

Examination of the Effectiveness of Silver Nanoparticles on the Growth, Development and Synthesis of Secondary Metabolites of *Kalanchoe blossfeldiana* Poelln. *In Vitro*

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Abstract

Plant secondary metabolites, which are produced under stress, are vital in pharmaceuticals, food and flavor industries. Plant tissue culture technique enables the production of large quantities of metabolites in controlled environments using elicitors. In this study, silver nanoparticles (AgNPs) were applied to *Kalanchoe blossfeldiana* cultures *in vitro* for two weeks to determine their effect on secondary metabolite production. The results exposed that the AgNPs influenced biomass production considerably, with the highest responses at 0.5 and 1 mg L⁻¹. A gas chromatography-mass spectrometry analysis revealed 42 secondary metabolites, 15 of which were found in the mother plant including Azulene (anti-inflammatory), Galbazine (anti-tumor), D-Limonene (antimicrobial), Palmitic acid, and Loliolide (both with anti-cancer properties). In contrast, the untreated *in vitro* control group produced 27 compounds, such as Piperidine, 2-pentyl- (CNS effects and Anticancer), α-Tocopherol (Cardiovascular protection.), Phytol (Analgesic and Anxiolytic) and γ-Sitosterol (antioxidant and antidiabetic). Meanwhile, Plants treated with AgNPs (0.5–2.5 mg·L⁻¹) synthesized 29–32 compounds. These included)DGLA((Vascular protection (Oleamide (antimicrobial and anti-allergic) cardioprotective fatty acids (Linoleic and Oleic acids), and Phytol (with antiparasitic, anticancer, and antimicrobial activities), along with Palmitic acid, D-Limonene, α-Piperidone, Galbazine and others). The findings indicate that Nano silver treatment enhanced biomass production and bioactive compound accumulation in *K. blossfeldiana*, which supports its potential for commercial applications.

Keywords: *Bryophyllum*; elicitor; biomass; plantform bioreactor; secondary metabolites

Introduction

Plant tissue culture is one of the major biotechnological approaches for plant micropropagation, production of secondary metabolites. Additionally, the elicitation process activates the secondary metabolite biosynthesis that acts as a protective agent for plant survival and productivity. In the recent years, developments in the field have improved efficiency of *in vitro* synthesis of this class of valuable metabolites, offering a sustainable and environmentally friendly alternative to conventional plant-derived yield. This advancement is beneficial for medicinal plant cell and organ cultures, which can produce higher concentrations of important metabolites than the whole plant in nature [1].

The ornamental plant *Kalanchoe*, which is native to Madagascar, is rich in bioactive compounds, such as flavonoids, bufadienolides, and triterpenoids that exhibit a high anticancer potential [2]. Yet, the conventional production of its active chemicals is insufficient for industrial demand. *In vitro* multiplication and biomass elicitation offer an efficient alternative for an enhanced secondary metabolite production. [3].

Very few methods are available for the regeneration of *Kalanchoe* or *Bryophyllum* spp. Kulus [4] used full-strength Murashige and Skoog (MS) medium enriched with benzylaminopurine (BAP) for propagation of this plant. By contrast, García-Pérez et al. [5] reported that reducing the macronutrient concentration of the MS medium to half-strength improved *in vitro* growth and multiplication of the same species. Winiarczyk et al. [6] induced the regeneration of direct and indirect adventitious shoots in *in vitro* cultures from three topophysical zones of leaf blade of *K. daigredistal*, on MS medium fortified with 0.8 mgL⁻¹ indole acetic acid (IAA) and 0.5 mgL⁻¹ BAP.

A Silver nanoparticle (AgNP) is highly useful in biology, agriculture, and medicine. In addition, it plays an important role in micropropagation by controlling the growth of epiphytic and endophytic contaminants in plant cells and improving biomass production, and the synthesis of secondary metabolites in *in vitro* cultures [7, 8]

According to Ulusoy et al. [9], AgNPs can improve plant tissue culture by boosting secondary metabolite synthesis, biomass production, and cell proliferation. *In vitro* studies have demonstrated that the concentration of AgNP affects plant growth, development, and metabolite synthesis [7, 9, 10].

Given these factors, the current study focused on developing novel approaches to employ AgNPs to increase the biomass and production of secondary metabolites in *Kalanchoe blossfeldiana* cultured *in vitro*.

Materials and Methods

The present study was carried out at the Tissue Culture Laboratory, Department of Biology, College of Science, University of Basrah, during the period 2022-2023.

Plant Materials

Plants were obtained from local nurseries and identified by the Department of Biology at the University of Basrah as *Kalanchoe blossfeldiana* Poelln.

In vitro Shoot Culture Initiation, and Multiplication

Nodal explants were wiped with 75% ethanol, followed by surface sterilization with 0.1% HgCl₂. Then, explants were rinsed five times with sterilized water. Sterilized nodal explants were cultured in 250-mL jars containing 50 mL of MS medium with 30 gL⁻¹

sucrose, 100 mg L⁻¹ myo-inositol, 80 mg L⁻¹ adenine sulfate, 120 mg L⁻¹ NaH₂PO₄·2H₂O, and 1 mg L⁻¹ each of kinetin and IAA. The cultures were then incubated at 27 °C under a 16-h light/8-h dark cycle at 30 μMm⁻²s⁻¹.

Next, the multiplied shoots grown in solid medium were shifted to a Plantform bioreactor containing the same medium (initiation medium) as described above, to enhance biomass production and to study the effect of AgNPs on fresh weight, dry weight, shoot numbers, and secondary metabolite accumulation. The bioreactor containers were incubated under the same temperature and previously mentioned light conditions for two months. Immersion cycles for the clumps were set to 5 min at 4-h intervals.

Spherical AgNPs measuring 20 nm from Hongwu International Group Ltd, China, were used at concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 mg L⁻¹. The AgNPs powder was dissolved in sterilized distilled water according to these concentrations. The prepared solution was then aseptically injected into plantform bioreactors containing two months old *Kalanchoe* biomass *via* an aeration tube using sterilized syringe under aseptic conditions.

Plant Materials and Extract Preparation

Greenhouse-grown mother plants and *in vitro* shoots were washed with tap water for 10 minutes, rinsed with distilled water, air-dried at room temperature for 21–30 days, oven-dried at 30°C for two weeks, and ground using coffee grinder.

The extraction procedure followed Harborne [11] protocol. A total of 5 g powdered leaves were immersed in petroleum ether using a Soxhlet apparatus for 24 h to dissolve and remove the fats from the sample, and then left to evaporate until dry. A mixture of 90% ethanol and 10% acetic acid was added, and extraction was continued for another 24 h. The solution was concentrated using a rotary evaporator, and the pH was adjusted to 9 with ammonium hydroxide.

The solution was filtered through a filter paper, added with chloroform, shaken several times, transferred to a separating funnel and then left undisturbed until two layers were formed. The lower layer, which contained the alkaloids dissolved in chloroform, was collected for further analysis.

The analysis was carried out at the Basra Oil Company Laboratory using an Agilent 7890B GC and an Agilent 5977A MS. The GC oven temperature ranged from 40°C to 300°C, with helium as the carrier gas. The injection was performed using a 0.5 μL sample in Pulsed Splitless mode at 290°C. Compounds were identified using NIST 2014 and 2020 libraries.

With the Use of MetaboAnalyst 4.0, raw data were uploaded without scaling, transformation, or normalization, and calibration curve parameters were tracked.

Data Recording and Statistical Analysis

A series of three *in vitro* experiments was conducted to examine shoot proliferation and induction in response to normal and nano-silver concentrations. Three containers Plantform bioreactors were used for each treatment. After 2 weeks, the data on shoot numbers, and biomass (fresh and dry weight) were recorded under normal and elicitor conditions. All data were subjected to analysis of variance (ANOVA) for a completely randomized design (CRD). The mean growth parameters (fresh and dry weights) were separated using the least significant difference (LSD) at 5% (13). Statistical analysis was performed using SPSS 24 software. Data were expressed as mean ± standard error (SE).

Results and Discussion

In vitro Shoot Culture Initiation, and Multiplication

The findings indicate that the treatment of propagated shoots with 0.5 mg L^{-1} AgNPs substantially increased biomass, which produced a maximum fresh weight of 98.43g. This followed by treatments using 1 and 1.5 mg L^{-1} AgNPs, which produced fresh weight come to 89.86 and 84.63 g respectively. On the other hand, the lowest fresh weights were recorded in media supplemented with 2, and 2.5 mg L^{-1} AgNPs, in addition to control treatment, with values of 76.54, 68.14, and 76.37 g, respectively (Table 1, Fig 1 and Plate 1A).

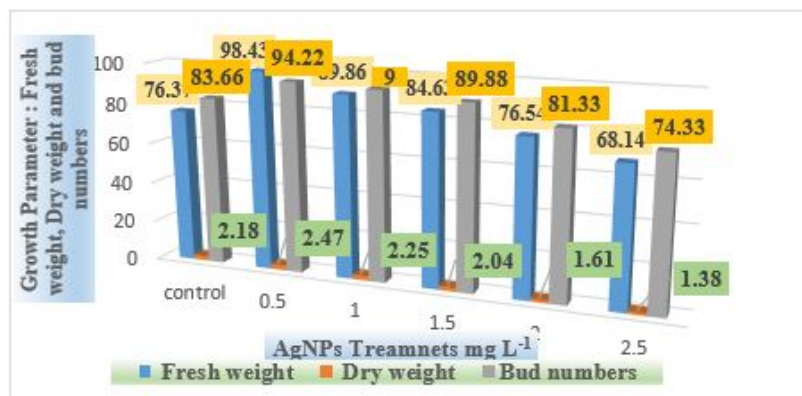


Figure 1: Effect of AgNPs Application at Different Concentrations on Growth Parameters

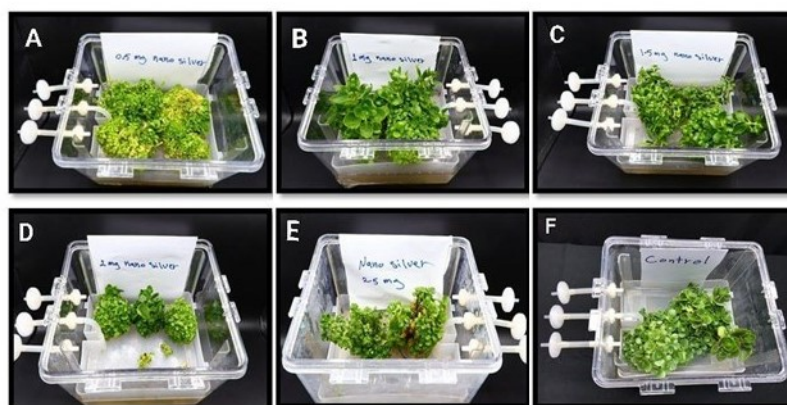


Plate 1 Multiplied shoots of *Kalanchoe blossfeldiana* grown in liquid medium with varying concentrations of AgNPs using a Plantform bioreactor. A: 0.5 mg L^{-1} AgNPs, B: 1 mg L^{-1} AgNPs, C: 1.5 mg L^{-1} AgNPs, D: 2 mg L^{-1} AgNPs, E: 2.5 mg L^{-1} AgNPs, F: Control (without AgNPs).

Table 1: Impact of AgNPs at various concentrations on growth parameters. The values that the various letters within the same group follow indicate a statistically significant difference

AgNPs Treatments mg. L^{-1}	Growth Parameters		
	Fresh weight g $\pm \text{SE}$	Dry weight g $\pm \text{SE}$	Buds number $\pm \text{SE}$

Control	76.37 ± 10.84 ^c 98.43 ± 6.28 ^a 89.86 ± 4.37 ^b 84.63 ± 3.84 ^{bc} 76.54 ± 5.01 ^c 68.14 ± 4.80 ^c	2.18 ± 0.51 ^{ab} 2.47 ± 0.52 ^a 2.25 ± 0.34 ^{ab} 2.04 ± 0.21 ^b 1.61 ± 0.22 ^c 1.38 ± 0.28 ^c	83.66 ± 9.21 ^b 94.22 ± 3.96 ^a 93.00 ± 3.80 ^a 89.88 ± 4.01 ^a 81.33 ± 3.80 ^b 74.33 ± 6.59 ^c
	LSD=8.40	LSD=6.22	LSD=0.42

Similarly, dry weight followed the same pattern as fresh weight, with the lowest values being recorded at 2, 2.5 mg L⁻¹, and the control (Table 1 and Plate 1B). The highest bud counts were observed at 0.5, 1, and 1.5 mg. L⁻¹ AgNPs (94.22, 93.00, and 89.88 buds, respectively). Meanwhile, the lowest bud counts were detected in the 2, 2.5 mg. L⁻¹, and control treatment (83.66, 81.33, and 74.3 buds, respectively) (Table 1).

The present study highlighted the effect of various concentrations of (AgNPs) on plant biomass yield responses in *K. blossfeldiana in vitro*, and the results show that low concentrations of AgNPs considerably enhanced growth parameters. This finding aligns with Elsayh [15], who observed improved callus induction and somatic embryo formation in date palm cultured at low AgNPs concentrations. In addition, [12, 13] suggested that AgNPs promote plant growth through nutrient uptake and cross-cellular barriers. Sadak [14] further suggested that AgNPs at different concentrations improve plant biomass production, and such finding may be due to the role of blocking ethylene signaling in plant. AgNPs can enhance plant cell uptake of nutrients and water from culture media through mutilation of the cell wall [7].

Identification and Analysis of Secondary Metabolites

Based on gas chromatography–mass spectrometry (GC-MS) analysis, the mother plant, and *in vitro* propagated plants, which were untreated and treated with AgNPs, produced substantially high metabolites (Fig 2).

GC-MS analysis identified 42 metabolic compounds, with 15 compounds detected in the mother plant and 27 in the *in vitro* control treatment plants (without AgNPs). Meanwhile, the application of AgNPs considerably enhanced metabolite production in *in vitro* propagated plants, which resulted in 29, 29, 30, 32, and 32 compounds observed at AgNPs concentrations of 0.5, 1, 1.5, 2, and 2.5 mgL⁻¹, respectively (Table 2 and Fig 4). The main compounds such as Pyranone (DDMP); Azulene; Myristic acid; Ethyl myristate; Loliolide; Pentadecanoic acid showed the highest significance value. Conversely, 3-hydroxy-2-pyranone; Galbazine; Palmitic acid; 1-Octadecanol; Dimethyl sulfone; Dimethyl Sulfoxide; DL-Proline, 5-oxo-, methyl ester exhibited the lowest significance value (Fig 3). However, not all secondary metabolites produced by mother plants were detected in *in vitro* plants and *vice versa*.

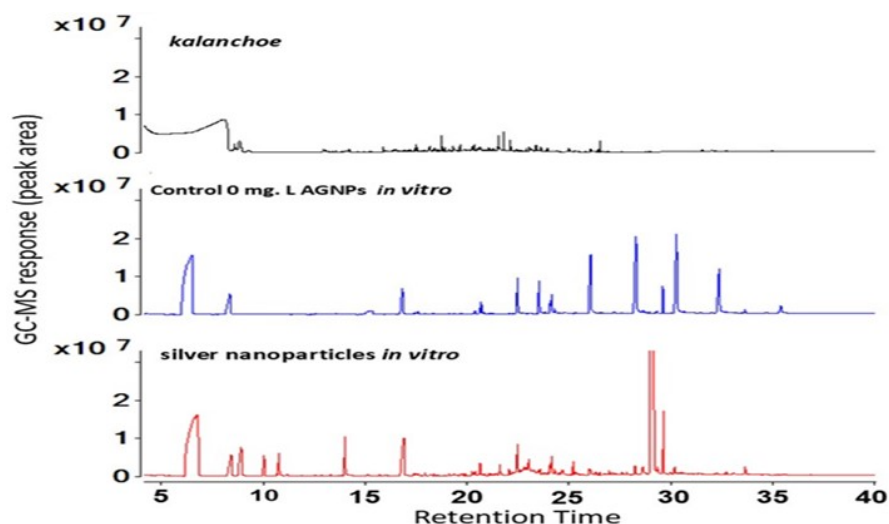


Figure 2: Chromatographic separation of secondary metabolites. The black color represents the mother plant (grown *ex vitro*), the blue represents the plant grown *in vitro* (control), and the red represents the plant grown *in vitro* on medium containing an elicitor (silver nanoparticles).

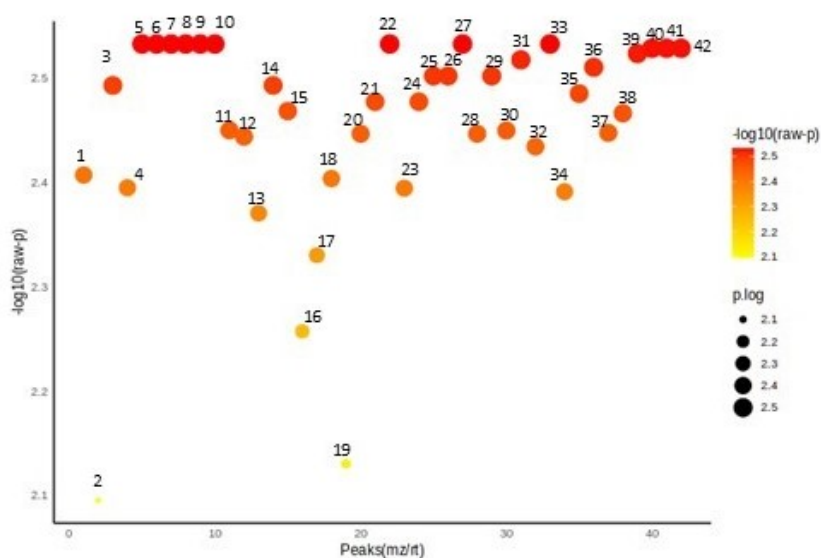


Figure 3: Identified secondary metabolites on mother plant, *in vitro*-propagated plants treated with silver nanoparticles, and a control, using p-values from GC-MS analysis. Red circles mark statistically significant compounds, while black dots indicate statistically non-significant ones. The log10 transformation highlights more significant compounds at the top. The x-axis shows compound numbers, and the y-axis displays log-transformed p-values.

Table 2: GC-MS analysis of the ethanolic extract of *K. blossfeldiana* from the mother plant and *in vitro*

No.	RT	Compound	RI	Peak Area)10 ⁶ (						
				Mother plant	<i>In vitro</i> AgNPs mg. L ⁻¹ Treatments					
					Control	0.5	1	1.5	2	2.5
1	9.03	3-hydroxy-2-pyrone	989.0	18.07±0.48	1.04±0.03	33.07±4.81	50.16±3.29	45.30±1.51	33.92±4.99	21.84±0.86
2	10.01	D-Limonene	1029.5	1.57±0.07	1.15±0.07	1.54±0.09	1.64±0.10	1.30±0.02	1.22±0.10	1.33±0.10
3	11.25	α-Piperidone	1168.0	1.60±0.01	0.13±0.01	3.41±0.14	7.54±0.18	8.00±0.13	6.48±0.12	5.54±0.13
4	12.02	Galbazine	1179.5	1.95±0.05	2.35±0.19	21.07± 1.08	29.99±1.03	1.04±0.03	1.19±0.11	1.15±0.08
5	14.31	Pyranone (DDMP)	1230.6	1.57±0.05	ND	ND	ND	ND	ND	ND
6	15.01	Azulene	1296.5	5.95±0.10	ND	ND	ND	ND	ND	ND
7	21.41	Myristic acid	1755.0	0.12±0.02	ND	ND	ND	ND	ND	ND
8	21.63	Ethyl myristate	1773.0	1.85±0.06	ND	ND	ND	ND	ND	ND
9	21.92	Loliolide	1789.0	1.01±0.01	ND	ND	ND	ND	ND	ND
10	22.47	Pentadecanoic acid	1869.2	2.05±0.02	ND	ND	ND	ND	ND	ND
11	22.91	Pentadecanoic acid, 14-methyl-, methyl ester	1890.4	2.05±0.02	2.92±0.03	4.03±0.05	5.29±0.23	4.16±0.11	3.14±0.15	3.30±0.19
12	23.15	Palmitic acid, methyl ester	1910.0	10.57±0.42	5.10±0.12	4.07±0.14	3.89±0.18	3.55±0.17	1.88±0.15	1.58±0.19
13	23.49	Palmitic acid	1970.2	5.12±0.05	1.51±0.04	1.54±0.08	1.86±0.05	2.45±0.06	2.45±0.07	2.08±0.10
14	24.62	1-Octadecanol	2069.0	65.53±1.25	86.67±8.96	5.51± 0.07	3.45±0.11	2.18±0.02	1.77±0.10	1.51±0.03
15	26.49	Stearic acid	2163.3	1.32±0.03	85.85±0.53	2.01±0.12	3.61±0.11	2.45±0.06	4.05±0.07	3.31±0.24
16	6.26	Dimethyl Sulfoxide	786.6	ND	184.38±16.96	179.26±2.01	274.76±22.53	303.31±5.66	267.49±2.98	297.48±12.24
17	8.38	Dimethyl sulfone	919.3	ND	47.95±2.69	24.19±5.63	49.67±2.85	45.47±1.18	24.38±1.06	92.49±1.97
18	12.91	Piperidine, 2-pentyl-	1194.3	ND	39.58±0.56	47.10±0.80	48.45±4.67	81.34±1.72	40.95±1.86	30.72±0.99
19	16.83	DL-Proline, 5-oxo-, methyl ester	1395.9	ND	3.15±0.60	3.08±0.08	3.60±0.18	2.23±0.10	3.19±0.15	9.70±0.26
20	18.73	Dihydroactinidiolide	1493.0	ND	1.97±0.03	2.57±0.24	2.64±0.16	1.71±0.05	1.44±0.08	1.23±0.10
21	20.92	1-Deoxy-d-arabitol	1660.0	ND	35.68±4.52	17.12±0.10	21.78±2.60	26.03±0.56	40.29±1.22	29.73±0.29
22	21.15	3-O-Methyl-d-glucose	1682.7	ND	0.91±0.07	ND	ND	ND	ND	ND
23	22.11	1,5-Anhydro-d-mannitol	1843.0	ND	11.35±0.74	9.16±11.94	1.78±0.08	1.66±0.09	1.54±0.08	1.26±0.09
24	23.31	Unknown	1955.1	ND	16.25±0.75	3.22±0.15	4.35±0.09	9.61±0.18	8.09±0.16	3.03±0.13
25	24.91	Methyl-9,10-octadecadienoate	2110.0	ND	4.02±0.09	4.32±0.16	7.45±0.19	13.13±0.12	11.92±0.13	5.40±0.33
26	25.15	Phytol	2122.1	ND	0.95±0.07	1.12±0.04	1.33±0.13	2.42±0.05	16.13±0.07	1.78±0.09
27	28.64	β-Monopalmitin	2475.0	ND	121.21±2.73	ND	ND	ND	ND	ND
28	29.63	Docosanoic acid, ethyl ester	2577.0	ND	1.43±0.06	3.90±0.32	7.71±0.20	9.86±0.12	6.16±0.08	4.15±0.20
29	30.58	Armid E	2625.0	ND	16.89±0.21	453.41±8.66	539.14±11.07	478.04±11.21	227.09±10.66	409.16±6.24
30	31.37	Campesterol	3105.5	ND	118.90±0.97	9.63±0.14	7.84±0.10	1.30±0.11	1.03±0.03	1.14±0.11
31	32.01	α-Tocopherol	3139.1	ND	69.84±0.41	5.67±0.09	3.18±0.10	1.08±0.05	ND	ND
32	33.62	β-Sitosterol	3220.0	ND	3.01±0.13	9.43±0.57	6.74±0.17	2.95±0.12	3.97±0.07	7.00±0.13
33	35.01	γ-Sitosterol	3290.0	ND	12.04±0.07	ND	ND	ND	ND	ND
34	24.29	Dihomo-gamma-linolenic acid (DGLA)	2050.7	ND	ND	1.22±0.03	1.46±0.09	1.37±0.11	1.51±0.02	1.71±0.02
35	26.81	Palmitamide	2189.0	ND	ND	4.35±0.13	7.06±0.07	6.89±0.12	7.88±0.13	4.06±0.06
36	26.99	Punicic acid	2202.0	ND	ND	3.82±0.24	4.86±0.06	5.36±0.17	2.17±0.12	1.02±0.21
37	27.67	Arachidonic acid	2324.3	ND	ND	20.99±3.01	20.07±2.03	13.43±0.48	6.03±0.11	3.23±0.16

38	28.19	Oleamide	2397.1	ND	ND	2.16±0.11	4.00±0.11	4.25±0.10	2.08±0.07	2.77±0.06
39	23.86	3-Eicosene, (E)-	1980.0	ND	ND	ND	ND	7.04±0.10	6.55±0.07	5.91±0.10
40	24.08	Octadecanal	1998.3	ND	ND	ND	ND	ND	6.91±0.10	22.84±1.39
41	25.95	Linoleic acid	2134.0	ND	ND	ND	ND	ND	1.46±0.04	5.11±0.12
42	26.17	Linolenic acid	2143.9	ND	ND	ND	ND	ND	7.08±0.07	4.09±0.11

RT: Retention time, RI: Retention index. Dimethyl sulfoxide (DMSO) and Dimethyl sulfone were detected in the GC-MS analysis, likely originating from their use as solvents in culture media preparation. The presence of 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester suggests contamination from plastic materials used during tissue culture procedures. Sequences 1–15:

Compounds detected in the mother plant, with partial overlap observed in the control and AgNPs-treated samples. 16–33: Compounds shared between the control and some of the AgNPs treatments. 34–38: Compounds detected exclusively in all AgNPs treatments. 39: Compound shared by the three highest AgNPs concentrations. 1.5; 2.5. 40–42: Compounds detected only at the two highest AgNPs concentrations (2.0 and 2.5 mg/L).

Table 2 shows that all *in vitro* treatments consistently produced the metabolic compounds (1-4, and 11-15). Conversely, secondary metabolites (5-10) were exclusively detected in the mother plants. The compounds arranged in Table 2 according to the sequence (16-21, 23-26, 28-30 and 32) were identified in all *in vitro* treatments, except (22) 3-O-Methyl-d-glucose, (27) β -Monopalmitin, (33) γ -Sitosterol which was detected in the control, and (31) α -Tocopherol, which was absent in plants treated with 2 and 2.5 mg L⁻¹ AgNPs.

In addition, compound 3-Eicosene, (E)- was detected in plants grown on media containing 1.5, 2.0, and 2.5 mg L⁻¹ AgNPs, and Octadecanal; Linoleic acid and Linolenic acid were detected in plants grown on media containing 2.0 and 2.5 mg L⁻¹ AgNPs.

Shoots treated *in vitro* with AgNPs exhibited tremendous production of key compounds. At 1 mg L⁻¹, the highest peak areas were observed for 3-hydroxy-2-pyranone (50.16), D-Limonene (1.64), Galbazine (29.99), Pentadecanoic acid, 14-methyl-, methyl ester (5.29), and Armid E (539). By contrast, the highest amounts of α -Piperidone (8.0), Piperidine, 2-pentyl- (81.34) and Docosanoic acid, ethyl ester (9.86) were recorded at 1.5 mg L⁻¹. while 1-Deoxy-d-arabitol (40.29) at 2.0 and DL-Proline, 5-oxo-, methyl ester (9.70) was recorded at 2.5 mg L⁻¹ (Table 2 and Fig 3).

Table 3 summarizes the percentage composition of the compounds identified in the GC-MS chromatograms. In the mother plant 1-Octadecanol was the most abundant compound (54.28%), followed by 3-hydroxy-2-pyranone (14.97%), whereas Loliolide (0.84%) and Myristic acid (0.10%) were the least abundant.

Table 3: Percentage of Identified Chemical Compounds *K. blossfeldiana* mother plant and *in vitro* culture

NO.	Compound	RI	Formula	Chemical Classification	Peak Area %						
					Mother plant	Control	In vitro AgNPs mg. L ⁻¹ Treatments				
							0.5	1	1.5	2	2.5
1	3-hydroxy-2-pyranone	989.0	C ₅ H ₄ O ₃	Lactone (Pyranone derivative)	14.97	0.12	3.75	4.46	4.16	4.51	2.21
2	D-Limonene	1029.5	C ₁₀ H ₁₆	Monoterpene hydrocarbon	1.30	0.13	0.17	0.15	0.12	0.16	0.13
3	α -Piperidone	1168.0	C ₅ H ₉ NO	Cyclic ketone (Lactam)	1.33	0.01	0.39	0.67	0.73	0.86	0.56

4	Galbazine	1179.5	C ₉ H ₁₄ N ₂ O	Aromatic amine derivative	1.62	0.27	2.39	2.67	0.10	0.16	0.12
5	Pyranone (DDMP)	1230.6	C ₆ H ₈ O ₄	Lactone (Pyranone derivative)	1.30	ND	ND	ND	ND	ND	ND
6	Azulene	1296.5	C ₁₀ H ₈	Aromatic hydrocarbon	4.93	ND	ND	ND	ND	ND	ND
7	Myristic acid	1755.0	C ₁₄ H ₂₈ O ₂	Saturated fatty acid	0.10	ND	ND	ND	ND	ND	ND
8	Ethyl myristate	1773.0	C ₁₆ H ₃₂ O ₂	Fatty acid ester	1.54	ND	ND	ND	ND	ND	ND
9	Loliolide	1789.0	C ₁₁ H ₁₆ O ₃	Monoterpenoid lactone	0.84	ND	ND	ND	ND	ND	ND
10	Pentadecanoic acid	1869.2	C ₁₅ H ₃₀ O ₂	Saturated fatty acid	1.70	ND	ND	ND	ND	ND	ND
11	Pentadecanoic acid, 14-methyl-, methyl ester	1890.4	C ₁₇ H ₃₄ O ₂	Branched fatty acid ester	2.01	0.33	0.46	0.47	0.38	0.42	0.33
12	Palmitic acid, methyl ester	1910.0	C ₁₇ H ₃₄ O ₂	Saturated fatty acid ester	8.76	0.58	0.46	0.35	0.33	0.25	0.16
13	Palmitic acid	1970.2	C ₁₆ H ₃₂ O ₂	Saturated fatty acid	4.24	0.17	0.17	0.17	0.23	0.33	0.21
14	1-Octadecanol	2069.0	C ₁₈ H ₃₈ O	Long-chain fatty alcohol	54.28	9.89	0.62	0.31	0.20	0.24	0.15
15	Stearic acid	2163.3	C ₁₈ H ₃₆ O ₂	Saturated fatty acid	1.10	9.80	0.23	0.32	0.23	0.54	0.34
16	Dimethyl Sulfoxide	786.6	C ₂ H ₆ OS	Sulfoxide	ND	21.04	20.33	24.42	27.85	35.60	30.15
17	Dimethyl sulfone	919.3	C ₂ H ₆ O ₂ S	Sulfone	ND	5.47	2.74	4.41	4.18	3.24	9.37
18	Piperidine, 2-pentyl-	1194.3	C ₁₀ H ₂₁ N	Alkylated piperidine	ND	4.52	5.34	4.31	7.47	5.45	3.11
19	DL-Proline, 5-oxo-, methyl ester	1395.9	C ₆ H ₉ NO ₃	Amino acid derivative	ND	0.36	0.35	0.32	0.20	0.42	0.98
20	Dihydroactinidiolide	1493.0	C ₁₁ H ₁₆ O ₂	Aromatic lactone (Terpenoid)	ND	0.23	0.29	0.23	0.16	0.19	0.13
21	1-Deoxy-d-arabitol	1660.0	C ₅ H ₁₂ O ₄	Sugar alcohol	ND	4.07	1.94	1.94	2.39	5.36	3.01
22	3-O-Methyl-d-glucose	1682.7	C ₇ H ₁₄ O ₆	Methylated sugar	ND	0.10	ND	ND	ND	ND	ND
23	1,5-Anhydro-d-mannitol	1843.0	C ₆ H ₁₂ O ₅	Sugar alcohol (Polyol)	ND	1.30	1.04	0.16	0.15	0.20	0.13
24	Unknown	1955.1	-	-	ND	1.85	0.37	0.39	0.88	1.08	0.31
25	Methyl-9,10-octadecadienoate	2110.0	C ₁₉ H ₃₄ O ₂	Unsaturated fatty acid ester	ND	0.46	0.49	0.66	1.21	1.59	0.55
26	Phytol	2122.1	C ₂₀ H ₄₀ O	Diterpene alcohol	ND	0.11	0.13	0.12	0.22	2.15	0.18
27	β-Monopalmitin	2475.0	C ₁₉ H ₃₈ O ₄	Monoglyceride	ND	13.83	ND	ND	ND	ND	ND
28	Docosanoic acid, ethyl ester	2577.0	C ₂₄ H ₄₈ O ₂	Long-chain fatty acid ester	ND	0.16	0.44	0.69	0.91	0.82	0.42

29	Armid E	2625.0	C ₂₂ H ₄₃ NO	Fatty acid amide	ND	1.93	51.41	47.91	43.90	30.22	41.47
30	Campesterol	3105.5	C ₂₈ H ₄₈ O	Phytosterol	ND	13.57	1.09	0.70	0.12	0.14	0.12
31	α-Tocopherol	3139.1	C ₂₉ H ₅₀ O ₂	Vitamin E (Tocopherol)	ND	7.97	0.64	0.28	0.10	ND	ND
32	β-Sitosterol	3220.0	C ₂₉ H ₅₀ O	Phytosterol	ND	0.34	1.07	0.60	0.27	0.53	0.71
33	γ-Sitosterol	3290.0	C ₂₉ H ₅₀ O	Phytosterol	ND	1.37	ND	ND	ND	ND	ND
34	Dihomo-gamma-linolenic acid (DGLA)	2050.7	C ₂₀ H ₃₄ O ₂	Polyunsaturated fatty acid	ND	ND	0.14	0.13	0.13	0.20	0.17
35	Palmitamide	2189.0	C ₁₆ H ₃₃ NO	Fatty acid amide	ND	ND	0.49	0.63	0.63	1.05	0.41
36	Punicic acid	2202.0	C ₁₈ H ₃₀ O ₂	Conjugated linolenic acid	ND	ND	0.43	0.43	0.49	0.29	0.10
37	Arachidonic acid	2324.3	C ₂₀ H ₃₂ O ₂	Polyunsaturated fatty acid	ND	ND	2.38	1.78	1.23	0.80	0.33
38	Oleamide	2397.1	C ₁₈ H ₃₅ NO	Fatty Amide	ND	ND	0.24	0.36	0.39	0.28	0.28
39	3-Eicosene, (E)-	1980.0	C ₂₀ H ₄₀	Alkene	ND	ND	ND	ND	0.65	0.87	0.60
40	Octadecanal	1998.3	C ₁₈ H ₃₆ O	Aldehyde	ND	ND	ND	ND	ND	0.92	2.32
41	Linoleic acid	2134.0	C ₁₈ H ₃₂ O ₂	Polyunsaturated fatty acid	ND	ND	ND	ND	ND	0.19	0.52
42	Linolenic acid	2143.9	C ₁₈ H ₃₀ O ₂	Polyunsaturated fatty acid	ND	ND	ND	ND	ND	0.94	0.41

RI: Retention index. Dimethyl sulfoxide (DMSO) and Dimethyl sulfone were detected in the GC-MS analysis, likely originating from their use as solvents in culture media preparation. The presence of 1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester suggests contamination from plastic materials used during tissue culture procedures. Sequences 1–15: Compounds detected in the mother plant, with partial overlap observed in the control and AgNPs-treated samples. 16–33: Compounds shared between the control and some of the AgNPs treatments. 34–38: Compounds detected exclusively in all AgNPs treatments. 39: Compound shared by the three highest AgNPs concentrations. 1.5;2.5. 40–42: Compounds detected only at the two highest AgNPs concentrations (2.0 and 2.5 mg/L).

In control shoots grown on MS medium without AgNPs, dimethyl sulfoxide (21.04%) and β-Monopalmitin (13.83%) were dominant. The application of AgNPs influenced metabolite composition substantially. At 0.5 mg L⁻¹ AgNPs, Armid E (51.41%) and dimethyl sulfoxide (20.33%) were the major compounds, Dihomo-gamma-linolenic acid (DGLA) (0.14%) and Phytol (0.13%), were the least abundant. Similar trends were observed across higher AgNPs concentrations (1.0–2.5 mg L⁻¹), with Armid E and dimethyl sulfoxide consistently dominating. However, at 2.5 mg L⁻¹ AgNPs Punicic acid; Campesterol and Galbazine present in minimal amounts (0.10%, 0.12% and 0.12%) Respectively.

The plant produces a large number of secondary metabolites essential for its survival in different environments. *In vitro* plant cell and organ culture has been proven to be advantageous for the production of secondary metabolites by using a suitable nutrient media [10]. Nonetheless, the advancement of nanotechnology has enabled the development of innovative nanoparticle applications in plant tissue culture, allowing for the production of bioactive secondary metabolites [1, 7, 15].

By optimizing the two factors (nutrients and elicitors) the culture growth and secondary metabolites production can be maximized.

Commonly, plants treated with different elicitors or signaling molecules produce secondary metabolites possessing distinct properties. According to Fakruddin [16], nanoparticles can effectively simulate secondary metabolite production in plants. Zhang *et al.* [17] emphasized that AgNPs have a strong elicitation potential for an increased secondary metabolite production.

Similarly, Ulusoy *et al.* [9] successfully stimulated secondary metabolites that are important for the food and pharmaceutical industries from the callus of anise (*Pimpinella anisum* L.). This outcome was accomplished using MS medium supplemented with 2 mg L⁻¹ BA, 2 mg L⁻¹ 2,4-D, and either 1 or 5 mg L⁻¹ AgNPs. Parallel with this finding, Fatima *et al.* [18] observed that the addition of varying concentrations of ZnO, CuO, and CoO nanoparticles to the MS medium supplemented with NAA resulted in an enhanced accumulation of phenolic content and antioxidant activity in callus derived from the root, shoot, and leaf of *Artemisia annua* L.

In another study carried out by Karakaş [8] the AgNPs at various concentrations in the leaves of indigo plant (*Isatis constricta*) grown *in vitro* were examined, to evaluate their effects on the production of secondary metabolite compounds. The research concluded that the inclusion of 2 mg.L⁻¹ AgNPs in the MS medium elevate tryptanthrin levels.

Furthermore, other workers have demonstrated the important effects of AgNPs on secondary metabolite production depending on the concentration and exposure time of plants [19, 20].

Oxidative stress plays a crucial role in production of reactive oxygen species (ROS) which act as key signalling molecules in stress responses. For crop plants to grow and yield under stress conditions, they must use ROS not only for the modulation of metabolic pathways, but to develop acclimatory phenotypes able to activate detoxification mechanisms in plants to reduce ROS damage. Therefore, biosynthesis of secondary metabolites activates a complex network of defense mechanisms, against ROS damage [21].

Exposure of plants to AgNPs leads to excessive production of ROS, which, in turn, act as signalling molecules that stimulate the production of secondary metabolites, these metabolites act as antioxidant against ROS [22].

Nevertheless, the present study on *Kalanchoe blossfeldiana* revealed several additional metabolites not previously documented in this species. These novel findings enrich our understanding of the genus's chemical diversity and pave the way for future exploration into the potential biological and pharmaceutical significance of these newly identified compounds.

Furthermore, the bioactive substances found in this study can help to explain the traditional therapeutic benefits of *Kalanchoe* species, particularly their usage in treating cancers, diabetes, bacterial infections, inflammation, and Alzheimer's disease [2]. Surprisingly, the existence of Galbazine (pyrazines and their derivatives), Loliolide, 2-pentylpiperidine, and limonene, is consistent with known pharmacological properties as anti-inflammatory, antibacterial, neuroprotective, and anticancer ones. Thus, the identification of these metabolites in *K. blossfeldiana* extracts supports the species' medicinal potential and offers a scientific justification for its ethnopharmacological significance.

All things considered, the results show that substances like phytol, Loliolide, and essential fatty acids are regular and distinctive secondary metabolites in *Kalanchoe* species, indicating their basic ecological and physiological roles. The present study of *Kalanchoe blossfeldiana*, however, identified a number of other metabolites that have not yet been identified in this species, increasing our knowledge of the chemical diversity of the genus and pointing to new avenues for examining the biological and medicinal significance of these recently identified substances. Crucially, a stress-responsive change in the plant's metabolic profile brought on by varying environmental conditions is suggested by the exclusive presence of certain metabolites in both the mother plant and the *in vitro* cells.

However, the *in vitro* cultures were maintained under sterile, regulated environments. This environmental contrast likely induced distinct physiological responses, including the suppression of some compounds and the *de novo* biosynthesis of others. Such differential metabolite profiles reflect the plant's adaptive metabolic plasticity in response to external stressors and artificial culture systems [23, 24].

Conclusion

This study demonstrated that low concentrations of AgNPs (0.5–1.5 mg L⁻¹) improved growth and bud development in *K. blossfeldiana*, and higher concentrations (2–2.5 mg L⁻¹) led to a reduced plant growth. AgNPs also increased the production of a variety of secondary metabolites, which indicates that they can be useful in enhancing plant metabolite production under laboratory conditions. Nevertheless, these results point out that AgNPs may serve as potential agents for the improvement of plant growth and metabolite production *in vitro*.

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