Research Article

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Synthesis, Characterization and Influence of Copper Nanoparticles on Growth of *Vigna mungo* L. Hepper CO-7 Variety

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ABSTRACT

The copper nanoparticle was synthesized by employing *Cnidoscolus aconitifolius* (Mill) leaf extract. The nanoparticles were characterized by UV, FTIR and SEM studies. The SEM image showed a spherical structure. The bio-stimulant effect of green synthesized copper nanoparticles on *Vigna mungo* L. Hepper was investigated. Seed germination, biochemical, antioxidant, and enzyme activities of the copper nanoparticles were studied. The germination and morphological characteristics were superior at 100 mg/L copper nanoparticle-treated seeds. The biochemical parameters such as chlorophyll (0.701±0.107 mg/g), carbohydrate (4.657±0.090 mg/g), protein (4.396±0.335 mg/g), amino acid (18.557±0.638 mg/g), phenol (10.824±0.53 mg/g), flavonoid (3.644±0.171 mg/g), alkaloid (6.529±0.45 mg/g), and tannin (12.159±0.218 mg/g) were higher at a nanoparticle concentration of 100 mg/L. As far as the enzymatic activities, the maximum activity of nitrate reductase (4.505±0.203 mg/g) and α-amylase (13.195±0.285 mg/g) were observed with 100 mg/L copper nanoparticle-treated seedlings. The in vitro antioxidant activities were also studied.

Keywords: *Cnidoscolus aconitifolius*; Bio-stimulant; Enzymatic activity; Antioxidant activity.

1. INTRODUCTION

Nanotechnology is one of the most promising new technologies for the twenty-first century. Nanoscience is the study of molecules and structures at nanoscales, which range from 1 to 100 nm (Mansoori and Soelaima, 2005). The manipulation of matter at the "nano" scale is seen as an important enabling technology (Satalkar *et al.* 2016). The unique properties of nanomaterials, such as improved thermal and electrical conductivity, catalytic activity, and biocompatibility, make them appealing for applications in biological labeling, optics, energy storage, catalysis, and cancer treatment (Ealia and Saravanakumar, 2017). When compared to other methods such as chemical reduction, photochemical reduction, electrochemical reduction, thermal breakdown, and so on, the green synthesis strategy was found to be superior (Al-Hakkani, 2020) for the synthesis of nanoparticles. Green synthesis employs natural and environmentally benign materials such as bacteria, algae, and fungi as reducing and end-capping agents, thereby conserving energy and avoiding dangerous chemicals. Green materials, such as proteins and polyphenols, can reduce metals. Green synthesized metal nanoparticles can be created under particular conditions, providing several advantages such as non-toxicity, pollution-free, ecologically friendly, cost-effectiveness, and sustainability (Ying *et al*. 2022). Plants can naturally produce metallic nanoparticles for detoxification and environmental protection. Plant extracts contain bioactive flavonoids, which help to reduce and stabilize metallic ions. The mix and concentration of these active biomolecules determine the diversity of nanoparticle sizes and forms. At room temperature, the process is straightforward, beginning with a sample of plant extract and reduction of metal salts. The plant extract stabilizes the nanoparticles, determining their most stable and energy-efficient form (Al-Hakkani, 2020). Although copper is a widely used metal in a variety of applications, its strong oxidation propensity makes it challenging to manufacture at nanoscale levels. Many industries, especially electronics, rely on copper as a good substitute for existing pricey metals and do not want copper oxides present on nanoparticle surfaces. When copper nanoparticles (Cu NPs) are polluted by oxide phases, their electrical conductivity significantly decreases (Saravanan *et al.* 2021).

Cnidoscolus aconitifolius (Mill) Johnson (Euphorbiaceae) is used in Africa to treat central nervous system diseases caused by alcoholism, convulsions, anxiety, and insomnia, as well as to improve memory and brain function. The Cnidoscolus genus includes approximately fifty species of woody trees, shrubs, subshrubs, and plants that grow in seasonally dry tropical climates. Members of the genus are used in traditional medicine in Africa, Asia, and Latin America to treat a wide range of diseases. *Cnidoscolus aconitifolius*, sometimes known as the spinach tree or chaya, is a popular green perennial shrub found throughout Central America and Brazil's Yucatan Peninsula. It is commonly consumed raw in salads and soups, but it is also used medicinally to treat a number of ailments. The potential for improving seed quality indices, especially in pulse crops, is considerable for NPs based on metals. Still, there are little investigations on NPs as nano-priming agents, especially in pulses for seed stimulation (Sripathy *et al*. 2023). A high rate of emergence, consistent seed germination, and rapid seedling growth are the main criteria for commercial agriculture, since seed priming is a viable method for rapidly increasing seed germination rates and encouraging early growth by modifying physiological factors (Banerjee *et al*. 2023).

2. EXPERIMENTAL APPROACH

2.1 Collection of Plants

The fresh leaves of *Cnidoscolus aconitifolius* (Mill) were collected from the forest campus at RS Puram in Coimbatore and then washed thoroughly 2-3 times with tap water and once with sterile water. The leaves were cleaned well.

2.1 Preparation of Plant Extract

About 10 g of fresh leaves of *Cnidoscolus aconitifolius* (s) were weighed in the weighing balance. The fresh leaves were cut into small pieces and soaked in 100 mL of deionized water in a 250 mL beaker. This was heated in the magnetic stirrer for 2 hours to obtain the leaf extract. After 2 hours, the extract was filtered using Whatman No. 1 filter paper, and the pure leaf extract was obtained. This leaf extract can be utilized only for 7 days (Mali *et al.* 2020).

2.2 Synthesis of Copper Nanoparticle

Copper sulphate (5 mM) was dissolved in 50 mL of deionized water, and 5 mL of the leaf extract was added to it. A light green solution was obtained. The pH was maintained at 7 by adding 1 N NaOH. This solution was kept at 60 °C for 30 minutes. After 30 minutes, a dark green precipitated solution was obtained. This solution was centrifuged at 7000 rpm for 10 minutes. The supernatant was discarded, and the green pellet in the centrifuge tube was cleaned by adding ethanol to remove the basicity. Again, it was centrifuged at 7000 rpm for 10 minutes. Again, the supernatant was discarded, and the green-coloured pellet in the centrifuged tube was cleaned by adding deionized water to it. The solution was again centrifuged at 7000 rpm for 10 minutes. After centrifugation, the supernatant was removed, and only 5-7 mL of deionized water was added and mixed with the pellet, which was then poured into the glass Petri dishes. The glass Petri dishes were kept in the hot air oven overnight at 30–60 °C. The green-coloured nanoparticles were harvested and stored for further studies and characterizations (Mali *et al*. 2020).

2.4. Effect of Copper Nanoparticles on Seed Germination of *Vigna mungo* L. Hepper

Vigna mungo L. Hepper seeds are commonly known as black gram. It belongs to the Legumes family. The name of the variety used was CO-7. The seeds were purchased from the Department of Pulses at TNAU in Coimbatore, Tamil Nadu, India. The black gram seeds were sterilized with 70% ethanol for 30 seconds, followed by rinsing with double-distilled water several times for the prevention of surface fungal or bacterial contamination. The healthy seeds were selected and treated with copper nanoparticles. The copper nanoparticles were taken in different concentrations (0 mg/L, 50 mg/L, 100 mg/L, 150 mg/L, and 200 mg/L) for seed germination. The seeds were soaked in different concentrations of biosynthesized copper nanoparticles overnight in dark conditions. The soaked seeds were sown on the double-layered tissue papers in the Petri dishes. The rate of germination was observed in the plant growth chamber for 7 days.

2.5 Effect of Copper Nanoparticles on Morphological Characterises of *Vigna mungo* L. Hepper

The germinated seeds were analysed for various growth parameters, like total length, shoot length, root length, fresh weight, and dry weight of the whole plant. The healthy plants were selected randomly, and the lengths were measured. The mean values are expressed in cm. The plants were kept in aluminium foil and weighed on an electronic weighing balance, and the fresh weight was recorded. Then the plants were placed in a hot air oven to dry at 70 °C for 1 day, and the dry weight was measured using a weighing balance. The values are recorded in mg/g.

2.6 Quantitative Analysis

The total carbohydrate was estimated by the method of Chandran *et al.* (2013), the protein was estimated as described by Krishna *et al*. (2014), the total free amino acid was estimated as described by Sadasivam and Manickam (2008), the total phenols were estimated as described by Makkar *et al*. (2003), the flavonoid was estimated as described by Zhishen *et al*. (1999), the alkaloids were estimated as described by Singh *et al*. (2004) and tannin was estimated as described by Makkar *et al.* (2003).

2.7 Enzymatic and *in vitro* Antioxidant Activities

The α-amylase inhibitory activity was estimated by the DNS method and the nitrate reductase activity was estimated as described by Lowe and Evans (1964). The DPPH scavenging activity was estimated by Blois (1958) method, the ABTS scavenging activity was estimated by Re *et al.* (1999) method, the phosphomolybdenum assay was carried out as described by Prieto *et al.* (1999) and the superoxide radicle scavenging activity was estimated as described by Beauchamp and Fridovich (1971).

2.8 Statistical Analysis

Statistical analysis was performed using Microsoft office Excel (2007). Mean Values for each treatment were calculated, and all the treatment means were compared via analysis of variance. All values were obtained from three independent analyses. The results are expressed as mean ± standard deviation. A one-way analysis of variance (ANOVA) test was used for statistical data analysis.

3. RESULTS AND DISCUSSION

3.1 Green Synthesis of Copper Nanoparticles from *Cnidoscolus aconitifolius* (Mill)

Nanotechnology is a collection of technologies that enable the study, manipulation, and utilization of incredibly small structures and systems typically less than 100 nanometers (Cheng, *et al.* 2016). Various methods were undertaken for the preparation of copper nanoparticles, like the chemical method (Kruk *et al.* 2015) and the water/acetonitrile mixed solvent (Abdulla-Al-Mamun *et al*. 2009), using L-ascorbic acid (Umer *et al.* 2014), ball milling, the pulse laser ablation method, the pulse wire discharge method, the chemical reduction method, electrochemical methods, thermal decomposition, microwave, microemulsion reduction, sonochemical and sonoelectrochemical (Tito *et al*. 2021). In the present work, the copper nanoparticles were synthesized with *Cnidoscolus aconitifolius* (Mill) leaves with the 5 mM copper sulphate. The colour changed to light green to dark green after continuous stirring of the reaction mixture (Fig.1).

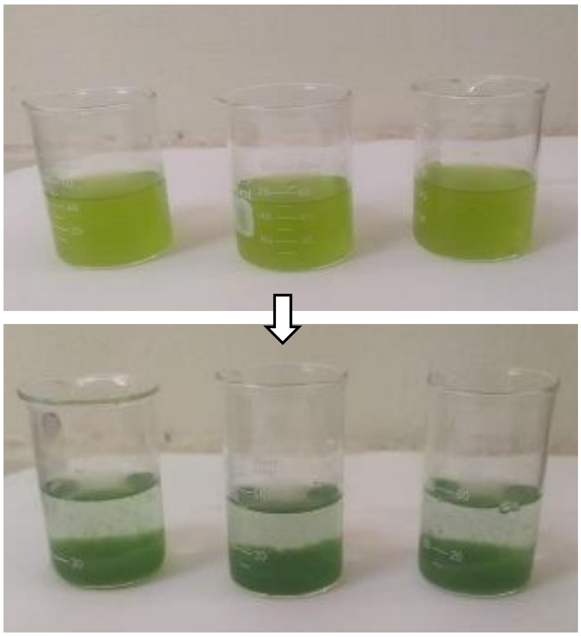

Fig.1: Green synthesis of copper nanoparticles from *Cnidoscolus aconitifolius* (Mill)

Table 1. FTIR spectrum analysis of copper nanoparticles synthesized using *Cnidoscolus aconitifolius* (Mill)

S. No.	Frequency (cm^{-1})	Absorption Range (cm^{-1})	Functional Group
1	3751.83	3584-3700	O-H Stretching
2	3648.66	3584-3700	O-H Stretching
3	3312.14	3200-3550	N-H Stretching
4	1747.19	1720-1740	C=O Stretching
5	1571.7	1566-1650	C=C Stretching
6	1390.42	1380-1390	C-H Stretching
7	1035.59	1030-1070	S=O stretching
8	908.308	860-900	C-H bending
9	779.101	760-800	C-H bending
10	696.177	680-720	C=C bending

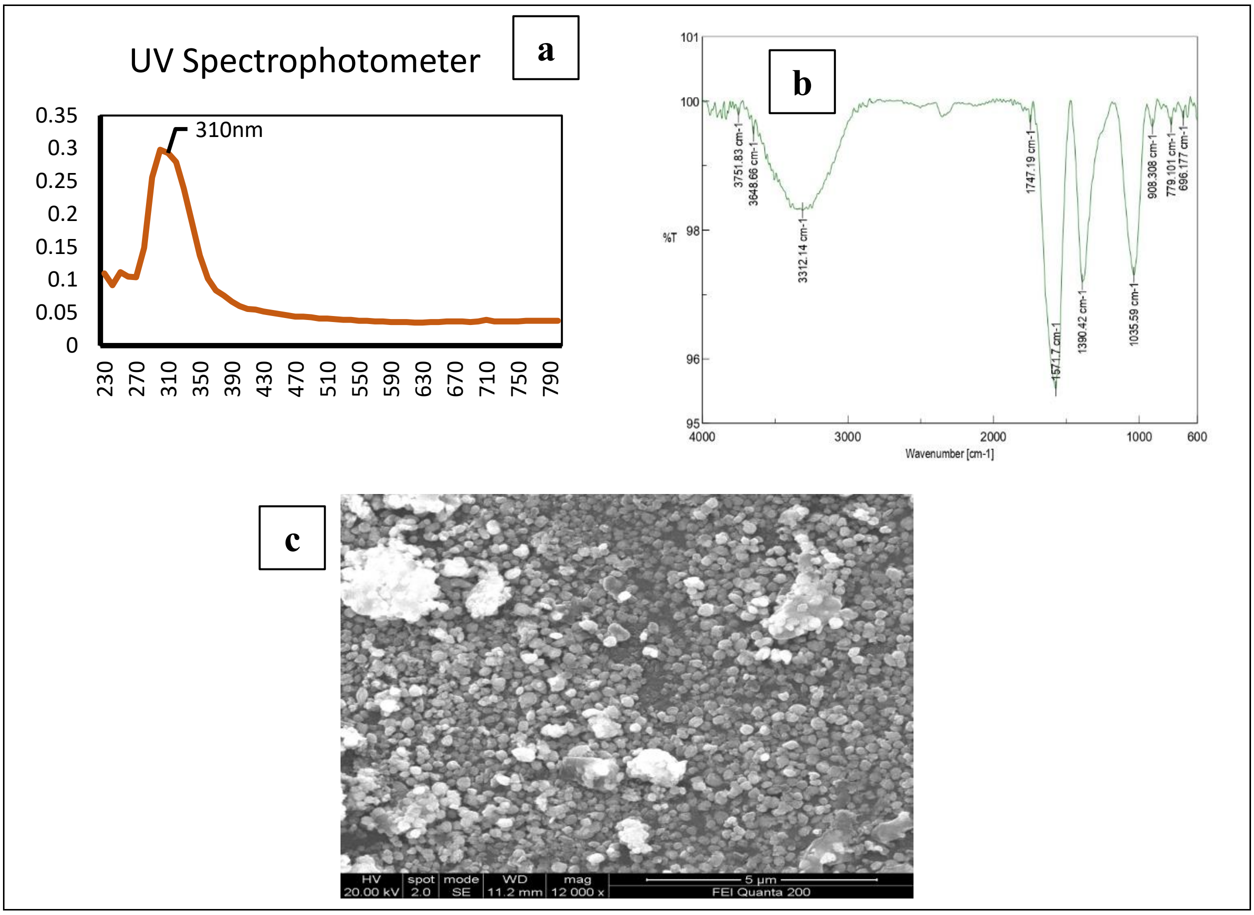

Fig.2: Characterization Studies of Synthesized Copper Nanoparticles from *Cnidoscolus aconitifolius* (Mill). (a) UV-visible, (b) FTIR, (c) SEM

3.2 Characterization Studies of Synthesized Copper Nanoparticles

In the present study, the synthesis of Cu NPs was confirmed by a distinctive UV-visible spectral peak measured at 310 nm (Mali *et al.* 2020) (Fig.2a). The FTIR spectrum of Cu NPs depicts the characteristic bands at 3751.83 and 3648.66 (O-H stretching), 3312.14 (N-H stretching), 1747.19 (C=O stretching), 1571.7 (C=C stretching), 1390.42 (C-H stretching), 1035.59 (S=O stretching), 908.308 (C-H bending), 779.101 (C-H bending) and 696.177 (C=C bending) (Fig.2b and Table 1). The morphology of the synthesized copper nanoparticles was analysed by scanning electron microscopy (SEM). Fig. 2c shows the image of the green synthesized copper nanoparticle (5 µm). The copper nanoparticles appeared to be solid, entirely developed, and randomly scattered. These observations are in line with literature studies. Joseph *et al.* (2016) have observed IR peaks at 1612 cm^{-1} and 1271 cm^{-1}, with carbonyl groups binding to the metal. The SEM images in earlier works showed spherical (Shikha *et al.* 2015), cubic, and hexagonal (Suárez-Cerda *et al.* 2017) nanoparticles.

3.3 Bio-stimulant Activity of Synthesized Copper Nanoparticles on *Vigna mungo* L. Hepper

The study examined the effects of different concentrations of copper nanoparticles on seed germination and early growth of *Vigna mungo* L. Hepper seeds. The seed germination of *Vigna mungo* L. Hepper was studied with different concentrations (0, 50 mg/L, 100 mg/L, 150 mg/L, and 200 mg/L) of synthesized copper nanoparticles. A significant effect of seed germination was recorded. The highest seed germination percentage was observed (97%) in 100 mg/L-treated nanoparticles. The lowest amount of germination was observed (64%) at 200 mg/L. The germination percentage decreased with increased nanoparticle concentrations (Fig. 3 and Table 2). The copper nanoparticles synthesized using *Cnidoscolus aconitifolius* leaf extract showed higher germination percentage as observed by Kumar *et al.* (2018).

The seeds were treated with different concentrations of copper nanoparticles (0, 50 mg/L, 100 mg/L,150 mg/L and 200 mg/L). The seeds were maintained constant photoperiod of 7 days. After the seedling stage, the morphological parameters were measured (Fig.3 and Table 2). The highest shoot length (9.077±0.711 cm), root length (4.811±0.879 cm), fresh weight (242.466±0.605 mg/g), and dry weight (0.148±0.004 mg/g) was observed with 100 mg/L concentration. The lowest shoot length (5.811±0.277 cm), root length (2.9±0.432 cm), fresh weight (145.9±0.368 mg/g) and dry weight (0.0176±0.001 mg/g) were measured at control. The findings suggest a concentration-dependent influence on *Vigna mungo* morphological traits (Singh *et al*. 2019). Copper nanoparticles stimulate seed germination by promoting water uptake and metabolic activities, while higher concentrations may inhibit it due to oxidative stress and cellular damage (Hong *et al*. 2015). Studies have shown that nanoparticles from *Cnidoscolus aconitifolius* leaf extract have higher growth parameters at 100 mg/L. Another study found that copper nanoparticles from *Cnidoscolus aconitifolius* leaf extract have a dose-dependent impact on soybean plant seed germination (Hong *et al.* 2015), with higher growth parameters at concentrations of 100 mg/L as observed in our work. Our findings are consistent with previous research on wheat seedlings (Ghafariyan, *et al*. 2019). They found that copper nanoparticles from *Cnidoscolus aconitifolius* leaf extract have a complex effect on root length during wheat seed germination. The study found that copper nanoparticles affect wheat seedlings' fresh weight and dry weight during seed germination. Fresh weight was maximum at 150 mg/L, involving stimulatory and inhibitory mechanisms like oxidative stress and cellular damage.

Table 2. Morphological characters of copper nanoparticle-treated *Vigna mungo* L. Hepper

S. No.	Concentration (mg/L)	Root Length (cm)	Shoot Length (cm)	Total Plant Length (cm)	Fresh Weight (mg/g)	Dry Weight (mg/g)
1.	Control	2.90±0.432	5.811±0.277	8.711±0.539	145.9±0.368	0.0176±0.001
2.	50	3.555±0.764	7.088±0.581	10.644±0.556	179.588±0.487	0.019±0.003
3.	100	4.811±0.879	9.077±0.711	13.888±0.528	242.466±0.605	0.148±0.004
4.	150	3.944±0.361	8.077±0.115	12.022±0.375	208.833±0.747	0.032±0.036
5.	200	2.944±0.385	6.588±0.831	9.533±1.08	165.7±0.259	0.029±0.040

Values were performed in triplicates determination (n=3) ± SD

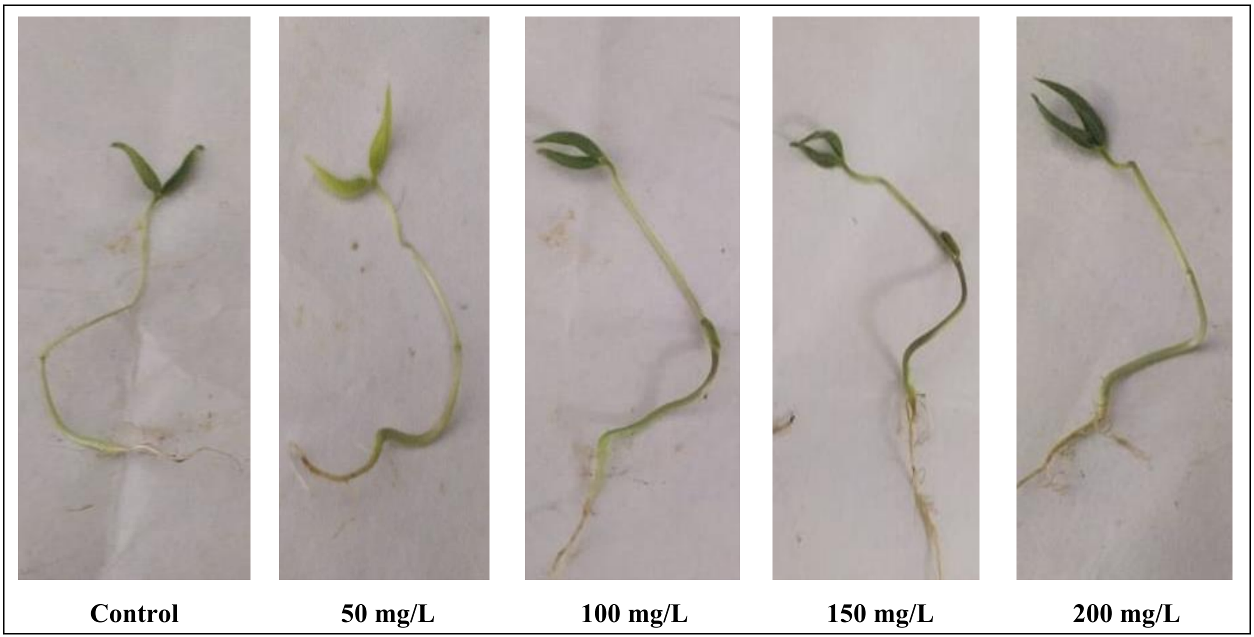

Fig. 3: Morphological characters of copper nanoparticle treated *Vigna mungo* L. Hepper

3.4 Biochemical Constituents on Copper Nanoparticles Treated *Vigna mungo* L. Hepper

The biochemical constituents of copper nanoparticle-treated *Vigna mungo* L. Hepper showed a significant amount of chlorophyll, carbohydrate, protein, free amino acids, phenols, flavonoids, alkaloids, and tannins (Tables 3 and 4). The maximum total chlorophyll content (0.701±0.107 mg/g sample) was observed in 100 mg/L. The lowest amount was observed in (0.347±0.037 mg/g sample) control (Fig. 4 and Table 3). The study found that the highest amount of carbohydrates, protein, and amino acids were found at 100 mg/L, while the lowest amount was recorded in the control sample (Fig.

4 and Table 4). Gogos *et al.* (2016) investigated the impact of copper nanoparticles on the carbohydrate, protein, amino acid, chlorophyll, phenol, flavonoid, alkaloid, and tannin content of treated maize seeds. The results showed that copper nanoparticles synthesized from *Cnidoscolus aconitifolius* leaf extract modulated carbohydrate metabolism, stimulate protein synthesis, and affect seed germination and growth. The optimal nanoparticle concentration was found to be 100 mg/L.

Table 3. Total chlorophyll content of copper nanoparticle treated *Vigna mungo* L. Hepper

S. No.	Concentrations	Chlorophyll-A (mg/g)	Chlorophyll-B (mg/g)	Total Chlorophyll (mg/g)
1	Control	0.142±0.013	0.205±0.025	0.347±0.037
2	50 mg/L	0.240±0.051	0.391±0.048	0.631±0.051
3	100 mg/L	0.259±0.002	0.442±0.105	0.701±0.107
4	150 mg/L	0.266±0.017	0.425±0.056	0.691±0.054
5	200 mg/L	0.195±0.037	0.262±0.034	0.458±0.071

Experiments were performed in triplicates determination (n=3) ± SD

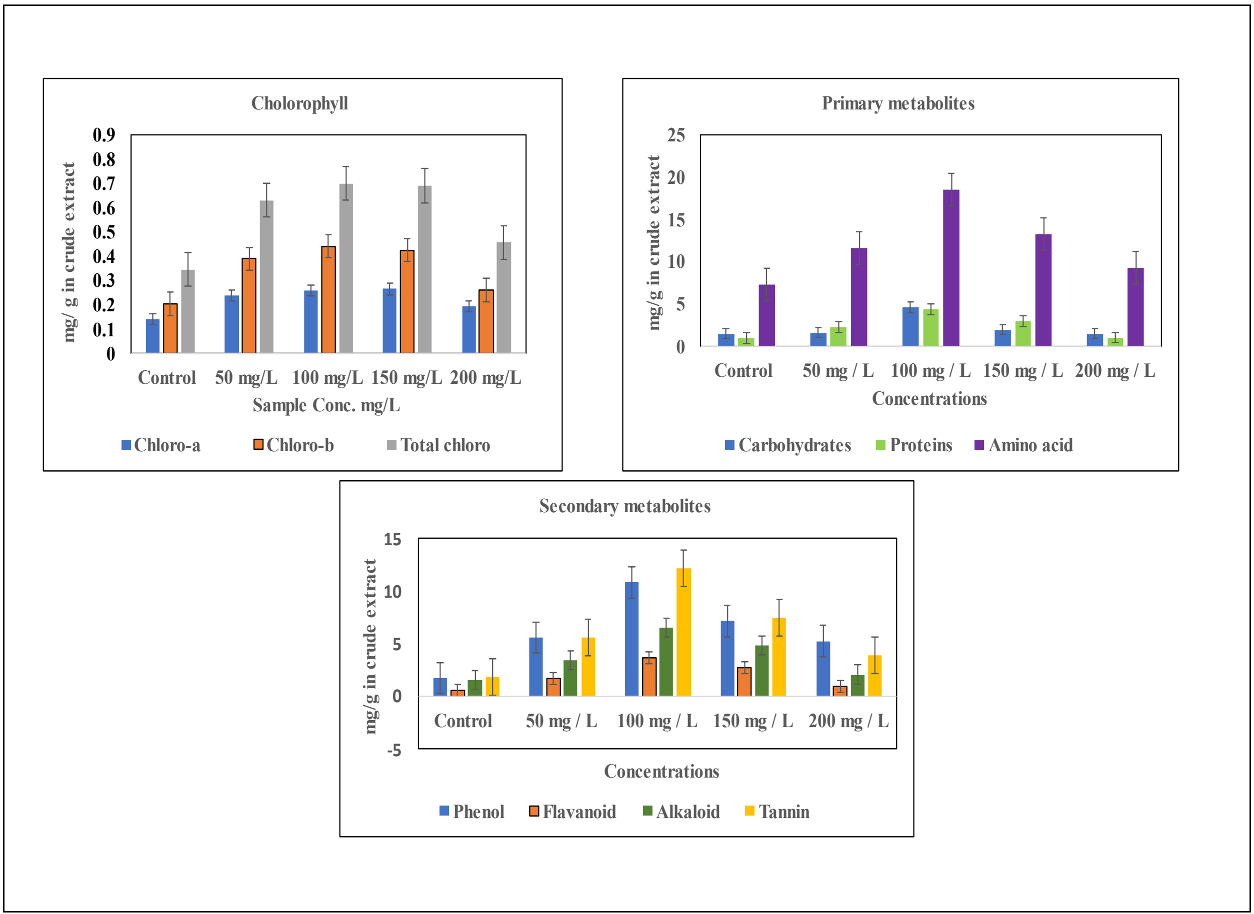

Fig. 4: Composition of biochemical compounds in copper nanoparticle treated *Vigna mungo* L. Hepper

3.5 Enzymatic and *in vitro* antioxidant activity of copper nanoparticles treated *Vigna mungo* L. Hepper

Copper nanoparticles can interact with cellular proteins and enzymes, potentially altering their structure and function. Gomes *et al.* (2021) reported that seed priming with bulk or nano-encapsulated Cu^{2+} improved maize seedling development by improving shoot growth and photosynthetic electron transport. Choudhary *et al.* (2017) found that α-amylase enzyme activity was highest in 150 mg/L copper nanoparticle-treated seeds during germination. The maximum nitrate reductase enzyme activity was observed in 150 mg/L (Ghafariyan *et al.* 2019). In the present investigation, the highest α-amylase enzymatic activity (13.195±0.285 mg/g) and nitrate reductase activity (4.505±0.203 mg/g) were observed in a sample with a concentration of and at 100 mg/L (Fig. 5 and Table 5).

Diphenyl picryl hydrazide is a useful reagent for assessing reducing compounds and their ability to scavenge free radicals (RajeshKumar and Rinitha *et al.* 2018). Quiterio-Gutiérrez *et al.* (2019) reported that hydrophilic compounds showed the highest antioxidant capacity when exposed to the ABTS radical. Copper nanoparticles may enhance antioxidant defence mechanisms in seeds during germination by upregulating enzymes like superoxide dismutase (SOD). The enzyme catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide, mitigating oxidative stress (Ghafariyan *et al.* 2019). The presence of copper nanoparticles can stimulate SOD production, leading to increased superoxide radical scavenging activity at 100 mg/L concentration. The present study found that the highest radical scavenging activity phosphomolybdenum (15.758±0.046 mg/g), superoxide (0.805±0.016 mg/g), followed by IC50 values of DPPH (0.758±0.072 µg/mL) and ABTS (0.606±0.055 µg/mL) was found with a nanoparticle concentration of 100 mg/L (Fig. 5 and Table 6).

Table 5. Enzymatic activities in copper nanoparticle treated *Vigna mungo* L. Hepper

S. No.	Concentration (mg/L)	Nitrate reductase (mg/g)	α-Amylase activity (mg/g)
1	Control	1.196±0.490	4.26±0.132
2	50	2.196±0.283	5.399±0.427
3	100	4.505±0.203	13.195±0.285
4	150	3.779±0.089	7.046±0.302
5	200	1.255±0.089	5.273±0.153

Values were performed in triplicates determination (n=3) ± SD

Table 4. Composition of biochemical compounds in copper nanoparticle treated *Vigna mungo* L. Hepper

Biochemical parameters (mg/g)	Control	50 mg/L	100 mg/L	150 mg/L	200 mg/L
Carbohydrate	1.53±0.156	1.628±0.066	4.657±0.090	1.991±0.115	1.513±0.115
Protein	1.02±0.277	2.276±0.582	4.396±0.335	3.015±0.201	1.077±0.226
Amino acid	7.345±0.481	11.648±0.457	18.557±0.638	13.284±0.555	9.284±0.457
Phenol	1.748±0.398	5.636±0.134	10.824±0.53	7.168±0.109	5.265±0.478
Flavonoid	0.560±0.180	1.718±0.561	3.644±0.171	2.700±0.048	0.953±0.024
Alkaloid	1.566±0.506	3.435±0.293	6.529±0.456	4.844±0.340	2.083±0.359
Tannin	1.885±0.428	5.611±0.179	12.159±0.218	7.492±0.285	3.945±0.322

Experiments were performed in triplicates determination (n=3) ± SD

Table 6. Antioxidant activity in copper nanoparticle treated *Vigna mungo* L. Hepper

S. No.	Concentration (mg/L)	Phosphomolybdenum (mg/g)	Superoxide radicle (mg/g)	DPPH (µg/mL)	ABTS (µg/mL)
1	Control	1.788±0.063	0.247±0.046	0.354±0.100	0.307±0.0013
2	50	7.304±0.092	0.484±0.011	0.482±0.075	0.377±0.050
3	100	15.758±0.046	0.805±0.016	0.758±0.072	0.606±0.055
4	150	8.344±0.271	0.648±0.061	0.532±0.126	0.473±0.027
5	200	5.142±0.060	0.349±0.029	0.477±0.090	0.323±0.108

Values were performed in triplicates determination (n=3) ± SD

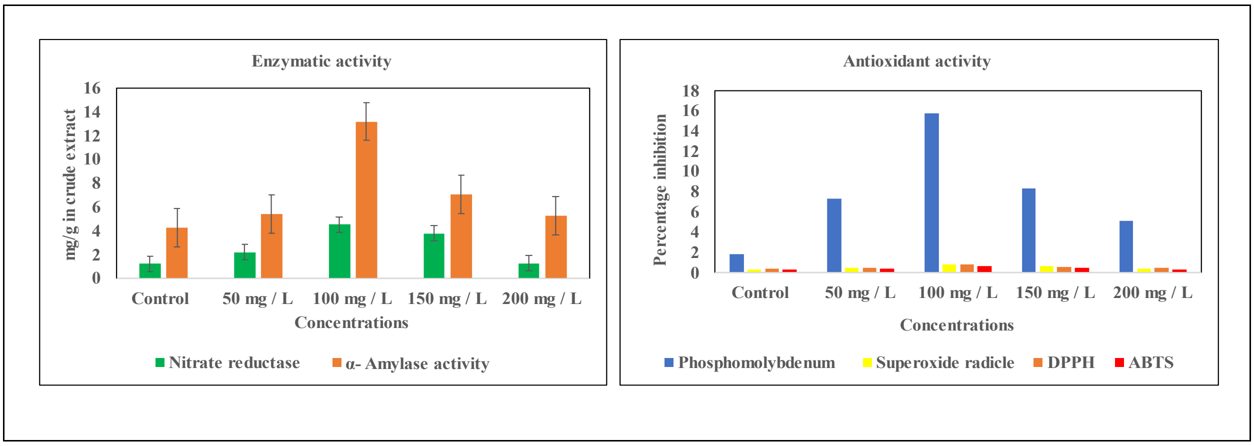

Fig. 5: Enzymatic and *invitro* antioxidant activity of copper nanoparticle treated *Vigna mungo* L. Hepper

CONCLUSION

The percentage of seed germination and morphological characteristics of *Vigna mungo* L. Hepper are improved by the copper nanoparticles green-synthesized using *Cnidoscolus aconitifolius* (Mill) leaves. According to the current study, the synthesized copper nanoparticles showed significant bio-stimulant activity. They increased the growth of *Vigna mungo* L. Hepper. However, more research needs to be done in order to increase the crop yield on a wide scale, and field tests are necessary to analyse the impact of copper nanoparticles on crop plants. Green synthesized copper nanoparticles are safe and harmless to the environment and they might be used in agricultural applications.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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