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Comprehensive Assessment of Morphological, Biochemical and Molecular Responses of *Melissa officinalis* subsp. *officinalis* L. Grown in Vitro Conditions to Copper Oxide Nanoparticles

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ABSTRACT

Copper oxide nanoparticles (CuO-NPs) were applied in vitro to Melissa officinalis L. subsp. officinalis seedlings (0.1, 1.0 and 10 mg L^{-1}) to probe morphological, biochemical and molecular responses. At 0.1 mg L^{-1}, shoot elongation rose markedly, whereas 10 mg L^{-1} stimulated root proliferation but curtailed shoot growth. The highest dose also maximised total phenolic and flavonoid contents and strengthened CUPRAC, DPPH and ABTS antioxidant activities. HPLC revealed elevated rosmarinic acid with concomitant declines in caffeic and p-coumaric acids, implying pathway redirection. Enzyme assays showed peak AChE inhibition at 1.0 mg L^{-1}, while MAO, urease and HIV-1 RT inhibition intensified with increasing doses. GC-MS demonstrated dose-dependent shifts in essential-oil composition, notably higher geranial and neral. qRT-PCR confirmed up-regulation of PAL, TAT and RAS transcripts, especially at 10 mg L^{-1}, corroborating enhanced phenylpropanoid biosynthesis. Overall, CuO-NPs act as concentration-dependent abiotic elicitors, boosting bioactive metabolite production and antioxidant capacity, and thus can improve lemon balm phytochemical quality under controlled conditions.

Keywords: Antioxidant activity, CuO Nanoparticles, Gene expression, In vitro propagation, *Melissa officinalis* subsp. *officinalis*, Phenolic compounds

In Vitro Koşullarda Yetiştirilen *Melissa officinalis* subsp. *officinalis* L.'nin Bakır Oksit Nanopartiküllerine Karşı Morfolojik, Biyokimyasal ve Moleküler Yanıtlarının Kapsamlı Değerlendirmesi

ÖZ

Melissa officinalis L. subsp. officinalis (oğul otu) fidelerine in vitro koşullarda 0.1, 1.0 ve 10 mg L^{-1} konsantrasyonlarında bakır oksit nanoparçacıkları (CuO-NP) uygulanarak morfolojik, biyokimyasal ve moleküler tepkiler değerlendirilmiştir. Düşük dozda (0.1 mg L^{-1}) sürgün uzaması belirgin şekilde artarken, 10 mg L^{-1} kök gelişimini teşvik etmiş ancak sürgün büyümesini baskılamıştır. En yüksek dozda toplam fenolik ve flavonoid içerikleri artmış, CUPRAC, DPPH ve ABTS testlerinde antioksidan aktiviteler güçlenmiştir. HPLC analizleri, rosmarinik asitte artış ve kafeik ile p-kumarik asitlerde azalma olduğunu göstermiştir; bu da metabolik yol yöneliminin değiştiğini düşündürmektedir. Enzim inhibisyon analizleri, 1.0 mg L^{-1}'de en yüksek AChE inhibisyonunu, daha yüksek dozlarda ise MAO, üreaz ve HIV-1 RT inhibisyonunun arttığını göstermiştir. GC-MS, geranial ve neral gibi bileşenlerin arttığı doz bağımlı uçucu yağ değişimlerini ortaya koymuştur. qRT-PCR sonuçları, özellikle 10 mg L^{-1}'de PAL, TAT ve RAS genlerinin yukarı yönlü düzenlendiğini göstermiştir. Genel olarak, CuO-NP'ler biyotik olmayan uyarıcılar olarak işlev görmekte ve fitokimyasal kaliteyi artırma potansiyeli sunmaktadır.

Anahtar Kelimeler: Antioksidan aktivite, CuO Nanopartikül, Fenolik bileşikler, Gen ekspresyonu, İn vitro propagasyon, *Melissa officinalis* subsp. *officinalis*

INTRODUCTION

Over the last few decades, the convergence of nanotechnology and plant biotechnology has given rise to new opportunities for the enhancement of beneficial chemical production in medicinal plants. Engineered nanomaterials-particularly metal and metal oxide nanoparticles-are increasingly utilized in plant tissue cultures due to their reactivity and biodegradation potential [1,2]. Among these, copper oxide nanoparticles

(CuO-NPs) are particularly noteworthy due to their distinct physical and chemical properties and their dual role as a vital nutrient as well as a potential stressor in plants. Copper (Cu) is a trace element that plays a vital role in several plant enzyme functions and redox reactions. It participates in vital activities such as photosynthesis, respiration, lignin synthesis, and antioxidant defense through major copper-dependent enzymes such as plastocyanin, cytochrome c oxidase, and superoxide dismutase [3].

In spite of the significance of Cu in the plant system, levels of Cu must remain strictly regulated. Too intense Cu levels within the plant can lead to oxidative stress through the enhanced production of toxic reactive oxygen species (ROS), damaging cell structures and hindering the process of photosynthesis [3,4]. Because of their minute size and huge surface area, CuO-NPs are capable of releasing easily assimilatable Cu^{2+} ions and directly interacting with plant tissues. Such interaction will largely depend on the concentration and properties of nanoparticles. Various studies confirm that low doses of CuO-NPs improve germination of the seeds, root growth, and absorption of nutrients in a variety of plant species [5,6]. In contrast to this, high concentrations are found to result in reduced plant growth, toxicity, and even DNA damage [1,7,8]. These impacts differ for plant species and nanoparticle characteristics in the form of size, shape, and surface properties. Still, we have a developing body of knowledge about how CuO-NPs affect overall plant metabolism-especially in medical plants that contain healing compounds.

Melissa officinalis L. subsp. *officinalis*, or lemon balm, is a perennial mint plant species (Lamiaceae) that has earned fame for its therapeutic properties in the form of antioxidant, antimicrobial, spasmodic, and neuroprotective activities [9,10]. These activities are due to naturally occurring compounds such as rosmarinic acid, caffeic acid, flavonoids, and other active compounds. Environmental stress in the form of oxidative conditions can affect the synthesis of these products [11].

The tissue culture of plants allows for precise plant growth and the increase in their bioactive compounds in a controlled laboratory setting. It also enables the selective application of nanoparticles to determine their impact [12]. Such in vitro systems are perfect for assessing how CuO-NPs impact plant growth, metabolism, and gene function. While several studies have examined CuO-NP toxicity and uptake in several crops and model organisms [6], we have little focused work in *M. officinalis*, particularly in a laboratory-controlling setting in which external influences are reduced. Further, we have little insight into how CuO-NPs affect the metabolic pathways and gene expressions that produce the secondary compounds in the form of phenylpropanoids.

This study aims to bridge that gap by conducting an in-depth analysis of the response of *Melissa officinalis* L. subsp. *officinalis* seedlings to varied levels of CuO-NPs in a laboratory condition. It targets the development of shoots and roots, antioxidant potential, phenolic and flavonoid content levels, inhibition of enzymes (acetylcholinesterase, urease, monoamine oxidase (MAO-A)), and the regulation of important genes (phenylalanine ammonia-lyase (PAL), tyrosine aminotransferase (TAT), and rosmarinic acid synthase (RAS)) involved in the synthesis of compounds. The information obtained both identifies the beneficial as well as stressful functions CuO-NPs might have and implies that nanotechnology has the potential to harness nanomaterials as high-value tools for enhancing plants' medical properties.

MATERIALS AND METHODS

Pre-Treatment and In Vitro Culture of *M. officinalis* Seeds

M. officinalis seeds were sterilized through a modified method by Bektaş and Sökmen (2016) [13]. The seeds were first soaked for 12 hours at room temperature in a 5% sucrose solution with a few drops of bleach. They were then briefly treated with a 30% hydrogen peroxide solution for five minutes. After three successive rinses with sterile distilled water, the seeds were placed on Murashige and Skoog (MS) medium, supplemented with vitamins, to promote germination. Cultures were incubated at 25 ± 1°C under a 16/8 h period. Once healthy seedlings had developed-after approximately four weeks-they were used for the next phase of experimentation.

Copper Oxide Nanoparticle (CuO-NP) Treatment

To assess the effect of copper oxide nanoparticles on *M. officinalis* growth, in vitro seedlings were transferred to fresh MS media containing CuO-NPs at concentrations of 0 (control), 0.1, 1.0, or 10.0 mg/L. The CuO-NPs used in this study were purchased from Nanografi Nanotechnology Company (Ankara, Turkey). The product consisted of high-purity (≥99.995%) CuO-NPs with a particle size distribution ranging from 15 to 45 nm. A small quantity of kinetin (0.1 mg/L) was also added to promote shoot development. The control group was grown on MS medium with kinetin, but without CuO-NPs. Although CuO-NPs were originally prepared in five concentrations (0.1, 1.0, 10.0, 100.0, and 1000.0 mg/L), seedlings exposed to the two highest concentrations (100.0 and 1000.0 mg/L) did not survive or show development under in vitro conditions. As a result, only the 0.1, 1.0, and 10.0 mg/L treatments were selected for further physiological, biochemical, and molecular evaluation.

Explants were cultured for 30 days under the same temperature and light conditions. At the end of the treatment period, plant growth parameters-including shoot length, leaf and node numbers, root count, root length, and both fresh and dry weights-were measured. Plant tissues were harvested, flash-frozen in liquid nitrogen, and stored at -80 °C until analysis. Before

testing, samples were freeze-dried and ground into a fine powder.

Phytochemical Analysis

Metabolite Extraction

The powdered plant samples were extracted using high-purity methanol at 40 °C for 12 hours with continuous stirring under reflux conditions to preserve volatile compounds. After the extraction, the solutions were cooled, filtered, and stored at -20 °C. These extracts were later analyzed to determine phenolic composition, total phenolics and flavonoids, antioxidant capacity, and enzyme inhibition activity.

HPLC Profiling

To identify individual phenolic compounds, the extracts were subjected to high-performance liquid chromatography (HPLC). A Thermo Fisher Dionex Ultimate 3000 system equipped with a C18 reversed-phase column was used. The mobile phase included a gradient of two solvents: one consisting of water with 2% acetic acid, and the other of acetonitrile with 0.5% acetic acid. Each chromatographic run lasted approximately 45 minutes, with a flow rate of 1.2 mL/min and a column temperature maintained at 30 °C. Eighteen standard compounds, including a variety of acids, flavonoids, and phenylpropanoids, were used to identify components in the samples. Concentrations were determined from calibration curves and expressed as micrograms per gram of dry plant weight.

Total Phenolic and Flavonoid Content

Total phenolic content was measured using the Folin–Ciocalteu method, with absorbance recorded at 760 nm [14]. Results were expressed in milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g DW). Total flavonoid content was assessed using the aluminum chloride method, measuring absorbance at 415 nm [15], and expressed in milligrams of quercetin equivalent per gram (mg QE/g DW).

Antioxidant Assays

Three different in vitro methods were used to evaluate the antioxidant activity of the plant extracts. The CUPRAC method was employed to measure the antioxidant capacity based on the reduction of copper(II) ions, following the procedure developed by Apak et al. (2004) [16]. In this assay, the formation of a colored Cu(I)-neocuproine complex was monitored at an absorbance of 450 nm, and results were expressed in micromoles of Trolox equivalents per gram of dry plant tissue.

To assess the radical scavenging ability of the samples, the DPPH assay was used with the stable DPPH• radical. The plant extracts were mixed with a methanolic DPPH solution and incubated at room temperature. Absorbance was then measured at 517 nm. The SC_{50} value, indicating the concentration of extract needed to reduce 50% of the radicals, was calculated using a logarithmic equation derived from a calibration curve [17].

Similarly, the ABTS assay measured the extract's ability to scavenge the $ABTS^{\bullet+}$ radical. The ABTS radical solution was prepared by reacting ABTS with potassium persulfate and incubating it in darkness for 16-18 hours. Before use, the solution was diluted to an absorbance of 0.700 ± 0.020 at 734 nm. The plant extracts were then added and incubated for 5 minutes before absorbance was recorded at 734 nm. The SC_{50} values were calculated as described by Re et al. (1999)[18]. Trolox served as the standard positive control in all three antioxidant assays.

Enzyme Inhibition Assays

The enzyme inhibitory effects of the plant extracts were evaluated using standard in vitro protocols. Acetylcholinesterase (AChE) inhibition was measured using Ellman's colorimetric method, where the formation of a yellow-colored complex was monitored at 412 nm [19]. A lower absorbance reading indicated stronger enzyme inhibition. Galantamine was used as the reference inhibitor in this assay.

Monoamine oxidase-A (MAO-A) inhibition was assessed using a commercial assay kit (Sigma MAK520-1KT). A decrease in absorbance at 490 nm was interpreted as enzyme inhibition, with clorgyline serving as the positive control [20].

Urease activity was measured by detecting ammonia released from urea, using phenol red as the pH indicator. A reduction in color intensity at 570 nm indicated effective inhibition [21]. Thiourea was used as the standard inhibitor for this assay.

The inhibition of HIV-1 reverse transcriptase (RT) was tested with a commercial ELISA kit from Roche. After a 1-hour incubation at 37 °C, absorbance was recorded at 405 nm. Nevirapine served as the positive control [22].

Analysis by GC-MS for Volatile Compounds

Volatile compounds in the freeze-dried *M. officinalis* seedlings were analyzed using gas chromatography–mass spectrometry (GC-MS) with a headspace sampling approach. Roughly 2 grams of powdered plant material were combined with a saturated sodium chloride (NaCl) solution and sealed in a vial, then incubated at 60 °C for 10 minutes to allow volatile collection.

The gas chromatograph was equipped with a BPX-90 high-polarity column, and an optimized temperature ramp was applied during the analysis. Mass spectra obtained were compared against the NIST library and confirmed with authentic chemical standards when available. The relative quantity of each volatile compound was determined based on its peak area in the chromatogram. Key compounds such as geranial and neral were identified and their levels compared across different treatment groups.

Gene Expression Analysis

To explore how CuO-NP treatment affects molecular responses, gene expression levels related to phenolic biosynthesis were measured using quantitative real-time PCR (qRT-PCR). Approximately 100 mg of frozen plant tissue was used to extract total RNA using the Spectrum™ Plant Total RNA Kit (Sigma, STRN50). The quality and integrity of the extracted RNA were checked through spectrophotometry and agarose gel electrophoresis.

One microgram of RNA was then used to synthesize cDNA using a standard reverse transcription kit and oligo(dT)$_{18}$ primers. The reaction took place at 42 °C for 60 minutes and was then heat-inactivated at 70 °C. Quantitative PCR was performed using SYBR Green chemistry on a Bio-Rad real-time PCR system.

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Table 1. Designed specific primers and their properties

Gene	Primer Name	Primer Sequence (5'-3')	Amplicon Size (bp)
PAL	PAL-F	AGCCTGGACTACGGCTTC	122
	PAL-R	ACATCCTGGTTATGCTGCTC	
TAT	TAT-F	GCTACCAGCCGACTCTGTC	122
	TAT-R	CGTAGATTGGGAAACACGGTC	
RAS	RAS-F	GTKGRRAACGAGCACCAC	122
	RAS-R	AGGGMSKSCGGTCGAAAG	
GAPDH	GAPDH-F	GCTAAGGCTGTTGGTAAAGTGC	121
	GAPDH-R	TGGCCTCTTTCTCTAGCCTGAC	

Primers were specifically designed to amplify three target genes: phenylalanine ammonia-lyase (PAL, GenBank AC: FN665700.1), tyrosine aminotransferase (TAT, GenBank AC: JN863949.1), and rosmarinic acid synthase (RAS, GenBank AC: KM575933.1) (Table 1). The GAPDH (Genbank AC: KF013133.1) gene served as the internal reference. Amplification was carried out over 40 cycles, and a melting curve analysis confirmed the specificity of the PCR products. Gene expression levels were calculated using the 2-ΔΔCt method

Statistical Analysis

All experiments were repeated at least three times independently. Results are reported as mean ± standard deviation. Statistical significance was determined using one-way ANOVA, followed by Tukey's post-hoc test ($p<0.05$). All analyses were performed using SPSS version 25.0.

RESULTS

Effects of CuO-NPs on In Vitro Plant Growth Parameters

The application of CuO nanoparticles (CuO-NPs) had notable, dose-dependent effects on various growth parameters of *M. officinalis* seedlings cultured in vitro (Table 2, Figure 1). Among the measured traits, shoot elongation exhibited the highest value at 0.1 mg/L CuO-NP treatment, reaching 101.73 ± 6.11 mm, which was significantly greater than that of the control group treated with kinetin (59.09 ± 2.04 mm; $p < 0.05$). However, higher CuO-NP concentrations led to reduced shoot length, with 1.0 and 10.0 mg/L treatments resulting in 64.02 ± 3.53 mm and 56.90 ± 1.09 mm, respectively.

Although five CuO-NP concentrations (0.1, 1.0, 10.0, 100.0, and 1000.0 mg/L) were initially tested, no seedling growth was observed at 100.0 and 1000.0 mg/L under in vitro conditions (Figure 1 D and E). As a result, only the 0.1, 1.0 and 10.0 mg/L concentrations were evaluated for further physiological and biochemical analysis.

Leaf number showed a slight decline with increasing CuO-NP concentration. While the highest number of leaves (10.88 ± 1.00) was recorded at 0.1 mg/L, it decreased to 8.00 ± 0.00 at 1.0 mg/L and 7.66 ± 0.75 at 10.0 mg/L. A similar trend was observed in nod number, which declined from 5.44 ± 0.50 (0.1 mg/L) to 3.83 ± 0.37 (10.0 mg/L), and was lower than the control (4.15 ± 0.31). The number of shoots per node (tillering) remained unchanged at 2.00 ± 0.00 across all treatment groups, including the control.

In terms of rooting performance, the highest number of roots was observed at 10.0 mg/L CuO-NPs (18.08 ± 0.96), followed by the control (15.14 ± 0.68), while the lowest values were recorded at 0.1 and 1.0 mg/L (11.36 ± 0.83 and 11.53 ± 0.84, respectively). In contrast, root elongation was most pronounced at 1.0 mg/L CuO-NPs (179.68 ± 10.30 mm), which significantly exceeded the control (69.11 ± 2.04 mm) and all other treatments ($p < 0.05$). Root length was shortest at 10.0 mg/L (34.66 ± 1.34 mm), suggesting a concentration-dependent suppression of elongation despite enhanced root proliferation.

Dry matter accumulation (% dry weight) also varied with CuO-NP dose. The control group exhibited the highest dry weight percentage (14.38 ± 0.57%), whereas values declined slightly with increasing nanoparticle concentration. Specifically, the 10.0 mg/L CuO-NP group reached 13.53 ± 0.85%, followed by 1.0 mg/L (12.13 ± 0.77%) and 0.1 mg/L (10.66 ± 0.64%).

Table 2. Growth responses of *M officinalis* seedlings to varying concentrations of CuO-NPs.

CuO-NP Concent. (mg/L)	Shoot Elongation (mm)	Number of Leaves (pcs)	Number of Nodes (pcs)	Shoot Multiplicatio	Number of Roots (pcs)	Root Lenght (mm)	% Dry Weight
0,1	101,73±6,11^{a}	10,88±1,00^{a}	5,44±0,50^{a}	2,00±0,00^{a}	11,36±0,83^{a}	116,00±3,17^{b}	10,66±0,64^{d}
1,0	64,02±3,53^{b}	8,00±0,00^{c}	4,00±0,00^{c}	2,00±0,00^{a}	11,53±0,84^{a}	179,68±10,30^{a}	12,13±0,77^{c}
10,0	56,90±1,09^{e}	7,66±0,75^{c}	3,83±0,37^{c}	2,00±0,00^{a}	18,08±0,96^{a}	34,66±1,34^{d}	13,53±0,85^{b}
Control*	59,09±2,04^{c}	8,40±0,67^{b}	4,15±0,31^{b}	2,00±0,00^{a}	15,14±0,68^{b}	69,11±2,04^{c}	14,38±0,57^{a}

Data are presented as mean ± SD (n=15). Different letters indicate significant differences at $p < 0.05$ according to Tukey's test. * Medium containing 0.1 mg/L KIN was considered as control group and all media containing nanoparticles were also supplemented with 0.1 mg/L KIN.

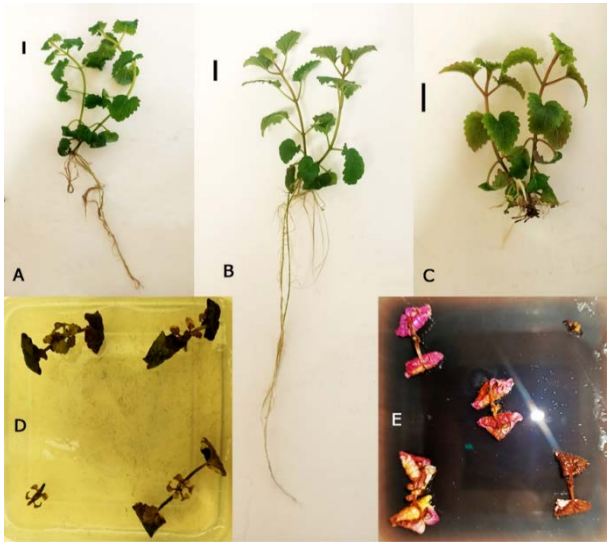

Figure 1. Representative images of *M. officinalis* seedlings after 30 days of in vitro culture under different concentrations of copper oxide nanoparticles (CuO-NPs). A: 0.1 mg/L, B: 1.0 mg/L, C: 10.0 mg/L, D: 100.0 mg/L, E: 1000.0 mg/L. Seedlings grown at 0.1 and 1.0 mg/L exhibited vigorous shoot and root development, while higher concentrations (100.0 and 1000.0 mg/L) led to severe growth inhibition and tissue necrosis. Scale bar = 1 cm.

These findings indicate that low-dose CuO-NP treatment (0.1 mg/L) promotes aerial growth, particularly shoot elongation and leaf formation, while moderate doses (1.0 mg/L) enhance root elongation. High concentrations (10.0 mg/L) appear to stimulate root induction but limit both root elongation and shoot development, demonstrating a biphasic response in plant morphogenesis.

Total Phenolic and Flavonoid Content

The total phenolic content (TPC) and total flavonoid content (TFC) of *M. officinalis* seedlings were significantly influenced by CuO-NP treatments in a dose-dependent manner ($p < 0.05$). results were presented in Table 3. The highest TPC was observed in the 10.0 mg/L CuO-NP group, reaching 43.453 ± 0.80 mg GAE/g plant, which represented a substantial increase compared to the control group (32.687 ± 0.32 mg GAE/g plant). Conversely, the lowest TPC was recorded at 0.1 mg/L CuO-NP (19.391 ± 0.17 mg GAE/g plant), indicating that low concentrations may not sufficiently trigger phenolic accumulation.

Similarly, TFC levels peaked in the 10.0 mg/L CuO-NP treatment group (1.716 ± 0.03 mg QE/g plant), followed by the control (1.612 ± 0.03 mg QE/g plant), while the lowest value was detected at 0.1 mg/L (1.579 ± 0.01 mg QE/g plant). Notably, both TPC and TFC levels were enhanced most markedly at the highest CuO-NP concentration, suggesting that stronger elicitation at elevated nanoparticle levels may stimulate the phenylpropanoid pathway and flavonoid biosynthesis. Statistically significant differences were observed

between all treatment groups ($p<0.05$), confirming the concentration-specific response of phenolic and flavonoid metabolism to CuO-NP exposure.

Table 3. Total phenolic content (TPC) and total flavonoid content (TFC) in methanolic extracts of *M. officinalis* treated with CuO-NPs.

Concentration (mg/L)		TPC (mgGAE/g plant)	TFC (mgQE/g plant)
CuO-NP	**0,1**	19,391±0,17^{d}	1,579±0,01^{b}
	1,0	23,036±0,75^{c}	1,632±0,03^{ab}
	10,0	43,453±0,80^{a}	1,716±0,03^{a}
Control*		32,687±0,32^{b}	1,612±0,^{03ab}

*Data are presented as mean ± SD (n=15). Different letters indicate significant differences at $p < 0.05$ according to Tukey's test. * Medium containing 0.1 mg/L KIN was considered as control group and all media containing nanoparticles were also supplemented with 0.1 mg/L KIN.

HPLC Profiling of Phenolic Compounds

HPLC analysis of *M. officinalis* seedlings exposed to CuO-NPs revealed considerable variation in the levels of individual phenolic compounds, indicating a compound-specific and concentration-dependent response to nanoparticle treatment. A total of ten phenolic compounds were identified and quantified, including phenolic acids, aldehydes, and flavonoids (Table 4).

Rosmarinic acid was the predominant compound across all treatments. Its concentration increased markedly in plants exposed to 10.0 mg/L CuO-NPs (20.796 ± 0.448 µg/g plant) compared to the control group (14.435 ± 0.365 µg/g plant), indicating enhanced biosynthesis under strong elicitor activity. A moderate increase was also observed at 1.0 mg/L (10.477 ± 0.744 µg/g), whereas the lowest value was recorded at 0.1 mg/L (9.148 ± 0.206 µg/g).

Caffeic acid content showed a clear decreasing trend in all CuO-NP-treated groups compared to the control. While control seedlings accumulated 41.92 ± 1.38 µg/g plant of caffeic acid, the levels dropped significantly to 17.49 ± 0.17, 16.67 ± 0.37, and 20.99 ± 2.42 µg/g in the 0.1, 1.0, and 10.0 mg/L CuO-NP groups, respectively. A similar reduction pattern was noted for p-coumaric acid, with a maximum concentration of 34.72 ± 0.92 µg/g in the control, and notably lower levels (13.87 ± 0.03 to 12.17 ± 0.25 µg/g) in treated plants.

Ferulic acid levels slightly increased at 10.0 mg/L CuO-NP (8.09 ± 0.24 µg/g), while remaining similar to the control (7.98 ± 0.16 µg/g). In contrast, gallic acid showed a peak at 1.0 mg/L (3.29 ± 0.05 µg/g), being higher than both the control (1.6 ± 0.05 µg/g) and the 10.0 mg/L group (1.32 ± 0.10 µg/g).

Table 4. Phenolic compound profiles of *M. officinalis* extracts determined by HPLC.

CuO-NP (mg/L)	Gallic acid	Protocatechuic acid	Protocatechuic Aldehyde	Caffeic acid	Syringic acid	Epicatechin	p-Coumaric acid	Ferulic acid	Benzoic acid	Rosmarinic acid
	µg phenolic/g plant									mg phenolic/g plant
0,1	2,53±0,02^{b}	n.d.	4,77±0,04^{b}	17,49±0,17^{bc}	1,89±0,02^{c}	n.d.	13,87±0,03^{b}	1,89±0,00^{d}	28,77±0,51^{b}	9,14± 0,20^{d}
1,0	3,29±0,05^{a}	n.d.	4,9±0,08^{b}	16,67±0,37^{c}	4,46±0,33^{b}	n.d.	12,17±0,25^{c}	3,82±0,09^{c}	28,77±0,39^{b}	10,47±0,74^{c}
10,0	1,32±0,10^{d}	n.d.	5,13±0,32^{b}	20,99±2,42^{b}	1,80±0,31^{c}	n.d.	13,55±0,42^{b}	8,09±0,24^{a}	32,3±1,89^{a}	20,79±0,44^{a}
Control	1,63±0,04^{c}	6,49±0,15^{a}	6,18±0,14^{a}	40,74±1,24^{a}	6,84±0,18^{a}	10,24±0,12^{a}	32,16±0,75^{a}	7,48±0,14^{b}	24,17±0,58^{c}	13,67±0,29^{b}

Data are presented as mean ± SD (n=15). Different letters indicate significant differences at $p < 0.05$ according to Tukey's test. * Medium containing 0.1 mg/L KIN was considered as control group and all media containing nanoparticles were also supplemented with 0.1 mg/L KIN. n.d: Not determined.

Interestingly, protocatechuic acid and epicatechin were only detected in control plants, indicating possible

suppression of their biosynthesis under CuO-NP stress. Protocatechuic aldehyde, however, was present across all treatments, with relatively stable values ranging between 4.77–5.13 µg/g.

The accumulation of benzoic acid remained fairly constant across all groups (25.49–32.3 µg/g), although a slight elevation was noted at 10.0 mg/L CuO-NP. These shifts in phenolic profiles suggest that CuO-NPs selectively affect the phenylpropanoid pathway, upregulating rosmarinic acid while downregulating precursors like caffeic and p-coumaric acids, potentially through pathway redirection or feedback inhibition mechanisms.

Antioxidant Activity

The antioxidant potential of *M. officinalis* seedlings treated with CuO-NPs was evaluated using three independent assays: CUPRAC, DPPH radical scavenging, and ABTS radical cation decolorization. All assays revealed significant differences among treatments ($p < 0.05$), indicating a concentration-dependent response to CuO-NP exposure (Table 5).

According to the CUPRAC results, antioxidant capacity increased markedly with CuO-NP concentration. The highest CUPRAC value was recorded at 10.0 mg/L CuO-NPs (504.3 ± 5.51 µmol TE/g plant), exceeding the control group (454.5 ± 2.04 µmol TE/g). Lower antioxidant activity was observed at 1.0 and 0.1 mg/L CuO-NPs, with values of 275.3 ± 0.58 and 256.3 ± 0.58 µmol TE/g, respectively, suggesting suboptimal elicitor effects at lower doses.

In the DPPH assay, lower SC_{50} values reflect higher radical scavenging capacity. CuO-NP-treated seedlings at 10.0 mg/L showed the most effective DPPH scavenging activity, with an SC_{50} of 68.67 ± 1.23 µg/mL, substantially better than the control group (83.97 ± 0.40 µg/mL). Moderate and low concentrations (1.0 and 0.1 mg/L) yielded SC_{50} values of 161.31 ± 8.73 and 231.88 ± 11.02 µg/mL, respectively, indicating reduced effectiveness.

Table 5. Antioxidant activities (CUPRAC, DPPH SC_{50}, ABTS SC_{50}) of extracts obtained from CuO-NP-treated *M. officinalis* seedlings.

CuO-NP Concentration (mg/L)	CUPRAC (µmol TE/g plant)	DDPH (SC_{50}: µg plant/mL)	ABTS (SC_{50}: mg plant/mL)
0,1	256,3±0,58^{d}	231,8±11,02^{b}	3,8±0,22^{d}
1,0	275,3±0,58^{c}	161,3±8,73^{c}	4,5±0,08^{bc}
10,0	504,3±5,51^{a}	68,6±1,23^{d}	4,0±0,18^{cd}
Control*	454,5±2,04^{b}	83,9±0,40^{d}	4,5±0,29^{b}
Trolox	n.t.	6,0±0,27^{a}	0,3±0,00^{a}

Data are presented as mean ± SD (n=15). Different letters indicate significant differences at $p < 0.05$ according to Tukey's test. Medium containing 0.1 mg/L KIN was considered as control group and all media containing nanoparticles were also supplemented with 0.1 mg/L KIN. n.t: Not tested.

A similar trend was observed in the ABTS assay. The lowest SC_{50} value was obtained at 10.0 mg/L CuO-NPs (4.05 ± 0.18 mg/mL), demonstrating enhanced antioxidant activity compared to the control (4.54 ± 0.29 mg/mL). Treatments with 0.1 and 1.0 mg/L resulted in ABTS SC_{50} values of 3.82 ± 0.22 and 4.51 ± 0.08 mg/mL, respectively, with the latter being comparable to the control.

These results collectively indicate that CuO-NPs, particularly at 10.0 mg/L, effectively enhance the antioxidant potential of *M. officinalis* seedlings, as reflected by increased reducing power and improved free radical scavenging capacity. The enhanced activities likely result from elevated phenolic compound accumulation, especially rosmarinic acid, as confirmed by HPLC analysis.

Enzyme Inhibitory Activities

The enzyme inhibitory activities of *M. officinalis* extracts obtained from CuO-NP-treated seedlings were evaluated against acetylcholinesterase (AChE), monoamine oxidase (MAO), urease, and HIV-1 reverse transcriptase (RT). All enzyme assays demonstrated a concentration-dependent response to CuO-NP exposure, with significant differences among treatment groups ($p < 0.05$) (Table 6).

For AChE inhibition, the extract obtained from plants treated with 1.0 mg/L CuO-NPs exhibited the strongest activity, with an IC_{50} of 50.40 ± 1.06 mg/mL, which was more potent than the control (57.45 ± 2.75 mg/mL). At 0.1 mg/L, inhibitory activity was weaker (65.76 ± 0.48 mg/mL), and a further decline in efficacy was observed at 10.0 mg/L (78.29 ± 2.70 mg/mL), indicating reduced AChE interaction at higher CuO-NP concentrations.

In terms of MAO inhibition, the most pronounced effect was seen at 10.0 mg/L CuO-NPs with an IC_{50} of 2.33 ± 0.07 mg/mL, followed by 1.0 mg/L (3.20 ± 0.02 mg/mL) and 0.1 mg/L (4.58 ± 0.18 mg/mL). These results suggest that CuO-NPs exert a dose-dependent inhibitory effect on MAO activity. The control group exhibited a moderate level of inhibition with an IC_{50} of 3.68 ± 0.11 mg/mL.

Urease inhibition also followed a clear dose-responsive trend. While the control extract showed an IC_{50} of 10.48 ± 0.90 mg/mL, CuO-NP treatment at 10.0 mg/L significantly improved inhibition, yielding the lowest IC_{50} value of 2.93 ± 0.06 mg/mL. Lower doses showed reduced activity, with IC_{50} values of 6.05 ± 0.17 mg/mL at 1.0 mg/L and 13.36 ± 0.78 mg/mL at 0.1 mg/L.

For HIV-1 reverse transcriptase (RT), all CuO-NP-treated groups exhibited mild to moderate inhibition. The highest activity was recorded at 10.0 mg/L (26.22 ± 1.87%), marginally higher than the control group (25.48 ± 1.11%). The lowest inhibition was observed at 0.1 mg/L (18.98 ± 1.70%), while 1.0 mg/L showed intermediate activity (21.04 ± 1.71%). Nevirapin, used as a positive control, displayed a high inhibition level (93.98

± 2.94%), but was marked as t.e. (tested but excluded from comparative analysis due to mechanistic differences).

Table 6. Inhibitory activities (IC_{50} values) against AChE, MAO-A, urease, and HIV-1 RT enzymes for extracts from CuO-NP-treated seedlings.

CuO-NP (mg/L)	Anti-AChE (IC_{50}:mg plant/mL)	MAO-A (IC_{50}: mg plant/mL)	HIV-1-RT (%inhibition)	Urease (IC_{50}: mg plant/mL)
0,1	65,76±0,48^{b}	4,58±0,18^{a}	18,98±1,70^{c}	13,36±0,78^{a}
1,0	50,40±1,06^{d}	3,20±0,02^{c}	21,04±1,71^{c}	6,05±0,17^{c}
10,0	78,29±2,70^{a}	2,33±0,07^{d}	26,22±1,87^{b}	2,93±0,06^{d}
Control*	57,45±2,75^{c}	3,68±0,11^{b}	25,48±1,11^{b}	10,48±0,90^{b}
Nevirapin	n.t.	n.t.	93,98±2,94^{a}	n.t.

Data are presented as mean ± SD (n=15). Different letters indicate significant differences at $p < 0.05$ according to Tukey's test. *Medium containing 0.1 mg/L KIN was considered as control group and all media containing nanoparticles were also supplemented with 0.1 mg/L KIN. n.t: Not tested.

Taken together, these findings indicate that CuO-NPs exert selective, concentration-dependent inhibitory effects on enzyme activity. While moderate concentrations (1.0 mg/L) were most effective for AChE, higher doses (10.0 mg/L) provided superior inhibition of MAO, urease, and HIV-1 RT. These bioactivities may be associated with CuO-NP-induced accumulation of specific polyphenolic compounds with known inhibitory properties, such as rosmarinic and ferulic acids.

Volatile Compounds (GC-MS Analysis)

The GC-MS analysis of *M. officinalis* essential oils revealed distinct qualitative and quantitative variations in volatile compounds among the control group (0.1 mg/L kinetin) and CuO-NP-treated groups (0.1, 1.0, and 10.0 mg/L) (Table 7). Various constituents were detected, with geranial, neral, verbenol, and limonene oxide being among the dominant compounds across all samples.

Table 7. GC-MS-identified essential oil components (%) of *M. officinalis* under CuO-NP treatments.

Compound	RI	Cont. *	0.1 mg/L	1.0 mg/L	10.0 mg/L
Valeraldehyde	625	n.d.	n.d.	0.23	n.d.
Hex-2(E)-enal	854	0.82	0.40	n.d.	n.d.
Nona-2(E),6(E)-dienal	859	n.d.	0.73	n.d.	n.d.
Hept-5-en-2-one <6-methyl->	986	0.36	0.62	0.50	n.d.
Geranyl acetone	994	n.d.	n.d.	n.d.	0.36
Myrcene	996	0.16	n.d.	n.d.	n.d.
Cymene <para->	1024	0.13	n.d.	n.d.	n.d.
Limonene	1029	1.19	n.d.	n.d.	n.d.
Linalool	1104	0.26	0.80	0.62	n.d.
Verbenol	1120	1.76	2.14	3.01	1.72
Citronellal	1159	0.14			
Limonene oxide <trans->	1171	1.20	1.07	1.81	1.11
Acetovanillone	1182	0.38	n.d.	0.92	n.d.
Pent-2(E)-enal	1208	0.07	n.d.	n.d.	n.d.
Oct-7-enol <3,7-dimethyl->	1240	0.24	n.d.	n.d.	n.d.
Caprylaldehyde	1241	n.d.	n.d.	0.24	n.d.
Neral	1250	29.16	32.57	32.36	29.65
Nerol	1264	4.48			
Geranial	1281	51.71	60.16	57.12	66.35
Citronellyl acetate	1288	0.13	n.d.	n.d.	n.d.
Lavandulol	1311	0.05	n.d.	n.d.	n.d.
Geraniol	1332	0.53	n.d.	n.d.	n.d.
Eugenol	1367	0.11	n.d.	n.d.	n.d.

Geranyl acetate	1392	0.74	n.d.	n.d.	n.d.
Verbenone	1428	0.23	n.d.	0.99	n.d.
Caryophyllene <beta->	1430	1.73	n.d.	n.d.	0.81
Aromadendrene	1472	0.11	n.d.	n.d.	n.d.
Isovalerate <allyl->	1511	0.10	n.d.	0.28	n.d.
Heptylidene acetone	1546	0.10	n.d.	n.d.	n.d.
Caryophyllene oxide	1584	n.d.	n.d.	0.62	n.d.
Bergamotol <(Z)-, alpha-trans->	1949	0.15	n.d.	n.d.	n.d.
Viridiflorol	2408	2.75	n.d.	n.d.	n.d.

*Medium containing 0.1 mg/L KIN was considered as control group and all media containing nanoparticles were also supplemented with 0.1 mg/L KIN.

Geranial was the major compound, accounting for 51.71% in the control and increasing with CuO-NP treatments, reaching a peak of 66.35% at 10.0 mg/L CuO-NP. Similarly, neral levels ranged between 29.16% and 32.57%, remaining relatively stable across treatments. Verbenol showed a dose-dependent increase, from 1.76% in the control to 3.01% at 1.0 mg/L CuO-NP, before slightly declining at 10.0 mg/L (1.72%).

A notable finding was that limonene oxide content increased from 1.20% in the control to 1.81% under 1.0 mg/L CuO-NP, while linalool and acetovanillone levels also peaked at this concentration (0.80% and 0.92%, respectively). Certain compounds such as nona-2(E),6(E)-dienal and caprylaldehyde appeared exclusively in CuO-NP-treated samples, indicating a possible CuO-NP-induced biosynthetic pathway activation.

In contrast, several monoterpenes (e.g., myrcene, cymene, citronellal, and geraniol) were only detected in the control group and disappeared with CuO-NP exposure. These observations suggest both upregulation and suppression of specific metabolic routes depending on the nanoparticle dose.

Gene Expression

To evaluate the molecular responses of *M. officinalis* to copper oxide nanoparticles (CuO-NPs), the relative expression levels of three key genes involved in phenylpropanoid metabolism-PAL (phenylalanine ammonia-lyase), TAT (tyrosine aminotransferase), and RAS (rosmarinic acid synthase)-were quantified via qRT-PCR under varying CuO-NP concentrations (0.1, 1, and 10 mg/L). Expression profiles of PAL, TAT, and RAS genes suggest a dose-dependent activation of phenylpropanoid metabolism under CuO-NP treatment. The highest CuO-NP concentration (10 mg/L) consistently led to the most pronounced upregulation across all genes, pointing toward a potential elicitor effect at higher doses. GAPDH was used as a reference gene for normalization.

PAL expression exhibited a concentration-dependent upregulation in response to CuO-NP exposure Figure 2). Compared to the kinetin control (0.1 mg/L KIN, set as 1.00), a modest increase was observed at 0.1 mg/L CuO (0.91-fold), followed by a significant elevation at 1 mg/L (set as baseline 1.00), and a striking ~4.9-fold induction at 10 mg/L CuO-NP. The strongest PAL induction under 10 mg/L suggests activation of phenylpropanoid biosynthesis, potentially as a defense mechanism against oxidative stress induced by higher nanoparticle concentrations.

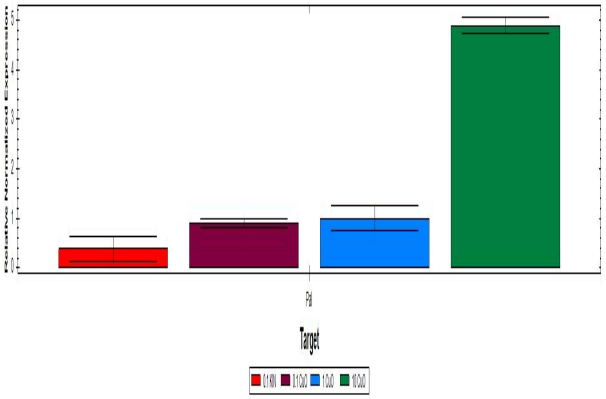

Figure 2. Expression profiles of PAL gene in *M. officinalis* seedlings.

The expression of RAS showed a pronounced upregulation only at the highest CuO-NP concentration (Figure 3). While expression levels remained minimal at 0.1 mg/L KIN (0.0057), 0.1 mg/L CuO (0.097), and 1 mg/L CuO (0.12), a marked ~10.6-fold increase was detected at 10 mg/L CuO-NP. This dose-dependent increase implies that RAS activation is responsive to elevated CuO-NP-induced stimuli and may reflect an upregulated biosynthetic pathway for rosmarinic acid under stress conditions.

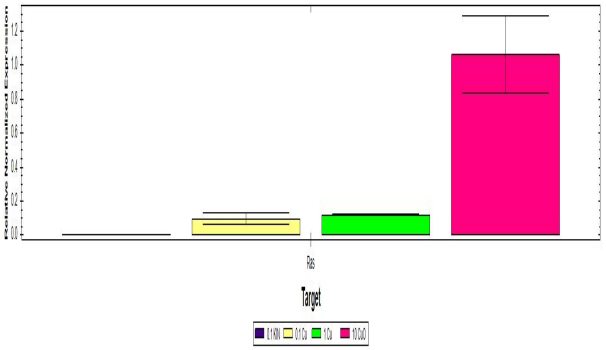

Figure 3. Expression profiles of RAS gene in *M. officinalis* seedlings.

TAT expression followed a biphasic pattern (Figure 4). Relative to the control (1.00), a strong downregulation was observed at 0.1 mg/L CuO (0.19-fold), followed by moderate activation at 1 mg/L (0.32-fold), and a substantial increase at 10 mg/L CuO-NP (1.52-fold). This

pattern indicates that TAT responds dynamically to increasing nanoparticle stress, with maximum.

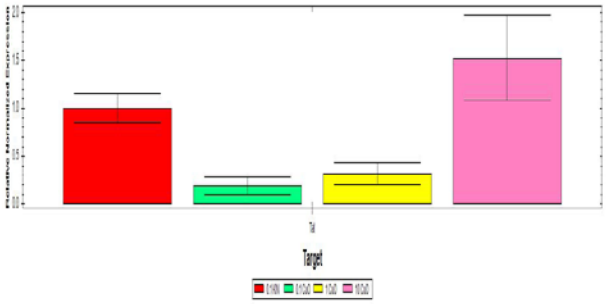

Figure 4. Expression profiles of TAT gene in *M. officinalis* seedlings.

DISCUSSION

This study showed that copper oxide nanoparticles (CuO-NPs) have a significant impact on the growth, metabolism, and overall physiology of lemon balm (*M. officinalis* subsp. *officinalis*) when grown in vitro. Depending on the concentration used, CuO-NPs acted either as growth stimulators or stress inducers, highlighting the importance of dosage in plant-nanoparticle interactions.

The observed growth responses of *M. officinalis* seedlings to CuO-NP treatments revealed a dose-dependent biphasic trend, a hallmark of many nanoparticle–plant interactions. At low concentrations (0.1 mg/L), CuO-NPs significantly enhanced shoot elongation and leaf formation compared to the control. This suggests that trace amounts of copper may act similarly to micronutrients, supporting cell division, chloroplast development, and hormonal balance essential for aerial organ growth [2]. However, at higher concentrations (10.0 mg/L), shoot parameters declined, likely due to Cu-induced oxidative stress that may impair cell elongation and metabolic homeostasis.

Remarkably, root-related parameters displayed an opposite trend. While low CuO-NP concentrations supported limited root development, 10.0 mg/L CuO-NPs significantly increased root number, suggesting that moderate stress conditions triggered an adaptive developmental shift favoring below-ground organogenesis. Such compensatory root proliferation is commonly reported under abiotic elicitor exposure and may reflect an effort by the plant to enhance water and nutrient uptake under perceived stress conditions [23,24]. Root elongation, on the other hand, was maximized at 1.0 mg/L CuO-NP, indicating that moderate concentrations may be optimal for promoting root cell expansion without imposing excessive stress. The decline in root length at 10.0 mg/L, despite the increase in root number, suggests a trade-off between root branching and axial growth, which may be driven by hormonal imbalances or ROS accumulation under higher nanoparticle load.

The percentage of dry weight, used as a proxy for biomass accumulation, also varied with CuO-NP concentration. Although 10.0 mg/L increased root biomass through proliferation, the highest dry weight percentage was recorded in the control group, indicating that excessive CuO exposure may impair overall carbon assimilation and storage efficiency despite localized organ expansion.

These findings confirm that CuO-NPs exert dual roles depending on dose-acting as stimulants at low levels and stressors at higher concentrations. Such dose-specific responses are consistent with hormetic effects observed in other plant species exposed to metal-based nanoparticles, where growth-promoting effects at sub-toxic doses are often followed by inhibitory or toxic effects beyond a certain threshold [25,26]. Understanding this balance is essential for optimizing nanoparticle-based elicitation strategies in plant biotechnology application.

The increase in total phenolic (TPC) and flavonoid content (TFC) observed in CuO-NP-treated *M. officinalis* seedlings highlights the nanoparticles' role as potent abiotic elicitors of secondary metabolism. A clear dose-dependent response was noted, with 10.0 mg/L CuO-NPs inducing the highest TPC (43.45 ± 0.80 mg GAE/g) and TFC (1.716 ± 0.03 mg QE/g) levels. These enhancements are consistent with prior findings that metal-based nanoparticles can trigger reactive oxygen species (ROS) formation, which in turn activates antioxidant defense mechanisms and upregulates phenylpropanoid biosynthesis [27,28].

HPLC analysis further clarified the impact of CuO-NPs on individual phenolic constituents. Rosmarinic acid, a known antioxidant and anti-inflammatory compound characteristic of *M. officinalis*, was significantly elevated in the 10.0 mg/L treatment group, reaching 20.796 µg/g compared to 14.435 µg/g in the control. This suggests a strong metabolic redirection toward terminal phenylpropanoid products under CuO-induced elicitation. Supporting this, a decrease in upstream precursors such as caffeic acid and p-coumaric acid was observed in CuO-treated samples, consistent with a feedback-driven channeling of intermediates into downstream branches of the biosynthetic pathway [25,29].

Notably, while low CuO-NP doses stimulated modest increases in some phenolics, higher concentrations appeared to suppress less stable intermediates, possibly due to oxidative degradation or feedback inhibition mechanisms. The concerted enhancement of specific end-products under CuO-NP elicitation aligns with previous studies showing that nanoparticles can influence enzymatic activity and gene expression involved in secondary metabolism [24,26].

Overall, these findings suggest that CuO-NPs modulate phenolic metabolism in a concentration-sensitive manner by promoting the biosynthesis of key bioactive compounds. Such modulation is valuable for phytopharmaceutical applications where enhanced production of select phenolics-especially rosmarinic acid-is desired. These results also provide a biochemical basis for the observed increases in antioxidant and enzyme inhibitory activities, establishing a strong link

between metabolite accumulation and functional bioactivity.

The antioxidant potential of *M. officinalis* extracts increased significantly in response to CuO-NP treatments, with the highest activity observed at 10.0 mg/L across all assays. This concentration yielded the greatest CUPRAC value (504.3 µmol TE/g) and the lowest SC_{50} values in both DPPH (68.67 µg/mL) and ABTS (4.05 mg/mL) assays, suggesting a strong enhancement of the plant's non-enzymatic antioxidant defense system. These improvements parallel the dose-dependent increases in total phenolic and flavonoid content, as well as the HPLC-confirmed accumulation of key antioxidant compounds such as rosmarinic and ferulic acids.

Phenolic compounds, particularly those with ortho-dihydroxy or methoxy functional groups, are known to efficiently scavenge free radicals and chelate metal ions [9]. The elevation of such compounds under CuO-NP elicitation likely contributes directly to the enhanced antioxidant profiles observed in this study. Rosmarinic acid, for instance, has been shown to neutralize reactive oxygen species (ROS) by donating hydrogen atoms and stabilizing phenoxyl radicals through resonance, making it a central player in antioxidant activity.

The results also indicate that antioxidant response is highly sensitive to nanoparticle dose. While 0.1 and 1.0 mg/L CuO-NPs moderately improved activity, maximum efficacy was reached at 10.0 mg/L. This supports the notion that a threshold concentration of nanoparticles is required to induce sufficient metabolic stress to activate the phenylpropanoid pathway and trigger defense responses without causing toxicity. Similar concentration-dependent trends have been reported in other medicinal plants treated with metal-based nanoparticles, such as ZnO, Fe_3O_4, and AgNPs, where elicitation of antioxidant pathways was linked to nanoparticle-induced redox perturbations [25,27].

Interestingly, although the CUPRAC assay provided a broad measure of reducing capacity, the radical scavenging assays (DPPH and ABTS) were more discriminative of nanoparticle dose effects, possibly due to the differing reactivity of specific phenolics toward distinct radical species. This highlights the importance of using multiple assays to comprehensively assess antioxidant behavior.

In summary, the enhanced antioxidant performance of CuO-NP-treated *M. officinalis* is strongly associated with nanoparticle-induced phenolic accumulation, particularly of rosmarinic acid. These findings support the utility of CuO-NPs as effective abiotic elicitors for improving the nutraceutical potential of medicinal plants through targeted enhancement of antioxidant capacity

The enzyme inhibition results clearly demonstrated that CuO-NP-treated *M. officinalis* extracts exert concentration-dependent bioactivity across a panel of clinically relevant targets, including AChE, MAO, urease, and HIV-1 reverse transcriptase (RT). Among these, the strongest AChE inhibition was observed at 1.0 mg/L CuO-NP, suggesting that this concentration may optimize the balance between secondary metabolite induction and phytotoxicity. Notably, higher doses such as 10.0 mg/L showed reduced AChE inhibitory activity, which may reflect a shift in metabolite profile or saturation of bioactive compound production.

In contrast, MAO and urease inhibition improved progressively with increasing nanoparticle concentration, with the lowest IC_{50} values recorded at 10.0 mg/L. These results suggest that enzymes involved in oxidative metabolism or microbial virulence may be more responsive to metabolite accumulation triggered by higher elicitor doses. The moderate yet consistent inhibition of HIV-1 RT across all concentrations, particularly at 10.0 mg/L, also points to a broader spectrum of bioactivity in CuO-NP-treated extracts.

These enzyme-inhibitory effects are likely mediated by the increased presence of polyphenolic compounds, especially rosmarinic acid, ferulic acid, and gallic acid, all of which have been reported to possess multi-target inhibitory potential due to their ability to bind enzyme active sites or metal cofactors [30,31]. The correlation between elevated phenolic content (as confirmed by HPLC and TPC/TFC assays) and improved inhibitory activity supports this biochemical linkage.

Notably, the differences in inhibition profiles across enzymes highlight the selective influence of CuO-NPs on metabolic pathways. For example, while AChE inhibition peaked at moderate dose levels, urease and MAO responded better to higher concentrations. This divergence could result from distinct structure–activity relationships among individual phenolic constituents, or from varying sensitivities of enzyme systems to stress-induced phytochemical changes.

Overall, the enzyme inhibition patterns observed in this study suggest that CuO-NPs can effectively modulate the production of functionally bioactive compounds in *M. officinalis*, with implications for developing phytochemical-based inhibitors for therapeutic or agricultural use. These findings align with previous studies demonstrating the potential of nanoparticle-elicited plant systems in generating natural enzyme inhibitors with diverse target selectivity [25,26].

The findings demonstrate that CuO nanoparticles significantly modulate the essential oil composition of *M. officinalis*, with both enhancing and suppressing effects on specific secondary metabolites. The marked increase in geranial and neral content in CuO-NP-treated groups, particularly at 10.0 mg/L, suggests a stimulation of the terpenoid biosynthetic pathway, possibly via stress-induced activation of terpene synthases.

The elevation in verbenol and limonene oxide at low to moderate CuO-NP concentrations further supports the hypothesis that CuO-NPs act as elicitors, promoting oxidative and enzymatic processes that favor monoterpene and sesquiterpene biosynthesis. However, the decline in certain volatile constituents such as myrcene and cymene indicates that some biosynthetic routes may be selectively repressed or that enzymatic competition occurs under nanoparticle stress.

Remarkably, the appearance of compounds not detected in the control group (e.g., nona-2(E),6(E)-dienal, caprylaldehyde) suggests that CuO-NP exposure may induce the de novo synthesis of stress-related volatiles or intermediates associated with lipid peroxidation.

These results align with previous studies reporting that metallic nanoparticles can serve as abiotic elicitors in aromatic and medicinal plants, enhancing secondary metabolite yield while reshaping their metabolic profiles. Therefore, the application of CuO-NPs, particularly at optimized low-to-moderate concentrations, may represent a promising strategy to enrich essential oil quality in *M. officinalis* cultivation.

At the molecular level, gene expression data confirmed these changes. Genes involved in phenolic biosynthesis-PAL, TAT, and RAS-were all upregulated under 1.0 mg/L CuO-NPs. PAL, for instance, is the key gateway enzyme in the phenylpropanoid pathway, and its activation sets the stage for many important secondary metabolites [29]. The coordinated upregulation of TAT and RAS suggests a targeted boost in rosmarinic acid production. In contrast, gene expression levels dropped under high nanoparticle stress, echoing the biochemical results.

In summary, this study shows that carefully dosed CuO-NPs can improve plant growth and boost production of beneficial bioactive compounds in *M. officinalis* by stimulating both metabolic and genetic pathways. However, too much of a good thing-like 10.0 mg/L-can backfire, causing stress and reducing productivity. This reinforces the importance of optimizing nanoparticle concentrations when using them as elicitors in plant science and biotechnology.

Looking ahead, future research should explore the signaling pathways involved, such as the roles of ROS, calcium, and plant hormones like jasmonic and salicylic acid. Broader "omics" approaches, including transcriptomics, proteomics, and metabolomics, could provide deeper insight into how nanoparticles influence plant systems at the molecular level.

CONCLUSION

This study provides clear evidence that copper oxide nanoparticles (CuO-NPs) significantly modulate the growth, secondary metabolism, and molecular responses of *M. officinalis* L. subsp. *officinalis* under in vitro conditions. The findings reveal a dose-dependent biphasic effect of CuO-NPs, where low concentrations (0.1 mg/L) enhance shoot development, while higher doses (10.0 mg/L) trigger root proliferation, secondary metabolite accumulation, and gene upregulation linked to phenylpropanoid biosynthesis.

Elevated total phenolic and flavonoid contents at 10.0 mg/L CuO-NP were associated with improved antioxidant potential and greater rosmarinic acid levels, as confirmed by HPLC and antioxidant assays. Enzyme inhibition results demonstrated the bioactivity of CuO-NP-treated extracts against key targets, including AChE, MAO, urease, and HIV-1 RT, reflecting the therapeutic potential of nanoparticle-elicited plant systems. Additionally, essential oil profiling and gene expression analysis provided further insights into CuO-NP-induced metabolic reprogramming and activation of defense-related pathways.

Taken together, these findings support the use of CuO-NPs as effective abiotic elicitors to enhance the phytochemical content and functional quality *M. officinalis*. However, the concentration of nanoparticles is critical, as excessive exposure may induce oxidative stress and suppress certain physiological traits. Future work should focus on elucidating the underlying signaling mechanisms and applying omics-based tools to better understand nanoparticle–plant interactions for optimized biotechnological applications.

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