



## **Green Synthesis of Copper Nanoparticles Using Mandarin (*Citrus reticulata*) Peel Extract and Antifungal Study**

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### **Authors' contributions**

*This work was carried out in collaboration between all authors. Authors NTHT, PTHV and PQT designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors PDD and LSN managed the analyses of the study. Author NTHT managed the literature searches. All authors read and approved the final manuscript.*

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### **ABSTRACT**

Copper nanoparticles were synthesized by the reduction method using copper (II) sulfate with the reducing agent (ascorbic) acid in aqueous mandarin (*Citrus reticulata*) peel extract characterisation, and protective polyvinyl alcohol (PVA, Mw = 85000 g/mol). The morphology and structure of the synthesized copper nanoparticles ranged from 10 – 40 nm by Dynamic Light Scattering (DLS), transmission Electronic Microscope (TEM) and Field Emission Scanning Electron Microscopy (FE – SEM). It can be controlled during synthesis by varying the reaction temperature, pH and relative ratio of copper sulfate to the surfactant. These synthesized copper nanoparticles were found to be effective in controlling the growth of pathogens viz. *Corticium salmonicola* and

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*Phanerochaete salmon color*. The antifungal activities of copper nanoparticles were enhanced by increasing their concentration and copper nanoparticles recorded higher events than those of copper sulfate.

**Keywords:** Copper nanoparticles; *Citrus reticulata*; PVA; *Corticium salmonicola*; *Phanerochaete salminicolor*.

## 1. INTRODUCTION

Copper is one of the most widely used materials in various fields of science, technology, and medicine [1,2]. It has a great significance in all industries, particularly in the electrical sector due to low cost. The antibacterial and antifungal properties of copper, silver, and zinc have been widely utilized in advanced coating technologies, such as the design of materials for biomedical devices, hospital equipment, food processing and storage equipment, household materials, and antifouling paints. The previous studies showed that the fungus inhibition efficiency of copper nanoparticles is better than other metal nanoparticles, such as Al, Ni, Zn, Ag, Au and products of bigger size copper particles. This advantage could offer the antifungal applications of the copper nanoparticles in agriculture [3,4].

Pink disease is one of many widespread and destructive diseases in many tropical and subtropical regions of the world where the heavy rainfall occurs. It is also known as “thread blight”, “brucellosis” and “cobweb”. This disease is often seen during or just after raining season. The anamorph and teleomorph are formed during wet climate. Pink disease is caused by *Erythricium salmonicolor* (Berk. and Broome) Burdsall [Syns. *Corticium salmonicolor* Berk. and Broome, *Phanerochaete salmonicolor* (Berk. and Broome) Julich, *Necator secret us* Masee] [5]. The fungus has a very wide host range viz cocoa (*Theobroma cacao*), coffee (*Coffea Arabica*, *C. liberica*), citrus (*Citrus* spp.), black pepper (*Piper nigrum*) and rubber (*Hevea brasiliensis*).

Nanoparticles are synthesized by physical and chemical methods, there are suffering from drawbacks like expensive reagent, hazardous reaction condition, longer time, tedious process to isolate nanoparticles. Development of green nanotechnology is generating interest of researchers toward eco-friendly biosynthesis of nanoparticles. The green synthesis of copper nanoparticles was achieved by using mandarin (*Citrus reticulata*) peel extract which can be a potential inexpensive reagent, less drastic reaction and eco-friendly. Plant microelements

as copper nanoparticles are known to play critical roles in plant disease resistance through enzyme activation for defence barrier production. Furthermore, the use of copper nanoparticles helps to reduce the number of chemicals in the prevention against fungal diseases [3]. Mandarin (*Citrus reticulata*) is a widely cultivated fruit tree in many subtropical or tropical areas such as Japan, Canada, the United States, Russia and Viet Nam. Mandarin peel, a waste product coming from juice production, is rich in vitamin C, aminoacids and natural antioxidants such as phenolic acids and flavonoids [6,7,8]. Ascorbic acid found in mandarin peel extract is a good reducing and capping agent for synthesis of copper nanoparticles.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Dried peels of mature mandarin (*Citrus reticulata*), the sample for the synthesis of the copper nanoparticles was purchased the local supermarket. Copper (II) sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ;  $\geq 99\%$ ), Polyvinyl alcohol (PVA; 99+%) ( $M_w = 85000$  g/m) and sodium hydroxide (NaOH;  $\geq 98\%$ ) were purchased from Sigma – Aldrich. *Corticium salmonicola* (Berk. & Broome) and *Phanerochaete salminicolor* were procured from Research Institute of Biotechnology and Environment of Nong Lam University – Ho Chi Minh City, Vietnam. The culture samples were maintained on potato dextrose agar.

### 2.2 Preparation of Mandarin Peel Extract (MPE)

Eight gram of dried peels were accurately weighed, thoroughly washed under running tap water followed by washing it with double de-ionized water to remove surface impurities. They were crushed using a blender and finely macerated. After homogenization 100 ml of double de-ionized water was added and heated over a water bath maintained at 80°C for 15 minutes. The aqueous extract was filtered through muslin cloth and then through Whatman's No. 1 filter paper (pore size 25  $\mu\text{m}$ )

and used immediately for the synthesis of copper nanoparticles [9-11].

### 2.3 Phytochemical Screening

The crude MPE was analyzed for the detection of various constituents using standard phytochemical methods [9].

### 2.4 Synthesis of Copper Nanoparticles (CuNps)

The four-step preparation scheme for CuNps starts with dissolving 0.15 g copper sulfate, in 50 ml de-ionized water to obtain a blue solution. Next, 1.5 g polyvinyl alcohol (PVA, Mw= 85000 g/mol) was dissolved in 50 ml water and added to the aqueous solution containing the copper salt with vigorous stirring. In this step, the solution colour changed from blue to white. In the third step, MPE was added to the copper sulfate solution containing PVA. The colour of the aqueous phase remains the same. Finally, 0.1 M sodium hydroxide was added in drops to the solution under continuous rapid stirring [9,11].

The instant colour change started to occur in the aqueous phase from white to yellowish green. The appearance of this colour indicates that the reduction has started. The formation of CuNps is confirmed by the colour change from yellowish green to pale brown when it is kept in a water bath at 60°C. The formation of CuNps is inferred by visual observation followed by recording UV-Visible spectrum. The synthesized CuNps are characterized by DLS, SEM, TEM studies.

### 2.5 pH Analysis

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

### 2.6 Characterization

The formation of CuNps is inferred by visual observation followed by recording UV-Visible spectrum. UV-Vis spectral analysis was done by using PerkinElmer dual beam spectrometer (model UV/Vis Spectrometer Lambda 25) at the range of 500 – 600 nm. The shape and size distribution of CuNps were characterized by Dynamic Light Scattering (Horiba – LB550 – Japan), Scanning Electron Microscopy (Hitachi-S 4800N – Japan), Transmission Electron Microscopy (JEM 1400 instrument).

### 2.7 Antifungal Activity

*Corticium salmonicola* and *Phanerochaete salminicolor* were isolated and incubated in potato dextrose agar. Different concentrations of CuNps (10, 50 and 100 ppm) were taken in different plates, a 100 ppm CuSO<sub>4</sub>. 5H<sub>2</sub>O solution and MPE were used as positive control and negative control in another plate, respectively. The plate was incubated at 37°C for three days and the growth of fungus was recorded during this period. Finally, we measured the diameter of fungal colonies and estimated the inhibition efficiency of CuNps at various concentrations. The diameter of colonies (mm) was read and taken as the activity of the extract against the test organisms. The inhibition percentage of fungal plant pathogens was calculated by using formula as suggested by Vincent [12]:

$$IP (\%) = \frac{C - T}{C} \times 100$$

Where,

IP = Inhibition percentage (%).

C = Average colony diameter in Check (control).

T = Average colony diameter in treatment.

### 2.8 Statistical Analysis

Analysis of variance (ANOVA) was used in analyzing the data generated by this study. All analysis was made with the statistical software Statgraphics Centurion XV. Results were expressed as means ± standard deviation. Values of P < 0.05 were regarded as being significant.

## 3. RESULTS AND DISCUSSION

The results of preliminary phytochemical analysis of MPE are shown in Table 1 which indicates the presence of carbohydrates, saponins, flavonoids, and alkaloid. The presence of ascorbic acid, polyphenols, and other phytonutrients in aqueous mandarin peel extract is mainly responsible for the reduction process [7,8]. From the literature, it has been found that the amount of ascorbic acid (natural vitamin C) present in MPE was found to be 723.18<sup>e</sup> ± 0.53 mg of ascorbic acid/100 gm of the peel. Ascorbic acid is well known to scavenge free radicals and thus provide an antioxidant action during copper nuclei formation. The photographs of samples are given in Fig. 1.

**Table 1. Phytochemistry of the aqueous mandarin peel extract**

Phytochemical test	Result
Carbohydrates	+
Tannin	+
Saponin	+
Flavonoid	+
Alkaloid	+
Anthraquinone	-
Anthocyanosides	-

Note: (-), indicates negative test result, (+), indicates the positive test result

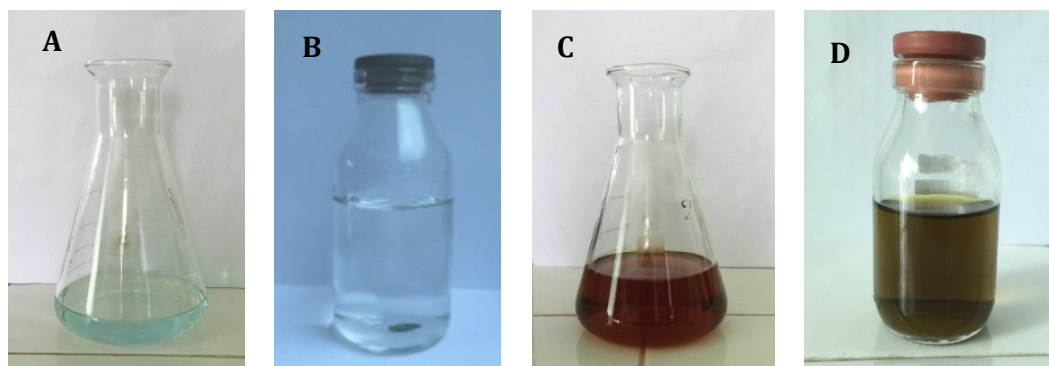
Synthesis of CuNps is quiet challenging due to its high tendency for oxidation. It is extremely sensitive to air, and the oxide phases are thermodynamically more stable. The high oxidation rate of CuNps may limit their applications. However, the capping agents or stabilizers can significantly reduce the oxidation but may not prevent it completely because of their molecular motion.

The use of various plants for metal nanoparticle synthesis has been studied by many researchers due to its low cost, high availability, and use of non-hazardous materials. Positive results have been obtained. Bio-extracts often include metabolites such as flavonoids, proteins, terpenoids, polyphenols, etc. Not only do these biomolecules act just as reducing agents, but also they are used as capping agents to minimize particles accumulation, control morphology and also protect and stabilize produced nanoparticles. Lee et al. used *magnolia Kobus* leaf extract as reducing agent and conversion of  $\text{Cu}^{+2} \rightarrow \text{Cu}^0$  for the synthesis of stable copper nanoparticles with a size of 40-100 nm. They used  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in aqueous solution and leaf extract to produce stable CuNps [13]. *Artabotrys Odoratissimus* (Nag Champa) has also been used as a reducing agent for the

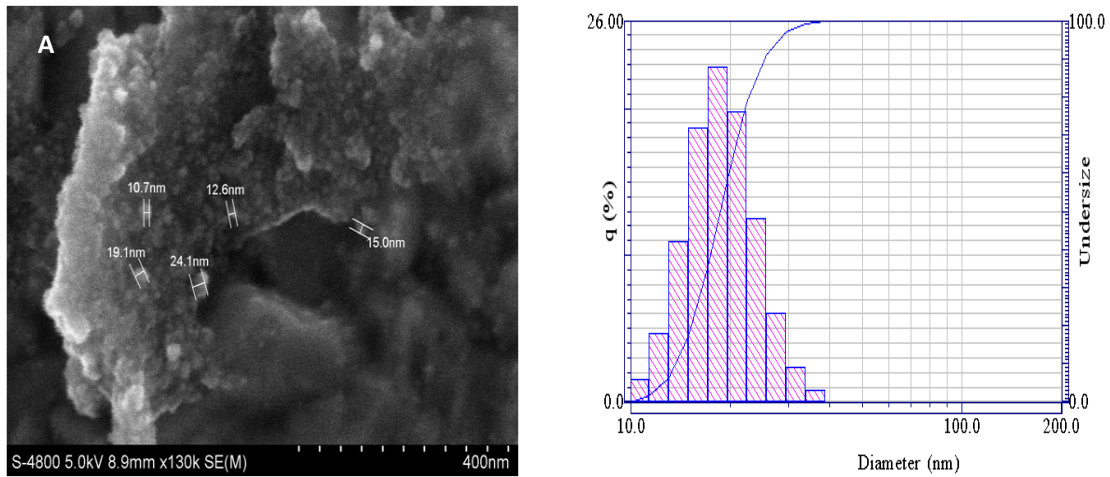
synthesis of CuNps from  $\text{CuSO}_4$  at  $95^\circ\text{C}$ , which resulted in particles from 109 to 135 nm in size [14]. The use of *Nerium Oleander* and L – ascorbic acid as stabilizing and reducing agent has been reported in the literature [15,16]. Subhankari and Nayak used Ginger (*Zingiber officinale*) to reduce copper sulfate and produce CuNps as a method for synthesis of CuNps, and they also analyzed the anti-bacterial effect of produced nanoparticles [17]. Also, Subhankari and Nayak in used *Syzygium aromaticum* (cloves) in an aqueous extract for the synthesis of spherical CuNps with 5-40 nm size.

At this moment the mechanism related to this phenomenon is not understood. Ascorbic acid present in MPE is well known to scavenge free radicals thus provides anti-oxidant action during CuNps formation. This provides the right condition for subsequent rapid reduction of phytonutrients, polyphenols along with ascorbic acid and hence CuNps formation. An important feature in the production of copper nanoparticles is to prevent agglomeration and oxidation processes. The stabilization of CuNps solution and the shape of nano material depend on PVA. PVA is frequently used as the stabilizer or capping agent for metal colloids because of its availability, low cost, and non-toxicity.

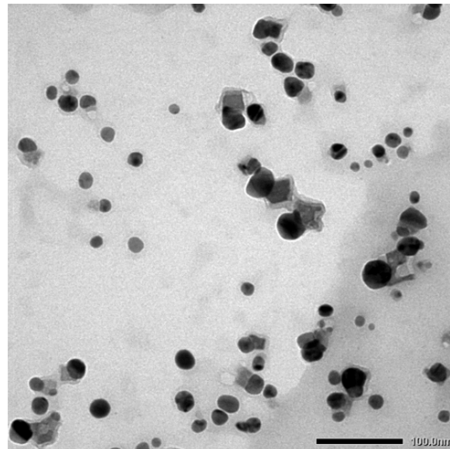
Fig. 2 shows the CuNps have been synthesized using MPE to produce stable nanoparticles ranging in size around 40 nm and a spherical to granular morphology. CuNps can be synthesized using *Citrus medica* Linn. (Idilimbu) juice [18]. These copper nanoparticles synthesized ranged from 10-60 nm with an average size of 33 nm. Furthermore, a study by Kaviya et al. using *Citrus sinensis* peel extract was able to synthesize highly stable spherical silver nanoparticles with a mean particle size of 10 nm [19].



**Fig. 1. Photographs of the sample: (A)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (aq), (B) Polyvinyl alcohol (aq), (C) Mandarin peel extract and (D) Synthesized copper nanoparticles**



**Fig. 2. (a) FE – SEM and (b) DLS image of synthesized copper nanoparticles**



**Fig. 3. TEM result of synthesized copper nanoparticles**

Temperature is an important factor that effects the size of CuNps and size distribution. The effect of temperature on the rate of formation of CuNps was studied for the 25 ml of the extract and 50 ml  $\text{CuSO}_4$  solution (Fig. 1). CuNps were formed after 2 hours at  $60^\circ\text{C}$ . However, above  $60^\circ\text{C}$  and under the boiling condition the solution becomes charred and no particle formation is seen. Hence, the reaction at  $60^\circ\text{C}$  favours the synthesis of CuNps using MPE. The pH of the solution has an influence on the progress of bio-reduction of copper sulfate solution. The pH of the mandarin peel extract,  $\text{CuSO}_4$ , and PVA on mixing was found to be 4.8. The solution pH was adjusted to 6.5 with the addition of 0.1 M NaOH solution. The Plasmon resonance is not clearly visible for pH 7.0 to 10. This probably indicates very small particles at such low pH. At the  $60^\circ\text{C}$ , CuNps was prepared with the size of particle

form 10 – 40 nm in DLS result (Fig. 2B). This result is suitable for the SEM result that CuNps are lower 40 nm (Fig. 2A). The sizes and shapes of CuNps were characterized by TEM images. Fig. 3 shows that the shapes of CuNps are spherical with uniform sizes. With a size range between 10 and 40 nm, we can say that those particles are the very small size (diameter <100 nm) and narrow distribution.

The stability of CuNps solution is a key factor in its application. The characteristic absorption peak at around 560 nm is due to the surface Plasmon band of CuNps solution (Fig. 4). This result indicated the rapid formation of the CuNps for 2-hour reaction. Formation of CuNps was indicated by a change in colour from yellowish green to pale brown which is supported by the UV absorption at 560 nm. The result was similar

to the previous studies that the peak at 550 – 600 nm of wavelength can be assigned to absorption of CuNPs [3,20,21]. The products have been stored at room temperature for 30 days without any decomposition or aggregation. Finally, there was no change in UV spectrum.

The antifungal activity of CuNPs was carried out on two plant pathogens such as *Corticium salmonicola* and *Phanerochaete salmon colour* (Table 2). The samples were incubated at 37°C for three days. All experiments were performed in triplicate. Similar studies have also shown that CuNPs exhibit both antifungal and antibacterial behaviour [3,10,18].

We survey the effects of CuNPs solutions on the development of *Corticium salmonicola* and *Phanerochaete salminicolor* by determining the diameter of the fungal colonies on the samples including negative control (MPE), positive control (100 ppm CuSO<sub>4</sub>.5H<sub>2</sub>O solution) and the various CuNPs solution concentrations (10, 50 and 100 ppm, respectively). The diameter of the fungal colonies was determined after the three days incubation. The images of colonies

according to incubation at various CuNPs solution concentrations are exhibited in Fig. 5. These results show that CuNPs inhibited the development of *Corticium salmonicola* and *Phanerochaete salmon color*. It demonstrated that the diameter of colonies in all samples was supplemented with CuNPs being smaller than the negative control. In three-day incubation, the diameters of *Corticium salmonicola* fungal colonies for additional formulations of 10, 50 and 100 ppm of CuNPs were measured about 2.50 mm, 1.87 mm and 0.33 mm, respectively. The fungi in the positive control were 4.33 mm while the negative control developed rapidly with the diameter of the fungal colony is about 57.30 mm. Meanwhile, the diameter of the *Phanerochaete salminicolor* colony was 48.80 mm at the CuNPs concentration of 10 ppm. In a comparison of the negative control was 57.30 mm. At this time the fungal colony diameter in the positive control was 6.23 mm.

In addition, Table 2 shows the significant decrease in the diameter of fungal colonies at 50 ppm at 3.33 mm and 100 ppm at 0.97 mm. These diagrams show that the more the CuNPs

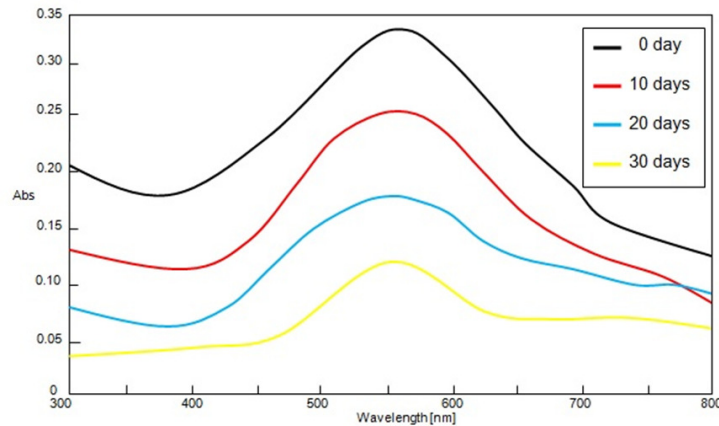


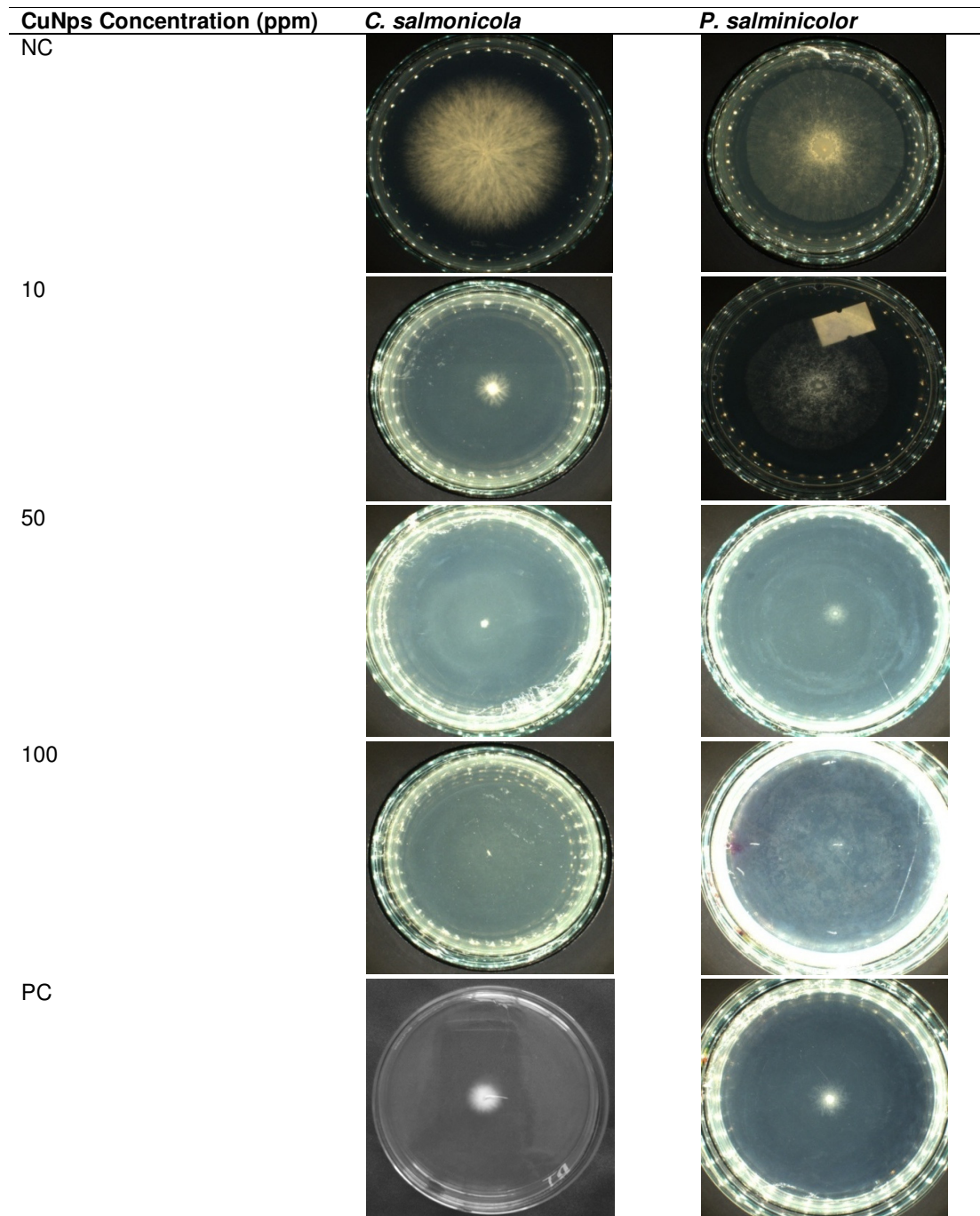
Fig. 4. UV-Visible spectra showing the stability of copper nanoparticles

Table 2. Antifungal activity of CuNPs against fungal test organism

Concentration (ppm)	<i>Corticium salmonicola</i> (mm)	Inhibition (%)	<i>Phanerochaete salmonicolor</i> (mm)	Inhibition (%)
NC	57.30 ± 0.95 <sup>h</sup>	-	57.30 ± 0.30 <sup>g</sup>	-
10	2.50 ± 0.27 <sup>c</sup>	95.64	48.80 ± 0.36 <sup>e</sup>	14.83
50	1.87 ± 0.53 <sup>bc</sup>	96.74	3.33 ± 0.15 <sup>c</sup>	94.18
100	0.33 ± 0.12 <sup>a</sup>	98.72	0.97 ± 0.15 <sup>a</sup>	98.31
PC	4.33 ± 0.15 <sup>d</sup>	92.44	6.23 ± 0.13 <sup>d</sup>	89.13

Note: Data were expressed as mean ± SD. Values with different superscripts within the column are significantly different at p<0.05 by Kruskal–Wallis test.

NC: Negative control and PC: Positive control



**Fig. 5. The diameter of *Corticium salmonicola* and *Phanerochaete salminicolor* colonies at various CuNps solution concentrations**

Note: NC: Negative control and PC: Positive control

concentration increases, the more the diameter of fungal colonies decreases. This result demonstrates the diameter of the fungal colony almost inhibits in the samples at the CuNps concentration of 100 ppm. These results show

that the inhibition efficiency of CuNps was good in the *in vitro* condition. Thus, the inhibition efficiency of CuNps increases according to the concentration used. In three-day incubation, CuNps solutions inhibited over 98% of the growth

of *Corticium salmonicola* and *Phanerochaete salmon color* at the concentration of 100 ppm. Meanwhile, CuNps solution could inhibit 14.83% of the *Phanerochaete salminicolor* fungal growth at 10 ppm concentration after being incubated for three days and reaches 95.64% if incubated for *Corticium salmonicola*.

At this time the results show that inhibition efficiency of CuNps solution increases significantly due to 100 ppm concentration dish; the fungi were inhibited intensively and no longer grew in a three-day incubation. In contrast, the fungi in the negative control continue to grow. This result illustrates copper nanoparticles synthesized in green synthesis method are showing more antifungal activities than copper sulfate solution and MPE. A similar result was reported by Essa et al. found that biological CuNPs demonstrated antifungal activities higher than those of the untreated copper sulfate [22]. The reason could be that smaller size of the nanoparticles which leads to tightly adsorbed on the surface of the microbial cells to disrupt the membrane which would lead to the leakage of an intracellular component, thus killing the microbial cells.

#### 4. CONCLUSION

Here we have reported a simple reproducible and low-cost approach for the preparation of stable CuNps by using aqueous extract of mandarin (*Citrus reticulata*) peel as the reducing, stabilizing and capping agent. The average size of these nanoparticles was formed to be in the range of 10 – 40 nm. The synthesized nanoparticles have been characterized by SEM, TEM, DLS and UV-VIS spectroscopy. The stability period is 30 days, which has been observed with no suspension or sedimentation. Copper nanoparticles synthesized in green synthesis method are showing more antifungal activities than copper sulfate solution and MPE after three-day incubation. As the synthesized copper nanoparticles showed excellent antifungal activity, which is another advantage using plant extract for metal nanoparticle synthesis over using the chemical method. This is a simple, economical and green method for the synthesis of CuNps with no toxic or hazardous effect.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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