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# Copper Nanoparticles and Culture Media Elicit Biomass, Secondary Metabolites, and Antioxidant Activity in the Cell Suspension Culture of *Momordica charantia* L.

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**Abstract**

Medicinal plants are characterized by their high content of secondary metabolites. They are known to possess pharmaceutical properties. Based on this, the present study describes the ability of laboratory-synthesized copper nanoparticles (Cu NPs) to influence the biomass properties, secondary metabolism, and biological activity of cell suspension culture (CSC) induced from the hypocotyl extirpated from sterilized seedlings of *Momordica charantia* L. CSC were grown in various cultures, including Linsmaier and Skoog (LS), Murashige and Skoog (MS), and Woody Plant Medium (WPM), supplemented with different concentrations of Cu NPs 0, 40, 80, 120, 160, and 200 µg $ml^{-1}$ respectively. The highest biomass accumulation for calli cultures was induced from the hypocotyl. CSC obtained from two-month-old calli cultures were subjected to Cu NPs treatments in different cultures for 21 days, which increased the biomass of the CSC. The increasing concentrations of Cu NPs caused a decreasing trend in biomass and cell vitality and an increase in the accumulation of Cu, hydrogen peroxide ($H_2O_2$), and malondialdehyde (MDA). The lowest values appeared for CSC grown in a WPM at a concentration of 200 µg $ml^{-1}$ Cu NPs. CSC grown in a WPM treated by 200 µg $ml^{-1}$ Cu NPs provided the highest total flavonoids production (TFP) and the most increased activity of phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and diphenyl-picrylhydrazyl (DPPH). On the other hand, exogenous 200 µg $ml^{-1}$ Cu NPs in MS medium improved total phenolic production (TPP). In conclusion, these results indicate the promising role of plant tissue culture technology in achieving sustainable biomass production and thus improving the production of secondary metabolites through the hypocotyl growth of *M. charantia* L. in specific cultures using Cu NPs as an effective elicitor.

**Keywords:** *Momordica charantia* L.; Cell Suspension Culture; Culture Media Elicitation; Phenolic compounds; Flavonoids compound.

## 1. Introduction

*Momordica charantia* L. is a medicinal plant that belongs to the cucurbit family. It has therapeutic properties because it contains many secondary metabolites, including phenols, alkaloids, saponins, triterpene glycosides, vicaine, momordin, charantin, triterpenes, momorcharin, and oleanolic acids (Grover and Yadav, 2004). In addition, it contains brevifolincarboxylic acid, margarolic acid, ascorbic acid, goyaglycoside G, 3-malonylmomordicin I, quercetin 3-O-glycoside, kuguacin H, and cucurbitacin E (Perumal *et al.,* 2021). Several previous reports on the biological activities of phenolic compounds and available secondary compounds have been previously reported as antioxidant, antimicrobial (Deshaware *et al.,* 2017), antidiabetic, anti-inflammatory, anticancer (Svobodova *et al.,* 2017), anti-ulcer, antibacterial, antiviral, antitumor, antifertility, antilipid, antimutagenic, immunomodulatory, anthelmintic, and hepatoprotective activities (Jia *et al.,* 2017).

Cell suspension culture (CSC) technology has provided alternative possibilities for enhancing in vitro production of secondary metabolites from natural plant sources (Sarkate *et al*., 2017; Setiowati *et al*., 2022). It contributes to the sustainable production of these compounds in a short period through the positive effects of different combinations of chemicals added to the culture medium. Moreover, CSC avoids the problems that the plant may be exposed to in the field like diseases, weeds, insects, and changes in climatic conditions. It also provides essential compounds in the pharmaceutical industry without being restricted by the growing season. CSC technology has succeeded in supplying the pharmaceutical industry with several secondary compounds, including vincristine, vinblastine, camptothecin, and Taxol (Wilson and Roberts, 2012). Added to this is the ability of CSC to produce compounds that are difficult to synthesize chemically (Kolewe *et al.,* 2008; Siahsar *et al.,* 2011; Chung *et al*., 2018).

Nanomaterials are materials as small as 1–100 nm in size with unique physicochemical properties (Veisi *et al.,* 2018). These characteristics can qualify them to activate

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many important biological processes of the plant cell, including eliciting the biological pathways responsible for synthesizing secondary metabolites (Sharafi *et al.*, 2013; Ghorbanpour and Hadian, 2015; Hatami *et al.*, 2016; Chung *et al.*, 2018; Bsoul *et al*., 2023). Moreover, nanomaterials are believed to have a role in protecting the plant cell from the negative effect of oxidative stress (Thiruvengadam *et al.*, 2015).

Copper is a trace element important of plant growth. It has many positive effects in improving the physiological characteristics of plant cell growth. In addition, it plays a role in plant biochemistry by increasing the activity of several enzymes. It is a catalyst for the synthesis of many proteins. Moreover, it is a structural component of regulatory proteins and is involved in many important physiological processes, including the electron transport chain, hormone signalling, and cell wall metabolism (Parida and Das, 2005). Previous literature has concluded that copper oxide nanoparticles (CuO NPs) are vital in promoting plant tissue growth and reducing oxidative stress (Anwaar *et al.*, 2016).

This study investigated the potential effects of laboratory-synthesized Cu NPs on biomass accumulation and production of phenolic compounds and their impact on improving the biological activities of CSC grown in different cultures of *M. charantia* L.

## 2. Materials and Methods

### *2.1. Chemicals*

Plant tissue culture media were purchased from Caisson labs company, USA. Sucrose and agar were purchased from Merck KGaA company. All other chemicals were purchased from Sigma Aldridge company Ltd.

### *2.2. Plant material*

This experiment was carried out in plant tissue culture laboratory, Center of Desert Studies, University of Anbar (33° 24′ 11″ N, 43° 15′ 43″ E). *M. charantia* L. seeds were surface-sterilized with 70% ethanol for 30 sec. Then, they were sterilized using sodium hypochlorite (NaOCl) 1.0% for 5 min. After that, they were rinsed five times with sterile distilled water. Sterilized seeds were grown in flasks under aseptic conditions in B5 media (Gamborg *et al.*, 1968). Moreover, the components and media, including 30 g $l^{-1}$ sucrose and 7.0 g $l^{-1}$ agar, were added to the B5 media. The pH of the medium was adjusted to 5.6. The cultures were incubated in a growth chamber at 25 ±1°C under an illumination intensity of 1000 lux for 8/16 h light/dark, respectively.

### *2.3. Calli cultures induction*

Media cultures were used to induce calli cultures such as LS (Linsmair and Skoog, 1965), MS (Murashige and Skoog, 1962), and WPM (Lloyd and McCown, 1980). They were supplemented with specific combinations of growth regulators naphthaleneacetic acid (NAA) at 0.1, 0.25, and 0.5 mg $l^{-1}$ and benzyladenine (BA) at 0.25, 0.5, and 1.0 mg $l^{-1}$. The media components included 30 g $l^{-1}$ sucrose and 7.0 g $l^{-1}$ agar. pH was adjusted to 5.6. The hypocotyl of 1 cm sterile seedlings was excised to be cultured in the media. The best combination for obtaining biomass was determined based on the fresh weight (FW) of calli cultures after growing for 28 days. Callus tissue was propagated based on the combination producing the highest biomass of explant.

### *2.4. Preparation of Cu NPs*

The colloidal material of Cu NPs was prepared according to the method described (Fernandez-Ariasa *et al.*, 2020). It was based on the liquid phase laser ablation technique covering the metal material at room temperature. Copper foil with a purity of 99.99% was used. It was immersed in methyl alcohol up to 2 mm. The laser source was a picosecond infrared (IR) at 1064 nm with an energy of 0.03 mJ and a pulse duration of 800 ps. The flask was covered with foil to protect it from light.

### *2.5. Characterization of Cu NPs*

The prepared Cu NPs were characterized using a UV-Vis spectrophotometer. The topographical properties were determined using a transmission electron microscope (Figure 1). The Cu content in the colloidal solution was estimated by atomic absorption.

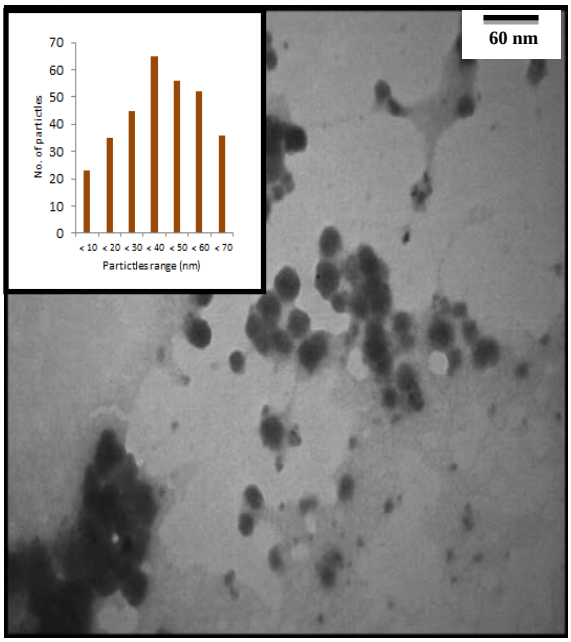


**Figure 1.** TEM image of Cu NPs with particle size distribution.

### *2.6. Establishment and treatment of CSC*

Calli cultures grown in media containing the optimum combination were used to obtain biomass. The suspensions were obtained from two-month-old calli cultures. CSC were cultured from 5.0 g of FW calli cultures after filtering by a sieve with a 300 µm hole in a 250 ml Erlenmeyer flask containing 50 ml medium consisting of LS, MS, and WPM cultures with 30 g $l^{-1}$ sucrose supplemented with 0, 40, 80, 120, 160, and 200 µg $ml^{-1}$ Cu NPs. The cultures were grown in a shaking incubator at 120 rpm at 25°C and 24 h illumination. CSC were harvested at 28 days of age for the quantification of the following parameters.

#### *2.6.1. Biomass accumulation, cell viability, and Cu content of CSC*

CSC growing in the cultures of the different treatments were harvested to determine biomass accumulation through FW determination. Moreover, the validity was determined based on previously described reports (Sahraroo *et al.*, 2016).

CSC samples were dried in a hot air oven at 60°C for weight stability. 250 mg of the dried sample was pre-dissolved in HCl and $HNO_3$ (1:3 v/v). The mixture was heated at 100°C for 3 h on a hot plate stirrer. The filtrate was adjusted to 25 ml with deionized distilled water. The copper ion content of the samples was estimated by ICP-OES (Arteaga *et al*., 2018).

*2.6.2. Hydrogen peroxide ($H_2O_2$) and malondialdehyde (MDA) content*

The $H_2O_2$ content was estimated using the spectrophotometric method described by Alexieva *et al.* (2001). Briefly, 500 mg of fresh samples of CSC was homogenized in 5.0 ml of 0.1% (w/v) trichloroacetic acid. Then, the centrifugation was carried out at 15000 rpm for 15 min. Then, 0.5 ml of the supernatant was supplemented into 0.5 ml potassium phosphate buffer (10 mM, pH 7.0) with 1.0 ml potassium iodide (1.0 M). The absorbance was recorded at 390 nm.

MDA content was determined in CSC by the method described by Heath and Packer (1968). Briefly, a mixture of 300 mg of a homogenized fresh sample of suspended cells was mixed in 1.5 mL of 1% (w/v) trichloroacetic acid. Then, centrifugation was performed at 10000 rpm at 4°C for 5 min. 1.0 ml of the supernatant was supplemented with 4 ml of 0.5% thiobarbituric in 20% trichloroacetic acid (TCA). The reaction mixture was heated at 95°C for 30 min. The absorbance of the supernatant was recorded at 532 and 600 nm by spectrophotometer (UV-Vis). The MDA value was determined from the absorbance difference using the 155 $mM^{-1}$ $cm^{-1}$ extinction coefficient.

*2.6.3. Antioxidant enzymatic activity*

Antioxidant enzymes were extracted according to the method described by Gapinska *et al.* (2008). Briefly, 500 mg of homogeneous fresh CSC was weighed. 5 ml of potassium phosphate buffer (pH = 7.0, 50 mM) was added, containing 1.0 mM of ethylenediamine tetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). The sample was centrifuged at 10000 rpm for 15 min at 4°C. The activity of polyphenol oxidase (PPO) and lipoxygenase (LPO) enzymes was estimated using the supernatant.

PPO activity was quantified by the protocol of Soliva *et al.* (2000). Briefly, 100 µL of the enzyme extract was mixed with 1900 µl of 0.1 M potassium phosphate (pH, M 0.1=7.0) containing 0.1 M catechol as a substrate. The absorbance was recorded at 410 nm. Phenylalanine ammonia-lyase (PAL) activity was estimated based on cinnamic acid production by the protocol of D'Cunha *et al.* (1996). Briefly, 100 µL of enzyme extract was added to 1000 µl of buffer extract, 500 µl of L-phenylalanine (10 mM), and 400 µl of deionized water. The mixture was incubated at 37°C for 1 h. Then, 500 µl of hydrochloric acid (6.0 M) was added. The absorbance was recorded at 290 nm.

*2.6.4. Secondary metabolites*

Phenolic compounds and flavonoids were extracted from homogenized samples of CSC according to the method described by Velioglu *et al.* (1998).

100 mg of powdered samples was dried in an oven at 50°C for 48 h in 20 ml aqueous methanol (80%) and added to room temperature for 20 min. Samples were centrifuged at 15000 rpm for 20 min. A vacuum evaporator dried the supernatant. 1 ml of methanol was re-added. The supernatant was carefully isolated and stored for quantification.

Total phenol production (TPP) was estimated using Folin-Ciocalteu (FC) and gallic acid. It is expressed by mg gallic acid/g dry weight (Ainsworth and Gillespie, 2007). Total flavonoid production (TFP) was estimated as rutin equivalents described in mg rutin $g^{-1}$ DW (Miliauskas *et al.*, 2004).

*2.6.5. Antioxidant activity*

Antioxidant activity was evaluated by diphenyl-picrylhydrazyl (DPPH) and 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Free radical scavenging activity was estimated based on the DPPH assay. Briefly, 100 mg of homogenized CSC had 0.02 ml of ethanol. Then, it was centrifuged at 10000 rpm for 20 min. The supernatant was isolated and added to 2.0 ml of DPPH (0.1 mM) in 95% ethanol. The reaction mixture was mixed and incubated at 25°C for 60 min. The absorbance was measured at 517 nm (Zhu *et al.*, 2006).

The MTT assay was determined according to the modified method described by Neamah (2016). Briefly, 2.0 ml of dimethyl sulfoxide (DMSO) was added to the supernatant of a 500 mg sample extracted by centrifugation at 10000 rpm for 20 min using 5.0 ml of methanol aqueous (80%). The sample was mixed by the vortex. It was dried by a vacuum evaporator. 10 µl of the reaction was added to the 190 µl MTT solution. Then, it was incubated at 37°C for 24 h. DMSO solution was added. Then, 200 µl was isolated and placed in a 96-well CSC. The absorbance was recorded using a universal microplate reader at a wavelength of 570 nm.

*2.7. Experimental design and statistical analysis*

A factorial experiment was conducted using a completely randomized design. It included 18 treatments with three replicates in each treatment. Standard deviation values were calculated for all mean values. Data were statistically subjected to two-way ANOVA using the GenStat package version 12 significant values were determined using L.S.D at $p \leq 0.01$.

## 3. Results

*3.1. Biomass accumulation of calli cultures*

The best biomass accumulation was determined for *M. charantia* L. calli cultures grown in diverse cultures, including LS, MS, and WPM containing different NAA and BA treatments. The aim was to obtain the most appropriate chemical components of the medium through which calli cultures can be grown for CSC. Media cultures did not show a significant difference in biomass accumulation. In contrast, it increased significantly ($p \leq 0.01$) by treatment with NAA and BA at concentrations 0.25+1.0 mg $l^{-1}$, respectively. It recorded the highest significant biomass accumulation for media cultures, which amounted to 2.04, 2.24, and 2.14 g, respectively (Figure 2).

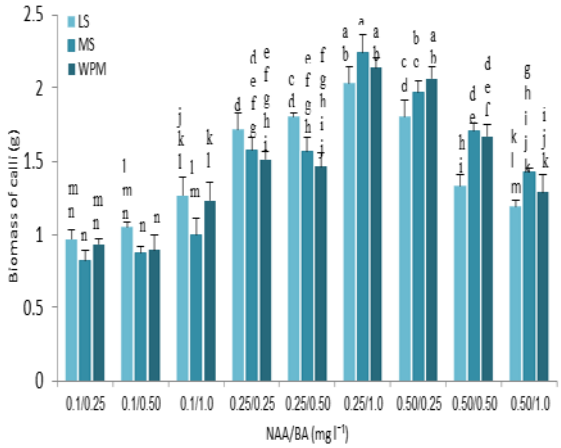


**Figure 2.** Assessment of biomass of calli cultures in *M. charantia* L. induced by different concentrations of NAA and BA. Vertical bars represent the mean ± standard deviation of three replicates. Different letter treatments indicate significant differences at $p \leq 0.01$ based on LSD test.

### *3.2. Biomass accumulation, cell viability, and Cu content of CSC*

Some morphological parameters of CSC derived from calli cultures were evaluated (Figure 3). Treatment with Cu NPs significantly increased the biomass accumulation of CSC ($p \leq 0.01$). The highest biomass accumulation was 5.27 g for MS medium treated with 120 µg ml$^{-1}$ of Cu NPs. In contrast, treatment with high concentrations caused a decrease in biomass to its lowest level at a concentration of 200 µg ml$^{-1}$ for culture media (Figure 4A).

Cell vitality also increased significantly as a result of treatment with Cu NPs, as treatment with a concentration of 80 µg ml$^{-1}$ increased the vitality of cells growing in LS medium by 93.92%, while treatment with a concentration of 200 µg ml$^{-1}$ led to recording the lowest vitality of cells growing in WPM amounting to 66.11% (Figure 4B).

The accumulation of Cu increased significantly with high concentrations of Cu NPs, and the increase in treatment caused an increase in the accumulation of Cu. The highest accumulation occurred for CSC of WPM, which reached 0.129 mg g$^{-1}$ DW in 200 µg ml$^{-1}$, while the accumulation was lower for lower concentrations of Cu NPs (Figure 4C)

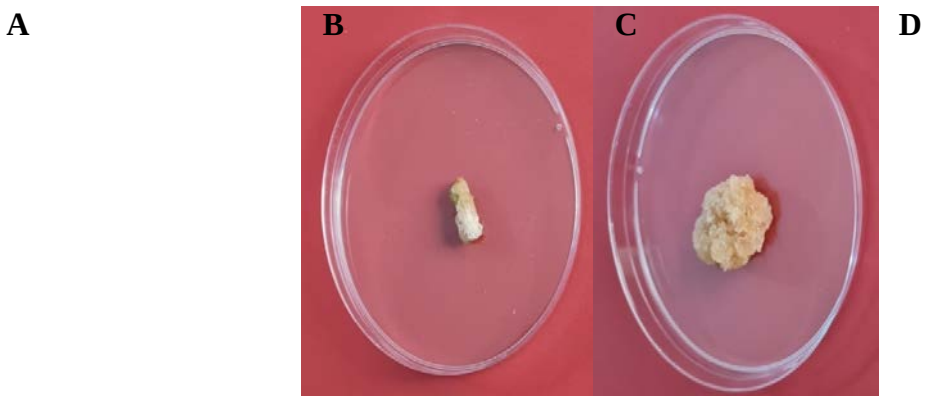


**Figure 3.** The stages of study on the hypocotyl derived from *M. charantia* L. under in vitro conditions on WPM with 0.25 mg l$^{-1}$ NAA+1.0 mg l$^{-1}$ BA. The hypocotyl extirpated from sterilized seedlings (A). Callus induction start (B). Callus formation complete (C). CSC (D).

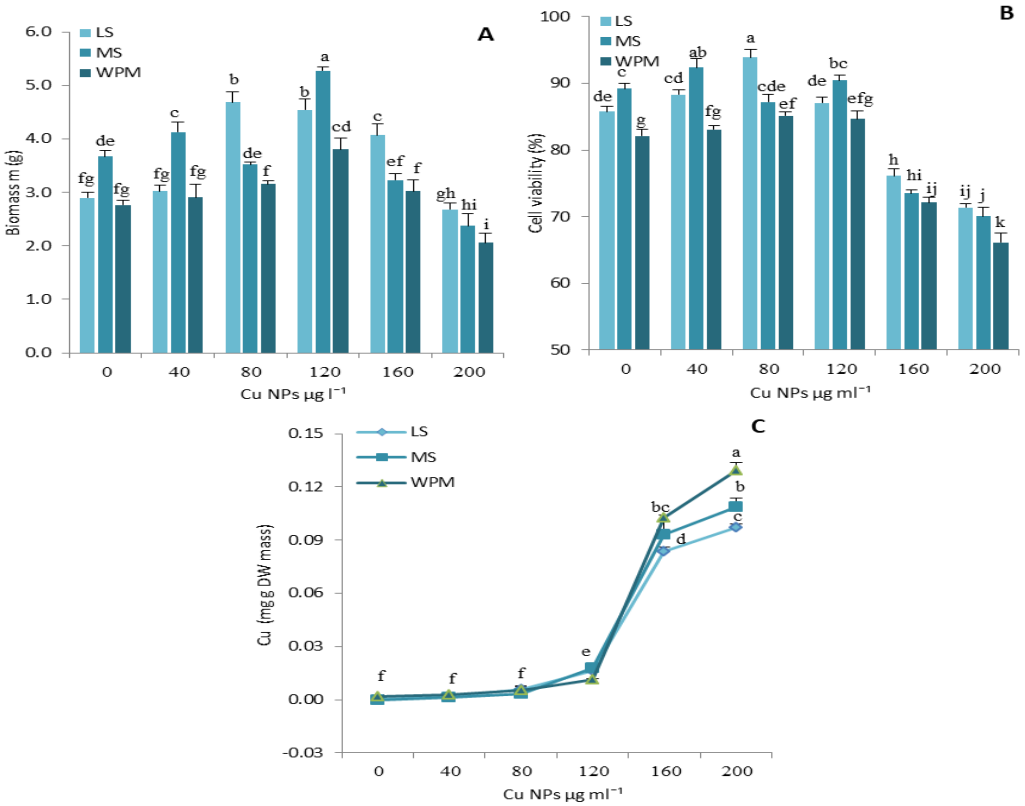


**Figure 4.** Assessment of mor-physiological parameters such as biomass accumulation (A), cell viability (B), and Cu content (C) of CSC in *M. charantia* L. elicited by different culture media and Cu NPs concentrations. Vertical bars represent the mean ± standard deviation of three replicates. Different letter treatments indicate significant differences at $p \leq 0.01$ based on L.S.D test.

### *3.3. H2O2 and MDA content of CSC*

$H_2O_2$ and MDA are fundamental indicators to determine the effect of treatments on stimulating the vital pathways responsible for the synthesis of secondary metabolites in the plant cell (Guo *et al*., 2018; Sarmadi *et al*., 2018).

The treatments significantly ($p \leq 0.01$) affected the $H_2O_2$ content of CSC. The treatment with 200 µg ml$^{-1}$ of Cu NPs recorded the highest content of $H_2O_2$ for CSC growing in WPM, equal to 361.67 µmol g$^{-1}$ FW. The increase was 3.2-fold compared to the control (Figure 5A).

The results showed a significant change in MDA content. The highest level of suspensions treated with a high concentration of Cu NPs appeared when added to the WPM by 6.4 µmol g$^{-1}$ FW. The control treatment of the MS medium recorded the lowest, equal to 0.67 µmol g$^{-1}$ FW (Figure 5B).

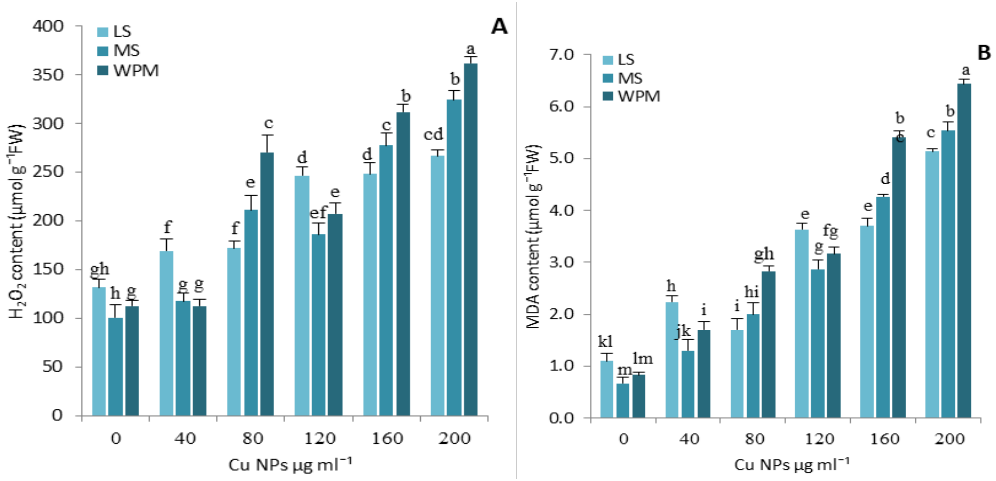


**Figure 5.** Assessment of oxidative stress parameters such as $H_2O_2$ (A) and MDA (B) of CSC in *M. charantia* L. elicited by different culture media and Cu NPs concentrations. Vertical bars represent the mean ± standard deviation of three replicates. Different letter treatments indicate significant differences at $p \leq 0.01$ based on L.S.D test.

### 3.4. Antioxidant enzymatic activity of CSC

PAL and PPO are essential enzymes in protecting cellular components from the effects of oxidative stress. Thus, they have a major role in sustaining the production of secondary metabolites in the cell (Sarmadi *et al.*, 2018). The different treatments caused a significant improvement ($p \leq 0.01$) in the enzyme activity.

The results showed an upward trend in PAL activity due to the change of cultures and treatment with Cu NPs. The concentration was 200 µg ml$^{-1}$ of Cu NPs with a mean WPM of 7.38 U mg$^{-1}$ protein on all treatments. MS medium untreated with Cu NPs showed the lowest enzyme activity of 1.10 U mg$^{-1}$ protein (Figure 6A).

As for PPO enzyme activity, the results showed the importance of treatments in improving its activity, and the cultures medium enriched with Cu NPs recorded a significant increase in activity, which reached a concentration of 200 µg ml$^{-1}$ with WPM 5.47 U mg$^{-1}$ protein. However, it was not significantly different with an MS medium of the same concentration. In contrast, we found the lowest activity of PPO when the control treatments of cells were cultured in an MS medium (Figure 6B)

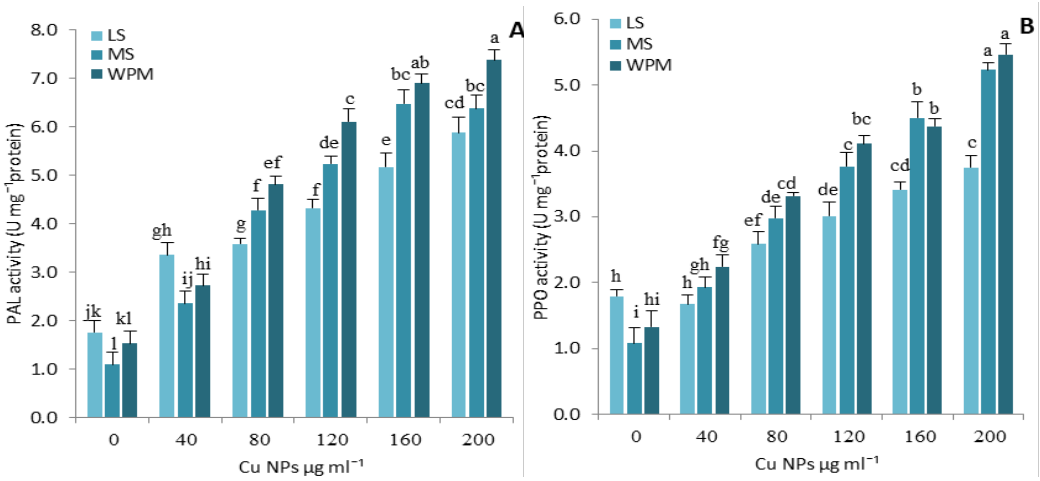


**Figure 6.** Assessment of antioxidant enzymatic activity parameters such as PPO (A) and LPO (B) of CSC in *M. charantia* L. elicited by different culture media and Cu NPs concentrations. Vertical bars represent the mean ± standard deviation of three replicates. Different letter treatments indicate significant differences at p ≤ 0.01 based on L.S.D test.

### 3.5. Secondary metabolites of CSC

Phenols and flavonoids are secondary metabolites available in *M. charantia* L., known for their therapeutic properties (Chung et al., 2018). The different treatments caused a significant improvement ($p \leq 0.01$) in the production of CSC from secondary metabolites.

The TPP increased due to different media cultures and Cu NPs treatment concentrations. The treatment of CSC with MS+200 µg ml$^{-1}$ recorded the highest yield of 8.23 mg g$^{-1}$ DW, which did not differ significantly from the production of a WPM of the same concentration. The control group showed the lowest production for all cultures (Figure 7A).

The change of cultures and the concentration of Cu NPs significantly affected the TFP. The treatment of 200 µg ml$^{-1}$ of Cu NPs with WPM recorded the highest yield with an increase of 4.4-fold over the control treatment of the same medium (Figure 7B).

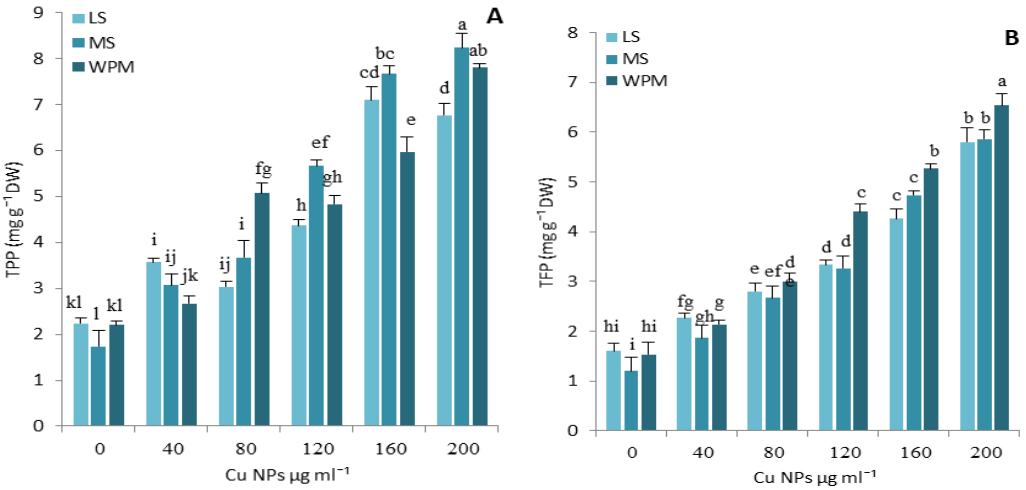


**Figure 7.** Assessment of secondary metabolites parameters such as TPP (A) and TFP (B) of CSC in *M. charantia* L. elicited by different culture media and Cu NPs concentrations. Vertical bars represent the mean ± standard deviation of three replicates. Different letter treatments indicate significant differences at p ≤ 0.01 based on L.S.D test.

*3.6. Antioxidant activity of CSC*

Antioxidant activities are the final result of evaluating the ability of different treatments to stimulate the production of secondary metabolites (Neamah and Almehemdi, 2017). MTT and DPPH assays are essential in evaluating antioxidant activity. The treatments significantly ($p \leq 0.01$) affected the antioxidant activities of CSC.

To assess antioxidant efficacy based on the MTT assay, the media under varying concentrations of Cu NPs differed significantly. CSC grown in the MS medium showed the highest activity under 160 µg ml$^{-1}$ of Cu NPs, which was 0.89. The level of difference was not significant with the MTT radical scavenging activity of CSC grown in WPM and MS medium under 200 µg ml$^{-1}$ concentration of Cu NPs (Figure 8A).

In the DPPH assay, WPM containing a high concentration of Cu NPs recorded the highest antioxidant activity with a significant increase of 9.0% and 13.67% over MS and LS cultures under the same concentration, respectively. In comparison, the control group showed the lowest mean values of antioxidant activity (Figure 8B).

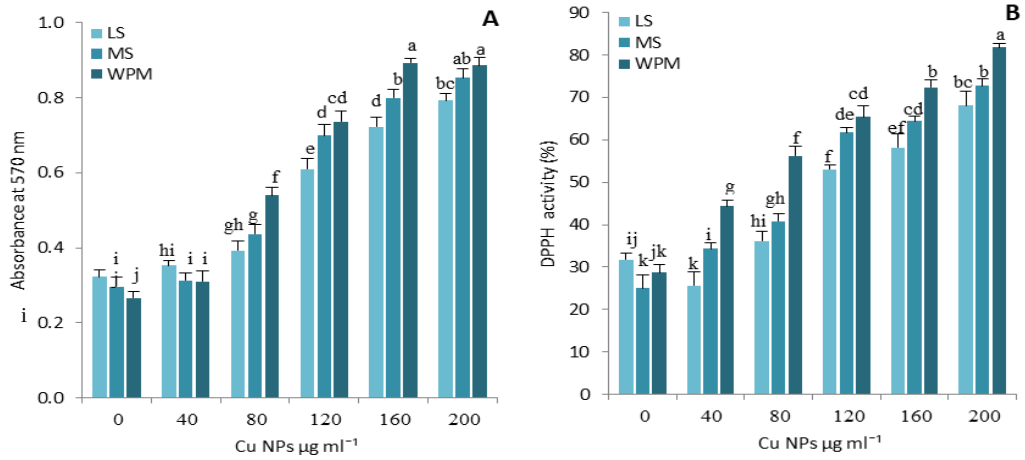


**Figure 8.** Assessment of antioxidant activity parameters such as MTT (A) and DPPH (B) of CSC in *M. charantia* L. elicited by different culture media and Cu NPs concentrations. Vertical bars represent the mean ± standard deviation of three replicates. Different letter treatments indicate significant differences at p ≤ 0.01 based on L.S.D test.

## 4. Discussion

There was a variance in the results of media cultures for the mor-physiological parameters. It may be due to the different components of the chemical cultures thus reflected on the characteristics of CSC. Copper nanoparticles may have the ability to penetrate cell walls and have high reactivity by increasing their surface area. Biomass accumulation and cell viability were enhanced by treatment with Cu NPs (Figures 4A and 4B). This may be due to the role of copper in synthesizing some essential enzymes in the vital activities of the plant. In contrast, over-treatment contributed to stress on the culture medium of CSC. This inhibited the growth of CSC biomass under high concentrations because of the toxicity generated by over-treatment. This led to copper accumulation in plant cells (Figure 4C). These results are similar to previous reports showing the role of treatment with CuO NPs in enhancing the induction of calli cultures in *Oryza sativa* L. (Anwaar *et al.*, 2016). The concentrations of Cu NPs enhanced the biomass of *Balanites aegyptiaca* L. (Ebad *et al.*, 2019). Hayat *et al.* (2021) reported that there was a significant difference in the biomass accumulation of *Artemisia absinthium* calli cultures treated with Cu NPs.

In general, the accumulation of metals in the plant cell is one of the leading causes of nutritional disorders (Neamah and Hamad, 2020). In our study, the oxidative stress indicators appeared due to the increased treatment of Cu NPs and the different media cultures. The accumulation of $H_2O_2$ increased (Figure 5A), contributing to cell membrane degradation in CSC. Hence, MDA content increased (Figure 5B).

On the other hand, the excessive increase in ROS and the rapid deterioration of cell membranes are controlled by defense systems. The plant cell has a defense mechanism that enables it to confront dangers resulting from various stresses, including metal stress. It is believed that $H_2O_2$ acts as a mediating signal that stimulates the synthesis of defense systems (Sanjari *et al.*, 2019).

PAL and PPO are two types of antioxidant enzymes (Figure 6). The PAL enzyme enhances the biosynthetic pathways responsible for synthesizing phenolic compounds (Asghari and Zahedipour, 2016; Manquián-Cerda *et al.*, 2016). In comparison, the PPO enzyme contributes to the oxidation of phenolic compounds under tensile conditions. Cu NPs played an essential role in membrane protection of vitals from damage, thus protecting the phenols stored in the cellular vacuoles from oxidative stress.

Phenolic compounds are classified as potent non-enzymatic antioxidants and an essential secondary metabolite (El Jabboury *et al.*, 2022). They mitigate ROS's toxic effects and can chelate minerals (Mierziak *et al.*, 2014).

There was a positive effect of the Cu NPs treatment (Figure 7). The effect of CSC by stress may have led to the elicitation of phenolic compounds.

MTT and DPPH assays assess antioxidant activity (Neamah, 2016; Chung *et al.*, 2018) based on their ability to accept an electron or hydrogen (Sharma and Ramawat, 2014). The results showed that the antioxidant activity could be increased by treatment with Cu NPs, as well as the different chemical components of CSC media cultures (Figure 8). Increased TPP or TFP can lead to enhanced antioxidant activity (Figure 7). These results are similar to

the report by Choudhary *et al.* (2011) on *Raphanus sativus* under Cu stress. This is consistent with the announcement by Hayat *et al*. (2021) of *Artemisia absinthium* L. callus cultures treated with Cu NPs.

## 5. Conclusion

The effect of media cultures and Cu NPs treatments on the CSC of *M. charantia* L. was reported for the first time. MS medium was more consistent in improving biomass accumulation compared to LS or WPM. In general, the exogenous Cu NPs at lower concentrations improved biomass accumulation, whereas the increasing concentrations of Cu NPs caused an increase in the accumulation of $H_2O_2$ and MDA. Thus, this enhanced the activity of non-enzymatic defense systems that contributed to the inhibition of phenolic oxidation and the elicitation of secondary metabolites by Cu NPs. These results can be exploited in the commercial production of secondary metabolites.

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