RESEARCH ARTICLE

Effect of MgO and CuO Nanoparticles on increasing Tannin and Phenol Compounds of *Punica granatum* L. using Shoot Tip *In vitro*

Manar F. Jassim Al-Khafagi*, Hashim K. Mohammed Al-Oubaidi

Mustansiriyah University, Iraq

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ABSTRACT

Secondary metabolites are the bioactive compounds in plants used pharmacologically for human health. For the development and growth, *Punica granatum* L. plants produce tannin and phenol compounds in addition to those primary metabolic and biosynthetic routes for associated compounds. Many such compounds are medicinal and play an important role in various treatments. However, these medicinal compounds are produced in small quantities within the plant; thus it is necessary to stimulate the plant to increase the amount of production. This research aimed at increasing the said compounds, *viz.*, secondary metabolites (tannins and phenols) in the *P. granatum* L. plant. In order to achieve this, different concentrations of MgO nanoparticles (NPs) (i.e., 3.5, 5.5, 8, and 12 mg/L) and CuO NPs (i.e., 4.5, 8, 13, and 18 mg/L) were added to the callus media *in vitro*. Then, using the high-performance liquid chromatography (HPLC) technique, the quantity of these compounds was measured, and a comparison was drawn with the quantity of these compounds present in the mother plant.

From the results, it was observed that using MgO and CuO NPs, highly significant differences were caused. It was found that the tannin compounds got increased using 8 and 12 mg/L, and 8, 13, and 18 mg/L of MgO and CuO NPs, respectively. Whereas the phenol compounds got increased using 3.5, 8, and 12 mg/L, and 4.5, 8, 13, and 18 mg/L of MgO and CuO NPs, respectively.

Keywords: Bitter Nutrition, HPLC, Phenol, *Punica granatum*, Tannin.

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INTRODUCTION

There has been a traditional use of various components of *P. granatum* L. plant, including its flower, leaf, shoots, roots, fruit, and fruit peel.[1] Pomegranate sauce prepared from the plant is also widely used. The components of *P. granatum* L. fruit are rich in tannins and have shown strong astringent effects.

P. granatum L. flower extracts have been used traditionally to prepare infusions and decotions to treat ordinary diarrhea. To relieve symptoms of pancreas inflammation, *P. granatum* L. flower extracts in combination with *P. granatum* L. fruit peels were gurgled.[2] Its fruit juice helps treat diseases, such as, dysentery, stomach, and gallbladder ailments.[2] The *P. granatum* L. plant has anti-bacterial and anti-inflammatory properties, and thus, its roots (both fresh and dried), barks, and ethanol extracts find use in traditional medicines.[3] Alkaloid substances found in them are used to get rid of intestinal parasites. The strong tannin found in *P. granatum* L. seeds has would healing properties and is bitter nutrition.[3]

Difficulties of vegetative propagation, including true type plants and rapid, mass production of planting materials, can be overcome with the use of micropropagation.[4] Through either organogenesis from shoot tip, cotyledons,[4,6] anthers,[7] or embryogenesis from various seeding explant and immature zygotic embryos,[5] new plantlets can be prepared *in vitro.*

Several reports give positive inputs on the use of nanotechnology in plant tissue culture. NPs have found wide acceptance and use in the improvement of germination of seeds, enhancement in the growth and yield of the plants. NPs enable modification of the plant genetics. They are useful to protect the plants, treat plant cells and tissue, organ culture, and improvement in the production of their bioactive compounds.[8,9] Copper oxide (CuO) and zinc oxide (ZnO) treatment upon the seedlings of licorice was found to enhance tannins, phenolic compounds, anthocyanins, flavonoids, and glycyrrhizin.[10] NPs act as a nutrient source and an elicitor when added to the plant culture *in vitro.*

Metal and metal oxide NPs eliminate various microorganisms.[11] NPs, including CuO, MgO, TiO_2, and ZnO, possess antimicrobial activities.[12]

Some parts of *P. granatum* L. plant are also used for leather tanning.[13] This research aims at micropropagation of *P. granatum* L. (pomegranate) and studies NPs effect on the increase of secondary metabolites in the shoot tip.

**Author for Correspondence: manar.alkhafagi@gmail.com*

MATERIAL AND METHODS

Plant Material

The explant of *P. granatum* L. from new branches was collected from Baghdad, Iraq.

Sterilization

The shoot tip of *P. granatum* L. was rinsed with running water for 20 minutes. Then, it was transferred to a laminar airflow cabinet. Thereafter, it was immersed in 75% ethanol for 2 minutes. Then, it was washed with sterilized with distilled water (DW) for 10 minutes, followed by rinsing with sodium hypochlorite solution (3% concentration). It was then washed five times using DW for 10 minutes. Then, it was cultured in universal tubes containing MS medium (Table 1).[14,15]

Growth Medium

Explant (shoot tip) of *P. granatum* L. was divided and cultured in universal tubes containing MS medium with BA in different concentrations (0.5, 1.5, 2.5, 3.5, and 4 mg/L). Thereafter, 10 replicas were created for each concentration. Then, it was incubated for 8 hours. The light photoperiod at illumination intensity was 1,000 lux and incubated at 30 ± 2°C. The results obtained were noted post 20 days.[16]

Weight Measurement of Shoot Tips Growth (Fresh and Dry)

A sensitive balance was used to measure the weight of fresh shoot tips. Thereafter, explants were dried in an oven at 75°C till such time the dry weight became stable. The resultant product was measured using a sensitive balance.[17]

Secondary Metabolite Analysis and Extraction from Callus and Shoot Tip of *P. granatum* L.

A 60 mg of *P. granatum* L. plant was dissolved in methanol. The extract of *P. granatum* L. plant was ultrasonicated. It was then centrifuged for 20 minutes. Each sample's clear supernatant was treated with charcoal and thereafter decantated. The solutions thus obtained were filtered using Whatman no. 1 filter paper. Then, the aqueous extracts were placed under vacuum and evaporated. The dried sampled were then vortexed in 2 mL HPLC grade methanol. The resultant mixture was filtered using 3 µm disposable filter paper. For further analysis, the filtrate was then stored at 3°C. As per the optimized separation condition, 30 µm of the sample was put into the HPLC system.[18,19]

Table 1: MS medium supplement with different concentrations of CuO or MgO NPs

S. No.	*Component*	*Concentration (mg/L)*
1	MS	45,000
2	Sugar	31,000
3	Myo-inositol	100
4	GA3	0.2
5	NAA	0.2
6	CuO NPs	0, 4.5, 8, 13, and 18
7	MgO NPs	0, 3.5, 5.5, 8, and 12
8	Agar-agar	9,000
9	BA	2

Estimation of Increase in Secondary Metabolite Compounds using HPLC Device

Under optimum conditions, the key compounds were segregated on a fast liquid chromatographic (FLC) column.

Column

Phenomenex C-18 (50 × 3 mm ID)

Particle Size

2.5 µm

Mobile Phase

Solvent A: 1.5% acetic acid in 3 pH distilled water, as a linear gradient.

Solvent B: Linear gradient of 1% in 98% acetonitrile.

Flow rate: 2 mL/min.

Detection: It was done using ultraviolet (UV) at 250 nm.

The formula for calculation of concentration is given as under:

$$\text{Concentration of sample}\left(\mu\frac{g}{mL}\right) = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{Concentration of standard} \times \text{Dilution factor}$$

Separation

It was done through liquid chromatography (LC), using Shimadzu 10AV-LC, coupled with a binary delivery pump (model: Shimadzu LC-10A).

Monitoring

UV-visible (UV-vis) 10A-SPD spectrophotometer was used to monitor the eluted peaks.[20]

Statistical Analysis

Completely randomized design (CRD) was put to use in the design of the experiments for studying various interactions and changes. Fisher's least significant difference (LSD) procedure was adopted to compare the difference in various experiments and their results, with a 6% probability.

RESULT AND DISCUSSION

Effect of Different Concentrations of BA on Weight and Length of Fresh and Dry Shoot Tips

The fresh shoot tip's highest weight with highly significant differences as compared to other BA concentration treatments, except control treatment, was recorded at 235.6 mg with 2.5 mg/L BA concentration. In comparison, the lowest fresh shoot tip weight was recorded at 125.8 mg with 4.5 mg/L BA concentration. It was further revealed that the dry shoot tip's highest weight was recorded at 69.5 mg for 2.5 mg/L BA concentration, without any significant difference as compared to other BA concentration treatments. Whereas the lowest dry shoot tip weight was recorded at 39.5 mg with 1.5 mg/L BA concentration (Table 2).

Highly significant differences were observed with 3.5 mg/L BA concentration for the shoot tip of length 4.62 cm and the least significant difference with 6.2 mg/L BA concentration for 3.29 cm shoot tip length.

Table 2: Effect of different concentrations of BA on shoot tip's fresh and dry, weight, and length of *P. granatum* L.

BA concentration (mg/L)	*Fresh weight (mg)*	*Dry weight (mg)*	*Length (cm)*
Control	225.3	62.6	2.76
1	233.7	68.8	2.82
2	162.1	38.4	3.42
3	197.5	53.2	2.62
4	123.9	38.8	2.6
LSD (0.05)	55.43	NS	0.36

Table 3: Effect of different concentrations of MgO NPs on shoot tip's fresh and dry weight of *P. granatum* L. cultured on MS medium

Dry weight (mg/L)	*Fresh weight (mg/L)*	*MgO concentrations (mg/L)*
25.3	116.2	Control
18.7	114.4	2.5
17.2	165	5
36.2	142.5	7.5
19.2	132.6	10
9.55	22.28	LSD (0.05)

Table 4: Effect of different concentrations of CuO NPs on shoot tip's fresh and dry weight of *P. granatum* L. cultured on MS medium

Dry weight (mg/L)	*Fresh weight (mg/L)*	*CuO concentrations (mg/L)*
25.3	116.2	Control
21.6	141.4	5
32	108.9	10
14.2	181.7	15
21.4	120.9	20
7.62	28.96	LSD (0.05)

Table 5: Effect of different concentrations of MgO NPs on shoot tip's tannin compound of *P. granatum* L. cultured on MS medium

	Concentrations of MgO NPs (mg/L)						
LSD (0.05)	*10*	*7.5*	*5*	*2.5*	*Control*	*Mother plant*	*Tannin compounds*
0.03	132.24	56.64	72.86	76.89	26.22	27.35	Gallic acid
0.05	208.53	164.64	127.45	122.75	49.97	17.54	Tannic acid
0.04	155.71	151.03	86.65	97.35	26.08	27.44	Ellagic acid
0.17	70.89	71.72	49.49	67.55	8.85	5.58	Brevifolin carboxylic acid

Effect of Different Concentrations of MgO and CuO NPs on Fresh and Dry Weight of Shoot Tip

The fresh shoot tip's highest weight with highly significant differences as compared to other treatments was recorded at 165 mg at a concentration of 5 mg/L, while the lowest fresh shoot tip weight was recorded at 114.4 mg at MgO NPs concentration of 2.5 mg/L (Table 3). From Table 3, it was further revealed that the dry shoot tip's highest weight was recorded at 36.2 mg for 7.5 mg/L concentration, with highly significant differences as compared to other treatments containing different MgO NPs concentrations. Whereas the lowest dry shoot tip weight was recorded at 17.2 mg with 5 mg/L MgO NPs concentration.

While, the fresh shoot tip's highest weight with highly significant differences as compared to other treatments was recorded at 181.7 mg, while the lowest fresh shoot tip weight was recorded at 108.9 mg at CuO NPs concentration of 9.5 mg/L (Table 4). Table 4 further revealed that the dry shoot tip's highest weight was recorded at 32 mg for 10 mg/L CuO NPs concentration, with highly significant differences as compared to other treatments containing different CuO NPs concentrations. Whereas the lowest dry shoot tip weight was recorded at 14.2 mg with 14.5 mg/L CuO NPs concentration.

Effect of Different Concentrations of MgO and CuO on Medical Compounds in Shoot Tips using HPLC Technique

With the increase in MgO NPs concentrations, concentration of medical compounds also increased in the shoot tips, as compared with the mother plant; the results are shown in Table 5. The highly significant differences of 132.24, 208.53, and 155.71 µg/mL for gallic acid, tannic acid, and ellagic acid, respectively, were observed at MgO NPs concentration of 9.5 mg/L as compared with other treatments. Whereas, the lowest significant difference average of 26.22 and 26.08 µg/mL was recorded for gallic acid and ellagic acid, respectively, while 17.54 µg/mL was recorded for tannic acid in the mother plant.

Table 6: Effect of different concentrations of CuO NPs on shoot tip's tannin compound of *P. granatum* L. cultured on MS medium

		Concentrations of CuO NPs (mg/L)					
Tannin compounds	*Mother plant*	*Control*	*5*	*10*	*15*	*20*	*LSD (0.05)*
Gallic acid	26.32	26.22	61.54	42.15	117.46	65.59	0.05
Tannic acid	17.54	49.97	47.61	45.91	59.7	154.45	0.04
Ellagic acid	27.44	26.08	75.93	37.46	128.54	94.72	0.05
Brevifolin carboxylic acid	5.58	8.85	24.80	42.04	39.48	16.73	0.06

Table 7: Effect of different concentrations of MgO NPs on shoot tip's phenol compound of *P. granatum* L. cultured on MS medium

		Concentrations of MgO NPs (mg/L)					
Phenol compounds	*Mother plant*	*Control*	*2.5*	*5*	*7.5*	*10*	*LSD (0.05)*
Chlorogenic acid	5.8	215.38	356.51	379.88	286.85	474.77	0.03
Catechin	1.3	172.78	212.06	189.54	287.57	217.09	0.05
Rutin	5.53	188.1	258.14	290.06	304.46	465.75	0.05
Coumaric acid	5.3	136.2	182.22	255.2	214.23	258.28	0.06
Ferolic acid	7.63	110.51	49.65	104.9	165.62	236.28	0.05
Benzoic acid	4.57	103.72	211.71	129.45	137.73	207.08	0.03
Acacetin	7.03	103.13	153.52	105.99	112.34	222.04	0.04
Cinnamic acid	8.21	74.05	99.43	80.31	109.36	202.76	0.05
Genistein	13.18	65.54	146.35	222.58	300.25	214.98	0.04
Kaempferol	3.32	63.24	174.79	140.74	201.17	192.78	0.05

Table 8: Effect of different concentrations of CuO NPs on shoot tip's phenol compound of *P. granatum* L. cultured on MS medium

		Concentrations of CuO NPs (mg/L)					
Phenol compounds	*Mother plant*	*Control*	*5*	*10*	*15*	*20*	*LSD (0.05)*
Chlorogenic acid	5.83	215.38	164.74	251.1	299.47	350.44	0.04
Catechin	1.3	172.78	273.97	349.47	128.58	255.75	0.04
Rutin	5.53	188.1	168.88	189.5	156.16	254.81	0.04
Coumaric acid	5.3	136.2	105.88	122.02	94.5	204.51	0.05
Ferolic acid	7.63	110.51	68.34	132.59	130.73	54.28	0.05
Benzoic acid	4.57	103.72	74.11	82.34	197.04	86.76	0.03
Acacetin	7.03	103.13	54.46	52.24	197.62	90.34	0.04
Cinnamic acid	8.21	74.05	23.63	89.19	114.64	80.89	0.04
Genistein	13.18	65.54	186.29	175.14	130.65	184.71	0.05
Kaempferol	3.32	63.24	162.75	66.73	83.78	267.94	0.06

The highly significant difference of 71.72 µg/mL was recorded for brevifolin carboxylic acid at MgO NPs concentration of 7.5 mg/L. The lowest concentration average of 6.58 µg/mL was recorded by the mother plant.

With the increase in CuO NPs concentrations, the concentration of medical compounds also increased in the shoot tips compared with the mother plant; the results are shown in Table 6. The highly significant differences of 117.46, 154.45, 128.54, and 42.04 µg/mL were recorded for gallic acid, tannic acid, ellagic acid, and brevifolin carboxylic acid, respectively, at CuO NPs concentration of 15, 20, 15, and 10 mg/L, respectively, as compared with other treatments. Whereas the lowest significant difference average of 26.22, 17.54, 26.08, and 5.58 µg/mL was recorded were recorded for gallic acid, tannic acid, ellagic acid, and brevifolin carboxylic acid, respectively, at CuO NPs concentration of 15, 20, 15, and 10 mg/L, respectively, for the control treatment.

With the addition of MgO NPs, the concentration of medical compounds, including chlorogenic acid, rutin, coumaric acid, ferolic acid, acacetin, and cinnamic acid also increased in the shoot tips compared with the mother plant; the results are shown in Table 7. The highly significant differences of 474.77, 465.75, 258.28, 236.28, 222.04, and 202.76 µg/mL were recorded for chlorogenic acid, rutin, coumaric acid, ferolic acid, acacetin, and cinnamic acid, respectively, at MgO NPs concentration of 10 mg/L, whereas the lowest significant difference of 6.9, 6.53, 5.3, 7.63, 7.03, and 8.21 µg/mL, respectively, were recorded for the above compounds.

The highly significant differences of 287.57, 211.71, 201.17, and 300.25 µg/mL were recorded for catechin, benzoic acid, genistein, and kaempferol, respectively, at MgO NPs concentration of 7.5, 2.5, 7.5, and 7.5 mg/L, respectively, whereas the lowest significant difference of 1.3, 4.57, 201.17, and 300.25 µg/mL, respectively, were recorded for the above compounds in the mother plant (Table 7).

With the addition of CuO NPs, the concentration of medical compounds, including chlorogenic acid, rutin, coumaric acid, and kaempferol, also increased in the shoot tips compared with the mother plant; the results are shown in Table 8. The highly significant differences of 350.44, 254.81, 204.51, and 267.94 µg/mL were recorded for chlorogenic acid, rutin, coumaric acid, and kaempferol, respectively, at CuO NPs concentration of 17.5 mg/L, respectively, whereas the lowest significant difference of 5.8, 6.23, 5.3, and 2.89 µg/mL, respectively, were recorded for the above compounds at the mother plant.

The highly significant differences of 197.04, 197.62, 114.64, 349.47, 132.59 µg/mL were recorded for benzoic acid, acacetin, cinnamic acid, catechin, ferolic acid, and genistein, respectively, at CuO NPs concentration of 15, 15, 15, 10, 10, and 5 mg/L, respectively, whereas the lowest significant difference of 3.49, 6.13, 8.21, 1.3, 6.63, and 13.18 µg/mL, respectively, were recorded for the above compounds in the mother plant.

The presence of several bioactive secondary metabolites in plants plays an important role in their survival. These metabolites can be produced in the shoot tip via *in vitro* culture. On supplying suitable culture conditions, optimization of the culture medium's composition, and combination of precursors/elicitors, considerably boosted these metabolites in the cell and organ cultures. For the purposes of nutrient source, the culture medium of the plant *in vitro* is supplemented with MgO and CuO NPs, who also act as an elicitor. The MS medium significantly increased the amount of essential oils in *Calendula officinalis* as reported by Al-Oubaidi H. K. M. *et al*.[21]

The amount of tannin and phenol compounds is considerably enhanced by the presence of MgO and CuO NPs, in the concentrations 2.5, 5, 7.5, and 10 mg/L for MgO and 6.5, 11.5, 16.5 and 20 mg/L for CuO NPs, respectively. Metal and metal oxide NPs have been shown to have a positive effect on plant growth and productivity in some studies, while many of them indicate their toxic effects.[22] The effects of CuO NPs have also been studied.[22]

CONCLUSION

It is observed that the secondary metabolite (phenolic and tannin) compounds concentration in *P. granatum* L. gets considerably enhanced upon the addition of various concentrations of CuO and MgO NPs.

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