Review paper
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COPPER NANOPARTICLES (CUNPS) FROM CITRUS (KINNOW) WASTE AND THEIR ANTIBACTERIAL ACTION AGAINST CANKER-CAUSING *XANTHOMONAS CITRI*

Ehsan-ul-Haque[1], Akbar Hayat[1], Sohaib Afzaal[1*]

[1]Citrus Research Institute, Rissala No. 05, Military Farm Road, 41000 Sargodha, Pakistan

[*]e-mail: sohaib.afzaal@hotmail.com

Abstract

Biologically synthesized nanoparticles are emerging as attractive alternatives to chemical pesticides due to the ease of their synthesis, safety, and antimicrobial activities in lower possible concentrations. In the present study, we have synthesized copper-based nanoparticles (CuNPs) using the aqueous extract of the citrus peel and tested them against the plant pathogenic bacterium *Xanthomonas citri*, the causative agent of citrus canker, via an *in-vitro* experiment.

Citrus (Kinnow) peel and rag after oven drying were subjected to prepare a peel extract in distilled water and purified by centrifugation. Anhydrous copper sulfate was supplemented to peel extract and CuNPs were synthesized by treating with 0.1 M NaOH/0.05 M H_2SO_4. The synthesized copper nanoparticles were characterized by techniques such as UV-Vis spectroscopy, energy-dispersive X-ray spectroscopy, and X-ray diffraction analysis. Moreover, the extracts were investigated for total phenolics by Folin Ciocalteu assay, total flavonoids by Aluminum chloride colorimetric assay, and antioxidant activity by DPPH assay, as well as antibacterial potential using the disc diffusion plate method.

The results confirmed the synthesis of centered cubic, spherical-shaped, and crystalline nanoparticles using standard characterization techniques. A qualitative and quantitative phytochemical analysis revealed the presence of phenolics (153.14 mg GAE/g) and flavonoids (34.2 mg QE/g). Different concentrations (1000 µg/mL to 15.62 µg/mL - 2-fold dilutions) of CuNPs and peel extract (PE) alone, and both in combination (CuNPs-PE), exhibited a differential inhibition of *X. citri* in a high throughput antibacterial assay. Overall, CuNPs-PE was superior in displaying significant antibacterial activity, followed by CuNPs alone. A remarkable anti-oxidant potential was observed along with excellent antibacterial activity. The observed antibacterial and antioxidant potential may be attributed to eight phenolic compounds identified in the extract.

The citrus peel-extract-induced synthesized CuNPs exhibited strong antibacterial activity against *X. citri*, which could be exploited as an effective alternative preparation against citrus canker.

***Key words**: Citrus immunity, Citrus infections, Green synthesis, Kinnow, Nano-formulations.*

1. Introduction

Citrus canker is a severe and widespread disease that poses a major threat to citrus crops worldwide, resulting in significant economic losses for citrus industry. The disease is caused by the bacterial pathogen *Xanthomonas citri*, which infects citrus plants, leading to the formation of characteristic lesions on leaves, stems, and fruits. These lesions are unsightly and can substantially diminish the market value of the produce, as consumers prefer blemish-free fruits. Furthermore, the disease can lead to a reduction in overall yield, overall consumer acceptance, and economic impact of the citrus crop [1]. The management of citrus canker has traditionally relied on the application of chemically synthesized pesticides having several significant challenges. One major issue is the development of resistant bacterial strains, which can render these treatments ineffective over time. This resistance reduces the ability of growers to manage the disease using traditional methods, leading to increased crop losses. Additionally, the use of chemically synthetic pesticides poses environmental concerns leading to environmental toxicity, affecting non-target organisms and the overall health of the ecosystem. The presence of pesticide residues in the environment can also pose risks to human health, particularly through the contamination of water supplies and food products [2].

The growing concerns over the environmental and health impacts of traditional chemical pesticides have led to an increased focus on developing sustainable and eco-friendly alternatives. Biologically synthesized nanoparticles have emerged as a promising solution, offering advantages such as ease of synthesis, biocompatibility, and potent antimicrobial properties at minimal concentrations. Among these, copper-based nanoparticles (CuNPs) stand out due to their broad-spectrum antimicrobial efficacy. Innovative and sustainable disease management strategies have become the need of time. This study aims to explore the synthesis of CuNPs using the aqueous extract of citrus peel and their potential application in combating citrus canker, a disease caused by the bacterium *Xanthomonas citri* [3].

The use of plant-derived materials in nanoparticle synthesis offers a green chemistry approach, leveraging natural compounds to reduce and stabilize metal ions. Citrus peels, particularly those of the Kinnow variety, are rich in bioactive compounds like phenolics and flavonoids, making them a valuable resource for nanoparticle synthesis. These bioactive compounds not only facilitate the reduction of copper ions but also enhance the antimicrobial properties of the resulting nanoparticles [4].

The biological synthesis of nanoparticles offers a green, eco-friendly alternative with fewer toxic effects. One cost-effective approach involves converting agricultural waste materials into beneficial products for environmental remediation. These agro-waste materials can be used to prepare metal or metal oxide nanoparticles to remove dispersed dyes from textile wastewater. Scientists have employed different physical and chemical methods for dye removal, including coagulation, flocculation, membrane filtration, and adsorption. Nanotechnology introduces a vast array of new applications that benefit the ecosystem. Recent studies indicate that dispersed dyes, when combined with copper nanoparticles, significantly aid in remediating textile wastewater polluted by harmful effluents [5].

Kinnow, a citrus tree found in warmer regions, produces fruit with a taste ranging from sour to sweet. Rich in vitamin C, pectin, and lycopene, Kinnow is also known to lower cholesterol levels in humans. The extracts and oil from Kinnow peel and even from seeds exhibit excellent antimicrobial potential against various groups of pathogenic microorganisms, especially bacteria [6]. Additionally, Kinnow peels are excellent reducing agents for synthesizing metal and metal oxide nanoparticles, such as silver, and nickel [7]. As said before, the current study aimed to synthesize the copper-based nanoparticles from citrus waste subjected to the green synthesis method, and analyses their efficiency against citrus canker-causing *Xanthomonas citri*.

2. Materials and Methods

2.1 Synthesis of copper nanoparticles (CuNPs) using peel extract

The peel of Citrus (Kinnow) was collected from the experimental orchard of the Citrus Research Institute, Sargodha. The peel was washed thoroughly with distilled water and subjected to cutting and trimming as soon as possible. After cutting, the peel was sieved and stored in an airtight container. The Citrus (Kinnow) peel and rag were oven-dried to remove moisture content before the extraction of nanoparticles. The dried material was subjected to extraction by soaking in distilled water (15 g dried peel in 500 mL distilled water). The mixture was heated using a hot plate at 70 ^{0}C for 18 minutes. The peel extract was then purified by centrifugation (6500 rpm for 20 min at 10 ^{0}C) to remove any solid residues and impurities. The purified peel extract was supplemented with 150 mL of anhydrous copper sulfate (0.1 M) and the pH of the solution was adjusted to 4.0 using 0.1 M NaOH/0.05 M H_2SO_4. The reaction mixture was again heated on a hot plate at 70 ^{0}C for 20 min. followed by cooling at room temperature for 72 h. After three days, brown precipitates were formed which confirmed the synthesis of copper nanoparticles (CuNPs) at the bottom of the flask. The particles were centrifuged at 5000 rpm for 15 min at 25 ^{0}C, filtered, and washed twice with deionized distilled water to remove impurities [8].

2.2 Characterization of copper nanoparticles

Synthesized nanoparticles were characterized using UV-Vis Spectroscopy, to analyze the optical properties and confirm the formation of copper nanoparticles. X-ray diffraction Analysis (XRD) was conducted to determine the crystalline structure and phase identification of the nanoparticles [9].

2.3 Phytochemical analysis of peel extract

Aluminium chloride colorimetric assay was performed for determination of total flavonoids using the method of Ali *et al.*, [10, 11]. Optical density at 510 nm was measured using the double beam spectrophotometer (Dynamica, Halo DB 20) instantly after the assay.

Folin-Ciocalteu assay was performed for phenolic contents using the method optimized by Ali *et al.*, [10, 11]. Optical density at 765 nm was measured after incubation of 2 hr to assay the mixture, using the double beam spectrophotometer (Dynamica, Halo DB 20) instantly after the chemical reaction.

2.4 Antioxidant activity

The DPPH assay (2,2-diphenyl-1-picrylhydrazyl) was performed to the evaluation of the antioxidant activity of CuNPs according to the method of Gil *et al.*, [12]. The sample was mixed with DPPH solution, and the decrease in absorbance was measured using a spectrophotometer at 517 nm.

2.5 Anti-microbial activity

The antibacterial activity of the synthesized CuNPs was assessed using the disc diffusion method as described by Hameed *et al.*, [13]. Bacterial cultures were inoculated on agar plates, and discs impregnated with the CuNPs were placed on the surface. Zones of inhibition around the discs were measured to determine antibacterial effectiveness.

3. Results and Discussion

3.1 Synthesis and characterization of CuNPs

The synthesis of copper nanoparticles (CuNPs) using Kinnow peel extract was confirmed through the formation of brown precipitates at the bottom of the flask after three days of reaction time, indicating successful nanoparticle formation. UV-Vis spectroscopy revealed characteristic absorption peaks at around 300 nm, which are indicative of the surface plasmon resonance of CuNPs. X-ray diffraction (XRD) analysis further confirmed the crystalline nature of the synthesized nanoparticles, revealing distinct peaks corresponding to the face-centered cubic (FCC) structure of copper. The Energy-dispersive X-ray spectroscopy (EDX) spectrum validated the presence of elemental copper, confirming the purity of the nanoparticles (Figures 1 and 2).

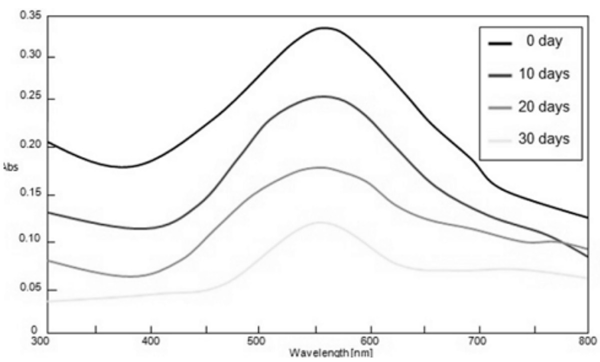

Figure 1. UV-Vis spectra showing the stability of copper nanoparticles

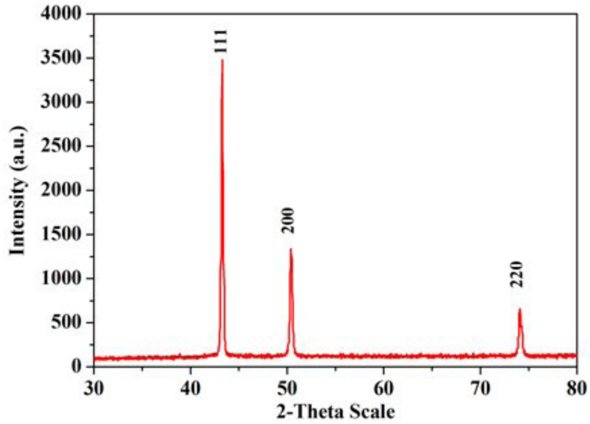

Figure 2. X-ray diffraction patterns of copper nanoparticles

3.2 Phytochemical composition of peel extract

Qualitative and quantitative phytochemical analyses of the Kinnow peel extract demonstrated substantial amounts of bioactive compounds. For flavonoids, the standard of quercetin standard, while for the phenolic, the standard curve of the gallic acid standard was optimized, and the formula for quantification of compounds was generated through regression analyses (Figures 3 and 4).

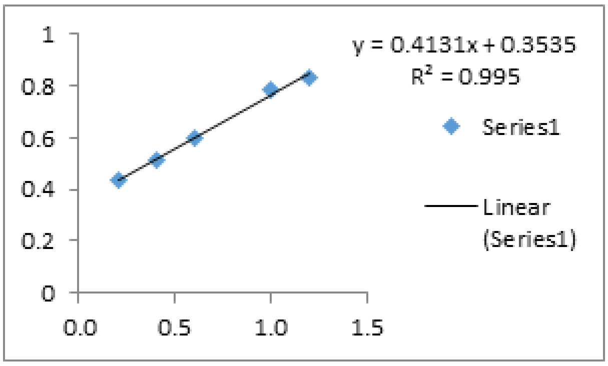

Figure 3. Standard curve of Quercetin for determination of total flavonoids

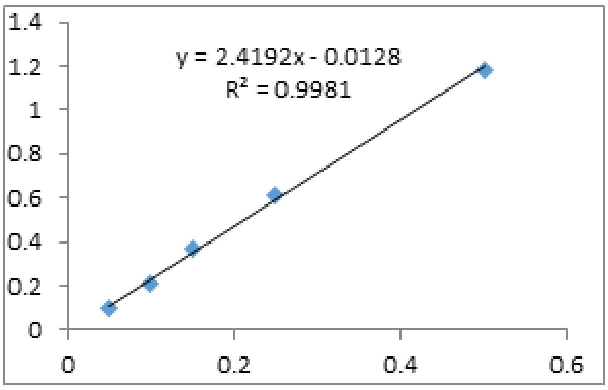

Figure 4. Standard curve of gallic acid for determination of total phenolic contents

The Folin-Ciocalteu assay revealed a high total phenolic content of 153.14 mg GAE/g, while the aluminum chloride colorimetric assay showed a total flavonoid content of 34.2 mg QE/g (Table 1). These results highlight the rich presence of antioxidant compounds within the peel extract, which are essential for the reduction and stabilization of copper ions during nanoparticle synthesis [14].

3.3 Antioxidant activity

The antioxidant potential of the synthesized Cu-NPs was evaluated using the DPPH assay. The CuNPs exhibited significant free radical scavenging activity, as evidenced by the reduction in DPPH absorbance at 517 nm. Ascorbic acid was used as a standard to construct a standard curve and results in terms of milimolar (mM) were calculated. Results revealed by increasing the concentration of CuNPs, antioxidant activity was increased (Figure 5). This strong antioxidant activity can be attributed to the high phenolic and flavonoid content in the peel extract, which are known for their potent antioxidant properties [14].

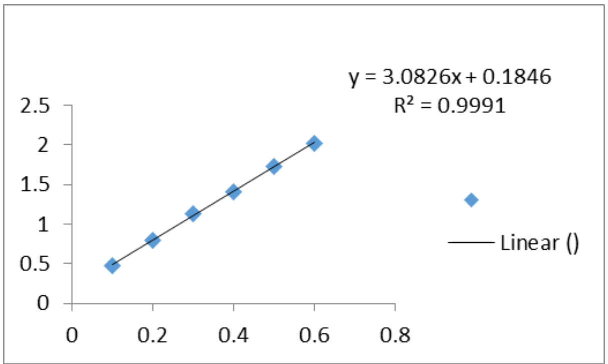

Figure 5. Standard curve of ascorbic acid for determination of the antioxidant activity of the CuNPs

3.4 Antibacterial activity against *Xanthomonas citri*

The antibacterial activity of CuNPs against *Xanthomonas citri* was assessed using the disc diffusion method. Different concentrations ranging from 25 ppm to 75 ppm showed a differential inhibition of *X. citri*. Results were directly proportional to CuNPs concentration. Notably, the CuNPs displayed the highest antibacterial activity, producing the largest zones of inhibition, followed by CuNPs alone, and then peel extract (PE) alone (Table 1). This enhanced antibacterial effect in the combined form suggests a synergistic interaction between the CuNPs and the bioactive compounds in the peel extract.

The remarkable antibacterial activity observed can be attributed to several factors. Copper nanoparticles are known to disrupt bacterial cell membranes, generate reactive oxygen species (ROS), and interfere with essential enzymatic processes within bacterial cells. The bioactive compounds present in the peel extract, particularly phenolics and flavonoids, further enhance these antibacterial mechanisms by providing additional oxidative stress and disrupting cellular function.

4. Conclusions

- The findings of this study underscore the potential of biologically synthesized CuNPs as an eco-friendly and effective alternative to conventional chemical pesticides for the management of citrus canker. The use of agricultural waste, such as Kinnow peel, not only provides a cost-effective raw material for nanoparticle synthesis but also aligns with sustainable agricultural practices by reducing waste and promoting the circular economy.
- Green synthesis of CuNPs using Kinnow peel extract presents a promising strategy for managing citrus canker, combining the antimicrobial efficacy of copper nanoparticles with the added benefits of plant-derived bioactive compounds. This approach offers a sustainable and environmentally friendly alternative to traditional chemical pesticides, addressing both agricultural productivity and environmental health.
- Prospects of the study include the field trials to evaluate the efficacy of CuNPs under the field conditions of citrus orchards, the long-term impacts on soil health and non-target organisms, and the development of formulations that maximize stability and ease of application. Additionally, exploring the molecular mechanisms underlying the observed synergistic effects between CuNPs and phytochemicals could provide deeper insights into optimizing their antibacterial properties against citrus canker and other infections.

Table 1. Analyses of CuNPs against *Xanthomonas citri*

Concentration	Total flavonoids mg QE/g	Total phenolics mg GAE/g	Antioxidant activity (mM)	Inhibition zone (mm)
25 ppm	13.02 ± 0.92	39.81 ± 0.32	11.05 ± 0.13	2.50 ± 0.27
50 ppm	21.72 ± 0.04	104.65 ± 0.19	12.86 ± 0.88	3.09 ± 0.43
75 ppm	34.29 ± 0.22	153.14 ± 0.41	14.41 ± 0.90	4.33 ± 0.25
PE	14.23 ± 0.61	86.17 ± 0.96	13.06 ± 0.71	2.71 ± 0.13

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