Original Research Article

GREEN SYNTHESIS OF COPPER NANOPARTICLES USING MANDARIN (*Citrus reticulata*) PEEL EXTRACT AND ANTIFUNGAL STUDY

ABSTRACT

Copper nanoparticles ~~was~~ synthesized by biological reduction method in water/PVA systems of copper (II) sulfate ($CuSO_4.5H_2O$) at 1000 ppm with the reducing agent ~~is~~ ascorbic acid in aqueous mandarin (*Citrus reticulata*) peel extract ~~chacracterisation~~, and protective polyvinyl alcohol (PVA, Mw = 85000 g/mol). The influence of parameters on the size of copper nanoparticles was studied: T= 60°C, pH = 6.5, aqueous extract of mandarin peel/ Cu^{2+} (2:1v/v), Cu^{2+}/PVA (1/10w/w). The morphology and structure of the synthesized copper nanoparticles were formed to be in the range of 10 – 40 nm by Dynamic Light Scattering (DLS), ~~Electronic Microscope~~ (TEM), Field Emission Scanning Electron Microscopy (FE – SEM) and Ultraviolet–Visible Spectrophotometry (UV–Vis). These biologically synthesised copper nanoparticles were found to be effective in controlling growth of pathogens viz. *Corticiumsalmonicola* Berk. and *Phanerochaetesalminicolor*. The synthesized copper nanoparticles showed stronger antifungal activities than copper sulphate.

Keyword: Copper nanoparticles, *Citrus reticulata*, PVA, *Corticiumsalmonicola* Berk., *Phanerochaetesalminicolor*

1. INTRODUCTION

Copper is one of the most widely used materials in various fields of science, technology and medicine. 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 [1, 2].

Pink disease is widespread and destructive in many tropical and subtropical regions of the ~~w~~world where heavy rain fall occurs. The disease is also known as “thread blight”, “rubellosis” and “cobweb”. The disease is seen during or just after raining season. The anamorph and teleomorph are formed during wet climate. Pink disease caused by *Erythricium salmonicolor* (Berk. and Broome) Burdsall [Syns. *Corticium salmonicolor* Berk. and Broome, *Phanerochaete salmonicolor* (Berk. and Broome) Julich, *Necator decret us* Massee] [3]. 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 defense barrier production. Furthermore, the use of copper nanoparticles helps to reduce the amount of chemicals in the prevention against fungal diseases [1]. 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 [4]. Ascorbic acid present in mandarin peel extract is a good reducing and capping agent for synthesis of copper nanoparticles. The purpose of ~~this investigation shows that~~ copper nanoparticles ~~are a significantly antifungal potential as bactericidal agents.~~

2. MATERIALS AND METHOD

2.1 Materials

Dried peels of mature mandarin *Citrus reticulata*, the sample for the synthesis of the copper nanoparticles was purchased ~~at~~ the local supermarket. Copper (II) sulphate, Polyvinyl alcohol (PVA) (Mw = 85000 g/m) and other reagents used in the study were

purchased from Sigma – Aldrich. The fungal strains (*Corticium salmonicola Berk.* and *Phanerochaete salminicolor*) employed in this work were procured from Research Institute of Biotechnology and Environment of Nong Lam University – Ho Chi Minh city, Vietnam.

2.2 Preparation of Mandarin Peel Extract (MPE)

8gm of dried peels were accurately weighed, thoroughly washed under running tap water followed by washing it with double de-ionised water to remove surface impurities. They were crushed using a blender and finely macerated. After homogenization 100ml of double de-ionised water was added and heated over a water bath maintained at 80^{o}C for 15 minutes. The extract obtained was filtered through muslin cloth and then through Whatman's No. 1 filter paper (pore size 25µm) and used immediately for the biosynthesis of copper nanoparticles [5, 6, 7].

2.3 Phytochemical Screening

The crude MPE was analyzed for the detection of various constituents using standard phytochemical methods [5].

2.4 Synthesis of copper nanoparticles (CuNps)

The four-step preparation scheme for CuNps starts with dissolving 0.15 g copper sulphate, in 50 ml de-ionised 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 color changed from blue to white. In the third step, MPE was added to the copper sulphate solution containing PVA. The color of the aqueous phase remains the same. Finally, 0.1 M sodium hydroxide was added in drops to the solution under continuous rapid stirring [5, 7].

The instant color change started to occur in the aqueous phase from white to yellowish green. The appearance of this color indicates that the reduction has started. The formation of CuNps is confirmed by the color change from yellowish green to pale brown when it is kept on a water bath at 60^{o}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 Characterization of Synthesized Copper Nanoparticles

2.5.1 Visual Inspection

The bio-reduction of the aqueous solution of copper sulphate using MPE was monitored and the appearance of brown color indicates the formation of CuNps. ~~Photograph of~~ CuNps at different ~~parameters.~~

2.5.2 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.

2.5.3 UV-Visible Spectroscopy

The reduction of copper sulphate to CuNps was monitored by recording UV-Visible spectrum of the reaction mixture after diluting a small aliquot of the sample with de-ionised water. The measurements ~~are~~ recorded on PerkinElmer dual beam spectrometer (model UV/Vis Spectrometer Lambda 25) operated at resolution of 1nm.

2.5.4 Dynamic Light Scattering Studies

Dynamic Light Scattering (DLS) measurement of the citrus reduced CuNps was carried out using light scattering techniques (Horiba – LB550 – Japan) at Laboratory for Nanotechnology – Vietnam National University**.**

2.5.5 Scanning Electron Microscopy (SEM)

The sample was prepared by placing a drop of colloidal solution of CuNps on carbon coated copper grid and subsequently drying in air, before transferring it into the microscope operated at an accelerated voltage of 130 Kv (Hitachi-S 4800N – Japan).

2.5.6 Transmission Electron Microscopy (TEM)

Morphologies include the sizes and shapes of CuNps which were recorded by Transmission Electronic Microscope (TEM) on a JEM 1400 instrument.

2.5.7 Pharmocognostic evaluation of synthesized copper nanoparticles

Determination of antifungal activity: The fungal strains (*Corticium salmonicola Berk.* 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 $CuSO_4$. $5H_2O$ solution and MPE were used as positive control and negative control in another plate, respectively. The plate was incubated at 37^{o}C for 3 days and the growth of fungus was recorded during this period. Finally, ~~we measured~~ diameter of fungal colonies and estimated the inhibition efficiency of CuNps at various concentrations. The diameter of colonies (mm) were 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 [8]:

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

Where:

IP = Inhibition percentage (%).

C = Average colony diameter in Check (control).

T = Average colony diameter in treatment.

2.5.8 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

3.1 Phytochemical Screening

The results of preliminary phytochemical analysis of MPE are shown in Table-1 which indicate the presence of carbohydrates, ~~tannis,~~ saponins, flavonoids and alkaloid.

Table 1. Phytochemistry of the aqueous mandarin peel extract

Phytochemical Test	Result
Carbohydrate	+
Tannin	+
Saponin	+
Flavonoid	+
Alkaloid	+
Anthraquinone	-
Anthocyanosides	-

Note: -: indicates positive test result

~~+~~: indicates negative test result

The presence of ascorbic acid, polyphenols and other phytonutrients in aqueous mandarin peel extract is mainly responsible for the bio-reduction process. From the literature it has been found that the amount of ascorbic acid (natural vitamin C) present in MPE was found to be $723.18^{e} \pm 0.53$ mg of ascorbic acid/100 gm of peel. Polyphenolic compounds are very important plant constituents ~~because of the~~ scavenging ability of their –OH groups.

The antioxidant property of polyphenolic compounds is mainly due to its redox property which allows them to act as reducing agents.

3.2 Synthesis of Copper nanoparticles (CuNps)

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.

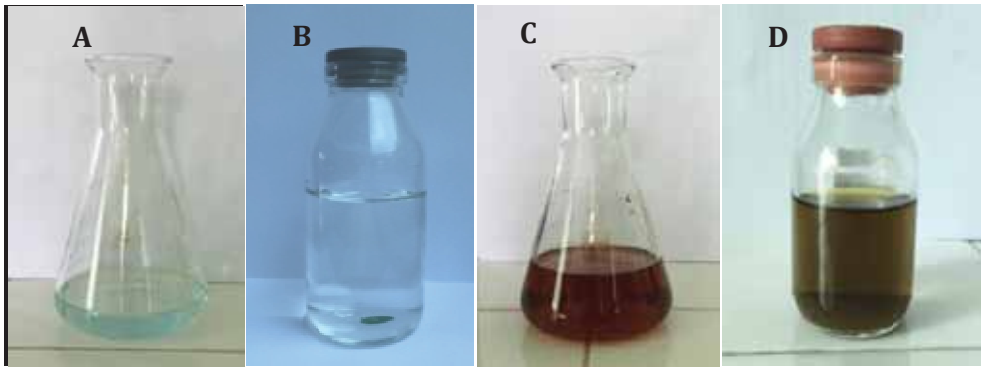


Fig. 1. Photographs of the sample: (A) CuSO4.5H2O (aq), (B) Polyvinyl alcohol (aq), (C) *Citrus* peel extract and (D) synthesised copper nanoparticles

Lee et al. used *magnolia kobus* leaf extract as reducing agent and conversion of $Cu^{+2} \rightarrow Cu^{0}$ for synthesis of stable copper nanoparticles with a size of 40-100 nm. They used $CuSO_4.5H_2O$ in aqueous solution and leaf extract to produce stable CuNps [9]. *Artabotrys Odoratissimus* (Nag Champa) has been also used as a reducing agent for the synthesis of CuNps from $CuSO_4$ at 95°C, which resulted in particles from 109 to 135 nm in size [10]. The use of *Nerium Oleander* and L – ascorbic acid as stabilizing and reducing agent has been reported in literature [11, 12]. Subhankari and Nayak used Ginger (*Zinngiber 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 [13]. In a distinct article, Subhankari and Nayak in used *Syzygium aromaticum* (cloves) in an aqueous extract for synthesis of spherical CuNps with 5-40 nm size.

At this moment the mechanism associate with this phenomenon is not clearly 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 by phytonutrients, polyphenols along with ascorbic acid and hence CuNps formation.

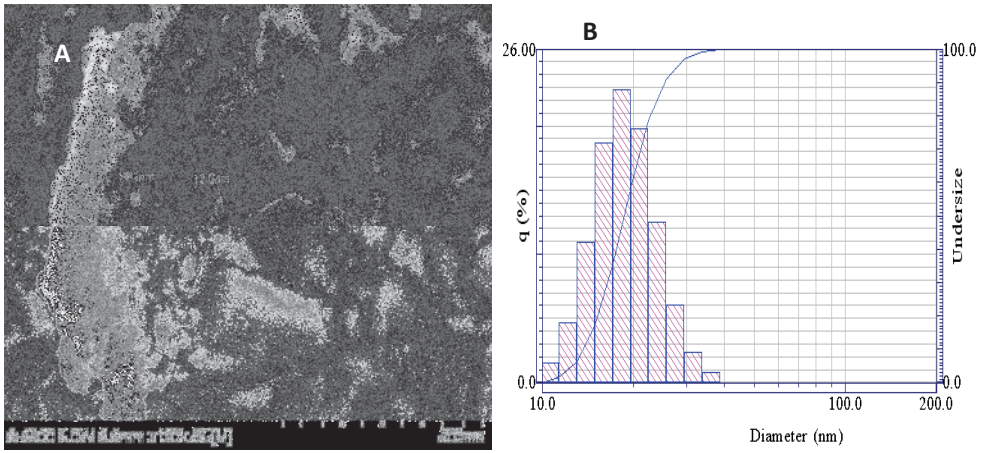


Fig. 2. (a) FE – SEM and (b) DLS result of copper nanoparticles was prepared at 60°C

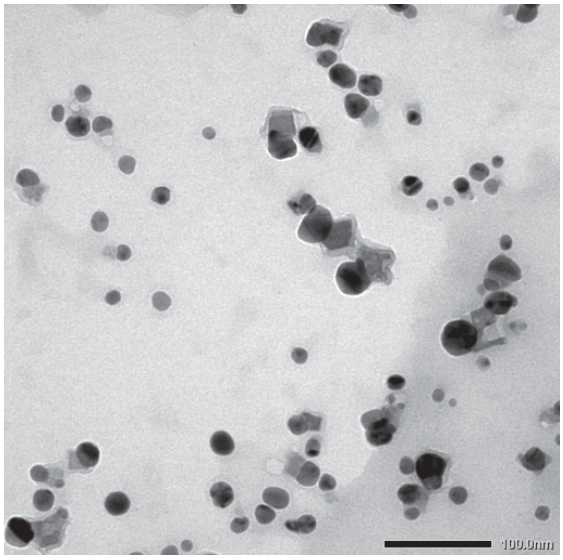


Fig. 3. TEM result of copper nanoparticles was prepared at 60°C

CuNps have been biologically 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 [14]. These copper nanoparticles synthesized were in the range of 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 [15].

Temperature is important factor that ~~effect to~~ 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 $CuSO_4$ solution. CuNps were formed within 2 hours at 60°C. Hence, the reaction at 60°C favours the synthesis of CuNps using MPE. The pH of CuNps solution was adjusted to be 6.5 with addition of 0.1 M NaOH solution. At the 60°C, CuNps was prepared with size of particle form 10 – 40 nm in SEM result. This result is suitable with the DLS result that CuNps are lower 40 nm. The sizes and shapes of CuNps were characterized by TEM images. Figure 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 large and widely dispersed.

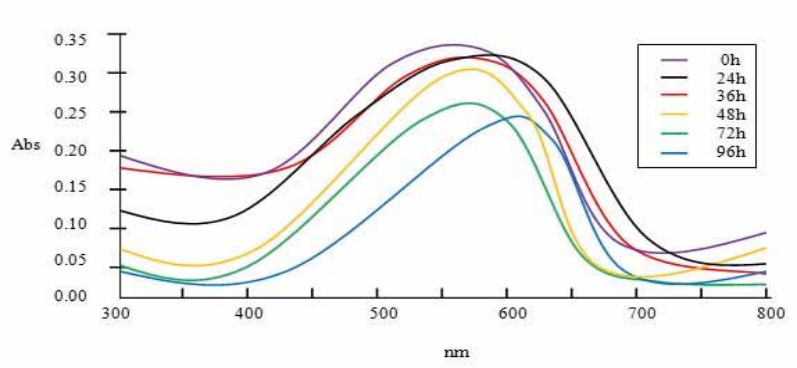


Fig. 4. UV-Visible spectrum of biosynthesized copper nanoparticles at 60°C

Table 2. Formation of copper nanoparticle at 60°C

Sample	Time (hours)	Color	OD (nm)	Λmax
1.	0	Violet	560	0.341
2.	24	Dark	595	0.325
3.	36	Red	575	0.316
4.	48	Yellow	575	0.302
5.	72	Green	575	0.261
6.	96	Blue	615	0.248

The effect of ascorbic acid concentration on the UV – Visible absorption spectroscopy of synthesized CuNps is shown in Figure 4. The characteristic absorption peak at around 575 nm is due to the surface Plasmon band of CuNps solution. The strong surface

Plasmon absorption band observed at 560 nm. This result indicated the rapid formation of the CuNps for 2-hour reaction. Our result agrees with previous studies that the peak at 550 – 600 nm of wavelength can be assigned to absorption of CuNps [1, 16, 17]. The products have been stored at room temperature for 30 days without any decomposition or aggregation.

3.3 Antifungal activity of copper nanoparticles

The samples were incubated at 37°C for 3 days. All experiments were performed in triplicate. The particles were found to have a significant antifungal activity against common pathogens such as *Corticiumsalmonicola* Berk. and *Phanerochaetesalminicolor*. Similar studies have also shown that CuNps exhibit both antifungal and antibacterial behavior [1, 6, 14].

Table 3. Antifungal activity of CuNps against fungal test organism

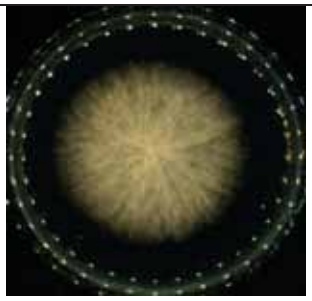
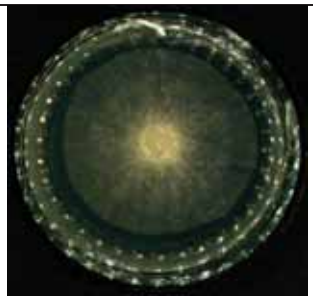
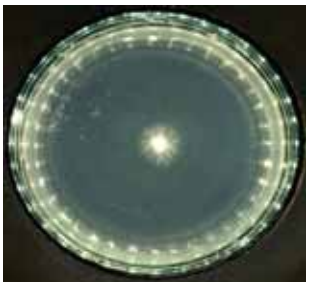
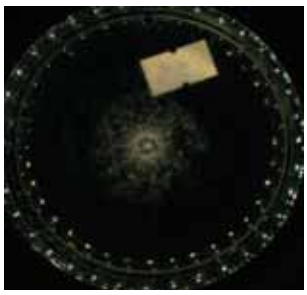
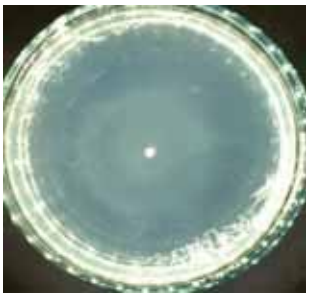
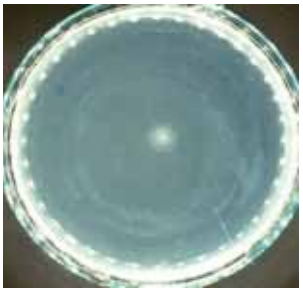
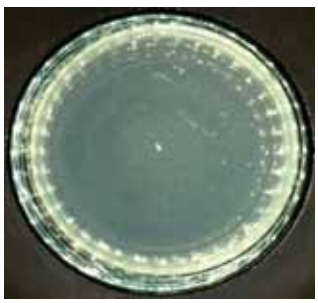
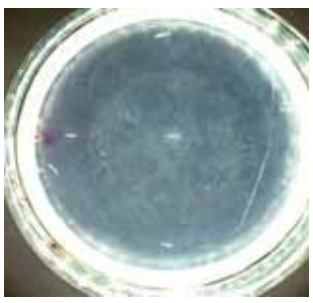
Concentration (ppm)	*Corticium salmonicola* Berk. (mm)	Inhibition (%)	*Phanerochaete salminicolor* (mm)	Inhibition (%)
NC	57.30 ± 0.95^{h}	-	57.30 ± 0.30^{g}	-
10	2.50 ± 0.27^{c}	95.64	48.80 ± 0.36^{e}	14.83
50	1.87 ± 0.53^{bc}	96.74	3.33 ± 0.15^{c}	94.18
100	0.33 ± 0.12^{a}	98.72	0.97 ± 0.15^{a}	98.31
PC	4.33 ± 0.15^{d}	92.44	6.23 ± 0.13^{d}	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

We survey the effects of CuNps solutions on the development of *Corticium salmonicola* Berk. and *Phanerochaete salminicolor* by determining the diameter of the fungal colonies on the samples including negative control (MPE), positive control (100 ppm $CuSO_4.5H_2O$ solution) and the various CuNps solution concentrations (10, 50 and 100 ppm, respectively). The diameter of the fungal colonies was determined after the incubation for 3 days. The images of colonies according to incubation at various CuNps solution concentrations are exhibited in Figure 5. These results show that CuNps inhibited the development of *Corticium salmonicola* Berk. and *Phanerochaete salminicolor*. It demonstrated that the diameter of colonies in all samples was supplemented with CuNps being smaller than the negative control. In 3-day incubation,

the diameters of *Corticium salmonicola* Berk. 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 being about 57.30 mm. Meanwhile, the diameter of *Phanerochaete salminicolor* colony was 48.80 mm at the CuNps concentration of 10 ppm that comparison with negative control ~~was~~ 57.30 mm. At this time the fungal colony diameter in the positive control was 6.23 mm.

CuNps Concentration (ppm)	***C. salmonicola Berk.***	***P. salminicolor***
NC		
10		
50		
100		

PC

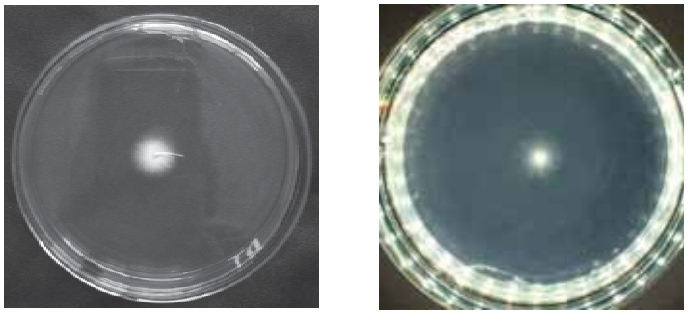


Fig. 5. The diameter of *Corticiumsalmonicola* Berk. and *Phanerochaetesalminicolor* colonies at various CuNps solution concentrations

Note: NC: negative control and PC: positive control

In addition, Table 3 shows ~~the~~ significant~~ly~~ decrease ~~of~~ the diameter of fungal colonies at 50 ppm and 100 ppm about 3.33 mm and 0.97 mm, respectively. These diagrams show that the more the CuNps concentration increases, the more the diameter of fungal colonies decreases. This result demonstrated that the diameter of the fungal colony almost ~~inhibit~~ 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 3-day incubation, CuNps solutions inhibited over 98% of growth of *Corticium salmonicola* Berk. and *Phanerochaete salminicolor* 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 3 days and reaches 95.64 % if incubated for *Corticium salmonicola* Berk. At this time the results show that inhibition efficiency of CuNps solution went up 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 still grew normally. Raman et al. has reported that antifungal activity of CuNps solution can be explained on the basis of Overtone's concept and chelation theory. Liposolubility is an important factor that controls the antifungal activity because the lipid membrane that surrounds the cell favors the passage of only lipid soluble matter and this is the Overtone's concept of cell permeability. Due to the overlap of the ligand orbital with the metal orbitals and the slight sharing of positive charge of the metal ion with the donor groups on chelation, the polarity of the metal ion is reduced to a greater extent. Chelation further increases the delocalization of π-electrons over the whole chelate ring and enhances the lipophilicity of the metal

complex. Thus the metal binding sites on the enzymes of microorganisms get blocked because of the increased lipophilicity, which in turn enhances the penetration of the metal complexes into lipid membranes. Copper nanoparticles also disturb the respiration process of the cell and thus block the synthesis of the proteins that restricts further growth of the organism [18]. This result demonstrated that copper nanoparticles synthesized in green synthesis method are showing more antifungal activities than copper sulphate solution and MPE.

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 were formed to be in the range of 10 – 40 nm. The biosynthesized 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 sulphate solution and MPE after a 3-day incubation. As the synthesized copper nanoparticles showed excellent antifungal activity, which is another advantage using plant extract for metal nanoparticle synthesis over using chemical method. This is a simple, economical and green method for the synthesis of CuNps with no toxic and hazardous effect.

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