Research Article

Autoclave-Assisted Mycosynthesis of Copper Nanoparticles from *Pleurotus ostreatus* Extract: Characterisation and Toxicity Analysis

Roslina Ainna Roslan[1], Nurul Aili Zakaria[1], Sharifah Aminah Syed Mohamad[1,2], Norfatimah Mohamed Yunus[1*] and Lyena Watty Zuraine Ahmad[1]

[1]School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia; [2]Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns), Level 9, FF3 Building, UiTM Puncak Alam Campus, 42300 Puncak Alam, Selangor, Malaysia.

Abstract | Copper nanoparticles (CuNPs) have gained significant attention due to their wide range of applications, particularly in antibacterial treatments. In this study, we synthesized CuNPs using an eco-friendly mycosynthesis approach with *Pleurotus ostreatus* (oyster mushroom) extract and employed an autoclave-assisted method to enhance the synthesis process. This green synthesis method presents distinct advantages, such as reduced toxicity, cost-effectiveness, and environmental sustainability. The copper nanoparticles were characterized using UV-Vis spectroscopy, field emission scanning electron microscopy (FESEM), and Fourier-transform infrared spectroscopy (FTIR). UV-Vis spectroscopy confirmed the formation of CuNPs with a peak at 374 nm. FESEM revealed irregular shapes and a wide size distribution, ranging from 41.56 nm to 131.9 nm. FTIR analysis demonstrated the presence of functional groups acting as capping agents, stabilizing the nanoparticles. The root elongation test and inter simple sequence repeat (ISSR)-PCR showed that the CuNPs exhibited no toxic effects on rice root growth. The findings suggest that autoclave-assisted mycosynthesis of CuNPs using *P. ostreatus* offers a sustainable and efficient route for producing CuNPs with potential applications in antimicrobial treatments and agricultural industry.

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***Correspondence** | Norfatimah Mohamed Yunus, Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia; **Email:** norfatimah9778@uitm.edu.my
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Introduction

Nanoparticles have gained significant attention among scientists due to their unique properties and wide applications across various fields, including medicine, electronics, and environmental science. Copper nanoparticles (CuNPs) show significant potential among various nanoparticle types, particularly in agricultural and antimicrobial domains. It is because CuNPs has demonstrated potent antibacterial activity, which has sparked interest in their potential use in combating bacterial pathogens (Abdel *et al*., 2024; Sreenayana *et al*., 2024; Obaidullah *et al*., 2017). Investigating the production and application of copper nanoparticles is crucial, particularly through eco-friendly methods.

Mycosynthesis is a method which refers to the use of fungi for nanoparticle synthesis (Šebesta *et al*., 2022). This biological route offers distinct advantages over conventional chemical methods, including lower toxicity (Šebesta *et al*., 2022), eco-friendliness, and cost-effectiveness (Eleonora *et al*., 2022). Fungi can effectively reduce nanoparticles by secreting bioactive compounds. The use of fungal organisms not only eliminates the need for hazardous chemicals but also enables large-scale production of nanoparticles under mild conditions.

P. ostreatus, also known as the oyster mushroom, has emerged as a promising candidate for nanoparticle biosynthesis among the various fungal species. This mushroom is well-known for its bio-reductive capabilities, and previous studies have shown its efficacy in synthesizing metal nanoparticles (Manimaran *et al*., 2023; Ahmed *et al*., 2022; Mkhize *et al*., 2022). The ability of *P. ostreatus* to mediate nanoparticle formation through its bioactive metabolites makes it an ideal organism for mycosynthesis, offering both efficiency and sustainability. The selection of *P. ostreatus* for this study is based on its proven track record in bio-reduction processes and its widespread availability.

Recently, researchers have investigated the autoclave-assisted method for improving the efficiency of synthesizing nanoparticles. The autoclave provides a controlled, high-pressure environment that accelerates reaction rates and improves the quality of nanoparticle synthesis (Ghanbari *et al*., 2018; Kalantari *et al*., 2018; Kora, 2023). The utilization of *P. ostreatus* extract in this method enables precise regulation of the reaction parameters, resulting in the synthesis of copper nanoparticles that exhibit exceptional stability and homogeneity. The presented methodology offers a novel and practical approach for efficiently synthesizing copper nanoparticles on a larger scale.

Rice is the third most significant crop produced in agricultural sector in Malaysia (Dorairaj & Govender, 2023; Zulkafflee *et al*., 2022). In an effort to augment crop yields and optimize financial returns, rice growers have utilized increasingly substantial quantities of synthetic fertilizers and other agrochemicals containing heavy metals such as copper (Zulkafflee *et al*., 2022). Due to the inability of heavy metals to biodegrade, paddy fields are prone to contamination by heavy metals, which has become a significant concern (Zakaria *et al*., 2021). The health risks that heavy metals pose to the environment have led to their recognition as one of the most significant chemical hazards (Mitra *et al*., 2022).

The primary objectives of this study are to investigate the synthesis of copper nanoparticles using *P. ostreatus* extract through an autoclave-assisted method, to characterize the resulting nanoparticles using various analytical techniques and to evaluate the toxic effect of the nanoparticles on rice plant (*Oryza sativa*). Inter simple sequence repeat (ISSR)-PCR will be utilized in this study to examine the variation of copper nanoparticles (CuNPs) in the genetic makeup of rice plants and how it affects the rice's growth. By exploring this eco-friendly and innovative approach, the study aims to advance the field of sustainable nanotechnology, with potential applications in industry and environmental remediation. The significance of using biological organisms like fungi for nanoparticle synthesis lies in their ability to promote greener synthesis methods, contributing to the growing need for environmentally conscious technological advancements.

Materials and Methods

Preparation of copper (II) sulfate powder

Copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) was procured from Bendosen. The copper sulfate was dried in an oven at 80°C for 24 hours until the light blue powder transitioned to a slightly paler hue. Subsequently, the dried $CuSO_4$ was ground using an A 11 basic Analytical mill (IKA, USA).

Extraction of the fungus sample

The oyster mushrooms (*Pleurotus ostreatus*) were purchased from the local market. The mushrooms were thoroughly washed to remove any impurities and then placed in an oven at 60°C for 24 hours to eliminate excessive moisture. After drying, the mushrooms were ground into a fine powder. This experimental procedure was adapted from methods described by Suh *et al*. (2017), Mohaddes-Kamranshahi *et al*. (2019), Mkhize *et al*. (2022), and Shaheen *et al*. (2024).

A stock solution was prepared by mixing 100 g of *P. ostreatus* with 1000 ml of sterilized distilled water. Then, the *P. ostreatus* stock solution was diluted into 5 different concentrations, 10%, 20%, 30%, 40% and 50%. The *P. ostreatus* extract was prepared by using the formula below where C_1 is the concentration of the stock solution, V_1 is the volume of the stock solution, C_2 is the working solution and V_2 is the volume for working solution.

$$C1V1 = C2V2$$

So, to make 10% of the *P. ostreatus* extract,
$C_1V_1 = C_2V_2$
(100%) V1 = (10%) (100ml)
V1 = 10 ml of 100% extract + 90 ml of sterilised distilled water

The 20%, 30%, and 50% working solution were calculated as above equation. *P. ostreatus* extract was produced using autoclave extraction methods at 121°C, 1kPa for 15 minutes (Pani *et al*., 2016; Suh *et al*., 2017). After autoclaving, the mixture was cooled to room temperature. The samples were then stored at 4°C until further analysis.

Autoclave-assisted synthesis

A total of one gram of copper powder was added to 10 ml of fungus extract and stirred with a glass rod until the copper powder was fully dissolved. The mixture was then subjected to autoclaving at 121°C and 1kPa for 15 minutes (Pani *et al*., 2016; Ghanbari *et al*., 2018; Kalantari *et al*., 2018). After autoclaving, the mixture was filtered using Whatman filter paper No 1. The purified CuNPs mixture was then poured into a sterile glass petri dish and stored in a dark environment at room temperature to allow the formation of CuNPs crystals. The test solutions were prepared and labelled as below.

11020 : 1g of Cu powder + 10ml of 20% *P. ostreatus* extract
11030 : 1g of Cu powder + 10ml of 30% *P. ostreatus* extract
11050 : 1g of Cu powder + 10ml of 50% *P. ostreatus* extract
21010 : 2g of Cu powder + 10ml of 10% *P. ostreatus* extract
21030 : 2g of Cu powder + 10ml of 30% *P. ostreatus* extract

Characterisation techniques

All prepared solutions underwent the characterization protocol; however, data in this section are presented only for the 11020 CuNPs as representatives.

UV-Visible spectroscopy

Ultraviolet-visible spectroscopy was employed to analyze the formation of the nanoparticles. The CuNPs suspension was loaded into a quartz cuvette, and the absorbance spectrum was recorded using a double-beam UV-Vis spectrophotometer (Perkin Elmer, USA) within the wavelength range of 200-700 nm. Distilled water served as the reference.

Field emission scanning electron microscopy (FE-SEM)

The size, shape and surface morphology of CuNPs were investigated after drying the CuNPs suspension and exposing it to Field Emission Scanning Electron Microscopy analysis. The CuNPs suspension was deposited onto a copper grid coated with amorphous carbon, allowed to dry, and then analyzed using field emission scanning electron microscopy (ZEISS Supra 40VP 7426, England) at an accelerating voltage of 80 kilovolts.

Fourier transform infrared spectroscopy (FTIR)

The FTIR method was used to identify functional groups such as amine, carbonyl, and hydroxyl involved in the synthesis in the spectrum 450–4000 cm− 1. A small amount of copper nano powder was utilized to create KBr pellet which was then subjected to FTIR (Perkin Elmer, USA) analysis using the instrument's built-in software.

Toxicity analysis of CuNPs

Preparation and exposure of oryza sativa seeds to CuNPs.

Rice (*O. sativa*) seeds were cultivated on moist tissue paper in warm and dark environment until the root's germination occur for at least 5.0 mm long. Then, the seeds were soaked in 5 ml CuNPs solution for 2 different duration treatments, 24 hours and 48 hours. The seeds were also soaked in the distilled water as negative control. The seeds were incubated at 25°C (± 1°C) in darkness. All the experiments were carried out for at least eleven replicates.

Root elongation test

The root length test was conducted to evaluate the phytotoxicity effect of CuNPs on the rice seeds. The root elongation test was executed according to the guideline from the US Environmental Protection

Agency with slight modifications. The rice seed roots length was measured in mm before and after the exposure to the five CuNPs solutions and distilled water by using a ruler. The data for the initial and final root length was recorded in Microsoft Excel.

$$\text{Change in root length} = \text{Final root length} - \text{Initial root length}$$

$$\text{Root elongation} = \left(\frac{\text{Final root length} - \text{Initial root length}}{\text{Initial root length}}\right) x\ 100$$

The change in root length and percentage of root elongation for each treatment group was calculated by using the formula above in Microsoft Excel. The mean difference between the control and CuNPs treatments were compared.

Statistical analysis

The mean ± standard deviation of the results was analysed using the analysis of variance (one-way ANOVA) test at SPSS software followed by a Tukey post hoc test, with $p < 0.05$ and a confidence level of 95%. The values are presented as the mean standard error of the mean (SEM) or percentage.

Genetic analysis

DNA Isolation and quality

The DNA of *O. sativa* was extracted using Qiagen DNeasy Plant Mini Kit. A total of 50 mg of *O. sativa* roots from each sample was homogenized using liquid nitrogen to fine powder. The protocol of the extraction followed the manual from the kit. The DNA yield's quality was evaluated using 1% agarose gel electrophoresis.

ISSR Primer selection

Six types of primers were used in the amplification of DNA using Inter Simple Sequence Repeat. The six types of primers which are IS4, IS20, IS21, IS22, IS28 and IS32 and their primer sequence (5'-3') with annealing temperature are shown in Table 1.

IS4, IS28 and IS32 have similar annealing temperatures, which is 54.2 °C. IS20 and IS21 share the same annealing temperature of 58.8 °C. The annealing temperature for IS22 is 47.8 °C.

DNA Amplification by using Polymerase Chain Reaction (PCR)

The DNA extracted was amplified using PCR protocol from Mohamad *et al.* (2017). The following contents in Table 2 were added in a 0.2 ml PCR tube.

Table 1: *List of primers with its sequence and annealing temperature.*

ISSR Primers	Primer sequence (5' – 3')	Annealing temperature (°C)
IS4	(CAC)7G (22mer)	54.2
IS20	(CA)8A (17mer)	58.8
IS21	(CA)8G (17mer)	58.8
IS22	(GT)8A (17mer)	47.8
IS28	(AC)8T (17mer)	54.2
IS32	(TG)8G (17mer)	54.2

Table 2: *PCR components for the ISSR-PCR.*

Component	Initial Concentration	Final concentration	Volume per reaction (µl)
Sterile dH2O			14.4
5x buffer	5x	1x	5
$MgCl_2$	25mM	1.5 mM	1.5
dNTP's	10 mM	0.2 mM	1
ISSR primer	10 µM	0.4 µM	1.0
Taq polymerase		0.1 unit/µl	0.1
DNA sample			2
Total			25.0

Table 3: *PCR amplification program for the ISSR-PCR.*

PCR Cycle	Temperature	Time	Cycles
Initial denaturation	94 °C	2 min	31 cycles
Denaturation	94 °C	1 min	
Annealing	47 °C – 59 °C (Optimized temperature)	50 sec	
Extension	72 °C	50 sec	
Final extension	72 °C	5 min	
End	10 °C		

The PCRs were run using the following amplification program as shown in Table 3 but with their optimal annealing temperature as stated in Table 1.

The PCR products were visualised using gel electrophoresis for genotoxicity evaluation. The ISSR banding patterns were interpreted to assess DNA polymorphisms and toxicity caused by CuNPs exposure.

Results and Discussion

The Copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) was dried before synthesizing nanoparticles to ensure that the precursor is in an optimal state for controlled and efficient nanoparticle formation as the copper powder has larger surface area than copper sulfate crystal. This large surface area allows more rapid dissolution in solvents and enhances the speed of reactions. A study by Ali *et al.* (2024) found that dry extract solutions are rich in antioxidants and contain a higher concentration of phytochemicals and nutrients per gram compared to fresh leaf extracts. This increased concentration is attributed to the lower moisture content in dry extracts.

From the observation, the copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) shifts color from light blue to pale blue then white (Figure 1). It showed that the water was removed from the $CuSO_4.5H_2O$. The anhydrous form of copper (II) sulfate was used for synthesizing nanoparticles to help reduce water interference.

Figure 1: *The color changes of copper (II) sulfate from light blue to white when dry in an oven at 80°C for 24 hours (1)*

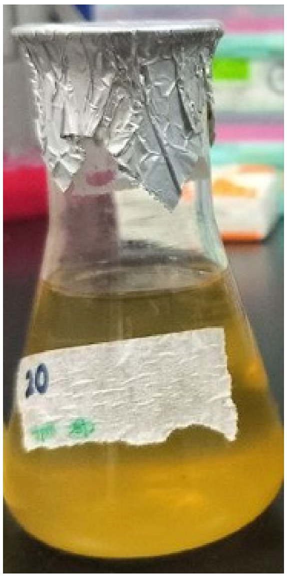

Figure 2: *The fungus extract after autoclaving show light brown in colour*

Mycosynthesis is one of the biological approaches in synthesizing nanoparticles which uses fungal biomass and metabolites. The chemical reaction, reducing metal salt into NPs is faster using fungus than bacteria because of various reducing enzymes and proteins (Sudheer *et al.*, 2022).

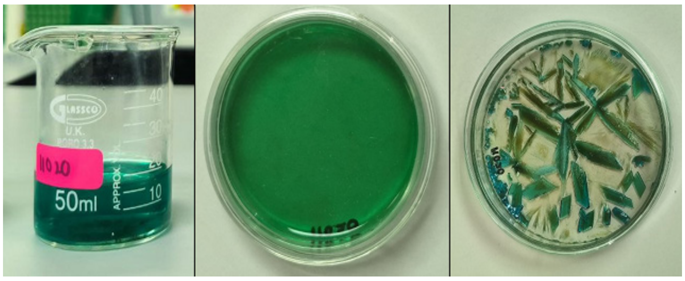

Figure 3: *The colour changes of CuNP before autoclave, after autoclave and forming crystal*

The light brown fungus extract changed to light green gradually after addition of 1g of copper powder (Figure 2 and Figure 3), representing the development of CuNPs. The color change in the reaction solution indicates the biosynthesis of metallic NPs (Abdelhai *et al.*, 2024). After autoclave, the green color became darker. When the CuNPs were left in the dark for three days, the CuNPs formed. All the CuNPs turned into crystals after 7 days.

Suh *et al.* (2017) stated that the autoclave extraction method is more efficient in extracting bioactive compound of *Opuntia ficus-indica* fruit extract than water and ethanol extraction. Autoclave extraction produced high solid extraction yield as elevated temperature enhanced the capacity of water to extract compounds by lowering the surface tension (Suh *et al.*, 2017). The high temperature and pressure in the autoclave caused the degradation of the cell membrane and protein denaturation. Thus, huge amounts of bioactive compounds can be extracted due to the cell permeability. Mohaddes-Kamranshahi *et al.* (2019) study showed that the autoclave extraction method extracted the highest amount of saponin from *Ziziphus spina-christi* leaves, compared to Bain-Marie and microwave heating extraction methods. Furthermore, using water as the solvent resulted in a higher saponin extraction yield than using methanol or ethanol.

Characterisation data

UV-Visible spectroscopy results

The study aimed to synthesize copper nanoparticles using *P. ostreatus* fungus extracts. Visual observation showed the fungus extract changed from light brown to green when mixed with 1g of copper powder, indicating a reaction between the mushroom extract's biochemical compounds and the copper powder. It showed that there was stimulation of the surface plasmon resonance (SPR) where the Cu^{2+} ion has reduced to Cu atom. The UV-Vis spectrophotometer was used to analyse the formation of the CuNPs. As shown in Figure 4, the UV-Vis spectroscopy analysis confirmed the formation of CuNPs, with a sharp peak at 374 nm after 24 hours of incubation. This peak demonstrated significant absorbance differences between the 0-hour and 24-hour samples. However, no peaks were observed for the copper sulfate solution or the mushroom extract alone. UV-Vis spectroscopy is an indispensable technique in characterizing synthesized nanoparticles, especially in confirming their formation and assessing their stability in solution (Ismail *et al.*, 2021).

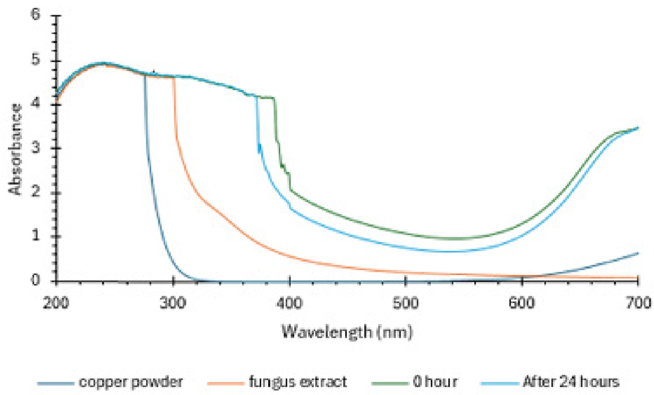

Figure 4: *UV–Vis spectra of synthesized CuNPs.*

The appearance of a Surface Plasmon Resonance band, typically within the 200-700 nm range, serves as a strong indicator of nanoparticle formation (Tito *et al.*, 2021). The position and shape of this band are sensitive to several factors, including the size and shape of the nanoparticles, the dielectric constant of the surrounding medium, and the presence of aggregation (Din & Rehan, 2016). In the context of copper nanoparticles, the UV-Vis spectrum usually exhibits a characteristic absorption peak in the range of 500-600 nm, contingent on the synthesis method and particle size (Calderón-Jiménez *et al.*, 2017). The observed peak at 374 nm in the current study suggests the successful formation of copper nanoparticles, with the specific wavelength indicative of their size and dispersion within the solution. The absence of peaks in the individual components (copper sulfate solution and mushroom extract) further supports the conclusion that the nanoparticles were formed through the interaction of these components, likely due to reduction of copper ions by bioactive molecules present in the mushroom extract.

Table 4: *The CuNPs absorption peak of UV-Vis from different type of capping agents.*

Capping agents	Absorption peak of UV-Vis (nm)	References
Pleurotus ostreatus	374	This study
Jatropha curcas leaves	337	(Ghosh *et al.*, 2020)
Dried ginger	300	(Devi *et al.*, 2020)
Cissus vitiginea leaves	340	(Wu *et al.*, 2020)
Ageratum houstonianum leaves	326	(Chandraker *et al.*, 2020)

Previous studies have reported similar CuNP surface plasmon resonance peaks, ranging from 300 to 400 nm, using extracts from *Jatropha curcas* leaves (Ghosh *et al.*, 2020), dried ginger (Devi *et al.*, 2020), *Cissus vitiginea* leaves (Wu *et al.*, 2020), and *Ageratum houstonianum* leaves (Chandraker *et al.*, 2020) (Table 4). The absorption peaks observed in these studies were similar to the ones seen in our work, which fell within the typical 300 to 400 nm range for CuNPs. These results align with existing research, highlighting the role of phytochemicals in the reduction of copper ions to form stable nanoparticles (Kamçı *et al.*, 2022; Pradhan *et al.*, 2020). The UV-Vis analysis therefore, validated the formation of CuNPs, indicating that the mushroom extract effectively reduced copper ions and stabilized the resulting nanoparticles (Gültekin *et al.*, 2017).

Researchers are increasingly interested in creating nanoparticles from natural resources (Jothiramalingam *et al.*, 2022). Green synthesis is becoming more well-known because it is a simple, non-toxic, and ecologically beneficial alternative to traditional physical and chemical approaches. In fact, studies have shown the effectiveness of copper nanoparticles in combating fungal infections, with nanoforms of copper demonstrating substantial antifungal capabilities (Oussou-Azo *et al.*, 2020). Copper nanoparticles stand out among metallic nanoparticles because of their distinct physical and chemical characteristics, including high thermal and electrical conductivity and biological activity (Jayarambabu *et al.*, 2021).

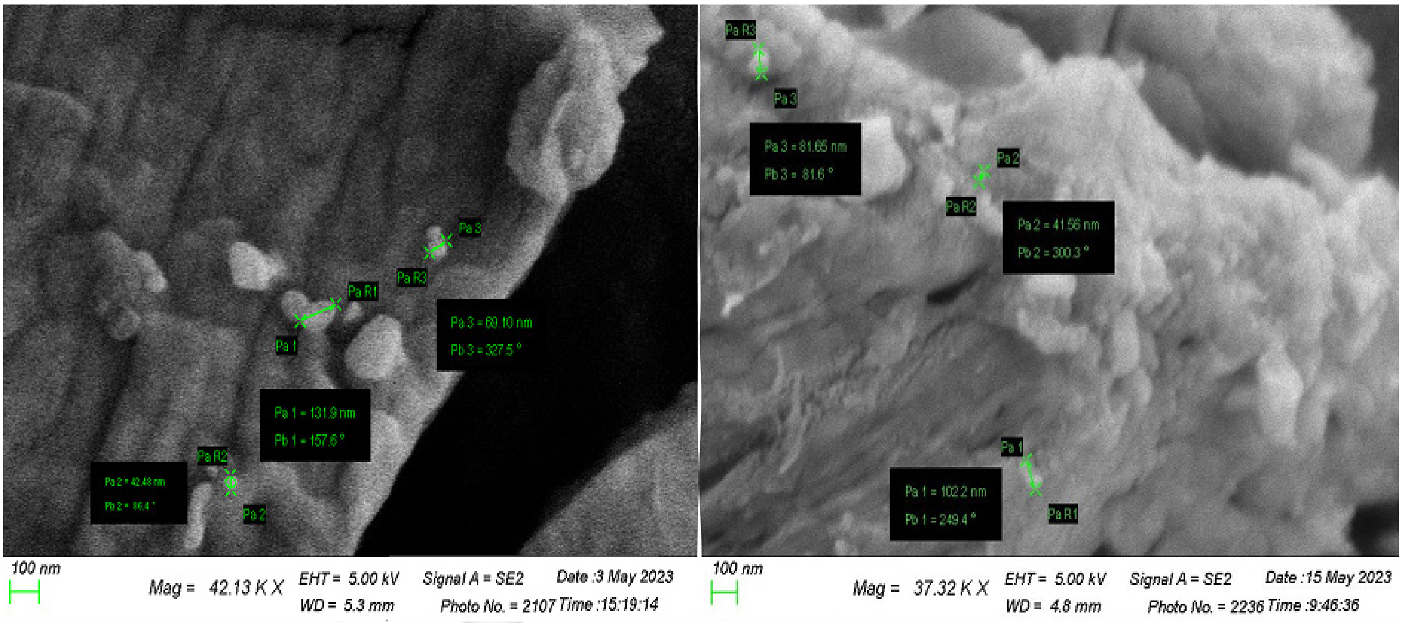

Figure 5: *Field emission scanning electron microscopy (FESEM) characterization of the synthesized CuNPs*

Field emission scanning electron microscopy (FE-SEM)
Field emission scanning electron microscopy imaging revealed that the synthesized copper nanoparticles exhibited a wide range of sizes, shapes, and surface morphologies. The smallest CuNPs measured 41.56 nm, while the largest were 131.9 nm in size. The FESEM images in Figure 5 illustrate the synthesized CuNPs exhibited irregular shapes. According to a previous study by Labaran *et al*. (2024) irregularly shaped nanoparticles, such as triangular, cylindrical, polygonal, and nearly spherical, often have a complex and heterogeneous structure. The formation of these varied nanoparticle shapes is influenced by synthesis methods and reaction conditions. Differences in nanoparticle shape can result in varied catalytic activities, optical properties, and biological interactions.

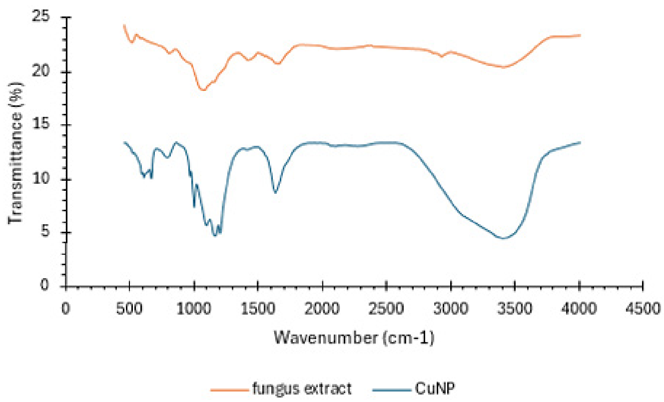

Figure 6: *The Fourier-transform infrared spectroscopy (FTIR) spectra of the Pleurotus ostreatus fungus extract and synthesized CuNPs.*

Fourier transform infrared spectroscopy (FTIR)
The Fourier Transform Infrared Spectroscopy (FTIR) analysis was used to identify the functional groups of biomolecules present in the *P. ostreatus* fungus extract, which likely acted as capping and stabilizing agents for the copper nanoparticles. The FTIR analysis was employed to measure the transmittance of infrared radiation over the wavenumber range of 450 to 4000 cm^{-1}, which corresponds to various vibrational modes within the molecular structures. The FTIR spectrum of the autoclave-assisted fungus extract was used as a control for comparison. The FTIR spectrum in Figure 6 revealed four obvious peaks. The broad CuNP's absorption peak at 3400cm^{-1} is due to O-H stretching vibrations (Alshammari *et al*., 2023). It showed the presence of surface hydroxyl groups (O-H stretching) adsorbed onto the surface of the CuNPs during synthesis (Mkhize *et al*., 2022). In addition, the absorption band of fungus extract at 1656 cm^{-1} shifted to 1628 cm^{-1} within CuNPs. The sharp absorption band in this region indicates C=O stretching, which suggests the presence of carbonyl groups from esters, aldehydes, or carboxylic acids (Manimaran *et al*., 2023) in the extract are used as stabilizers or capping agents used to prevent the aggregation of CuNPs (Mkhize *et al*., 2022). The presence of C–O band of the primary alcohols in the fungus extract were observed at peak 1076 cm^{-1} (Labaran *et al*., 2024). This absorption band was slightly shifted to 1160 cm^{-1} in CuNPs which showed the stretching and bending NH of amino groups (Alshammari *et al*., 2023). The band present at 800cm-1 in the fungus extract indicates that CuNP

was stabilised using bioactive compound of the extract that serve as capping agent (Hajizadeh *et al*., 2022). Secondary metabolites from the fungus extract such as flavonoids or phenolic compounds might be present during the synthesis to reduce and cap nanoparticle from aggregation. These biomolecules appear due to the bending or stretching vibrations of C-O, C-H, or C-N bonds. The band then shifted to 996 cm^{-1} in CuNP due to the vibrations of Cu-O stretching in copper oxide species. This indicates that the surface of the nanoparticles has been oxidized, forming a stable layer of oxide, which is expected as CuNPs were synthesized using biological extracts (Hajizadeh *et al*., 2022). Copper nanoparticles tend to form a thin layer of copper oxide (CuO or Cu_2O) on their surface when exposed to air or moisture.

Toxicity analysis of CuNPs

The effect of copper nanoparticles from oyster mushroom extract were investigated using *O. sativa* as the model organism in this study. A root elongation test on *O. sativa* seeds was successfully conducted to examine the impact of potential toxicity associated with the CuNPs. Two distinct data sets, obtained through macroscopic and molecular techniques involving PCR-based Inter Simple Sequence Repeats analysis, were analyzed and compared.

Table 5: *The change in root length and percentage of root elongation of O. sativa for 24 hours and 48 hours of exposure to different concentrations of CuNPs. Values are expressed as mean ± standard error of the mean (SEM) (n = 11/17); * (p < 0.05) show significant difference when compared to the control group; one-way ANOVA followed by Tukey post hoc test, considering (p < 0.05) with a confidence level of 95%.*

Treatment	Change in root length ± SE (mm)		Root elongation (%)	
	24 hours	48 hours	24 hours	48 hours
Control	3.15 ± 0.45[a]	3.38 ± 0.49[a]	6.47	10.77
11020	4.00 ± 1.02[a]	4.14 ± 0.44[a]	14.75	19.60
11030	4.50 ± 0.55[a]	5.88 ± 0.78[a]	11.57	15.90
11050	5.09 ± 0.90[a]	6.91 ± 1.58[a]	11.28	27.64
21010	4.47 ± 0.54[a]	4.50 ± 0.88[a]	11.38	12.86
21030	4.75 ± 0.60[a]	6.54 ± 1.22[a]	15.52	21.11

The roots of the *O. sativa* plant were subjected to distilled water and solutions containing copper nanoparticles at five different concentrations, with treatments lasting for 24 and 48 hours, respectively. This duration was chosen as the average time needed for eukaryotic cells to complete their full cell cycle is 24 hours. Extending the treatment period to 48 hours allowed for observation of the effects of the prolonged exposure on the cell cycle.

Root elongation test: macroscopic analysis

The effect of CuNPs on the roots of *O. sativa* was assessed by measuring the mean changes in root length 24 and 48 hours after exposing the plants to the test medium, allowing for an analysis of root growth.

The root elongation test results in Table 5 showed a positive trend for changes in root length. There was an increase in root length after 24 and 48 hours of incubation for all the CuNPs and control sample. The percentage of root elongation increased with longer incubation time. The *O. sativa* roots exposed to the liquid CuNPs at five different concentrations (11020, 11030, 11050, and 21010, and 21030), exhibited a substantial increase in mean root length after both 24 and 48 hours. The 11050 CuNPs recorded the lowest percentage root elongation for 24-hour incubation with 11.28% but the highest percentage of root elongation for 48-hour incubation, 27.64%.

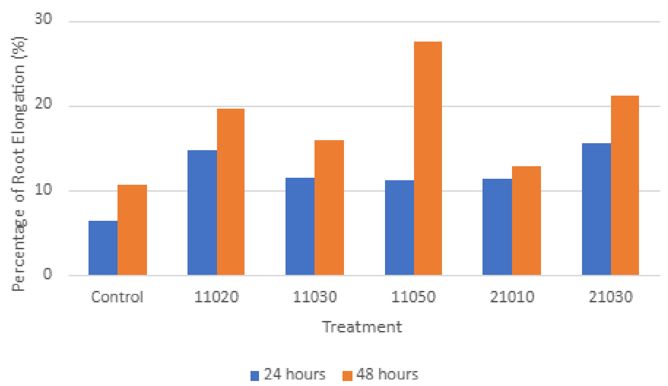

Figure 7: *The percentages of root elongation at various concentrations for 24-hour and 48-hour incubation time (1)*

The changes in root length increased with the increment of CuNPs concentration. One-way ANOVA analysis showed that there were no statistically significant mean differences ($p < 0.05$) correlations between the results of CuNPs treatment and control for both 24-hours and 48-hours incubation time.

As shown in Figure 7, all the sample concentrations showed a higher percentage of root elongation compared to the control for the 24-hour and 48-hour incubation period. This indicates that the CuNPs synthesized from *P. ostreatus* are not toxic to the rice and can enhance root growth. This statement was supported by the study from Chen *et al*. (2022) mentioned that the growth and development of plants were affected by the copper as it is one of the vital micronutrients for plants growth. In addition, the root elongation test demonstrates that CuNPs contribute to the development of root growth. After being treated with CuNPs, the roots of the *O. sativa* plant were seen to have grown in length. It was shown that the increase was greatest at a concentration of 21030 for both 24 hours and 48 hours. The fact that the length of the roots has increased is a strong indicator that the copper nanoparticles have assisted in the growth of the roots. According to the study by Tortella *et al*. (2024), *L. esculentum* root length, shoot length, biomass, and seedling vigor index were increased after treated with 20 mg L^{-1} copper-based NPs. Copper-based NPs also improve photosynthesis in *L. esculentum* shown by increasing in leaf pigments, chlorophyll and carotenoid. Similar increases in root length have been observed in *Cajanus cajan* and *Medicago sativa* when treated with copper-based NPs. This study has demonstrated copper's ability to encourage longer root growth in *Oryza sativa*.

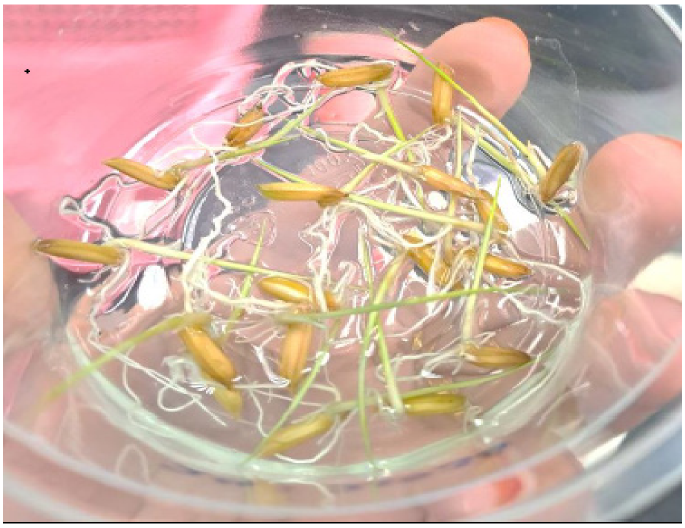

Figure 8: *Oryza sativa root after 24 hours of treatment*

Apart from that, from the observation, the roots from 24-hour incubation time samples experienced minimal physical changes, where the root's colour changed from white to slightly greenish blue after 24 hours of exposure to five different CuNPs solution (Figure 8). However, this change in root colour is not terminal, as the roots still appear turgid and healthy. The colour of the roots becomes darker after 48-hour incubation time (Figure 9). It showed that the rice plant roots had absorbed the colour of the test solutions. The rice plant roots exhibited a brittle texture after being incubated for 48 hours and could be easily broken. As the roots developed in direct contact with the test solutions, any alterations in their appearance can be attributed to those solutions. The colour of the roots remained the same for the control both 24 hours and 48 hours of exposure.

Figure 9: *Oryza sativa root after 48 hours of treatment*

Genetic analysis

All *Oryza sativa* roots that have been exposed to distilled water and test solution of five concentrations containing copper nanoparticles for 24 hours and 48 hours have gone through the DNA extraction process. In this study, the ISSR-PCR technique has been used to investigate genotoxic effect of CuNPs towards rice seeds. Previous study also using ISSR-PCR technique to study the genotoxicity in plants caused by abiotic stress (drought) (Coşkun, 2023), wastewater treatment (El-Kholy *et al*., 2023) and ionizing radiation (IR) effect (Sorrentino *et al*., 2023). The banding pattern profile of ISSR fingerprinting in rice seeds was observed.

Table 6 summarized the PCR results using ISSR markers. At the 24-hour incubation period, multiple bands appeared, while for the 48-hour treatments, only one band was observed at a concentration of 11050. All six primers were successfully amplified for sample 21010, with DNA bands results comparable to the control sample. However, for the 21030, only primer IS22 did not reveal any bands, while the rest appeared. The results for 11020, 11030, and 11050 were the same, except that at 11030, IS21 amplified the DNA. The amplified band sizes for all the primers were between 400 and 600 base pairs.

Table 6: *The summary of PCR results using ISSR primers. (√) show the presence of DNA band while (-) indicates no presence of band.*

		ISSR primers					
		IS4	IS20	IS21	IS22	IS28	IS32
24-hour	11020	√	-	-	-	√	√
	11030	√	-	√	-	√	√
	11050	√	-	-	-	√	√
	21010	√	√	√	√	√	√
	21030	√	√	√	-	√	√
	C48	√	√	√	√	√	√
48-hour	11020	-	-	-	-	-	-
	11030	-	-	-	-	-	-
	11050	-	-	-	-	-	√
	21010	-	-	-	-	-	-
	21030	-	-	-	-	-	-

As predicted, there is no DNA bands visualised for 48-hour sample. A study by Abu and Mba (2011) state that both concentration of the treatment and incubation time had significant effect towards root growth. However, in this study, the concentration of the treatments had no adverse effect towards the root growth. The effect of these treatments is time-dependent as no DNA bands appeared after 48-hour incubation time.

The results showed the bands were stable in the control group, but they were absent in some other treatments. This suggests that DNA alterations may have occurred due to changes in the oligonucleotide priming sites. These changes can impact the activity and interaction of DNA polymerase with altered DNA (El-Kholy *et al*., 2023). Both findings, root elongation test and ISSR-PCR, suggest that the CuNPs synthesized from *P. ostreatus* have no significant phytotoxic and genotoxic effects, as the DNA alteration does not affect the growth of seeds.

Conclusions and Recommendations

This study successfully established a novel and sustainable method for synthesizing copper nanoparticles using *Pleurotus ostreatus* extract. This eco-friendly and cost-effective mycosynthesis process, enhanced by an autoclave-assisted method, yielded highly stable and uniform CuNPs. This approach provides a significant advancement in sustainable nanomaterial synthesis, offering a viable alternative to traditional methods that often rely on hazardous chemicals.

Thorough characterization confirmed the nanoparticles' desirable properties, including their size, structure, and potent antibacterial efficacy. The phytotoxicity and genotoxicity tests suggested that the nanoparticles produced are safe to use. These findings highlight the potential of these biologically synthesized CuNPs for various industrial and biomedical applications. CuNPs synthesized from *P. ostreatus* extract may be suitable for crop growth and act as fertilizer; however, their application should not exceed 24 hours, as prolonged use might damage the crops.

Given the promising results, future research should focus on optimizing the reaction parameters to further enhance the efficiency and scalability of this mycosynthesis process. Additionally, further investigations should explore the full scope of applications for these CuNPs, including their potential as nano-fertiliser, antibacterial applications, environmental remediation, and beyond.

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Novelty Statement

This manuscript reports a novel, eco-friendly, and cost-effective method for synthesizing stable and uniform copper nanoparticles using an autoclave-assisted mycosynthesis process with *Pleurotus ostreatus* extract. This sustainable approach eliminates the use of harmful chemicals, advancing the field of green nanotechnology for various industrial and agricultural applications.

Authors' Contribution

Roslina Ainna Roslan: The conceptualization and

experimental design of this study were developed, carried out the experiments and analyzed the resulting data and manuscript was written and edited
Norfatimah Mohamed Yunus: The conceptualization and experimental design of this study were developed, and manuscript was written and edited
Nurul Aili Zakaria, Sharifah Aminah: The conceptualization and experimental design of this study were developed, Carried out the experiments and analyzed the resulting data.
Lyena Watty Zuraine Ahmad: Assisted in the data analysis.

Generative AI or AI assisted technology statement
Generative AI tools such as Grammarly and QuillBot were used only for minor language refinement and grammar correction during manuscript preparation. The authors reviewed and verified all AI-generated text to ensure accuracy and originality.

Conflict of interest
The authors declare no conflict of interest.

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