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In vitro increasing medical compounds (tannins and phenols) of *punica granatum* L. in callus using MgO NPs and CuO NPS

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Abstract
The present research was conducted in order to increase some secondary metabolites (tannins and phenols) compounds of *punica granatum* L. *in vitro* by adding different concentrations of MgO NPs (2.5, 5, 7.5 or 10) mg/l and CuO NPs (5, 10, 15 or 20) mg/l to callus media.
Quantitative and qualitative analysis of tannins and phenols compounds were estimated by using high performance liquid chromatography (HPLC) and compared with those in mother plant.
The results showed that the concentration (10) mg/l of MgO NPs for increasing tannin compounds and the concentrations (2.5, 5 and 10) mg/l of MgO NPs for increasing phenol compounds and the concentrations (5, 20) mg/l of CuO NPs for increasing tannin compounds and the concentrations (15, 20) mg/l CuO NPs for increasing phenol compounds and cause highly differences.

INTRODUCTION

pomegranate (*punica granatum* L.) belong to the family punicaceae is one of the important medicinal plants in traditional therapies (1) it's used for medicinal purpose for centuries (2) its contain significant amount of acid, sugar, vitamins, polysaccharides, polyphenol and minerals (4) carbohydrate, ascorbic acid, pectin, cellulose, tannin, flavonoid, (5) natural product are the source of traditional and synthetic herbal medicine (6) all secondary metabolites have specific function such as saponins have anti-fungal activity (9) phenols compounds are the major group that act as antioxidants (7) antibacterial activity (8) some alkaloid may be useful against HIV infection (10) flavonoids have strong anticancer activity (11) and tannin anti-microbial activity

The potential therapeutic properties of plant are including treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, diarrhea, folliculitis, and allergic dermatitis (12).

Plant tissue culture are alternative techniques were established to propagate a large number of *in vitro* plants. A rapid and efficient in vitro regeneration system has been developed nodal segments (13). By using PTC technique genetically similar plantlets can be produced in relatively short time , speeding up conventional breeding and propagation , reducing space and lab our requirement and achieving manipulative goal that can't be carried our via in vivo conditions

The main importance for culturing techniques is to obtaining secondary metabolites, such as active compounds for pharmaceuticals and enzymes, proteins, antigens, food additives and natural pesticides from the harvest of the cultured cells or tissues (3) Secondary metabolites can be produced by using different biotechnological approaches, such as cell suspension cultures and/or organ cultures that production of secondary metabolites is generally higher in differentiated plant tissues, there were attempts to cultivate whole plant organs, i.e. shoots, embryos or roots in in vitro conditions with the aim to produce medicinally important compounds (20).

In modern material sciences, research on nanoparticles (NPs) is one of the most attractive and active areas of research. NPs have wide applications due to their size, structure, and physiochemical properties in industries and agriculture (21). Nanotechnology, a new emerging and interesting field of science is currently applied in many areas. Nanoparticles (NPs) are commonly accepted as materials with at least two dimensions between 1-100 nm (24) Among all reported NPs so far, CuO-NPs MgO-NPs being a significant kind of metal oxide NPs, are used in biomedical applications (22).

The present study was conducted to determine and examine the suitable strength of MS media, and the effect of different auxins and cytokinin at various concentrations on callus induction, then study the effect of some nanoparticles on increasing medical compounds to comparing it with mother plant and control

MATERIAL AND METHOD

Plant material and sterilization

The explants of ***punica granatum*** from newly branches were collected from gardens in Baghdad Iraq on 1/10/2017. leaves of ***punica granatum*** were rinsed with running water for 30 minutes then transfer to laminar air flow cabinet where submerged in 70% ethanol for 1 minutes then washed with sterilized D.W for 5min then rinsed with sodium hypochlorite at concentration 2% then washed with sterilized D.W for 5min three times and culture on universal tubes which contain MS medium (14, 27)

Callus induction

Explants (leaves) of *punica granatum* were dissected and cultured on universal tubes contain MS medium with different concentrations of the auxin 2,4-D(0,1,2,3 or 4)mg/l then distributed in to 10replicates for each concentrations which incubated in dark conditions at a temperature 25±2°C the results recorded after 21 day(15)

Measuring fresh and dry weight of callus

The fresh weight of callus was measured by using the sensitive balance then the callus was dried using oven at 70°C until the dry weight is stable then measured by sensitive balance (16)

Extraction and analysis of secondary metabolite from callus and shoot tip of *punica granatum*

50mg of callus were mixed and dissolved in methanol the extract was subjected to the ultra-sonication followed by centrifugation for 15min .the clear supernatant of each sample was subjected to charcoal treatment then decantation and filtered through filter paper watman no.1 then the aqueous extraction were evaporated under vacuum. Dried samples were re-suspended in 1.0 ml HPLC grade methanol by vortexing, the mixture were passed through 2.5 µm disposable filter paper, and stored at 4°C for farther analysis, then 20µm of the sample injected into HPLC system according the optimum separation condition previously optimized. (17, 18)

Estimation the increase in secondary metabolites compound by device High performance liquid chromatography (HPLC)

The main compound were separated on FLC (fast liquid chromatographic) column under the optimum condition

Column: phenomenex C-18, 3µm particle size (50×2.0mm I.D) column,

Mobile phase: linear gradient of, solvent A 0.5% acetic acid in distilled water PH2.5, solvent B was 0.5% in 99.5% acetonitrile , linear gradient program flow rate 1.2ml/min.

Detection: UV 245nm

concentration of sample $\mu g/ml = \frac{area\ of\ sample}{area\ of\ standard} \times concentration\ of\ standard \times dillution\ factor$

The separation occurred on liquid chromatography shimadzu 10AV-LC equipped with binary delivery pump model LC-10A shimadzu, the eluted peaks were monitored by UV-Vis 10A-SPD spectrophotometer. (19)

Statistical analysis and experimental design

Experiments are designed according to the completely randomize design (CRD) to study the effect of various transactions in the studied traits and compared the differences between the treatments according to least significant differences (LSD) probability of 5%

Table (1): components of media which used of stimulation (tannin and phenol) compounds by adding CuO NPs or MgO NPs

No.	Component	Concentration(mg/l)
1.	MS	44000
2.	Sugar	30000
3.	L-asparagine	150
4.	Glycine	100
5.	2,4,D	2
6.	CuO NPs	0,5,10,15 or 20
7.	MgO NPs	0,2.5,5,7.5 or 10
8.	Agar-agar	8000
9.	Kinetin	0.2

Table (2) effect of different concentration of 2,4-D on percentage of callus induction from leave of *punica granatum* L.

Concentration of 2,4-D (mg/l)	callus induction
Control	0%
0.5	100%
1.5	80%
2.5	90%
3.5	90%
4.5	90%

Table (3): Fresh and dry weight for callus which cultured on (MS) medium supplemented with different concentrations of MgO NPs

MgO concentrations Mg/l	Fresh weight(mg)	Dry weight(mg)
Control	241.20	30.70
2.5	236.90	24.80
5	233.00	24.90
7.5	248.30	21.70
10	347.40	23.70
LSD(0.05)	56.23	N.S

RESULT AND DISCUSSION

Effect of different concentrations of 2, 4-D on percentage of callus induction

In table 2 showed that the concentration of 0.5mg/l 2, 4-D gave the highest percentage 100% for callus induction from leaves while the lowest percentage was found in control 0%

The effect of different concentrations of MgO and CuO on fresh and dry weight in callus

The result in (table 3)showed the concentration (10)mg/l had the highest callus fresh weight (347.40 mg) that had high significant than the other treatments while the lowest callus fresh weight (233mg) at concentration 5mg/l of MgO NPs .the results at same table showed that the highest callus dry weight was the control (30.70 mg) without significant differences than another treatments with different concentrations of MgO NPs while the lowest callus dry weight was (21.70mg) at concentration 7.5mg/l of MgO NPs.

While the result in (table 4) showed that the control had the highest callus fresh weight (321 mg) without significant differences than the other treatment while the lowest callus fresh weight (254mg) at concentrations (10, 15, 20)mg/l of CuO NPs .The result at same table showed that the highest callus dry weight was the control (30.70 mg) that found high significant than another treatment with different concentration of CuO NPs while the lowest callus dry weight was (18.70mg) at concentration 15mg/l of CuO NPs.

The result in (table 5) showed different concentrations of medical compounds were increase depending on the increasing concentration of MgO NPs compared with mother plant. The Gallic acid gave high significant difference (151µg/ml) at concentration 7.5 mg/l MgO NPs while the lowest average at mother plant that measured (27.35µg/ml). The tannic acid gave high significant difference (333.53µg/ml) at concentration 10 mg/l of MgO NPs than the other treatment while the lowest average at mother plant which gave (17.54µg/ml).The Ellagic acid gave high significant difference at concentration 10 mg/l MgO NPs that measured (153.60 µg/ml) while the lowest average at control treatment (11.75 µg/ml). Finally the Brevifolin carboxylic acid measured the high significant difference (52.70µg/ml) at concentration (10 mg/l) MgO NPs where the control treatment measured (5.53µg/ml) the lowest average of Brevifolin carboxylic acid

Table (4): Fresh and dry weight for callus which cultured on (MS) medium supplemented with different concentrations of CuO NPs

CuO concentration Mg/l	Fresh weight(mg)	Dry weight(mg)
Control	321.0	30.7
5	283.0	25.7
10	254.0	25.0
15	254.0	18.7
20	254.0	25.4
LSD(0.05)	N.S	N.S

Table (5): Tannin compounds for callus which cultured on MS medium supplemented with different concentration of MgO Nps (mg/l)

tannin compounds	Mother plant	Concentrations of MgO NPs (mg/l)					LSD(0.05)
		Control	2.5	5	7.5	10	
Gallic acid	27.35	27.38	61.92	85.69	151.78	115.25	0.03
Tannic acid	17.54	35.15	172.74	153.67	124.16	333.53	0.06
Ellagic acid	27.44	11.75	89.06	73.82	149.38	153.60	0.05
Brevifolin carboxylic acid	5.58	5.53	18.06	36.79	44.98	52.70	1.71

Table (6): Tannin compounds for callus which cultured on MS medium supplemented with different concentration of CuO NPs (mg/l)

tannin compound	Mother plant	concentrations of CuO NPs (mg/l)					LSD(0.05)
		control	5	10	15	20	
Gallic acid	27.35	27.38	29.84	41.89	51.28	81.62	0.04
Tannic acid	17.54	35.15	92.05	38.41	56.07	60.36	0.06
Ellagic acid	27.44	11.75	41.45	49.63	39.29	82.82	0.04
Brevifolin carboxylic acid	5.58	5.53	5.19	21.33	21.93	76.28	0.06

Table (7): Phenol compounds in callus which cultured on MS medium supplemented with different concentrations of MgO NPs (mg/l)

phenol compounds	Mother plant	Concentrations of MgO NPs (mg/l)					LSD(0.05)
		control	2.5	5	7.5	10	
Chlorogenic acid	6.90	176.15	342.72	338.14	316.47	285.92	0.04
Catechin	1.30	98.69	183.52	128.56	195.15	253.52	0.03
Rutin	5.53	91.85	186.92	332.94	282.94	316.40	0.05
Coumaric acid	5.30	61.50	77.71	262.25	237.44	208.98	0.88
Ferolic acid	7.63	42.92	78.08	204.09	172.45	199.60	0.05
Benzoic acid	4.57	12.02	21.26	123.45	70.89	164.38	0.05
Acacetin	7.03	35.07	131.22	63.25	70.46	174.07	0.06
Cinnamic acid	8.21	23.38	139.64	138.73	55.93	130.09	0.05
Genistein	13.18	41.37	169.13	96.89	83.85	128.77	0.04
Kaempherol	3.32	96.63	65.71	92.91	157.76	172.08	0.03

Table (8) Phenol compounds in callus which cultured on MS medium supplemented with different concentrations of CuO NPs (mg/l)

phenol compounds	Mother plant	concentrations of CuO NPs (mg/l)					LSD(0.05)
		control	5	10	15	20	
Chlorogenic acid	6.90	176.15	238.81	216.26	231.52	331.23	0.04
Catechin	1.30	98.69	237.27	85.97	271.92	221.82	0.03
Rutin	5.53	91.85	158.31	136.96	104.16	335.80	0.04
Coumaric acid	5.30	61.50	95.54	70.09	311.39	195.67	0.06
Ferolic acid	7.63	42.92	14.09	12.43	241.29	170.88	0.05
Benzoic acid	4.57	12.02	42.96	12.18	263.08	158.20	0.03
Acacetin	7.03	35.07	49.49	27.29	54.56	27.99	0.03
Cinnamic acid	8.21	23.38	33.77	15.69	135.43	102.94	0.04
Genistein	13.18	41.37	49.66	16.91	140.48	147.83	0.03
Kaempherol	3.32	96.63	98.87	192.33	80.68	229.66	0.05

The effect of different concentrations of MgO NPs and CuO NPs on medical compounds in callus using HPLC technique

The result in (table 6) showed different concentrations of medical compounds were increase depending on the increasing concentration of CuO NPs compared with mother plant. The Gallic acid gave high significant difference (81.62 µg/ml) at concentration 20 mg/l CuO NPs while the lowest average at mother plant that measured (27.35µg/ml).The tannic acid gave high significant difference (92.05µg/ml) at concentration 5 mg/l of CuO NPs than the other treatments while the lowest average at mother plant which gave (17.54 µg/ml). The Ellagic acid gave high significant difference at concentration 20 mg/l CuO NPs that measured (82.82 µg/ml) while the lowest average at control treatment (11.75 µg/ml). Finally the Brevifolin carboxylic acid measured the high significant difference (76.28µg/ml) at concentration (20 mg/l) CuO NPs where the control treatment measured (5.53µg/ml) the lowest average of Brevifolin carboxylic acid.

From table 7 adding MgO nanoparticles resulting in high significantly differences in Chlorogenic acid reach up to (342.72 µg/ml) in concentration (2.5 mg/l) MgO NPs treatment and lowest average in mother plant was recorded (6.90 µg/ml). Catechin showed high significant difference with treat (10 mg/l) MgO NPs that recorded (253.52µg/ml) and the lowest average recorded in mother plant (1.30µg/ml).

Rutin, Coumaric acid, Ferolic acid gave high significance differences in concentration (5 mg/l) MgO NPs that recorded (332.94, 262.25, 204.09) µg/ml and the lowest average showed in mother plant that recorded (5.53, 5.30, 7.63) µg/ml respectively.

While the Benzoic acid, Acacetin were recorded the high significance differences in concentration (10 mg/l) MgO NPs that

measured (164.38, 174.07) µg/ml where the lowest average showed in mother plant (4.57, 7.03) µg/ml respectively.

But in Cinnamic acid and Genistein showed that the treated (2.5 mg/l) MgO NPs that have high signification difference where recorded (139.64, 169.13) µg/ml and lower average in mother plant (8.21, 13.18) µg/l respectively.

Finally Kaempherol compound have lowest concentration in mother plant (3.32µg/l) and high concentration recorded in treatment (10mg/l) of MgO NPs where recorded (172.08µg/ml)

In table 8 the result showed that different concentration of CuO NPs cause increasing in medical compound comparing with mother plant that what seen in Chlorogenic acid, Rutin, Genistein and Kaempherol gave a highly significant differences (331.23, 335.80, 147.83, 229.66) µg/ml at concentration 20mg/l of CuO NPs while the lowest avarage at mother plant which gave (6.90, 5.53, 13.18, 3.32) µg/ml respectively.

While the Catechin, Coumaric acid, Ferolic acid, Benzoic acid, Acacetin, Cinnamic acid recorded high significant differences that reach (271.92, 311.39, 241.29, 263.08, 54.56, 135.43) µg/ml respectively in concentration (15 mg/l) of CuO nanoparticles and the lowest average in mother plant (1.30, 5.30, 7.63, 4.57, 7.03, 8.21) µg/ml respectively.

CuO, MgO each alone could make a significant increase in secondary metabolites, with regards to more effect of MgO with tannin compounds than phenol compounds which may be due to a higher share of its in cell division process (25) and because the more particles at nanoscale means the more contact surface leading to increase in the contacts of molecules and velocity of chemical reactions inside the cell, including increase phenols and tannins compounds of *punica granatum*, it is because the magnesium ions are essential for cell division (26) The effect of treatments (cont., 2.5, 5, 7.5, 10) mg/l MgO NPs and (cont., 5, 10, 15, 20) mg/l CuO NPs on producing secondary metabolites from callus by HPLC techniques was very high significant comparing with mother plant MgO NPs and CuO NPs

Increasing tannin compounds and phenol compounds in *punica granatum* L. due to changing the physicochemical properties of materials at nanoscale. The effect of nanoparticles on increasing secondary metabolites studied by many researchers, (28) said the positive role of NPs in callus induction, organogenesis, somatic embryogenesis, somaclonal variation, genetic transformation and secondary metabolite production (23) said that the nanoparticles have the ability to increasing secondary metabolites in callus due to its effect on gene expression

CONCLUSION

Adding different concentration of (CuO, MgO) nanoparticles for callus media cause high significant increase in all study medical compound (phenolic and tannin) compounds of *punica granatum* L.

RECOMMENDATION

Utilize other elicitors to increase secondary metabolites that used as medicinal plant

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