrane permeability, leading to a decrease in bacterial viability in copper oxide solutions. However, the exact mechanism behind the antimicrobial effect of copper nanoparticles is still not fully understood and requires further investigation.

Toxicity Issues

The increasing production and use of metal nanoparticles in various applications have raised concerns regarding their potential adverse effects on human health (Galdiero et al., 2011). Compared to silver and gold nanoparticles, the cytotoxicity of copper nanoparticles has been less extensively studied (Valodkar et al., 2011). Kim et al. (2012) conducted a comparative toxicity study of laser-generated silver, gold, cobalt, and copper nanoparticles. Their findings indicated that ultra-pure nanoparticles with nascent surfaces exhibit moderate cytotoxicity to human cells, depending on the cell type.

Copper is an essential nutrient required for normal physiological functioning and is regulated within the body through homeostasis. However, when copper intake exceeds the body's tolerance limits, it can cause toxic effects such as hemolysis, jaundice, and even death. Similarly, excessive exposure to copper nanoparticles—whether through ingestion, inhalation, or skin contact—can lead to toxic effects in the respiratory and gastrointestinal tracts, as well as in other tissues (Chen et al., 2006).

Chen et al. (2006) investigated the acute toxicity of micro-sized (17 µm) and nano-sized (23.5 nm) copper particles in mice and found that the nanoparticles were significantly more toxic. This increased toxicity is attributed to the nanoparticles' ability to easily penetrate the body via skin contact, inhalation, or ingestion (Prabhu et al., 2010). Furthermore, copper nanoparticles have been reported to cause pathological damage to the liver, kidneys, and spleen (Chen et al., 2006).

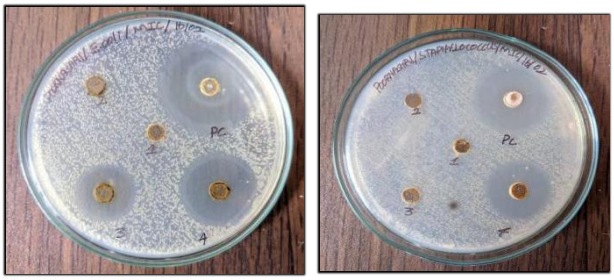
RESULTS

Table-1: Minimal Inhibitory concentration of Herbal-Metal nanocomposite (HM_{NC}) against bacterial pathogens

S. No	Test organism	Zone of inhibition (mm)				
		1	2	3	4	PC
1	*Escherichia coli*	0	0	13.3±0.26	16.3±0.57	19.6±0.57
2	*Staphylococcus aureus*	0	0	0	14.6±1.25	17.3±1.03

1, 2, 3, 4 - 100µg/ml, 200µg/ml, 300µg/ml, and 400µg/ml of nanocomposite
PC - Positive control (ciprofloxacin+tinidazole)

Figure Table-1: Minimal Inhibitory concentration of Herbal-Metal nanocomposite (HM_{NC}) against bacterial pathogens



Escherichia coli ***Staphylococcus aureus***

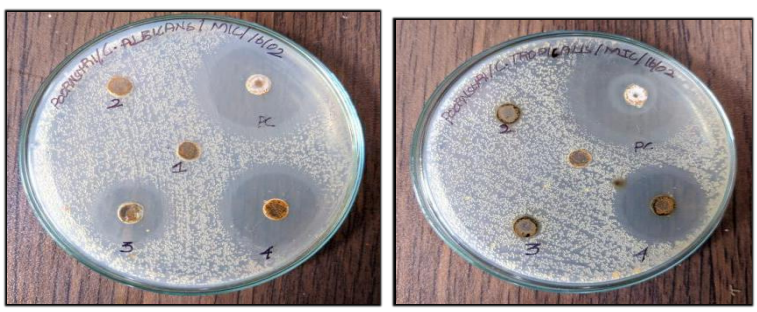
RESULTS

Table-2: Minimal Inhibitory concentration of Herbal-Metal nanocomposite (HM_{NC}) against fungal pathogens

S. No	Test organism	Zone of inhibition (mm)				
		1	2	3	4	PC
1	*Candida albicans*	0	0	13.6±0.57	16.5±1.25	18.6±0.25
2	*Candida tropicalis*	0	0	0	15.9±0.57	19.3±1.25

1, 2, 3, 4 - 100µg/ml, 200µg/ml, 300µg/ml, and 400µg/ml of nanocomposite
PC - Positive control (ciprofloxacin+tinidazole)

Figure Table-2: Minimal Inhibitory concentration of Herbal-Metal nanocomposite (HM_{NC}) against fungal pathogens



Candida albicans ***Candida tropicalis***

RESULTS

Table-3: Qualitative Antibacterial activity of HM_{NC} against bacterial pathogens

S. No	Test organism	Zone of inhibition (mm)			
		1X	2X	3X	PC
1	*Escherichia coli*	0	0	13.6±1.25	16.9±1.03
2	*Staphylococcus aureus*	0	0	14.9±0.57	17.6±1.25

1X – 10ul, 2X – 20ul and 3X – 30ul of nanocomposite
PC - Positive control (ciprofloxacin+tinidazole)

Figure Table-3: Qualitative Antibacterial activity of HM_{NC} against bacterial pathogens

Escherichia coli ***Staphylococcus aureus***

RESULTS

Table-4: Qualitative Antifungal activity of HM_{NC} against fungal pathogens

S. No	Test organism	Zone of inhibition (mm)			
		1X	2X	3X	PC
1	*Candida albicans*	0	0	15.9±1.03	17.3±0.57
2	*Candida tropicalis*	0	0	14.6±0.57	16.9±1.25

1X – 10ul, 2X – 20ul and 3X – 30ul of nanocomposite
PC - Positive control (ciprofloxacin+tinidazole)

Figure Table-4: Qualitative Antifungal activity of HM_{NC} against fungal pathogens

Candida albicans ***Candida tropicalis***

CONCLUSION

Investigations into the antimicrobial effects of the as-synthesized copper nanoparticles performed against pathogenic bacteria and fungi reveal the high efficacy of copper nanoparticles as strong antimicrobial agents.

Various studies have shown that copper nanoparticles can be synthesized through chemical, physical, and biological routes. Among these, physical and chemical methods are often time-consuming and tedious. Moreover, some chemical methods involve the use of hazardous substances, which may pose health risks to users. Therefore, there is a growing demand for eco-friendly, simple, and rapid methods of synthesis. Biological synthesis represents a promising solution in this direction. Research on the bioactivity of copper nanoparticles has confirmed their effectiveness against a wide range of pathogenic bacteria, fungi, algae, and viruses. They have also been reported to possess anti-parasitic and anticancer properties. Since metallic copper salts are less expensive than those of silver, the overall technology is cost-effective. As a result, copper nanoparticles present a viable and affordable alternative to silver nanoparticles.

However, it must be noted that using copper nanoparticles in concentrations exceeding safety limits can lead to serious cytotoxic effects. Despite numerous studies, the exact mechanism by which copper nanoparticles exert antimicrobial action remains unclear. Hence, further extensive studies are necessary to better understand the interaction of copper nanoparticles with microbial cells.

In conclusion, the greener approach to copper nanoparticle synthesis using plant leaf material as a reducing and capping agent offers several advantages. These include scalability, economic feasibility, environmental sustainability, and the elimination of the need for high pressure, high energy, high temperatures, or toxic chemicals. The potential applications of eco-friendly copper nanoparticles in bactericidal treatments, wound healing, and various medical and electronic fields are promising. Their proven toxicity against human pathogenic bacteria opens the door to a new class of antibacterial agents.

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