Sustainable and Eco-Friendly In Vitro Production of Natural Compounds in Kalanchoe blossfeldiana: Role of Silver Nanoparticles in Growth and Secondary Metabolite Biosynthesis

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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 35 secondary metabolites, 18 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 25 compounds, such as Oleamide (antimicrobial and anti-allergic), α-Tocopherol (Cardiovascular protection.), Phytol (Analgesic and Anxiolytic) and γ-Sitosterol (antioxidant and antidiabetic), meanwhile, Plants treated with AgNPs (0.5–2.5 $mg \cdot L^{-1}$) synthesized 29–31 compounds. These included Piperidine, 2-pentyl- (CNS effects and Anticancer), Neophytadiene (anticancer, anxiolytic), Isoledene (antibacterial and anti-inflammatory activities) cardioprotective fatty acids (Linoleic and Linolenic acids), along with Palmitic acid, D-Limonene, 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 (1). 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 (2).

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 (3). 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. (4).

Very few methods are available for the regeneration of *Kalanchoe* or Bryophyllum spp. Kulus, (5) used full-strength Murashige and Skoog (MS) medium enriched with benzyl aminopurine (BAP) for propagation of this plant. By contrast, García-Pérez et al. (6) 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. (7) induced the regeneration of direct and indirect adventitious shoots in in vitro cultures from three topo physical 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 (AgNPs) 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 (8; 9).

According to Ulusoy et al. (10), 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 AgNPs affects plant growth, development, and metabolite synthesis (8; 11; 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*.

PMaterials 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% $HgCl_2$. 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 mgL^{-1} myo-inositol, 80 mg L^{-1} adenine sulfate, 120 mg L^{-1} $NaH_2PO_4 \cdot 2H_2O$, 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 $\mu Mm^{-2}s^{-1}$.

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 (12) 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)

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). 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, (Plate 1A). This finding aligns with Elsayh, (13), who observed improved callus induction and somatic embryo formation in date palm cultured at low AgNPs concentrations. In addition, Aqeel *et al.* (14) and Al-Khayri *et al*., (15) suggested that AgNPs promote plant growth through nutrient uptake and cross-cellular barriers. Sadak, (16) 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 (8).

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).

The analysis identified a total of 35 metabolic compounds. Quantitatively, the total number of compounds increased from 18 in the mother plant and 25 in the control to (31,30,31,29,29) compounds across the AgNPs treatments (0.5, 1.0, 1.5, 2.0, 2.5 mg L^{-1}), indicating an enhanced diversity of the metabolite profile. Qualitatively, fifteen core compounds, including Galbazine, Azulene, Palmitic acid, Phytol, and Stearic acid, were common to all treatments. However, the compounds Armid E, α-Tocopherol, β-Sitosterol, and γ-Sitosterol were found exclusively in the control, whereas α-Piperidone, Linoleic acid, and Linolenic acid were produced only in the five AgNPs treatments. Furthermore, the analysis demonstrated a dose-dependent effect, with the highest peak areas for most compounds occurring at various AgNPs concentrations. Margaric acid peaked at 0.5 mg L−1. Palmitic acid, Dimethyl Sulfoxide, and Linoleic acid registered their highest peaks at 1.0 mg L^{-1}. Niacinamide, Arachidonic acid, and Linolenic acid peaked at 1.5 mg L^{-1}. The 2.0 mg L^{-1} treatment showed the highest production of 3-hydroxy-2-pyranone, Galbazine, and α-Piperidone. Notably, the 2.5 mg L−1 treatment elicited the highest concentrations for a large group of metabolites, including D-Limonene, DL-Proline, 5-oxo-, methyl ester, Lauric acid, Myristic acid, and Loliolide. This differential maximization of compound production at distinct AgNPs concentrations confirms the role of silver nanoparticles as a potent elicitor in the Kalanchoe system, targeting specific metabolic pathways based on the applied dose (Table 2).

Table 3 summarizes the percentage composition of the compounds identified in the GC-MS chromatograms showed distinct patterns across treatments. In the Kalanchoe mother plant, Ethyl myristate was dominant at 34.50%, with D-Limonene recording the minimum percentage at 1.14%. For the control treatment, β-Sitosterol recorded the highest percentage at 17.39%, while Niacinamide, Dihydroactinidiolide, Margaric acid, and Ethyl behenate all registered the lowest percentage at 0.18% each. Across all five silver nanoparticle (AgNPs) treatments, the compound Dimethyl Sulfoxide consistently showed the highest percentage, with values ranging from 12.05% to 31.52%. Conversely, the lowest percentage values varied by treatment: 0.17% was the minimum in the 0.5 mg L−1 treatment (for Methyl linoleate and Ethyl behenate) and the 1.5 mg L−1 treatment (for 3-hydroxy-2-pyranone, D-Limonene, Galbazine, Azulene, and Phytol), and the 2.5 mg L−1 treatment (for Phytol, Stearic acid, and Methyl linoleate). Phytol registered the absolute minimum of 0.14% at 1.0 mg L−1, while Isoledene recorded 0.25% as the minimum in the 2.0 mg L−1 treatment.

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 (11). 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 (8; 2; 13).

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 *et al*. (17), nanoparticles can effectively simulate secondary metabolite production in plants. Zhang *et al*. (18) emphasized that AgNPs have a strong elicitation potential for an increased secondary metabolite production.

Similarly, Ulusoy *et al*. (10) 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.* (19) 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, (9) the AgNPs at various concentrations in the leaves of indigo plant (*Isatis constricta*) grown in vitro were examined, to evaluated 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 (20; 21).

Oxidative stress plays a crucial role in production of reactive oxygen species (ROS) which act as key signaling 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 developed acclimatory phenotypes able to activation of detoxification mechanisms in plants to reduce ROS damage. Therefore, biosynthesis of secondary metabolites activates a complex network of defense mechanisms, against ROS damage (22).

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 (23).

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 (3). 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, Neophytadiene is promising due to its anticancer, anxiolytic (anti-anxiety), anticonvulsant (anti-seizure), and anti-inflammatory properties (24). Isoledene shows therapeutic value through its antibacterial and anti-inflammatory activities (25). Finally, while Conhydrine itself is a toxic compound, its greatest pharmaceutical value lies in serving as a chemical scaffold for synthesizing less-toxic piperidine derivatives that are highly promising as antivirals, including anti-HIV agents, and antitumor compounds (26). 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 (27; 28).

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.

These naturally occurring metabolites hold a significant advantage over many synthetic pharmaceuticals due to their inherently eco-friendly nature. As plant-derived compounds, they are biodegradable and generally pose minimal environmental risk. Their utilization aligns with the principles of Green Chemistry, offering a sustainable path to drug discovery by reducing the reliance on petrochemical synthesis and minimizing the generation of persistent, non-degradable environmental pollutants. This makes them highly desirable as environmentally responsible alternatives in modern medicine.

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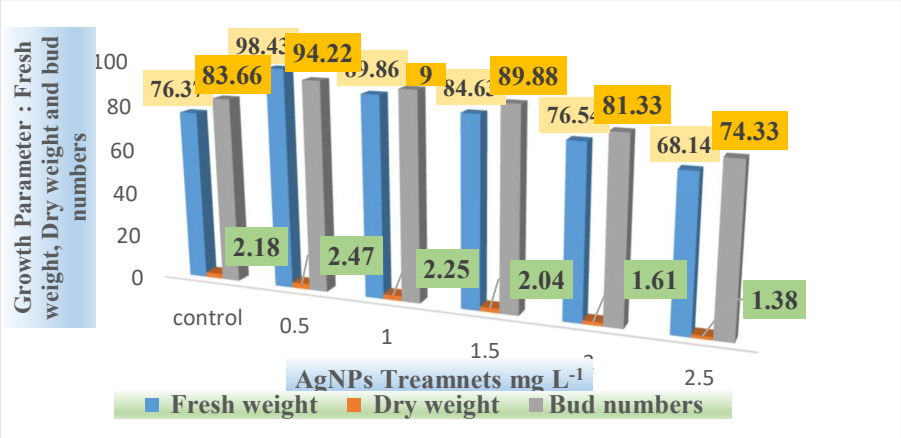

Fig 1 Effect of AgNPs Application at Different Concentrations on Growth Parameters.

Plate 1 Multiplied shoots of *Kalanchoe blossfeldiana* grown in liquid medium with varying concentrations of AgNPs using a Plantform bioreactor. 1: Control (without AgNPs) 2: 0.5 mg L^{-1} AgNPs, 3: 1 mg L^{-1} AgNPs, 4: 1.5 mg L^{-1} AgNPs, 5: 2 mg L^{-1} AgNPs, 6: 2.5 mg L^{-1} AgNPs

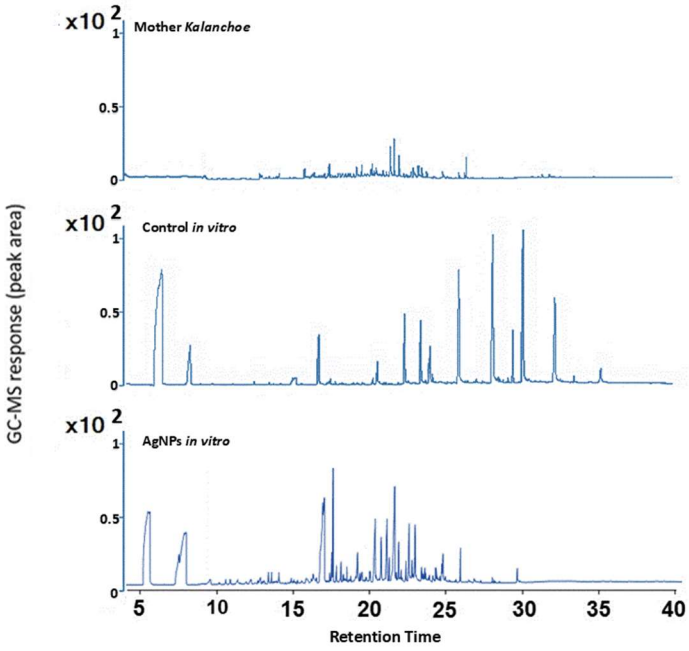

Fig 2 Chromatographic separation of secondary metabolites. The first represents the mother plant (grown *ex vitro*), the second represents the plant grown *in vitro* (control), and the third represents the plant grown in vitro on medium containing an elicitor (silver nanoparticles).

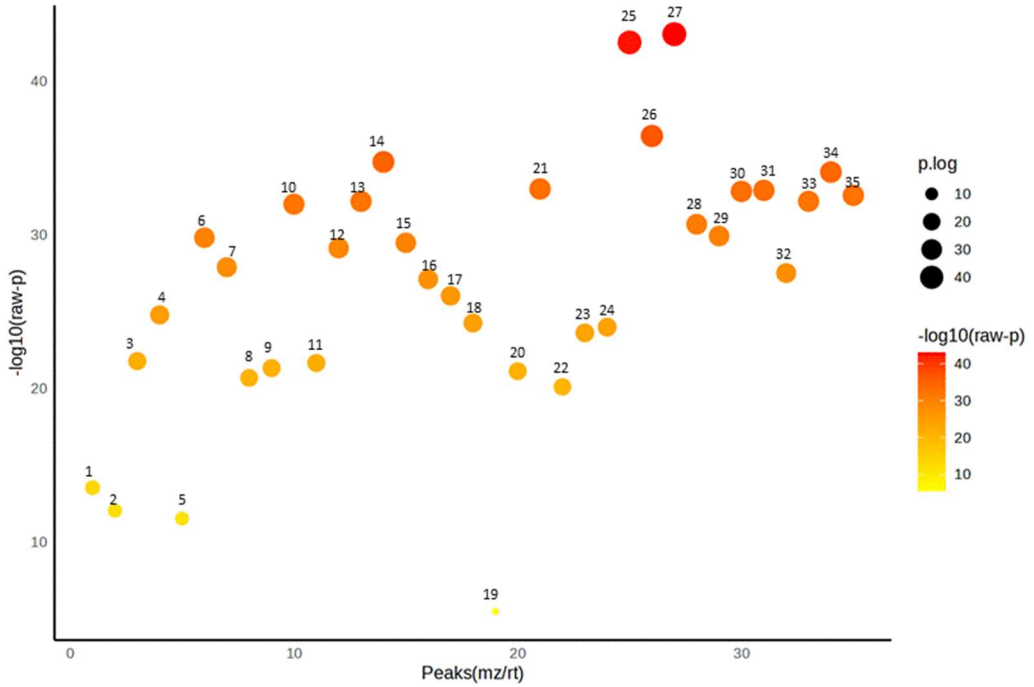

Fig 3 Identified secondary metabolites on mother plant, in vitro-propagated plants treated with silver nanoparticles, and a control, using p-values from GC-MS analysis. Red circles mark statistically significant compounds, while black dots indicate statistically non-significant ones. The log10 transformation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

Table 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 ± SE	Dry weight g ±SE	Buds number±SE
Control	76.37 ± 10.84^{c}	2.18±0.51^{ab}	83.66 ± 9.21^{b}
0.5	98.43 ± 6.28^{a}	2.47 ±0.52^{a}	94.22 ± 3.96^{a}
1.0	89.86 ± 4.37^{b}	2.25±0.34^{ab}	93.00 ± 3.80^{a}
1.5	84.63 ± 3.84^{bc}	2.04±0.21^{b}	89.88 ± 4.01^{a}
2.0	76.54 ± 5.01^{c}	1.61±0.22^{c}	81.33 ± 3.80^{b}
2.5	68.14 ± 4.80^{e}	1.38± 0.28^{c}	74.33 ± 6.59^{c}
	LSD=8.40	LSD=6.22	LSD=0.42

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

No.	RT	Compound	RI	Peak Area (10^6)						
				Mother plant	*In vitro* AgNPs mg. L^{-1} Treatments					
					Control	0 .5	1	1.5	2	2.5
1	9.03	3-hydroxy-2-pyranone	989.0	1.12	ND	1.12	1.07	1.02	1.18	ND
2	10.01	D-Limonene	1029.5	1.19	ND	1.23	1.20	1.06	1.10	2.16
3	12.02	Galbazine	1179.5	1.53	1.22	3.23	4.12	2.77	9.78	4.12
4	14.31	Pyranone (DDMP)	1230.6	1.62	1.30	2.72	10.99	10.44	3.87	11.62
5	15.01	Azulene	1296.5	1.13	2.48	1.07	1.03	1.02	1.11	1.40
6	16.83	DL-Proline, 5-oxo-, methyl ester	1399.7	1.49	36.22	74.21	78.21	57.43	19.32	98.70
7	17.15	Niacinamide	1422.0	1.66	1.09	40.98	51.32	59.73	15.29	2.86
8	18.73	Dihydroactinidiolide	1493.0	1.20	1.11	10.70	16.48	11.53	2.18	19.72
9	19.32	Lauric acid	1562.0	2.08	1.34	12.09	15.25	1.12	2.39	22.74
10	20.92	1-Deoxy-d-arabitol	1660.0	1.83	3.22	8.60	39.67	29.81	19.87	39.81
11	21.41	Myristic acid	1755.0	26.93	22.38	31.06	53.61	30.17	17.12	54.23
12	21.63	Ethyl myristate	1773.0	35.74	ND	11.94	16.85	30.93	19.16	21.64
13	21.92	Loliolide	1789.0	14.51	1.21	42.79	29.60	10.85	8.03	89.84
14	22.47	Pentadecanoic acid	1869.2	2.31	54.28	1.31	3.15	3.88	2.59	45.28
15	23.49	Palmitic acid	1970.2	2.01	1.21	53.93	54.72	42.53	24.25	10.13
16	24.73	Margaric acid	2069.0	2.23	1.08	68.76	51.72	52.77	12.72	30.14
17	25.15	Phytol	2122.1	2.71	40.42	20.90	0.99	1.05	1.08	1.17
18	26.49	Stearic acid	2163.3	2.32	25.43	10.98	2.04	14.09	11.50	1.20
19	6.26	Dimethyl Sulfoxide	786.6	ND	70.32	100.63	136.42	73.41	131.56	100.41
20	8.58	Dimethyl sulfone	919.3	ND	10.97	49.66	52.41	42.16	46.84	52.72
21	24.91	Methyl linoleate	2110.0	ND	70.28	1.03	21.52	40.26	9.75	1.20
22	27.67	Arachidonic acid	2324.3	ND	5.42	6.45	2.64	11.17	9.57	2.89
23	28.19	Oleamide	2397.1	ND	1.38	1.14	ND	8.47	ND	ND
24	29.63	Ethyl behenate	2577.0	ND	1.10	1.02	1.04	1.21	1.33	12.06
25	30.58	Armid E	2625.0	ND	96.04	ND	ND	ND	ND	ND
26	32.01	α-Tocopherol	3139.1	ND	35.28	ND	ND	ND	ND	ND
27	33.62	β-Sitosterol	3220.0	ND	104.92	ND	ND	ND	ND	ND
28	35.01	γ-Sitosterol	3290.0	ND	13.71	ND	ND	ND	ND	ND
29	11.25	α-Piperidone	1168.0	ND	ND	1.72	2.40	1.86	20.41	3.19
30	13.05	Piperidine, 2-pentyl-	1194.3	ND	ND	1.08	1.09	9.72	6.15	1.26
31	15.45	Conhydrin	1330.0	ND	ND	1.09	2.52	1.26	1.22	6.03
32	16.51	Isoledene	1375	ND	ND	17.17	19.06	12.35	1.05	19.33
33	22.01	Neophytadiene	1819.0	ND	ND	5.38	1.10	9.14	7.14	13.23
34	25.95	Linoleic acid	2050.7	ND	ND	6.11	31.61	27.16	9.80	30.53
35	26.25	Linolenic acid	2189.0	ND	ND	1.08	1.20	8.81	ND	1.51

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 1–18: Compounds detected in the mother plant, with partial overlap observed in the control and AgNPs-treated samples. 19–28 Compounds shared between the control and some of the AgNPs treatments. 28–35: Compounds detected exclusively in all AgNPs treatments.

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	$C_5H_4O_3$	Pyranone	1.08	ND	0.19	0.15	0.17	0.28	ND
2	D-Limonene	1029.5	$C_{10}H_{16}$	Monoterpene	1.14	ND	0.21	0.17	0.17	0.26	0.31
3	Galbazine	1179.5	$C_9H_{14}N_2O$	Pyrazine	1.47	0.20	0.55	0.58	0.45	2.34	0.59
4	Pyranone (DDMP)	1230.6	$C_5H_4O_2$	Pyranone	1.56	0.22	0.46	1.56	1.71	0.93	1.66
5	Azulene	1296.5	$C_{10}H_8$	Aromatic hydrocarbon	1.09	0.41	0.18	0.15	0.17	0.27	0.20
6	DL-Proline, 5-oxo-, methyl ester	1399.7	$C_6H_9NO_3$	Lactam & Ester	1.44	6.00	12.55	11.09	9.43	4.63	14.08
7	Niacinamide	1422.0	$C_6H_6N_2O$	Amide	1.60	0.18	6.93	7.28	9.81	3.66	0.41
8	Dihydroactinidiolide	1493.0	$C_{11}H_{16}O_2$	Lactone	1.16	0.18	1.81	2.34	1.89	0.52	2.81
9	Lauric acid	1562.0	$C_{12}H_{24}O_2$	Saturated fatty acid	2.01	0.22	2.04	2.16	0.18	0.57	3.24
10	1-Deoxy-d-arabitol	1660.0	$C_5H_{12}O_4$	Sugar alcohol	1.77	0.53	1.45	5.63	4.89	4.76	5.68
11	Myristic acid	1755.0	$C_{14}H_{28}O_2$	Saturated fatty acid	26.00	3.71	5.25	7.60	4.95	4.10	7.73
12	Ethyl myristate	1773.0	$C_{16}H_{32}O_2$	Ester	34.50	ND	2.02	2.39	5.08	4.59	3.09
13	Loliolide	1789.0	$C_{11}H_{16}O_3$	Lactone	14.00	0.20	7.24	4.20	1.78	1.92	12.81
14	Pentadecanoic acid	1869.2	$C_{15}H_{30}O_2$	Saturated fatty acid	2.23	9.00	0.22	0.45	0.64	0.62	6.46
15	Palmitic acid	1970.2	$C_{16}H_{32}O_2$	Saturated fatty acid	1.94	0.20	9.12	7.76	6.98	5.81	1.45
16	Margaric acid	2069.0	$C_{17}H_{34}O_2$	Saturated fatty acid	2.15	0.18	11.63	7.34	8.66	3.05	4.30
17	Phytol	2122.1	$C_{20}H_{40}O$	Alcohol	2.61	6.70	3.54	0.14	0.17	0.26	0.17
18	Stearic acid	2163.3	$C_{18}H_{36}O_2$	Saturated fatty acid	2.24	4.21	1.86	0.29	2.31	2.76	0.17
19	Dimethyl Sulfoxide	786.6	$(CH_3)_2SO$	Sulfoxide	ND	11.65	17.02	19.35	12.05	31.52	14.32
20	Dimethyl sulfone	919.3	$(CH_3)_2SO_2$	Sulfone	ND	1.82	8.40	7.43	6.92	11.22	7.52
21	Methyl linoleate	2110.0	$C_{19}H_{34}O_2$	Ester	ND	11.65	0.17	3.05	6.61	2.34	0.17
22	Arachidonic acid	2324.3	$C_{20}H_{32}O_2$	Unsaturated fatty acid	ND	0.90	1.09	0.37	1.83	2.29	0.41
23	Oleamide	2397.1	$C_{18}H_{35}NO$	Amide	ND	0.23	0.19	0.00	1.39	ND	ND
24	Ethyl behenate	2577.0	$C_{24}H_{48}O_2$	Ester	ND	0.18	0.17	0.15	0.20	0.32	1.72
25	Armid E	2625.0	$C_{18}H_{35}NO$	Amide	ND	15.92	ND	ND	ND	ND	ND
26	α-Tocopherol	3139.1	$C_{29}H_{50}O_2$	Vitamin	ND	5.85	ND	ND	ND	ND	ND
27	β-Sitosterol	3220.0	$C_{29}H_{50}O$	Phytosterol	ND	17.39	ND	ND	ND	ND	ND
28	γ-Sitosterol	3290.0	$C_{29}H_{50}O$	Phytosterol	ND	2.27	ND	ND	ND	ND	ND
29	α-Piperidone	1168.0	C_5H_9NO	Lactam	ND	ND	0.29	0.34	0.31	4.89	0.46
30	Piperidine, 2-pentyl-	1194.3	$C_{10}H_{21}N$	Alkylated piperidine	ND	ND	0.18	0.15	1.60	1.47	0.18
31	Conhydrin	1330.0	$C_8H_{17}NO$	Alkaloid	ND	ND	0.19	0.36	0.21	0.29	0.86
32	Isoledene	1375	$C_{15}H_{24}$	Sesquiterpene	ND	ND	2.90	2.70	2.03	0.25	2.76
33	Neophytadiene	1819.0	$C_{20}H_{38}$	Diterpene	ND	ND	0.91	0.16	1.50	1.71	1.89
34	Linoleic acid	2050.7	$C_{18}H_{32}O_2$	Unsaturated fatty acid	ND	ND	1.03	4.48	4.46	2.35	4.35
35	Linolenic acid	2189.0	$C_{18}H_{30}O_2$	Unsaturated fatty acid	ND	ND	0.18	0.17	1.45	ND	0.22

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.. **Sequences 1–18:** Compounds detected in the mother plant, with partial overlap observed in the control and AgNPs-treated samples. **19–28**: Compounds shared between the control and some of the AgNPs treatments. **28–35:** Compounds detected exclusively in all AgNPs treatments.