Effects of Heavy Metal Stress on Secondary Metabolite Production in Pot Marigold (*Calendula officinalis* L.) Under in vitro Conditions

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INTRODUCTION

Medicinal and aromatic plants, which are widely used in the pharmaceutical, food, cosmetic, traditional medicine and spice industries, represent valuable natural resources. Throughout history, humans have explored different parts of plants (roots, leaves, flowers, and fruits), observed their healing effects on health and transmitted this knowledge across generations. It is reported that the earliest written records on herbal therapy date back to the Mesopotamian civilization around 5000 BC and that more than 250 herbal drugs were used for medicinal purposes during this period. Today, medicinal plants have considerable economic and therapeutic value in many fields, including herbal teas, pharmaceutical products, cosmetic formulations and spices (Demirezer, 2010).

Pot marigold (*Calendula officinalis* L.), commonly used today for medicinal and cosmetic purposes, is an annual herbaceous plant belonging to the genus *Calendula* within the Asteraceae family, typically reaching 30–60 cm in height (Ashwlayan et al., 2018) (Figure 1). Native to Central and Southern Europe, Western Asia and America, this species has long been cultivated and utilized in various regions. In Türkiye, it is known by several local names, including “aynısefa”, “tıbbi nergis” and “portakal nergisi” (Baytop, 1984; Deniz et al., 2010).



Figure 1. General appearance of *Calendula officinalis* L. (Pot marigold) plant
Source: Original Photo, S. Beyza BUÇAN

The rich chemical composition of *Calendula officinalis* enables its use in many fields, including medicine, pharmacy, the food industry and the cosmetic industry. This plant contains numerous biologically active constituents and its main chemical groups include terpenoids, flavonoids, coumarins, quinones, essential oils, carotenoids and amino acids (Ashwlayan et al., 2018). In traditional and complementary medicine, these constituents are widely used for the management of various health conditions, such as the common cold, wound healing, febrile illnesses, insect bites, diaper rash, gingival disorders, hemorrhoids, stomach cramps, constipation and hypertension. In addition, extracts obtained from *C. officinalis* are incorporated into the formulations of numerous pharmaceutical and cosmetic products, including tea preparations, shampoos, tinctures, soaps, ointments, creams and lotions (Bruneton, 1995).

To survive and adapt to environmental conditions, plants synthesize not only primary metabolites but also secondary metabolites, which constitute the basis of their defense mechanisms. Secondary

metabolites are specialized compounds that do not directly participate in growth and development processes; instead, they play critical roles in plant adaptation to abiotic stress factors (e.g., drought, salinity, heavy metal contamination and UV radiation) as well as biotic challenges such as pathogen attacks and herbivory (Selmar, 2013; Isah, 2019). Under stress conditions, these compounds activate the plant defense system, confer protective effects against environmental pressures and enhance survival capacity (Selmar, 2013; Isah, 2019).

In recent years, increasing urbanization and industrialization have led to a marked rise in environmental pollution. Among these pollutants, heavy metals exert toxic effects in both terrestrial and aquatic ecosystems and have become major sources of biological stress for all living organisms, including plants. Heavy metal compounds such as silver nitrate ($AgNO_3$) induce oxidative stress in plant tissues by triggering the production of reactive oxygen species (ROS); this disrupts physiological homeostasis and causes substantial changes in secondary metabolite biosynthesis (Al-Oubaidi et al., 2014).

In this context, investigating the secondary metabolite responses of *Calendula officinalis* L. under heavy metal–induced stress is of great importance for understanding the plant's biological resilience and metabolic adaptive capacity. Moreover, such studies provide new insights into how environmental stressors regulate the production of plant-derived bioactive constituents, thereby establishing a scientific basis for phytocosmetic, phytopharmaceutical and biotechnological applications (Yurteri and Buçan, 2025).

1. MATERIALS VE METHODS

1.1. Plant Material

Seeds of *Calendula officinalis* L. (pot marigold) were used as the plant material. The seeds were obtained from the Atatürk Horticultural Central Research Institute (Yalova, Türkiye).

The seeds were established in vitro under sterile conditions and aseptic plantlets obtained after germination were multiplied using tissue culture techniques to generate the experimental material. Stress treatments were then applied to the propagated in vitro plant material according to the experimental design and post-stress samples were prepared for analyses aimed at determining volatile and phenolic constituents. All stages of the tissue culture procedures and stress applications were carried out under sterile conditions in the Tissue Culture Laboratory and the tissue culture room of the Faculty of Agriculture, Recep Tayyip Erdoğan University.

1.2. Method

1.2.1. Seed Sterilization, Germination and Stress Application

The ridged and irregular surface structure of *Calendula officinalis* (pot marigold) seeds made the sterilization procedure challenging. Several approaches were tested to achieve effective surface sterilization; however, due to the characteristic morphological and structural properties of the seeds, certain difficulties were encountered in both the sterilization and germination stages. Therefore, the sterilization procedure was performed on seeds separated from the seed coat.

Based on preliminary trials, the following protocol yielded satisfactory results: seeds removed from the seed coat were treated with absolute ethanol for 40–50 s and then rinsed three times with sterile distilled water to eliminate residual alcohol. Subsequently, the seeds were immersed for 5 min in a diluted household bleach solution containing 10% sodium hypochlorite (NaOCl) prepared at a final

concentration of 6%. Finally, a last rinse was performed to ensure complete removal of chemical residues, after which the seeds were transferred onto a Petri dish lined with sterile drying paper and prepared for sowing (Figure 2a,b).

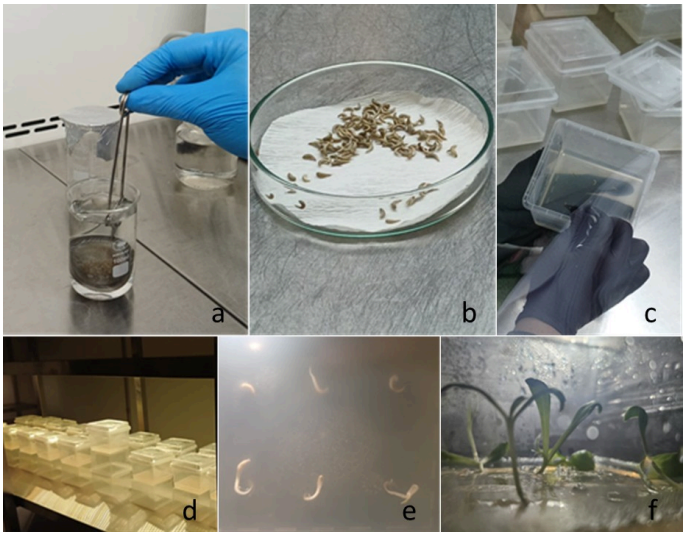


Figure 2. The procedural steps are as follows: seed sterilization (a,b), inoculation onto the culture medium (c), transfer to the tissue culture growth room (d) and the germination and growth period (e,f)

Following completion of surface sterilization, the seeds were inoculated onto MS (Murashige and Skoog, 1962) medium to promote germination and seedling development. The culture medium was prepared to contain 30 g/L sucrose and 0.7% (w/v) agar and the pH was adjusted to 5.7. All medium components were mixed prior to autoclaving and sterilized at 121 °C for 20 min.

After inoculation (Figure 2c) and transfer to the tissue culture room (Figure 2d), the seeds began to germinate within approximately 4–5 days (Figure 2e) and reached the seedling stage within 10 days (Figure 2f). Healthy seedlings were selected for the experimental treatments and

individually transferred to stress media containing different doses of silver nitrate ($AgNO_3$).

Silver nitrate ($AgNO_3$) was used to induce heavy metal stress. First, an $AgNO_3$ stock solution was prepared at a concentration of 1 mg/mL. For this purpose, a pre-weighed amount of $AgNO_3$ was placed in an Erlenmeyer flask, sterile distilled water (dH_2O) was added and the compound was completely dissolved. The stock solution was then sterilized by passing it through a sterile syringe filter, transferred into sterile Falcon tubes and stored at −20 °C for subsequent addition to the culture medium after autoclaving. Because $AgNO_3$ is heat-sensitive, it was not added prior to autoclaving; instead, it was aseptically supplemented to the medium after sterilization.

To establish heavy metal stress, media containing $AgNO_3$ at different concentrations and a fixed 2,4-D concentration were prepared and an unstressed control group was also included (Table 1).

Table 1. Composition of control and heavy metal ($AgNO_3$) stress media used in tissue culture

Medium	**Culture Medium (4.4 gr)**	**Sakkaroz (gr/L)**	**$AgNO_3$ (mg/mL)**	**2,4-D (mg/mL)**	**Agar (%)**	**pH**
(Kontrol)	MS	30	-	-	0,7	5.7
2	MS	30	0,1	0,5	0,7	5.7
3	MS	30	0,1	1	0,7	5.7
4	MS	30	0,5	0,5	0,7	5.7
5	MS	30	0,5	1	0,7	5.7
6	MS	30	1	0,5	0,7	5.7
7	MS	30	1	1	0,7	5.7
8	MS	30	1,5	0,5	0,7	5.7
9	MS	30	1,5	1	0,7	5.7

Each treatment was conducted with three replicates, with one plant used per replicate. After the seedlings were transferred to the stress media, they were exposed to stress in a growth chamber for

approximately 8 weeks (2 months). Throughout this period, plant growth rate, morphological characteristics and changes in leaf coloration were monitored regularly, while identical environmental conditions were maintained across all groups (Figure 3a,b).

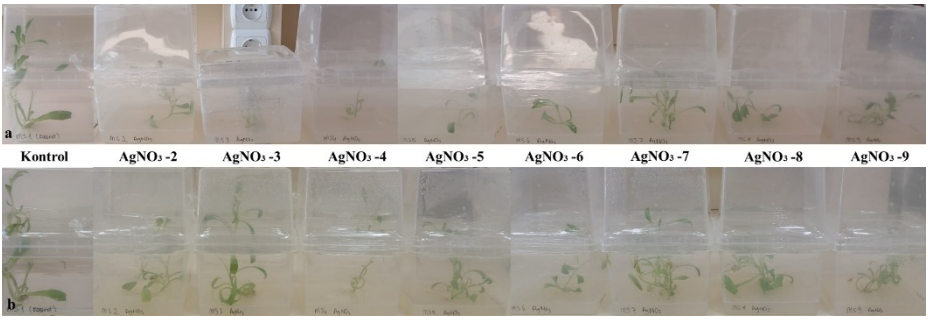


Figure 3. Plants transferred to the control medium and heavy metal stress media containing different $AgNO_3$ concentrations (a) and their development over a 2-month period (b)

1.2.2. Preparation of Samples

At the end of the stress period (2 months), the plants were carefully removed from the culture medium and agar residues remaining on the root and stem surfaces were rinsed off with sterile distilled water. Each sample was coded and labeled, then dried in an oven at 37 °C until a constant weight was reached. The dried samples were ground in a sterile mortar to obtain a homogeneous powder and stored at +4 °C until analysis.

1.2.3. Chemical Analysis

To determine the volatile and phenolic constituents, qualitative analyses were performed using GC–MS (gas chromatography–mass spectrometry) and HPLC (high-performance liquid chromatography).

1.2.3.1. GC–MS Analysis

From the dried plant samples, 0.20 g was weighed into GC tubes and one drop of hexane was added to each tube. The samples were analyzed at the Plant Analysis Laboratory of the Faculty of Agriculture, Recep Tayyip Erdoğan University, using a Shimadzu GC–MS (GCMS-2010 Plus) system (Figure 4a,b). The determination of volatile constituents was performed by modifying the SPME (solid-phase microextraction) method described by Yurteri et al. (2021).



Figure 4. Preparation of samples for GC–MS analysis (a) and GC–MS measurement/analysis (b)

1.2.3.2. HPLC Analysis

Quantitative determination of phenolic and flavonoid constituents was performed by HPLC. For each sample, 0.1 g was placed into Falcon tubes, 10 mL of methanol was added and the mixture was extracted in a 40 °C water bath for 1 h. After extraction, the samples were centrifuged at 4000 rpm for 7 min and the supernatant was filtered through a 0.45 µm syringe filter and transferred into HPLC vials. The prepared vials were placed into the autosampler tray and the samples were then injected and analyzed (Figure 5a,b,c).



Figure 5. Following extraction for HPLC analysis, centrifugation of the samples (a), transfer into vials (b) and HPLC measurement/analysis (c)

2. RESULTS AND DISCUSSION

In this study, the effects of heavy metal stress induced by silver nitrate ($AgNO_3$) at different concentrations on secondary metabolite production in *Calendula officinalis* L. (pot marigold) were investigated at both the volatile and phenolic levels. GC–MS and HPLC analyses demonstrated that increasing $AgNO_3$ concentrations promoted the accumulation of phenolic metabolites in *C. officinalis* L., while causing pronounced fluctuations in the composition of volatile constituents.

As a result of gas chromatography–mass spectrometry (GC–MS) analysis, a total of 60 different volatile compounds were identified (Table 2). The predominant compounds were δ-cadinene (19.75%), γ-cadinene (19.27%), α-muurolene (9.76%) and methyl palmitate (8.85%) (Figure 6).

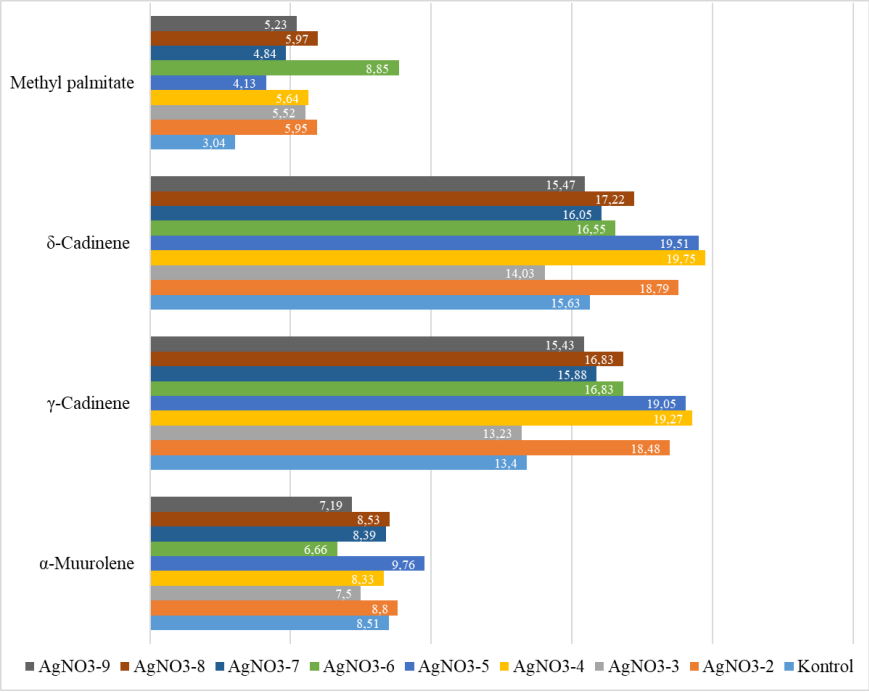


Figure 6. Major volatile compounds highlighted in the GC–MS analysis under heavy metal ($AgNO_3$) stress

Depending on the increasing $AgNO_3$ concentrations, dose-specific fluctuating changes were observed in the proportions of sesquiterpene compounds (particularly δ-cadinene, γ-cadinene and α-muurolene). In contrast, marked increases were recorded in compounds such as methyl palmitate, daucol and cedrol in parallel with the $AgNO_3$ dose. These findings indicate that $AgNO_3$-induced stress substantially reshapes the volatile profile, particularly through changes in constituents such as methyl palmitate, cedrol and daucal.

Table 2. Volatile compounds detected in plants under heavy metal ($AgNO_3$) stress using GC-MS analysis (%)

RI *	Compound Name	Control	$AgNO_3$-2	$AgNO_3$- 3	$AgNO_3$-4	$AgNO_3$-5	$AgNO_3$-6	$AgNO_3$-7	$AgNO_3$-8	$AgNO_3$-9
1308	Myrtenyl acetate	—	—	0.29	—	—	—	—	—	—
1345	α-Cubebene	1.65	—	1.57	0.98	—	0.77	1.59	1.64	0.95
1371	α-Copaene	3.94	4.2	4.26	3.46	5.38	2.79	4.8	5.02	3.4
1393	Tetradecane	—	—	—	—	—	—	—	0.31	—
1405	α-Gurjunene	0.43	0.42	0.98	0.32	0.57	—	0.58	0.43	—
1415	β-Caryophyllene	1.98	1.06	3.51	—	1.43	0.62	1.3	1.55	0.99
1419	Methyl eugenol	—	—	0.25	—	—	—	—	—	—
1442	β-Cedrene	1.3	1.55	1.5	1.14	1.84	0.96	1.63	1.61	1.1
1446	α-Patchoulene	1.35	0.77	0.81	0.59	0.95	0.45	0.86	0.85	0.53
1449	α-Humulene	4.44	2.78	6.94	0.94	3.39	1.86	3.42	3.43	2.71
1451	(E)-β-Farnesene	—	—	—	—	—	—	—	0.98	—
1457	α-Himachalene	0.68	—	—	—	—	—	—	0.95	—
1459	10βH-Cadina-1(6),4-diene	2.33	3.73	3.33	3.01	4.21	1.96	3.85	3.01	2.78
1472	α-Amorphene	5.92	6.33	6.84	5.48	7.01	4.06	6.05	6.33	4.95
1477	Germacrene D	—	0.73	2.59	—	1.14	2.89	1.01	0.83	0.5

Table 2. (Continued)

1482	Bicyclogermacrene	0.64	—	—	—	—	—	—	—	—
1483	Viridiflorene	—	—	1.57	—	—	—	—	—	—
1485	Lauryl alcohol	—	—	0.61	—	—	—	—	0.44	—
1490	α-Zingiberene	1.92	—	2.4	—	—	—	—	—	—
1483	(E)-β-Ionone		1.08	—	0.59	1.58	0.95	1.49	0.93	0.3
1496	α-Muurolene	8.51	8.8	7.5	8.33	9.76	6.66	8.39	8.53	7.19
1504	β-Bisabolene	0.55	—	—	—	—	—	—	0.56	—
1511	γ-Cadinene	13.4	18.48	13.23	19.27	19.05	16.83	15.88	16.83	15.43
1521	δ-Cadinene	15.63	18.79	14.03	19.75	19.51	16.55	16.05	17.22	15.47
1526	Tridecyl aldehyde	—	—	—	—	—	—	—	—	0.23
1556	(E)-Tridec-2-enal	—	—	—	—	—	—	0.42	—	—
1557	Spathulenol	—	0.26	—	—	0.46	—	—	—	—
1563	γ-Undecalactone	—	0.24	0.26	—	0.34	0.41	0.35	—	—
1565	Germacrene B	—	—	0.45	—	—	—	0.34	—	—
1550	Nerolidol	0.3	0.22	—	—	—	—	—	0.33	—
1591	Hexadecane	0.94	0.67	0.76	1.07	1.14	1.89	1.72	2	1.46

Table 2. (Continued)

1531	Citronellyl butyrate	—	—	—	0.49	—	0.62	—	—	—
1606	Cedrol	3.48	3.13	3	5.81	4.13	5.19	3.52	2.26	5.85
1634	Daucol	4.95	3.84	4.32	6.96	5.47	—	3.64	2.12	7.92
1643	Cedryl methyl ether	—	—	—	—	—	—	—	—	0.43
1646	Hedione	—	0.25	—	—	0.43	—	0.41	—	—
1647	(E)-Citronellyl tiglate	—	—	—	—	—	0.73	—	—	0.22
1649	β-Eudesmol	—	0.37	0.4	0.45	0.53	—	—	—	—
1649	α-Bisabolol oxide B	—	—	—	—	—	0.75	—	—	—
1637	α-epi-Muurolol	3.32	2.62	2.67	2.25	—	2.01	3.06	1.84	1.65
1655	Methyl dihydrojasmonate	—	—	—	0.52	—	—	—	—	—
1639	Octyl furan-2-carboxylate	—	0.27	—	1.26	1.52	1.65	1.36	1.27	1.27
1684	Myristyl alcohol	—	—	—	—	—	0.49	—	—	—
1691	Heptadecane	0.81	0.46	0.34	0.56	0.41	1.56	0.44	0.46	—
1736	Cis,cis-Farnesol	—	—	—	—	—	7.33	4.1	—	—
1750	Farnesal	0.35	3.29	1.73	4.09	3.39	—	0.51	2.18	1.49
1762	Octyl caprylate	—	—	—	—	0.34	—	—	—	0.45

Table 2. (Continued)

1790	Octadecane	0.79	0.22	—	—	—	0.76	—	—	0.49
1817	Isopropyl myristate	2.09	0.46	0.62	0.52	0.32	0.61	0.38	0.77	0.81
1830	Neophytadiene	10.71	5.76	6.54	4.39	—	6.75	5.73	6.73	14.42
1837	Phytone	1.07	1.47	0.9	1.68	1.16	2.07	1.88	1.54	1.54
1848	6-Acetyl-1,1,2,4,4,7-hexamethyl-tetralin	1.88	—	—	—	0.38	—	0.39	1.06	—
1889	Nonadecane	0.38	—	—	—	—	0.99	—	—	—
1916	Methyl palmitate	3.04	5.95	5.52	5.64	4.13	8.85	4.84	5.97	5.23
1956	Isophytol	—	—	—	—	—	—	—	—	0.25
1985	Ethyl palmitate	—	0.22	—	—	—	—	—	—	—
2014	Isopropyl palmitate	0.4	—	—	—	—	—	—	—	—
2257	Tributyl citrate acetate	—	—	—	0.47	—	—	—	—	—
2271	5α-Androst-16-en-3-one	—	—	0.28	—	—	—	—	—	—
2386	Tetracosane	0.82	—	—	—	—	—	—	—	—

* Kovats Retention Index (RI)

High-performance liquid chromatography (HPLC) analyses were conducted to evaluate how increasing $AgNO_3$ stress intensity (from $AgNO_3$-1 to $AgNO_3$-9) affected the accumulation of phenolic constituents. In this context, five major phenolic compounds (gallocatechin, catechin, chlorogenic acid, caffeic acid and quercitrin) were detected (Table 3). Marked changes in the levels of these compounds were observed depending on the $AgNO_3$ treatment.

In the control group, chlorogenic acid (51.3 mg/g) and catechin (46.4 mg/g) were identified as the predominant compounds, whereas caffeic acid was detected at a moderate level (6.9 mg/g). Quercitrin was present at a low level (0.4 mg/g) and gallocatechin remained at trace level (Tr.).

Under $AgNO_3$ stress, the amounts of chlorogenic acid and catechin were generally suppressed compared with the control. Chlorogenic acid decreased to 0.6 to 17 mg/g in the stress groups and did not approach the control level at any stress stage. Similarly, catechin concentrations ranged from Tr. to 16.3 mg/g and remained markedly below the control, not exceeding approximately one-third of the control level even at the highest values. These findings indicate that increasing $AgNO_3$ stress severity suppresses the accumulation of chlorogenic acid and catechin and suggest that phenolic metabolism may have been redirected toward the formation of alternative phenolic products.

In contrast, quercitrin exhibited the most pronounced stress-associated response. Compared with the control, quercitrin levels

increased substantially under $AgNO_3$ treatments, rising to 1.8 to 37.1 mg/g, with the highest value recorded at the highest stress level ($AgNO_3$-9: 37.1 mg/g). Caffeic acid displayed a more variable pattern; although it generally remained below the control (0.2 to 4.1 mg/g), partial recoveries were observed at some stages ($AgNO_3$-8: 3.9 mg/g). Another notable compound, gallocatechin, was present at trace level in the control but became quantitatively detectable at certain stress intensities ($AgNO_3$-5: 7.4 mg/g).

Table 3. Components detected by HPLC analysis in plants exposed to heavy metal ($AgNO_3$) stress (mg/g)

Compound Name	Gallocatechine	Chlorogenic Acid	Catechine	Caffeic Acid	Quercitrin
Kontrol	Tr.	51.3	46.4	6.9	0.4
$AgNO_3$-2	5.5	0.6	Tr.	1.7	2.6
$AgNO_3$-3	1.3	10.7	16.3	4.1	29.9
$AgNO_3$-4	Tr.	Tr.	Tr.	0.4	Tr.
$AgNO_3$-5	7.4	Tr.	13.6	2	1.8
$AgNO_3$-6	Tr.	Tr.	Tr.	0.2	15.3
$AgNO_3$-7	1.8	17	3.7	3.4	20
$AgNO_3$-8	2.7	4.6	4.9	3.9	25.4
$AgNO_3$-9	2.4	12	5.4	2.7	37.1

* Tr.: traces

When these results are considered collectively, $AgNO_3$-induced heavy metal stress applied under in vitro conditions affected phenolic

accumulation and altered the quantitative distribution of the phenolic profile. As stress intensity increased, certain compounds (chlorogenic acid and catechin) were markedly suppressed, whereas a strong increase was observed in flavonol derivatives such as quercitrin.

In our study, the prominence of δ-cadinene (19.75%) and γ-cadinene (19.27%) among the major constituents of the volatile profile indicates that sesquiterpenes may constitute core components of the volatile composition in *Calendula officinalis*. This finding is consistent at the level of predominant compounds with the report of Gazim et al. (2008), who identified δ-cadinene (22.5%, 22.1% and 18.4%, respectively) and γ-cadinene (8.9%, 25.4% and 24.9%, respectively) as major constituents in the volatile profiles of flower samples obtained using different extraction approaches (SD, HS-SPME and HS-CF). However, a direct quantitative match should not be expected because that study was based on flower material, whereas our study analyzed samples obtained from plants grown under tissue culture conditions. Plant organ and developmental stage, the influence of in vitro conditions on metabolic fluxes and the extraction approach are key factors that can substantially affect the relative abundances of major constituents. Nevertheless, the recurrence of δ-cadinene and γ-cadinene as prominent constituents in both studies suggests that these compounds may represent a reproducible chemical backbone of the *C. officinalis* volatile profile across different materials and conditions.

Okoh et al. (2008) reported that sesquiterpenoids can be predominant in essential oils obtained by hydrodistillation from fresh material of *C. officinalis* leaves and flowers, whereas the composition may change markedly after drying. In that study, δ-cadinene was reported among the major constituents in both fresh and dried leaves (11.08% and 9.0%) as well as in flowers (13.1%) (Okoh et al., 2008). In our study, samples were derived from tissue culture conditions and volatile constituents were determined using an SPME-based approach; therefore, it is plausible that the relative abundances do not directly coincide with those reported in hydrodistillation-based studies.

Sabir et al. (2015) reported that hot-water extracts obtained from the flowers and leaves of *C. officinalis* exhibit strong antioxidant potential and that luteolin, caffeic acid, apigenin, rosmarinic acid and kaempferol were shared major constituents in the HPLC profiles of both flowers and leaves (Sabir et al., 2015). In our study, the detection of caffeic acid supports the view that this compound may be among the recurring phenolics of the species. The lack of prominence of other major flavonoids in our profile may be attributed to differences in extraction conditions, the type of plant material and the directing effects of stress conditions on metabolic flux.

Overall, our findings demonstrate that heavy metal stress induced by $AgNO_3$ under in vitro conditions reorganized the secondary metabolite profile of *Calendula officinalis* at both the volatile (GC–MS) and phenolic (HPLC) levels. Most previous studies have been conducted

on plant material grown under natural or field conditions and have often focused on different organs such as flowers and leaves. In contrast, our study was performed using samples derived from tissue culture conditions, which limits direct quantitative comparisons. Moreover, the use of an SPME-based approach for volatile analysis and methanolic extraction followed by HPLC for phenolic profiling should be considered, as differences in solvents and analytical methods can affect the relative abundances of major constituents when compared with studies employing alternative methodologies.

It has been emphasized that changes in chemical profiles are associated with many parameters, including abiotic and biotic factors as well as post-harvest processing, extraction methods and storage conditions (Fokou et al., 2020). Therefore, although our results show similarities with the literature at the level of predominant constituents, a one-to-one correspondence in relative percentages is not expected due to the originality of the experimental setup and methodological differences. Accordingly, our findings contribute to the literature by demonstrating that heavy metal stress can modify metabolite distribution in *C. officinalis* under in vitro conditions. However, because studies using an identical experimental design are limited, the observed agreement is better interpreted at the profile level and in terms of dominant constituents, rather than as a strict quantitative match.

3. CONCLUSION

Increasing urbanization and industrialization in recent decades have contributed to a substantial rise in environmental pollution. Among these pollutants, heavy metals exert toxic effects in both terrestrial and aquatic ecosystems and have become major sources of biological stress for all living organisms, including plants. Heavy metal compounds such as silver nitrate ($AgNO_3$) induce oxidative stress in plant tissues by triggering the production of reactive oxygen species (ROS). This process disrupts physiological homeostasis and can lead to significant alterations in secondary metabolite biosynthesis (Al-Oubaidi et al., 2014).

In the present study, the effects of $AgNO_3$ application on the secondary metabolite profile of *Calendula officinalis* L. were evaluated using both GC–MS and HPLC analyses. The results indicate that silver ions markedly reprogram metabolic activity in the plant. In the GC–MS analyses, dose-dependent increases and decreases were observed in the relative abundances of certain constituents, including δ-cadinene, γ-cadinene and α-muurolene. In contrast, the levels of methyl palmitate, cedrol and daucol increased under $AgNO_3$ treatments.

Consistently, the HPLC results revealed pronounced differences in phenolic constituents. With increasing stress intensity, some compounds (chlorogenic acid and catechin) were strongly suppressed, whereas a pronounced increase in flavonol derivatives such as quercitrin was observed. This pattern suggests that, under stress conditions, phenolic metabolism may shift toward compounds associated with protective and

antioxidant responses. Accordingly, $AgNO_3$ application appears to influence not only the overall level of the phenolic profile in *C. officinalis* L. but also its composition. These findings indicate that stress intensity should be considered a key variable shaping the balance among phenolic constituents.

Overall, the results suggest that *Calendula officinalis* L. develops biochemical adaptation to $AgNO_3$-induced heavy metal stress and that this adaptation is accompanied by a reorganization of both volatile and phenolic constituents.

In conclusion, the data demonstrate that environmental stress factors play an important role in directing plant secondary metabolite production. Understanding stress-driven metabolic alterations provides important scientific contributions by elucidating biological resilience mechanisms in plants and by supporting the identification of new active constituents that may be utilized in phytocosmetic and phytopharmaceutical applications.

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