



Eco-friendly Phytosynthesis of Copper Nanoparticles Using *Medicago sativa* Extract: A Biological Activity and Acute Toxicity Evaluation

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ABSTRACT

This investigation aimed to study the antioxidant and anti-inflammatory effects of the aqueous extract of *Medicago sativa* and copper nanoparticles CuNPs. Biosynthesized CuNPs were characterized by analytical methods. Qualitative phytochemical analysis was carried out by standard protocols. Quantitative Phytochemical analyses were analyzed by using HPLC methods. Moreover, in vivo intraperitoneally acute toxicity testing of nanoparticles was carried up. Results of qualitative phytochemical and quantitative HPLC analysis results revealed that *M. sativa* contains most of bioactive compounds, especially Naringin, Rutin, and Vanillic Acid compounds with high antioxidant and anti-inflammatory activity. Characterization of CuNPs confirmed the involvement of biological molecules in CuNPs synthesis with size ranged from 19.8 to 92.8 nm. In this study, the intraperitoneal toxicity test showed no mortality and minor behavioral variations up to 20 mg/kg of CuNPs in Wistar rats. We concluded that *M. sativa* L has potential properties as biocatalyst stabilizers for CuNPs synthesis and MsE-CuNPs revealed good activity as a potent antioxidant, and anti-inflammatory agent. Further in vivo studies are needed to explore them as good therapeutic agents.

Keywords: *Medicago sativa*, CuNPs, SEM, FTIR, Antioxidant, Acute toxicity

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INTRODUCTION

The creation of metal and metal oxide nanoparticles (NPs) has significantly improved the biomedical area in recent years in terms of biosensing, imaging, diagnosis, and therapy. The three most often used metals and their oxides are copper, silver, and gold (Cu). (Letchumanan *et al.*, 2021). Due to their unusual physical and chemical characteristics and ease of manufacture, nanoparticles have recently attracted a lot of attention (Derouiche *et al.*, 2022). Today, production of nanoparticles (NPs) using biosynthetic techniques, has been considered as a valuable method with increasing attraction (Keyhani *et al.*, 2018). Cu NPs are effective catalysts that have excellent yields, simple product separation, and may be employed repeatedly. The human body may be harmed by Cu - free ions at the cellular, organ, and systemic levels. Cu ions in living things should therefore be controlled. Inorganic NPs called copper oxides (CuOs) can be produced readily from copper nanoparticles (Cu NPs). Experiments have demonstrated an anti-inflammatory, anti-bacterial, and oxidative stress protective impact for both Cu and CuO NPs, which are both

widely utilized as anticancer agents (Ouidad *et al.*, 2020; Derouiche *et al.*, 2022). The ability of NPs to interact with the biological system at the cellular level for numerous reactions and functions is largely responsible for this (Chetehouna *et al.*, 2020). CuO NPs are used in a variety of processes, including catalysis, gas sensing, magnetic phase transitions, antimicrobial activity, and superconductivity (Zahrah, 2022). Current research into the therapeutic effects of plant extracts has revealed several effects of great importance to modern medicine, pharmacy, and industry (Zerrouki & Riaz, 2021). Numerous plant extracts have been used to make copper oxide nanoparticles in large quantities. The copper salts are decreased as a result of the plant extract's production of electrons. In the SEM investigation, the CuNPs image was found. Copper oxide nanoparticles are created when phytochemicals combine with copper ions, causing reduction (Siddiqi & Husen, 2020). The nanoparticles, which were biosynthesized from plant extract, are spherical and less than 90 nm in size (Malik *et al.*, 2022). In this paper, we will choose the *Medicago sativa* aqueous extract as a base for CuO nanoparticles synthesis (MsE-CuO-NPs); their characterization; anti-oxidant and anti-inflammatory analysis.

MATERIALS AND METHODS

Plant materials and Aqueous extract preparation

The plant (*Medicago sativa*) used in this study was purchased from the market. These herbs were mechanically ground into a fine powder before being used. Until the trial started, the *Medicago sativa* powders were kept at room temperature in airtight containers away from strong light. The aqueous extract was made by mixing 50 g of plant powder dry at 50°C for two hours with 500 ml of distilled water. The mixture was filtered using Whatman paper after 24 hours of maceration at room temperature, and then it was evaporated using a rotary evaporator.



Figure 1. Leave *Medicago sativa*

Green synthesis of MSE- Cu Nanoparticles

The appropriate reaction mixture was made for the biogenesis of phyto-copper nanoparticles by adding 2g of copper sulfate into the specified quantity of prepared *Medicago sativa* aqueous extract (20 ml), and the reaction solutions were combined using a heater-stirrer. Both flasks were incubated for 1:30–2 hours in the rotary shaker at 60°C, after which the samples were placed in an electric furnace set to 200°C for 2 hours. Later, using continuous centrifugation (3900 rpm; 10 min; 70°C) with double-distilled water and ethanol, the created phyto-copper nanoparticles (CuNPs) were separated and purified. For additional characterization and bioactivity research, the dried CuNPs were maintained at 60°C (Asemani & Anarjan, 2019).

Characterization of MSE-Cu Nanoparticles

UV-Visible Spectroscopy

UV-visible (UV-Vis) spectroscopy makes it simple to detect the synthesis of copper nanoparticles in *Medicago sativa* solution. By routinely sampling aliquots (1 mL) of the aqueous component and determining the solution's UV-Vis spectra, the bio-reduction of the Cu⁺ ions in solutions was seen. These aliquots' UV-Vis spectra were observed on a JENWAY 6705 UV-Vis spectrophotometer in the 200-700 nm region operating at a solution of 1 nm as a function of reaction time.

Scanning electron microscope (SEM)

A high-energy electron beam is used to scan a sample using a raster scan pattern in a Scanning electron microscope (SEM).

The VERTIV-MODEL 6390 machine was used to perform the SEM and EDX analyses. An amount of the sample was used to create thin films on a carbon-coated copper grid. The extra solution was then blotted off with a piece of paper, and the films on the SEM grid were then dried for five minutes under a mercury lamp.

X-ray diffraction (XRD)

XRD was used to identify the copper nanoparticle's size and makeup. Shimadzu XRD-6000/6100 model with 30 kv, 30 mA, and CuK α a radian at 2 θ angles were used for this. A quick analytical method that can reveal the dimensions of unit cells is X-ray powder diffraction, which is primarily used to determine the phase of crystalline materials. The average bulk composition of the studied material is found after it has been finely processed. On the copper nanoparticles, the particle or grain size of the particles was measured using Debye Sherrer's equation:

$$D = 0.94\lambda \times B \cos\theta \quad (1)$$

FTIR spectroscopy

The reduction of Cu ions with a spectrum range of 400–4000 cm⁻¹ was subjected to FTIR analysis to identify the biomolecules that were present in the extract. Here, the sample was dried in a hot air oven and pulverized with KBr to create a pallet before being centrifuged at 3900 rpm for 10 minutes. The pellet was then examined using an FTIR instrument using the Cary 630 model.

Phytochemical analysis, total phenols, and flavonoids compounds

On the aqueous extracts made from the plant using the qualitative characterization method, the phytochemical analysis was done. The Folin-Ciocalteu (FC) method was used to determine the total amount of polyphenols (Boizot & Charpentier, 2006). The procedure given was used to determine the total flavonoids (Dehpour et al., 2009).

Method of chromatographic analysis by HPLC

Before injection, the *Medicago sativa* extracts were filtered. The following experimental conditions were utilized with an HPLC system, with a detector at = 280 nm for the polyphenols and 360 nm for the flavonoids: other experimental condition: The column used is of length 150 mm and diameter 4.6 mm, the stationary phase C18; Mobile phase: A: acetonitrile; B: 2% glacial acetic acid solution (pH = 2.6); Gradient: 0-5min: 5 % A ; 25-30min: 35% A ; 35-45min: 70%A; Debit: 0.5 ml / min; Injection volume: 20 μ L; Temperature: 30 °C.

Antioxidant activity

Add 1.25 ml of the buffer solution (0.2 M, PH = 6.6) to 500 l of a sample. 1.25 potassium ferrioxalate should be added. then 20 minutes of incubation at 50 ° C in a water bath. Add 1.25 cc of the 10% aqueous TCA solution after cooling to terminate the reaction. Centrifugation at 3000 rpm for 5 minutes. After that, 1.25 ml of supernatant, 1.25 ml of distilled water, and 250 l of FeCl₃ (0.1%) are combined. At 700 nm, the absorbance was calculated in comparison to a blank. Following the computation of the inhibition percentage values in accordance

with (Oyaizu., 1986), the following results were obtained by IC50:

$$IP(\%) = 100 - \frac{OD\ control}{OD\ sample} \times 100 \quad (2)$$

Hemolysis assay

A hemolysis assay was performed in accordance with (Vinjamuri et al., 2015). Healthy participants provided 5mL of blood, which was drawn into tubes containing 5.4 mg of EDTA to stop clotting and centrifuged at 1000 rpm for 10 min at 40 °C. The white buffy layer was meticulously aspirated with a pipette after the plasma had been thoroughly removed. The erythrocytes were then washed three more times with 1X PBS, pH 7.4, each time for five minutes. Erythrocytes that had been washed were kept at 4°C and used for the hemolysis assay within 6 hours. The hemolysis assay required 50 L of 10 erythrocytes, which were kept at 4 oC and used within 6 hours. 50 uL of 10 dilutions of erythrocyte suspension (100 uL Erythrocytes suspension: 900 uL 1XPBS) were combined with 100 uL of test samples of *Medicago sativa* (48 g/mL), 100 uL of 1% SDS, and 100 uL of 1XPBS as positive controls. For 60 minutes, the reaction mixture was incubated in a water bath at 37°C. One mL of the reaction mixture's volume was added by adding 850 L of 1XPB. Finally, the mixture was centrifuged at 300 rpm for 3 minutes, and the amount of hemoglobin in the supernatant was determined at 560 nm using a spectrophotometer. Following are the calculations for percentage of hemolysis inhibition (IH):

$$IH(\%) = 100 - (Abs\ control)/(Abs\ sample) \times 100 \quad (3)$$

Acute toxicity testing

The CuNPs were subjected to an acute toxicity test using Lorke's method. All rats were kept fasting for 12 hours before receiving a single intraperitoneal injection of CuNPs (Control, 10 and 20 mg/kg body weight) into each of the three groups of four (n = 5). There were fifteen (15) rats in total. The rats were watched for 24 hours to track both their behavior and mortality. The department of cellular and molecular biology at El-Oued University's ethics committee examined and gave its blessing to all animal experimentation protocols (approval number: 12 EC/DCMB/FNSL/EU2022).

RESULTS AND DISCUSSION

Phytochemical study of *M. sativa* aqueous extract

The findings of phytochemical studies indicate that *M. sativa* aqueous extract is rich in numerous significant chemical components, including flavonoids, phenols, saponins, and terpenoids, but that our plant's extract is made from an alkaloids-reducing ingredient. On the other hand, the findings shown in **Table 1** indicated that the aqueous extract of *M. sativa* was rich in both total phenols and flavonoids.

Table 1. Phytochemical essays for aqueous extract of *M. sativa*

Compounds	Aqueous extract <i>M. sativa</i>
Flavonoids	+

Terpenoids	+
Phenols	+
Tannins	+
Reducing compound	-
Alkaloids	-
Saponins	+
Polyphenols (mg GAEq/g of extract)	322.15 ± 22
Flavonoids (mg QEq/g of extract)	271.3 ± 17.9

(+): Present, (-): Absent

HPLC analysis of *M. sativa* aqueous extract

The results of the HPLC chromatographic study demonstrate that *M. sativa* extract contains a variety of polyphenols in varying amounts, with naringin, rutin, and vanillic acid serving as the primary ingredients (**Figure 2** and **Table 2**).

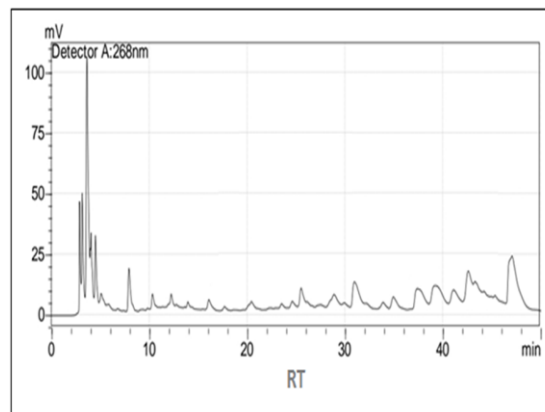


Figure 2. HPLC chromatogram of phenolic compounds of *M. sativa*

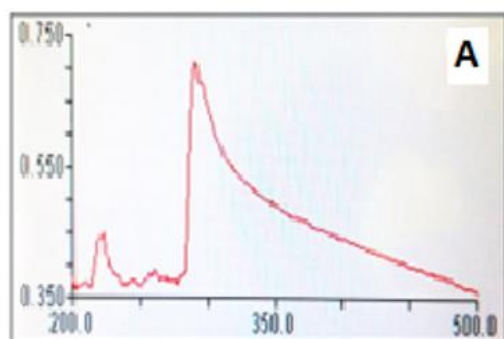
Table 2. Phenolic compounds concentration of *M. sativa* aqueous extract

Composition	Retention time (min)	Concentration (µg/ml)
Gallic Acid	5,29	
Chlorogenic Acid	13,392	1.50
Vanillic Acid	15,531	2.43
Caffeic Acid	16,277	1.940
Vanilin	21,46	0.65
p-Coumaric Acid	23,817	0.3
Rutin	28,37	3.87
Naringin	34,788	12.21
Quercetin	45,047	2.36

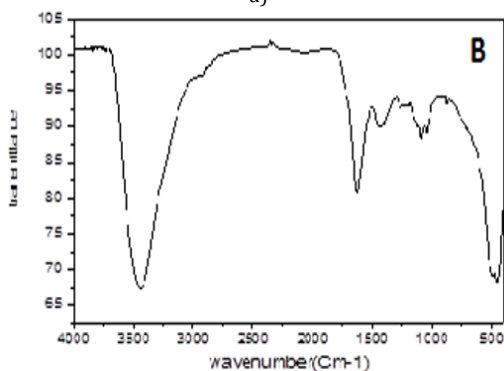
Characterization of copper nanoparticles

By utilizing a UV-visible spectrophotometer to obtain a spectrum in the visible range of 200 nm-700 nm, the presence

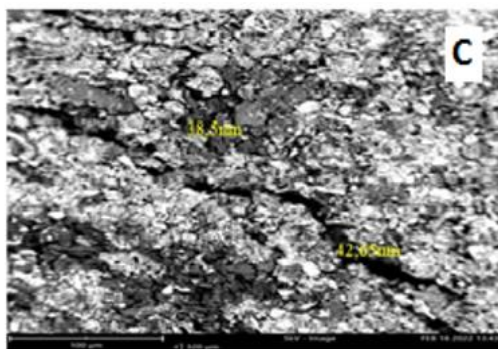
of nanoparticles was confirmed (**Figure 3a**). This investigation revealed an absorbance peak at about 300 that was unique to copper nanoparticles. The chemical make-up of the CuNPs surface was identified and characterized using FTIR spectroscopy. The peak at 3400 cm⁻¹ demonstrated the water and OeH absorption frequency, as can be observed in (**Figure 3b**). It was done using the SEM (JEOL MODEL 6390) technology to see how big and how shaped the copper nanoparticles were (**Figure 3c**). The creation of CuNPs and their morphological characteristics in the SEM study showed that the inter-particle spacing was 38.5 nm on average. The copper nanoparticles' shapes showed to be varied. CuO's XRD analysis demonstrated (**Figure 3d**) the peaks at 18.13°, 19.87°, 22.28°, 25.37°, 25.89°, 26.34°, 36.44°, 78.09° that symbolized by (0).



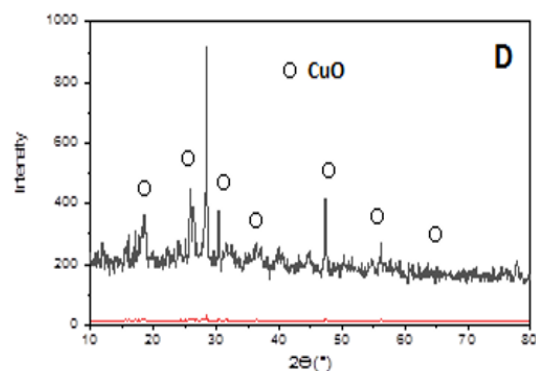
a)



b)



c)

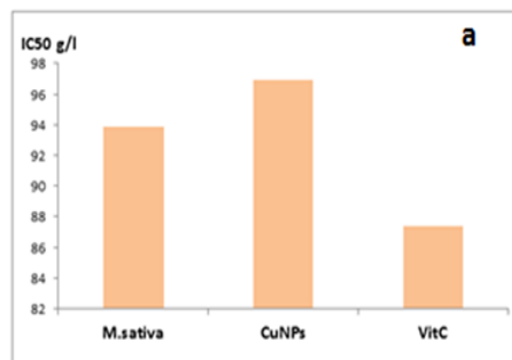


d)

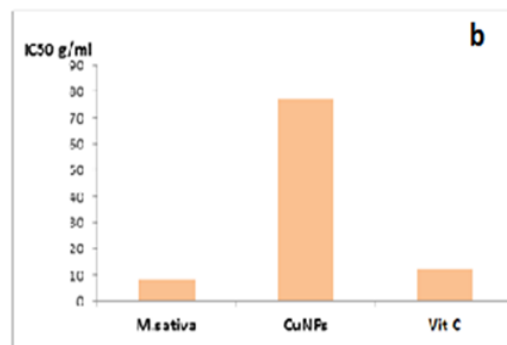
Figure 3. Characterization of copper nanoparticles UV-VIS spectrum (a), FTIR spectrum (b), SEM image (c), and X-ray diffraction diffractograms (d).

Antioxidant activity

Figure 4a reports on the FRAP assay's outcomes. Vitamin C showed the strongest inhibitory efficacy, up to a maximum of 90.68% at a concentration of 0.7 mg/mL, while *M. sativa* Reported a concentration of 93.85% at 0.8 mg/ml and 98.63% at 0.8 mg/ml for Cu NPs. The amount of *M. sativa* extract needed to inhibit free radicals by 50% (IC₅₀) was 17.32 g/ml. IC₅₀ values for *M. sativa* aqueous extract with regression coefficients ($R^2=0.99$). High regression coefficients and IC₅₀ values for the common vitamin C were 4.12 g/mL.



a)



b)

Figure 4. IC₅₀ values for *M. sativa* aqueous extract, CuNPs, and Vitamin C, a for FRAP test and b for hemolysis test

Hemolysis protection

Human blood erythrocyte membrane dehydration and delayed proton equilibria caused by phosphate buffer (XPBS, pH= 7.4) result in membrane degradation and eventual hemolysis. The antihemolytic properties of the different concentrations (0.2-1 mg/mL) of *M. sativa*. The effects of *M. sativa* and CuNPs on human blood erythrocytes were shown in **Figure 4b**. The maximum antihemolysis activity of *M. sativa*'s aqueous extract was 8.60% at a concentration of 1 mg/mL, while CuNPs' antihemolysis activity peaked at 91.11% at the same dose. Contrary to popular belief, antihemolysis action increased with decreasing CuNP content at different concentrations. On the other hand, we observed the exact opposite: at different concentrations of *M. sativa* aqueous extract, the antihemolysis activity increased with increasing concentration. Aqueous extract of *M. sativa* has anti-hemolysis activity with a high regression coefficient ($R^2 = 0.9184$), and regression was also present in CuNPs ($R^2 = 0.5692$).

Acute toxicity study

The test's findings (**Table 3**) demonstrated that physiological parameters were normal throughout the experiment, which involved treating albino Wistar rats with different doses of CuNPs (0, 10, and 20 mg/kg) to affect their eyes, sleep, movement, and diarrhea. Additionally, no unusual symptoms or side effects appeared, and no fatality cases were reported before 14 days. These outcomes supported research demonstrating the medicinal and safe uses of these environmentally friendly Cu nanoparticles.

Table 3. Acute toxicity test of CuNPs on physiological parameters of Wistar albino rats

Parameters	0 h		3 h		24 h		Day- 7		Day-14	
	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
Dead rats	0	0	0	0	0	0	0	0	0	0
Eyes	N	N	N	N	N	N	N	N	N	N
Sleep	N	N	N	N	N	N	N	N	N	N
Movement	N	N	N	N	N	N	N	N	N	N
Diarrhea	N	N	N	N	N	N	N	N	N	N

N: Normal

The findings of phytochemical investigations indicate that *M. sativa*'s aqueous extract contains a variety of significant secondary metabolic compounds, such as flavonoids, phenols, carbohydrates, saponosides, and terpenoids, but does not include tannins or alkaloids. Bora and Sharma (2012) found that the phytochemical finding was identical to our result. The biological features of secondary plant metabolites include antioxidant activity, antibacterial activity, antidiabetic activity, anti-inflammatory activity (Chetehouna *et al.*, 2021), hypolipidemic activity, anxiolytic activity, dermatological activity, immunological activity, and cytotoxic activity (Ali Esmail *et al.*, 2021). According to a prior study, a lot of focus is placed on the effects of saponins found in alfalfa. Studies using

macaques failed to find any evidence of the harmful effect of alfalfa saponins (Raeeszadeh *et al.*, 2021). Saponins have been linked to increased production of bile, gastric, pancreatic, and intestinal fluids as well as a reduction in total cholesterol levels (Samir *et al.*, 2020). The research showed that they may exhibit antimycotic and antibacterial activities depending on their chemical structure, mostly against certain yeast harmful to humans. As a result, they might potentially stimulate the immune system. Alfalfa spawning was found to be most active against Gram-positive bacteria like *Staphylococcus aureus* and *Enterococcus faecalis*. The in-vitro tests proved their effectiveness as anticancer drugs and established their capacity to stop the growth of human leukemia cell lines. The amount of total phenolic and flavonoid components in the aqueous *M. sativa* extract is larger than the amounts noted by Karimi *et al.* (2013), indicating that the concentration is based on the sample's features and the extraction process used.

According to our research, the combination turned black when CuSO₄ was added to green-synthesized CuNPs made from *M. sativa* extract. This, in our opinion, points to the production of CuO. Furthermore, that is comparable to a study (Ouidad *et al.*, 2020). The phenolic components, flavonoids, saponins, carbohydrates, and nargenin that are abundant in our plant extract have antioxidant characteristics and biological reductivity in the creation of nanoparticles. We propose that hydrogen ions are divorced during net diversion because it changes the copper ion status to copper condition while also oxidizing it to generate nanoparticles (Bezza *et al.*, 2020). Compared to the following research, for the enol form of flavonoids to the quito shape, reducing copper ions and the synthesis of CuNPs nanoparticles (Mroczek-Sosnowska *et al.*, 2013).

One of the crucial methods for confirming the initial presence of metal nanoparticles in a liquid media is UV-Vis spectroscopy (Djouadi & Derouiche, 2021). The UV-Vis spectrophotometer was used to further describe the color shift that indicated the presence of copper nanoparticles, and readings were taken at a specific temperature to detect it. The UV-Vis spectra of copper nanoparticle production utilizing *Medicago sativa* nanoparticle extract are visible in the absorption peaks. Peaks in the 200–700 nm spectral lines in the UV-visible spectra were seen as a result of copper colloid's surface plasma resonance at various temperatures. Around 435 nm, there were 300 strong SPR bands. Our findings support the observations that copper nanoparticle formation occurs. Results regarding the FTIR spectra of CuNPs showed peaks at 3500, 1600, and. The band at 3452 cm⁻¹ is said to correspond to the polymeric OH stretching mode. Amido and open chain amino (C N) are represented by the absorption at 1629 cm⁻¹. According to methyl C-H, 1388 cm⁻¹ is the Siam bend. This evidence suggests that the ability to arrange and regulate CuNPs in an aqueous media could be performed by protein particles (Popa *et al.*, 2022). The crystallinity of the synthesized particle structures were revealed by X-ray diffraction (XRD) analysis, which revealed the existence of several sharply pronounced reflections (Shutov *et al.*, 2017). The XRD methodology demonstrated in the work by Emmalai and Velmurugan (2015), which utilized *Azadirachta indica* (L.) for the synthesis of CuO nanoparticles, confirms the environmentally acceptable approach of nanoparticle synthesis. The SEM scan revealed nanoparticles with diameters between 38.5 and 65.5 nm that

were substantially spherical. Memo *et al.*, 2020, who create copper nanoparticles (CuNPs) using an aqueous leaf extract of *Ziziphus mauritiana* L., reported a similar result. Copper nanoparticles demonstrate extraordinary performance as antibacterial and antimicrobial agents (Xinzhen *et al.*, 2021). Because *M. sativa* plants contain a lot of phenolic compounds, flavonoids, and saponins, all of which have high antioxidant potential, their extract has a strong antioxidant impact, according to studies on anti-oxidant activity (Michalak, 2022). According to FRAP experiments, they are powerful free radical scavengers because they reduce iron by proportionally donating hydrogen to the extract concentration (Rafiska *et al.*, 2017). Free radicals are known to quietly contribute to the oxidation of unsaturated fat in foods, but they are also combated by phenolic compounds, flavonoids, and saponins. Our extract has contributed to antioxidant activity because it contains a lot of these components. As CuNPs were used to treat the albino Wister rats' eyes, sleep, movement, and diarrhea during the study period, the results of the acute toxicity test revealed that there were normal physiological parameters during that time. No unusual symptoms, negative side effects, or cases of mortality were recorded before 14 days, either. The uses of these green-synthesized Cu nanoparticles for both medicinal and safe purposes were demonstrated by this study's results, which were confirmed (Atoussi *et al.*, 2020).

CONCLUSION

In conclusion, we were successful in synthesizing high-quality, pure monodispersed CuO nanoparticles using alfalfa extract. Additionally, both the created CuNPs and the alfalfa extract were able to provide powerful antioxidant and anti-inflammatory capabilities.

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ETHICS STATEMENT: The study had the approval of the ethics Committee of the El-Oued University (12 EC/CMB/FNSL/EU2022).

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