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Impact of zinc oxide nanoparticles on callus growth, antioxidant activity, and secondary metabolite content in Moringa oleifera

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Abstract

This study was carried out in the laboratories of the Biology Department and aims to know the role of 2,4-D and nano-zinc concentrations on callus induction and growth of *Moringa oleifera* Lam. Its seeds were grown in media containing different concentrations of 2,4-D, and the concentration of 0.75 was chosen as it gave the best callus induction. Then, we made four concentrations of the nano-ZnO compound in addition to the comparative concentration to know their effect on the biomass of callus cultures, the accumulation of some pharmaceutical compounds, and antioxidant activity. Results showed that a maximum callus fresh weight was seen at 10.0 $mg.L^{-1}$, with a mean weight of 999.74 mg, indicating that this concentration is especially effective at promoting callus growth. The concentration of 25.0 $mg.L^{-1}$ was found to exhibit the highest level of antioxidant activity, with an IC50 value of 96.78 ± 3.28 $mg.L^{-1}$. This suggests that this dosage is the most efficient in promoting antioxidant qualities. Results pointed to the potential of Nano-ZnO as a helpful agent in promoting alkaloid and phenolic synthesis in plant tissue culture. Finally, it can be said that stimulation by the nano-ZnO compound leads to an increase in vital biological materials.

Keywords: Antioxidant activity, Moringa oleifera, Nano-ZnO, pharmaceuticals, seed callus.

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1. Introduction

Moringa (*Moringa oleifera* Lam.) is the only genus of the family Moringaceae, which is native to Asia [1]. *M. oleifera* is widely cultivated and flourishes in all soil types [2]. *M. oleifera* is a plant that grows to a medium size of 5-12 meters, with elliptical-shaped leaves with bi- and tri-pinnate arrangements [3]. It serves as both an economic and medicinal plant, as its nutritional and medicinal properties have tremendous potential for managing malnutrition and treating many diseases. It is considered a functional plant in the agricultural system in many countries due to its ability to thrive in all types of soils and difficult climatic conditions, which has given it great economic importance, as it is grown as a crop for the production of food, fodder, and fuel. On the other hand, it is an indispensable plant for promoting health, where it works in treating many different diseases such as anemia, rheumatism, hyperthyroidism, arthritis, epilepsy, herpes simplex virus, blindness, sexually transmitted diseases, and gout [4, 5]. Many pharmacological studies report that various extracts of *M. oleifera* show significant biological activities, such as anti-microbial, antioxidant, anti-inflammatory, cardiovascular, anti-cancer, analgesic, and anti-ulcer [6]. *M. oleifera* is rich in nourishment because it contains essential phytochemicals in its leaves such as essential minerals, tannins, vitamins, anthraquinones, flavonoids, terpenoids, sterols, and alkaloids [7]. These phytoconstituents have anti-inflammatory, antidiabetic, and anti-ulcer properties [8]. Anticancer [9]. Antioxidant, antimicrobial, and antifungal properties [10].

Nowadays, attention has turned towards tissue culture techniques that encourage the growth and biosynthesis of many secondary metabolites in plant tissue and they have been considered as one of the most promising technologies in the pharmaceutical industries [11] and for this goal, there are many various explants can be entering in induction callus such as leaves [12] seed [13] cotyledons [14] shoots and roots [15].

In the current century, nanotechnology is considered one of the important techniques that are widely used by researchers to manufacture nanoparticles, especially the metallic inorganic oxide nanoparticles such as copper, titanium, and zinc oxide NPs [10]. Mainly, this field relies on the use of ultrafine particles, which range between 1 to 100 nanometers in size [16]. Zinc oxide is one of the metal nanoparticles that has garnered much interest from researchers because of its excellent characteristics in environmental sustainability [17]. Zinc oxide NPs have received significant attention due to their antimicrobial and highly catalytic actions [18]. Many studies have reported the elicitor role of ZnO NPs in producing secondary metabolites and improving metabolism and the growth of plants through different ways such as altering phytohormonal balances, improving nutritional status, modifying enzymatic activity, affecting nitrogen assimilation, and influencing gene expression [19].

The present study aims to understand the role of 2,4-D and different concentrations of nano-zinc oxide on the induction and growth of callus from *Moringa oleifera* Lam. seeds and its content of secondary metabolites represented by alkaloids and phenolic compounds, as well as measuring the antioxidant activity of the extract from these calli.

2. Materials and Methods

2.1. Preparation and Culturing of Explants on MS Medium

This study was carried out in the Laboratories of the Biology Department, Al-Iraqia University through the year 2023–2024. The medium of Murashige and Skoog (MS) [20] was used in the experiments produced by CASSON Labs/USA. In the laminar air-flow cabinet, and after removing the outer covers of the seeds, they were surface sterilized by immersing them in 0.01% $HgCl_2$ for three minutes. Then, the seeds were rinsed with sterilized distilled water three times (2 min. each) to remove residues of the sterilization material. The sterilized seeds were cultured on MS medium supplemented with 2,4-D at 0.0, 0.75, 1.5, 2.25, or 3.0 $mg.L^{-1}$, and the cultures were incubated for 30-45 days in a growth room under controlled conditions (temperature of 25 ± 2 °C and 16 h light/8 h dark at 1000 Lux). Callus induction percentage (%) and fresh weight of callus (mg) were calculated to choose the optimal concentration of 2,4-D for subsequent experiments.

2.2. Sub-culturing of Callus and Accumulation of Pharmaceuticals Elicited with Nano-ZNO Concentrations

Induced callus was cut and sub-cultured on the optimal concentration of 2,4-D (0.75 mg. L^{-1}) to obtain adequate quantities of callus. Then, MS medium containing the optimal 2,4-D concentration was supplement with nano-ZnO in different concentrations (0.0, 5.0, 10.0, 15.0 or 25.0 $mg.L^{-1}$). Cultures were then incubated under the same conditions mentioned previously for 21 days. Callus fresh weight and antioxidant activity were measured, as well as the determination of the alkaloids and phenolic compounds in the callus that was grown on nano-ZnO concentrations.

2.3. Measurement of Antioxidant Activity Using the DPPH Method

The radical 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) was prepared at a concentration of 400 $\mu g.mL^{-1}$ [21]. Standard solutions (5000 ppm) of vitamin C and the sample were prepared by dissolving 500 mg of each in 100 ml of methanol and distilled water, and by using the dilution law, other concentrations (30, 60, 120, 250, 500 ppm) were prepared from vitamin C and the sample [22]. After incubating the reaction mixture for 30 min in the dark at room temperature, the absorbance was measured at 517 nm using a spectrophotometer (UV-VIS Shimadzu) [23]. The IC_{50} values of the samples were determined using the log-dose inhibition curve. The higher free radical activity is represented by the lower absorbance of the reaction mixture [24]. The DPPH scavenging effect was calculated using the following equation: DPPH scavenging effect (%) = (A - B) / A * 100, where A is the absorbance of the blank and B is the absorbance of the test sample.

2.4. Determination of Pharmaceuticals using HPLC Conditions

The phenolic compounds were extracted from homogenized callus growing on different concentrations of nano-ZnO. One hundred milliliters of chloroform was added to 20 g of the callus after grinding it well and placing it on the electric

vibrator for 3 hours to remove the fat from the sample. Then, the chloroform layer was removed, and after drying the sample at a temperature of 50 ̊C to ensure that no residues of chloroform remained, 10 g of the dried sample was taken to extract using a solvent (70/30) of ethanol/water. The extraction process was achieved using an Ultrasonic Bath (USA) for 1 hour at room temperature. After filtration, 5 ml of liquid extract was used for extraction yield determination. The solvent was removed using a rotary evaporator under vacuum (Slovenia) and was dried to a constant mass at 40 ℃. To prevent oxidative damage to dry extracts, they should be stored in glass vials at 4 ℃ until analysis. The individual phenolic compounds were analyzed using a SYKAM (Germany) HPLC chromatographic system equipped with a UV detector (280 nm). The separation column was –C18-OSD (25 cm * 4.6 mm) at a flow rate of 0.7 ml.min^{-1} with a temperature of 30 ℃. The gradient elution method, with eluent A (methanol) and eluent B (1% formic acid in water (v/v)), was performed as follows: initial 0-4 min, 40% B; 4-10 min, 50% B; the injected volume of standards and samples was 100 µL for each [25].

For the determination of alkaloids, the extraction was done by taking the dried powdered (2g) of *M. oleifera* samples and mixing them with 25 mL of a 60% (v/v) ethanol aqueous solution by incubating at 30 ºC for 24 hours. Each extract was filtered, and the solvent was removed under vacuum at 40 ºC. The residue was redissolved in 3 mL of HPLC-grade methanol. HPLC model SYKAMN (Germany) chromatography was carried out using a mobile phase of methanol: sodium dihydrogen phosphate: acetic acid buffer (pH 3.8, 0.1 M) (20%:80%) (v/v). The flow rate was 0.7 ml.min^{-1} during the analysis, and peaks were detected at 220 nm [26].

2.5. Statistical Analysis

The experiments of induction and multiplication of callus were performed with 10 replicates using a completely randomized design (CRD), whereas DPPH and HPLC analysis were assessed with 3 replicates. The ANOVA test was used for comparison among groups at $P \leq 0.05$ [27] and followed by Duncan`s test using IBM SPSS statistics (Version 27 for Windows).

3. Results and Discussion

3.1. Induction of Callus and Production of Biomass

Results presented in Table 1 and Figure 1 showed the influence of 2,4-D concentrations on the percentage of callus induction from *Moringa oleifera* Lam. seeds and its fresh weight. The findings indicated that there was no induction of callus at the treatment of 0.00 mg.L^{-1} (control), suggesting that the absence of 2,4-D had the influence on the lack of response of explants to produce callus, which may be also due to the loss of sufficient internal contents of hormones in explants [28]. On the other hand, the capacity of explants to form callus increased when the medium was supplied with the auxin 2,4-D, where the percentage of callus induction reached to 100% at of 0.75 mg.L^{-1}, 1.50 mg.L^{-1}, and 2.25 mg.L^{-1}, indicating that these levels were ideal for stimulating callus formation. However, callus induction dropped to 50% at the concentration of 3.00 mg.L^{-1}, suggesting a possible inhibitory action at this higher concentration. These results highlighted the benefit of using 2,4-D concentrations between 0.75 mg.L^{-1} and 2.25 mg.L^{-1} for generating callus formation, whereas higher concentrations may have an adverse effect. This highlights the significance of precisely adjusting growth regulators' levels in plant tissue culture techniques to get successful results [29].

Table 1
Influence of 2,4-D concentrations on induction percentage (%) and fresh weight (mg) of callus from *Moringa oleifera* Lam. seeds.

2,4-D Concentration (mg.L^{-1})	Callus Induction Percentage (%) Mean ± S.D.	Callus fresh weight (mg) Mean ±S.D
0.00	0 ± 0 c	0±0 c
0.75	100 ± 0 a	1069.17±197.347a
1.50	100 ± 0 a	468.33±129.838 b
2.25	100 ± 0 a	683.50±364.45 b
3.00	50 ± 54.77 b	493.50±250.667 b
ANOVA test P. Value	20.00 < 0.001 HS*	17.879 < 0.001 HS*

Note: *HS: High significant difference among groups ($p < 0.05$).
-Groups have different letters are different statistically.

Figure 1.
Callus induction from *M. oleifera* seed on MS medium with 0.75 mg.L^{-1}2,4-D.

From the same table above, the data showed that at the control treatment (0.00 mg.L^{-1}), there was no fresh weight of callus (0 mg) due to the lack of callus formation on explants in the absence of 2,4-D. In contrast, at 0.75 mg.L^{-1} of 2,4-D, the fresh weight of callus increased dramatically to 1069.17 mg, which is the greatest weight reported in the study, demonstrating that this concentration is quite effective for callus formation. Meanwhile, concentrations of 1.50 mg.L^{-1} (468.33 mg), 2.25 mg.L^{-1} (683.50 mg), and 3.00 mg.L^{-1} (493.50 mg) resulted in lower, but still significant weights when compared to the control treatment. Overall, the results indicated that 0.75 mg.L^{-1} of 2,4-D was the best for maximizing callus fresh weight, whereas higher concentrations provided varied results, emphasizing the importance of concentration in optimizing callus development in *M. oleifera* tissue culture from different explants [30].

3.2. Effect of Nano ZnO on Callus Multiplication

Results shown in Table 2 summarize the effects of different Nano-ZnO concentrations on the *M. oleifera* Lam. callus fresh weight, where the control treatment gave an average of 109.93 mg, demonstrating that the absence of Nano-ZnO led to limited callus growth. In contrast, the maximum callus fresh weight was seen at 10.0 mg.L^{-1}, with an average of 999.74 mg, indicating that this concentration is especially effective at promoting callus growth (Fig.2). Other concentrations, such as 5.0 mg.L^{-1} (857.93 mg) and 25.0 mg.L^{-1} (876.36 mg), resulted in considerable weights, whereas 15.0 mg.L^{-1} yielded a lower weight of 534.74 mg. Overall, these findings indicated that 10.0 mg.L^{-1} of Nano-ZnO was best for maximum callus fresh weight, whereas lower and higher concentrations have different degrees of efficiency, stressing the relevance of nanoparticle concentration in optimizing callus development in *M. oleifera* tissue cultures [31]. The use of nanoparticles, including nano-metal oxides as abiotic elicitors, has enhanced plant growth and productivity both under normal field conditions or in *in vitro* culture programs, depending on concentrations that enhance the accumulation of biomass for cultures. However, concentrations that exceed the optimal value may cause severe toxicity to cultured tissues [32].

Table 2.
Influence of Nano-ZnO concentrations on callus fresh weights (mg) of *M. oleifera* Lam.

Nano-ZnO concentration (mg.L^{-1})	Callus weights (mg) Mean ± S.D.	ANOVA test p-value
0.0	109.93 ± 1.83 c	5.825 0.001 HS*
5 .0	857.93 ± 63.18 ab	
10 .0	999.74 ± 46.96 a	
15 .0	534.74 ± 44.36 bc	
25 .0	876.36 ± 53.81 ab	

Note: *HS: High significant difference among groups ($p < 0.05$).
-Groups have different letters are different statistically.

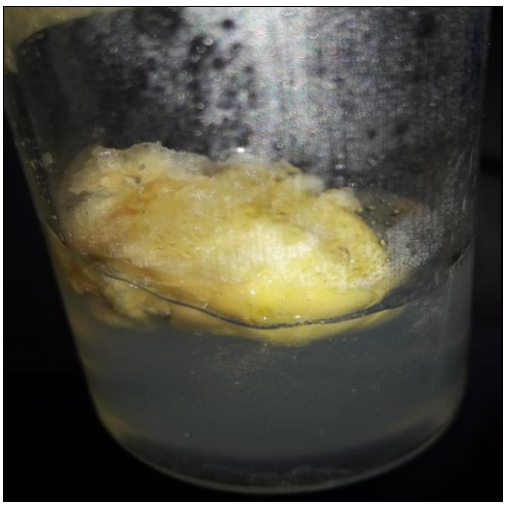

Figure 2.
Callus of *M. oleifera* grown on 10.0 mg. L^{-1} of nano ZnO.

3.3. Antioxidant Activity Through Using DPPH Assay

Table 3 showed how different Nano-ZnO concentrations affected the antioxidant activity of *M. oleifera* Lam. callus to inhibit 50% of DPPH radical. The findings showed that there were little antioxidant activities at concentrations of 0.0 and 5.0 $mg.L^{-1}$, with IC50 values of 100.36 ± 2.42 $mg.L^{-1}$ and 100.03 ± 4.09 $mg.L^{-1}$, respectively (Figure 3). Nevertheless, there was a noticeable increase in antioxidant activity at concentrations of 10.0 $mg.L^{-1}$ (98.83 ± 3.46 $mg.L^{-1}$) and 15.0 $mg.L^{-1}$ (98.03 ± 6.17 $mg.L^{-1}$), with IC50 values showing better efficacy in comparison to the lower concentrations. The concentration of 25.0 $mg.L^{-1}$ was found to exhibit the highest level of antioxidant activity, with an IC50 value of 96.78 ± 3.28 $mg.L^{-1}$. This suggests that this dosage is the most efficient in promoting antioxidant qualities. Whereas, the Vit. C considered a positive antioxidant, but lower than the other crude callus extracts from different nano-ZnO treatments. A significant difference was found among the groups using the ANOVA test and Duncan's test. These results highlighted the significance of concentration in optimizing antioxidant capacity in plant tissue culture by indicating that Nano-ZnO concentrations, especially at 25.0 $mg.L^{-1}$, increase the antioxidant activity of *M. oleifera* callus, this maybe indicates that nano-ZnO may stimulates the increasing in the accumulation of effective antioxidant compounds in the methanolic crude extract of callus grown at the concentrations of this nano-elicitor and finally leads to an increase in the free radical scavenging activity of DPPH [33]. Previous studies have indicated the positive effect of adding nano-elicitors to callus cultures on antioxidant activity. For example, the addition of nano-silver particles to callus cultures of fenugreek resulted in significant differences in their antioxidant content, as they showed significant activity in scavenging free radicals [34, 35].

Table 3.
Influence of Nano-ZnO concentrations on antioxidant activity of *Moringa oleifera* Lam. callus using DPPH assay.

Nano-ZnO concentrations ($mg.L^{-1}$)	**IC50 ($mg.L^{-1}$)**
Vit. C	133.36 ± 3.39 d
0.0	100.36 ± 2.42 c
5 .0	100.03 ± 4.09 c
10 .0	98.83 ± 3.46 b
15 .0	98.03 ± 6.17 b
25 .0	96.78 ± 3.28 a
ANOVA test	5.592
p value	0.023 S*

Note: *S: significant difference among groups (p value < 0.05).
The Numerical values are the (means ± standard deviation) of 3 replicates.
In each column, according to Duncan's test (p < 0.05), significant differences were referred to by different letters.

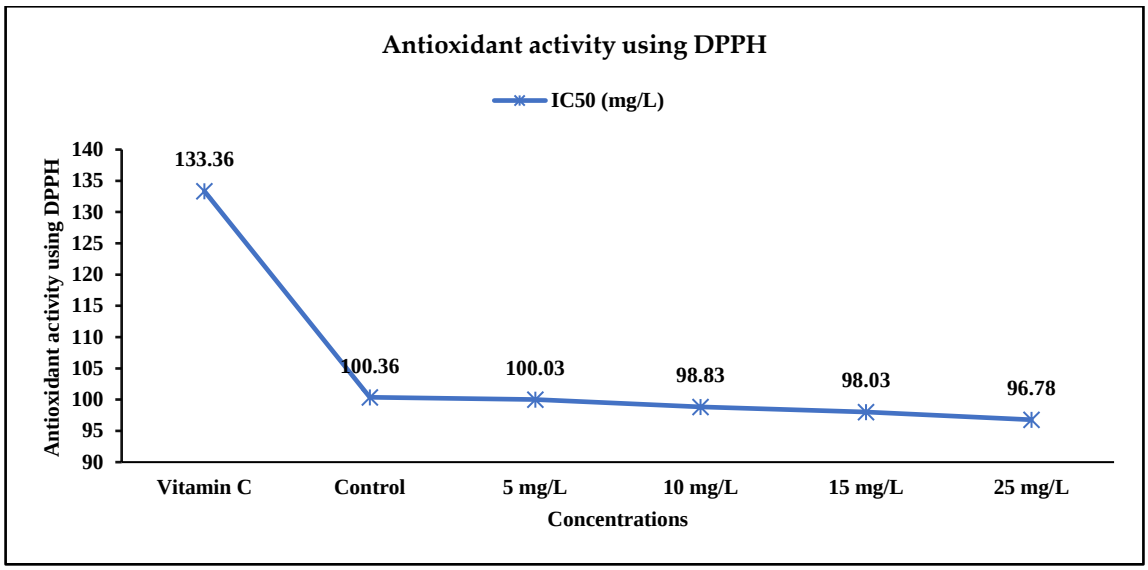

Figure 3.
IC 50of antioxidant activity of crude extract of *M.oleifera* callus samples grown on different concentrations of nano- ZnO.

3.4. Effect of Nano-ZnO on Pharmaceutical Accumulation

The effects of different Nano-ZnO concentrations on the alkaloids content of *M. oleifera* Lam. callus are summarized in Table 4, with an emphasis on the levels of niazirin and vincosamide as determined by HPLC. Baseline values of these alkaloids were found to be 396.48 ± 12.09 µg.mg^{-1} for niazirin and 172.48 ± 15.29 µg.mg^{-1} for vincosamide at control treatment (0.0 mg.L^{-1}). Both alkaloids concentrations exhibited a noticeable and significant rising trend as the concentration of Nano-ZnO rose. Niazirin reached 422.59 ± 14.25 µg.mg^{-1} and vincosamide reached 186.26 ± 25.52 µg.mg^{-1} at 5.0 mg.L^{-1}. With niazirin reaching 585.78 ± 39.13 µg.mg^{-1} and vincosamide at 244.56 ± 39.14 µg.mg^{-1}, the greatest amounts were recorded at 25.0 mg.L^{-1}, indicating a definite significant increase in alkaloid synthesis with increasing Nano-ZnO concentrations. These results pointed to the potential of Nano-ZnO as a helpful agent in promoting alkaloids synthesis in plant tissue culture by indicating that increasing concentrations of Nano-ZnO positively influenced the formation of both niazirin and vincosamide in *M. oleifera* callus. This confirms the importance of stimulating plant tissue culture to accelerate the biosynthesis and accumulation of active metabolites [36]. on the other hand, Ramezannezhad et al. [37] indicated that supplemented the cell suspension cultures of *Lactuca undulate* with silver nanoparticles led to a significant stimulating the assembly of effective metabolites in these suspensions.

Table 4.
Influence of Nano-ZnO concentrations on contents of alkaloids (niazirin and vincosamide) (µg.m g $^{-1}$ of crude extract of *Moringa oleifera* Lam. callus) detected with HPLC.

Nano-ZnO Concentration (mg.L^{-1})	Niazirin (µg.mg^{-1}) Mean ± S.D.	Vincosamide (µg.mg^{-1}) Mean±S.D.
0.0	396.48 ± 12.09 e	172.48 ± 15.29 e
5.0	422.59 ± 14.25 d	186.26 ± 25.52 d
10.0	509.80 ± 19.13 c	210.02 ± 18.43 c
15.0	550.13 ± 15.28 b	230.99 ± 24.97 b
25.0	585.78 ± 39.13 a	244.56 ± 39.14 a
ANOVA test p value	8.405 0.011 S*	6.939 0.019 S*

Note: *S: significant difference among groups (p value < 0.05).
The numerical values (means ± standard deviation) of 3 replicates.
In each column, according to Duncan's test (p < 0.05), significant differences were referred to by different letters.

Results of Table 5 demonstrated the impact of varying concentrations of Nano-ZnO on the phenolic content of *M. oleifera* Lam. callus, specifically assessing the levels of hesperidin, rosemarinic acid, kaempferol, rutin, and quercetin using HPLC which indicated a clear trend of increasing phenolic compound concentrations with higher Nano-ZnO levels. The content of hesperidin increased significantly with higher concentrations of Nano- ZnO. At control (0.0 mg.L^{-1}), the mean concentration was 134.56 ± 14.56 µg.mg^{-1}, which rose to 247.07 ± 17.06 µg.mg^{-1} at 25.0 mg.L^{-1}. Similar to hesperidin, the levels of rosemarinic acid also increased significantly with increasing Nano-ZnO concentrations, starting from 144.4 ± 17.39 µg.mg^{-1} at 0.0 mg.L^{-1}, the concentration reached 210.78 ± 10.78 µg.mg^{-1} at 25.0 mg.L^{-1}, reinforcing that Nano-ZnO effectively enhances rosemarinic acid synthesis in *M. oleifera* callus. The kaempferol content showed a marked increase from 45.96 ± 4.04 µg.mg^{-1} at 0.0 mg. L^{-1} to 115.18 ± 15.18 µg.mg^{-1} at 25.0 mg.L^{-1}. This demonstrated the positive impact of Nano-ZnO on kaempferol production, emphasizing its role in promoting this phenolic compound.

Also, rutin levels increased significantly from 93.9 ± 16.1 µg.mg^{-1} at 0.0 mg.L^{-1}(control treatment) to 148.72 ±18.72 µg.mg^{-1} at 25.0 mg.L^{-1}, suggesting that the presence of Nano-ZnO plays a crucial role in optimizing rutin content in *M. oleifera* callus. Quercetin content also demonstrated a significant increase, starting at 77.82 ± 6.18 µg.mg^{-1} at 0.0 mg.L^{-1} and reaching 133.96 ± 33.95 µg.mg^{-1} at 25.0 mg.L^{-1}. This underscored the potential of Nano-ZnO to enhance phenolic profiles in *M. oleifera* callus. These conclusions indicated the importance of nanoparticles as abiotic elicitors to increase the accumulation of secondary metabolites *in vitro* cultures of plants [31, 37]. In a previous study by Zanella et al. [12], they confirmed that the highest content of phenolic compounds in callus cultures has a correlation with the increasing of antioxidant activity of the crude extracts of these calli, this also confirmed in our study (see Table 3).

Overall, the data from Table 5 collectively suggested that increasing concentrations of Nano-ZnO significantly enhanced the production of various phenolic compounds in *M. oleifera* callus, demonstrating its potential as a beneficial elicitor in *in vitro* culture for enhancing the increase of effective metabolites.

Table 5.
Influence of Nano-ZnO concentrations on contents of phenols (µg.mg^{-1} of crude extract of *Moringa oleifera* Lam. callus) detected with HPLC.

Nano-ZnO Concentration (mg.L^{-1})	Hesperidin Mean ± S.D.	Rosemaric acid Mean ± S.D.	Kaempferol Mean ± S.D.	Rutin Mean ± S.D.	Quercetin Mean ± S.D.
0.0	134.56 ± 14.56 e	144.4 ± 17.39 e	45.96 ± 4.04 e	93.9 ± 16.1 e	77.82 ± 6.18 e
5.0	164.44 ± 16.44 d	174.74 ± 7.73 d	59.85 ± 14.15 d	99.09 ± 9.1 d	101.51 ± 15.07 d
10.0	203.33 ± 13.33 c	184.47 ± 8.47 c	89.99 ± 10.01 c	128.11 ± 28.11 c	114.03 ± 14.03 c
15.0	219.86 ± 19.85 b	196.26 ± 16.25 b	101.45 ± 11.45 b	131.98 ± 31.98 b	124.43 ± 24.42 b
25.0	247.07 ± 17.06 a	210.78 ± 10.78 a	115.18 ± 15.18 a	148.72 ± 18.72 a	133.96 ± 33.95 a
ANOVA test p value	4.465 0.036 S*	4.956 0.029 S*	5.029 0.017 S*	4.492 0.029 S*	3.983 0.041 S*

Note: *S: significant difference among groups (p value < 0.05).
The numerical values (means ± standard deviation) of 3 replicates
In each column, according to Duncan's test (p < 0.05), significant differences were referred to by different letters.

4. Conclusion

Results of the current study showed that the influence of using concentrations of 2,4-D on the induction and growth of callus from *M. oleifera* Lam. seeds was clear, as the concentration of 0.75 mg.L^{-1} was the best in increasing callus biomass. On the other hand, the addition of different concentrations of nano-ZnO to the callus growth medium has proven its importance in increasing the biomass of callus via increasing fresh weight, where the concentration of 10.0 mg.L^{-1} was optimal in this regard, while the increase in the nano-ZnO concentrations led to an increase in the accumulation of alkaloids and phenolic compounds with a positive increase in the antioxidant activity of callus growing on these concentrations of nano-ZnO.

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