EFFECT OF CHEMICAL MEDIA COMPONENTS AND SILVER NANOPARTICLES ON THE INDUCTION OF CALLUS CULTURE AND ACCUMULATION OF SECONDARY COMPOUNDS IN A *Trigonella foenum-graecum* L.

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ABSTRACT

This study was investigate the effect of components media and silver nanoparticles (Ag-NPs) synthesized in the laboratory on the induction of biomass and secondary metabolites of calli cultures induced from the cotyledons of *Trigonella foenum-graecum* L. The culture media LS and B5 were tested with the growth regulators 2,4-Dichlorophenoxyacetic acid (2,4-D) at a concentration of 1.0, 2.0 and 3.0 mg l^{-1} and 6-benzyladenine (BA) at a concentration of 0.0, 0.5, 1.0, 1.5 and 2.0 mg l^{-1}, with the aim of studying its effect on callus induction. In comparison, Ag-NPs were used at a concentration of 0.0, 0.5, 1.0, 1.5 and 2.0 mg l^{-1} to study its effect on the production of some secondary compounds. The results showed that the mixture consisting of the culture media LS with 3.0 mg l^{-1} of 2,4-D and 1.0 mg L^{-1} of BA the highest percentage of callus induction and the minimum days to complete callus formation. While, the same media with 2.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} of BA recorded the highest dry weight (DW) and relative water content (RWC). The combination B5×2.0 mg l^{-1} 2,4-D×1.5 or 2.0 mg l^{-1} showed the highest fresh weight (FW) and the lowest time required to start callus induction, respectively. As for the production of SC, the B5 media with 2.0 mg l^{-1} of Ag-NPs achieved the highest concentration of alkaloid compounds represented by trigonelline and diosgenin, and flavonoids expressed by qurcetine, vitxein, rutin, apigenin, isovitexin, and kaemferol.

Key words: in vitro, nanoparticles, flavonoids, alkaloids.

مجلة العلوم الزراعية العراقية 2024 -:55(عدد خاص):111-121 جاسم ونعمة

تأثير مكونات الوسط الكيميائية وجسيمات الفضة النانوية في إستحثاث مزارع الكالس وتراكم المركبات الثانوية في الحلبة

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المُستخلص

نفذت الدراسة الحالية لمعرفة تأثير مكونات الوسط الغذائي وجسيمات الفضة النانوية المُصنعة في المختبر في استحثاث الكتلة الحيوية ومركبات الأيض الثانوي لمزارع الكالس المُستحثة من الورقة الفلقية لنبات الحلبة *Trigonella foenum– graecum* L. . أختبر الوسطين الغذائيين LS و B5 مع منظمي النمو Dichlorophenoxyacetic acid (2,4–D) بالتركيز 1.0 و 2.0 و 3.0 ملغم لتر$^{-1}$ و 6–benzyladenine (BA) بالتركيز 0.0 و 0.5 و 1.0 و 1.5 و 2.0 ملغم لتر$^{-1}$, بهدف دراسة تأثيرها في استحثاث كالس نبات الحلبة. بينما استُعملت الفضة النانوية بالتركيز 0.0 و 0.5 و 1.0 و 1.5 و 2.0 ملغم لتر$^{-1}$ لدراسة تأثيرها في إنتاج بعض مركبات الأيض الثانوي. بينت النتائج تحقيق التوليفة المتكون من الوسط الغذائي LS مع التركيز 3.0 ملغم لتر$^{-1}$ من 2,4–D و التركيز 1.0 ملغم لتر$^{-1}$ من BA أعلى نسبة مئوية لإستحثاث الكالس وأدنى مدة لازمة لإكتمال تكوين الكالس. بينما سجل ذات الوسط مع التركيز 2.0 ملغم لتر$^{-1}$ من 2,4–D والتركيز 1.0 ملغم لتر$^{-1}$ من BA أعلى وزن جاف ومحتوى مائي نسبي. في حين أظهرت التوليفة B5×2.0 ملغم لتر$^{-1}$ من 2,4–D مع كل من 1.5 أو 2.0 ملغم لتر$^{-1}$ اعلى وزن طري وأدنى مدة لازمة لبدء استحثاث الكالس بالتتابع. أما بالنسبة لانتاج المركبات الثانوية فقد حقق الوسط الغذائي B5 مع التركيز 2.0 ملغم لتر$^{-1}$ من Ag–NPs اعلى تركيز لمركبات القلويدات المُمثلة بِـ trigonelline و diosgenin ولمركبات الفلافونيد المتمثلة بِـ qurcetine وvitxein و rutin و apigenin و isovitexin و kaemferol.

الكلمات المفتاحية: خارج الجسم الحي, جسيمات نانوية, فلافونيدات, قلويدات.

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INTRODUCTION

Trigonella foenum- graecum L., an annual herbaceous plant, belongs to the Fabaceae family. It has been used as a spice since ancient times (1). It is used in many medical and industrial fields, as it is used in manufacturing beverages, perfumes, and cosmetics (4). It characterized by its high content of secondary metabolite compounds, including alkaloids, phenolic acid, flavonoids, and saponins (10, 23, 24, 33, 35). The compounds have anti-oxidant, anti-carcinogenic, and glycemic-regulatory properties (29) and anti-inflammatory (22), as well as a role in the treatment of cardiovascular disease, hypercholesterolemia, liver disease, and testosterone disorders (1, 25). Implanting tissue culture technology is one of the promising methods in the commercial production of secondary metabolite compounds. Producing these compounds is at a lower cost and higher purity than their field counterpart. Therefore, this technology was used to protect diminishing wild plants known for their high content of secondary compounds and to develop strains characterized by their high content of these compounds, in addition to the possibility of producing these compounds from different plant parts based on the technique of culturing callus or cell suspensions (17, 18, 34). The culture medium prepared for crops outside the living body differ in their chemical components and ability to develop tissue cultures on the one hand and produce secondary compounds on the other. Many studies contributed to the crystallization of different ideas regarding the role of these media in the production of secondary compounds in vitro (8). The scientific communities followed up the field of botany with great interest and the possibilities offered by nanotechnology. They demonstrated the essential roles that can be exploited in the development of living cells through their unique physical and chemical properties, which allow for improving the vital activities of the plant cell (34). Silver nanoparticles (Ag-NPs) represent one of the available types of nanomaterial, which has attained wide interest from researchers due to its remarkable physiological properties (30, 31) including its potential to influence plant cell growth and thus biomass accumulation and production of metabolic compounds (2, 5, 7). Based on that, this study was aimed to find the optimal method to obtain the highest possible response to callus induction from the cotyledons by testing two culture mediums, including LS and B5, under different combinations of growth regulators 2,4-Dichlorophenoxy acetic acid (2,4-D) and Benzyl adenine (BA) as well as an ability to test culture media (LS or B5) and Ag-NPs as effective tools that enable increased accumulation of plant secondary compounds in vitro.

MATERIALS AND METHODS

Sterilization: The work requirements, distilled water, and the medium prepared for cultivation were sterilized in an autoclave at 121 °C for 15 min and a pressure of 1.04 kg cm^2. The laminar airflow cabinet was sterilized by 70% of ethanol.

Seed germination

The seeds to be cultured were sterilized using sodium hypochlorite (NaOCl) at a concentration of 2% for 5 min. Then they were washed with distilled water three consecutive times. The cultivation was in vials containing of Murashige and Skoog (MS) media (16). The media was prepared by adding 4.43 g MS, 30 g sucrose, and 7.0 g Agar. The pH was adjust to 5.7 ± 0.1. The vials were placed inside the incubator at a temperature of 1 ± 25ºC, under a light intensity of 1000 lux, 16 h of light and 8 h of darkness.

Induction of callus culture

Ready-made was used, Caisson-produced Linsamir and Skoog (LS) and Gamborg (B5) culture media devoid of growth regulators described by Linsmair and Skoog (13) and Gamborg et al. (6). The dietary media used in the study were prepared independently by adding 4.73 and 3.21 g to LS and B5 media, respectively. Moreover, 20 g sucrose and 7.0 g agar were added to the media. 2,4-Dichlorophenoxyacetic acid (2,4-D) at a concentration (1.0, 2.0, and 3.0 mg l^{-1}), and (0, 0.5, 1.0, 1.5, and 2.0 mg l^{-1}) of 6-benzyladenine (BA) was added, after which the pH was adjusted to 5.6, after which the media was sterilized according to the method shown in seed germination. The cotyledons were the explant, it enucleate from the

sterilized seedlings by cutting a part of them with a length of 1 cm using the surgical blade with a scratch or wound to be cultured in the two prepared media. The cultures were incubated under the same previous physical conditions.

Study parameters

Callus formation response (CFR): It was calculated from the explant that formed calli to those that did not formed calli.

Start callus induction (SCI): Data were recorded based on the appearance of white color on the explant grown in the culture medium (Figure 1).

Figure 1. The SCI of callus induction from the cotyledon of *T. foenum-graecum* L

Complete callus formation (CCF): The number of days required for the completion of callus formation was recorded.

Fresh weight (FW) and Dry weight (DW) of callus culture: The FW was calculated using a sensitive balance. Then the callus was dried in oven at 50 ºC until the weight stabilized to record the DW of the callus.

Relative Water Content (RWC): The RWC of callI cultures was measured based on the method described by Karimi et al. (9)

Establishment of callus culture

Based on the results of the callus induction experiment, the best combination of growth regulators 2,4-D and BA was chosen to obtain a sufficient amount of plant material (calli cultures). After reaching the appropriate size, the explant enucleated from the sterilized seedlings. It was cultured in LS and B5 culture media equipped with the appropriate combination of growth regulators 2,4-D and BA in the amount of 2.0 and 1.5 mg l^{-1} for both regulators, respectively. The cultures were left in the growth chamber according to the previously indicated physical conditions. The maintenance phase was 8 weeks to reach the required amount of calli cultures (Figure 2).

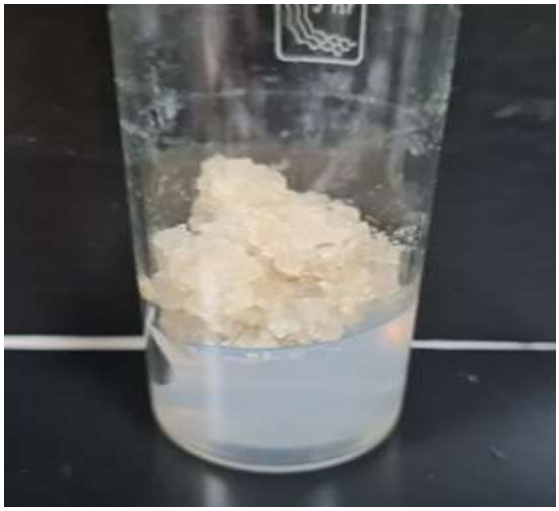

Figure 2. Callus induced from the cotyledons of *T. foenum- graecum* L. cultured in LS media treated with the optimized combination

Nanomaterial preparation

The colloidal material of silver nanoparticles was prepared according to the method of laser ablation in the liquid phase covering the metallic material (15, 27).

Nanomaterial characterization

The nanomaterial was characterized to ensure that it reaches the nanoscale level of 1-100 nm using a spectrophotometer, and its topography was determined using a transmission electron microscope (Figure 3).

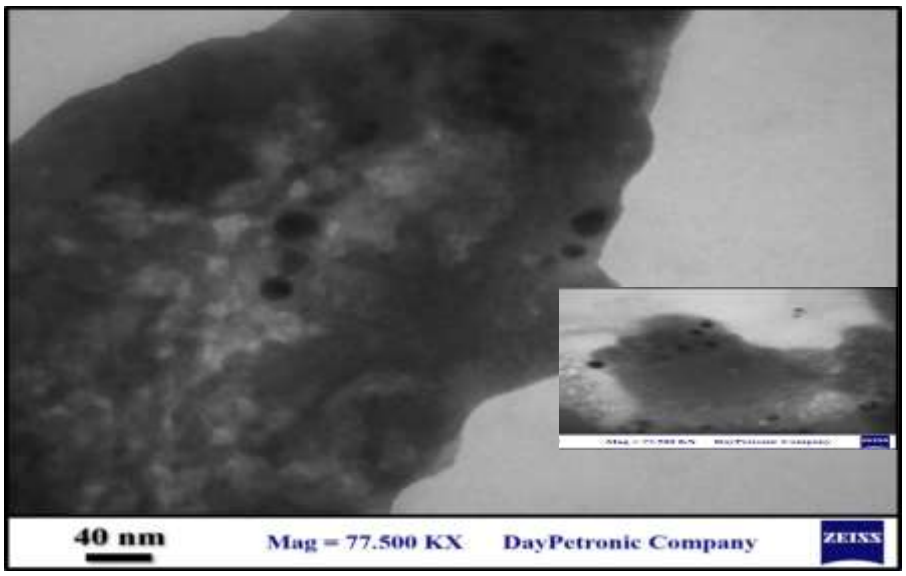

Figure 3. Size description of nanoparticles using TEM scanning.

Stimulation of secondary compounds media

LS and B5 media were prepared to grow callus cultures using the same method and physical conditions mentioned previously, taking into consideration the use of the optimal combination obtained from the induction experiment for growth regulators and the addition of concentrations of Ag-NPs (0, 0.5, 1.0, 1.5 and 2.0) mg l^{-1}. The cultures were incubated in the growth chamber for 4 weeks.

Alkaloid compounds

The extraction and quantification of the alkaloid compounds in calli cultures were carried out according to the proven method by Li et al. (12). Figure (4), shows the standard compounds that were measured in the plant extract of calli cultures.

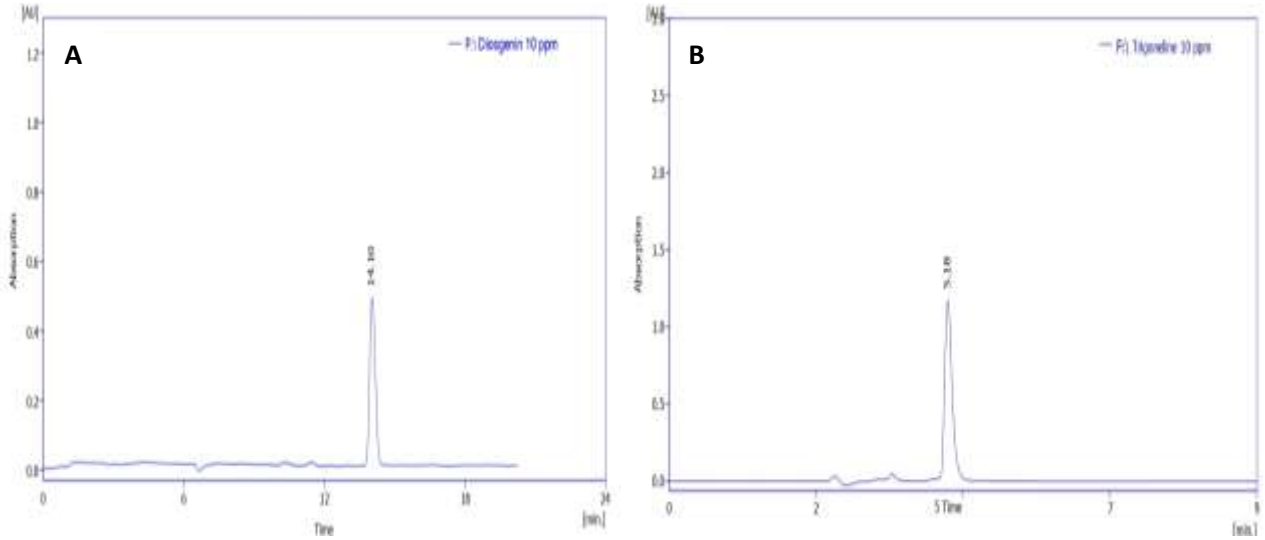

Figure 4. Standard curve for alkaloid compounds (A) Trigonelline (B) Diosgenin.

Flavonoid compounds

The extraction and quantification of flavonoids were carried out according to the method proven by Radovanovic et al. (28). Figure (5), shows the standard compounds estimated to the plant extract of calli cultures.

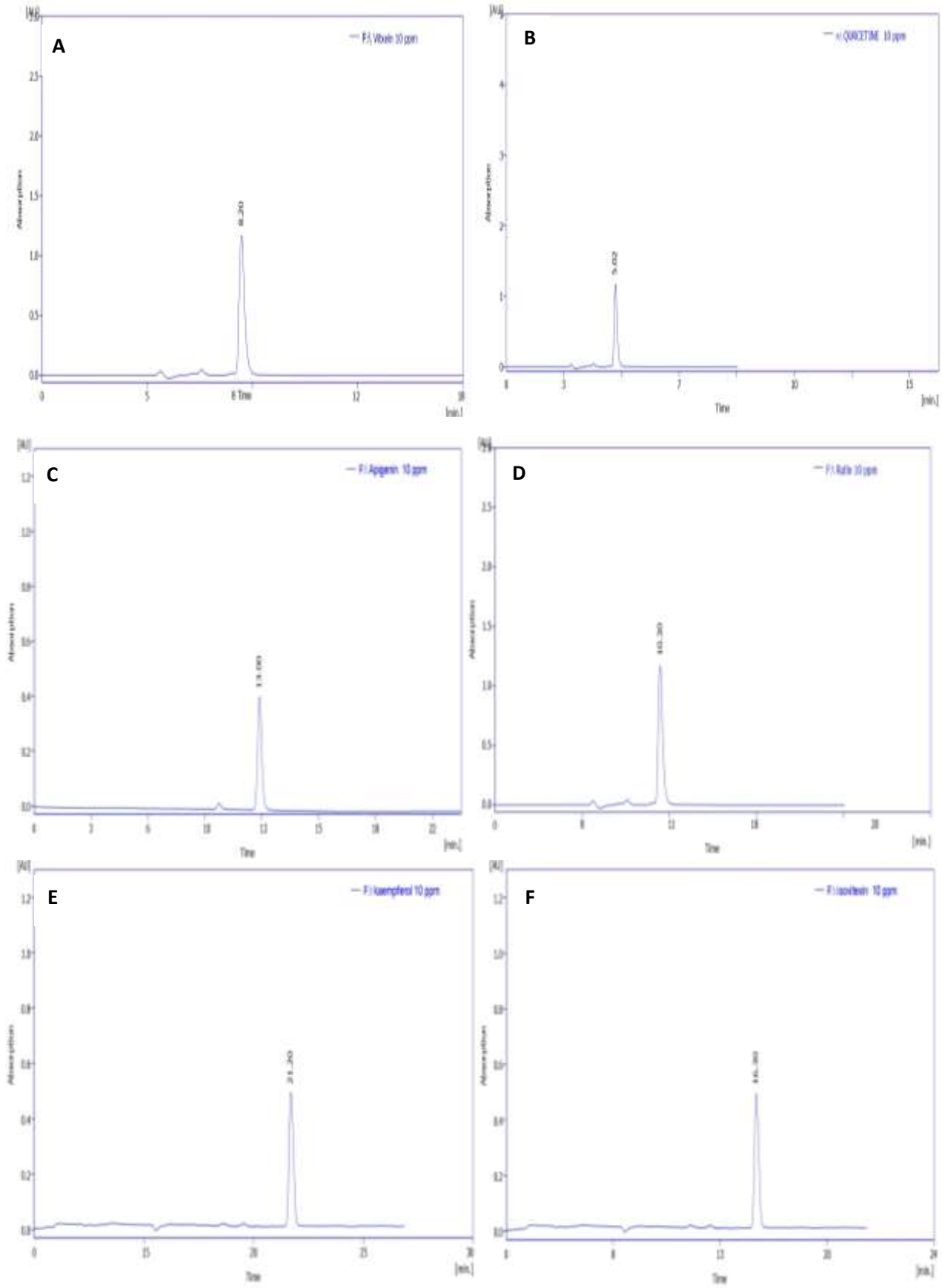

Figure 5. Standard curve for flavonoids (A) Qurcetine (B) Vitxein (C) Rutin (D) Apigenin (E) Isovitexin (F) Kaempferol

Experimental design and statistical analysis

A complete randomized design (CRD) was followed with 10 replications, and the data were analyzed statistically using GenStat version 12. The least significant difference

(LSD) value was calculated at the 0.05 probability level.

RESULTS AND DISCUSSION

Callus induction: The results in Table (1) show that there was a significant effect of the different combinations on some indicators of inducing calli cultures from the cotyledonous of *T foenum- graecum* L. plant, as the damage was achieved between LS medium with a concentration of 3.0 mg l^{-1} of 2,4-D with a concentration of 0.5 or 1.0 mg l^{-1} of BA. The highest induction rate was 100%. In contrast, when damaged with a concentration of 1.0 mg l^{-1} of 2,4-D, the same medium and the comparison treatment of BA achieved the lowest of induction of 10%. The mixture between B5 media with 2.0 mg l^{-1} of 2,4-D and 2.0 mg l^{-1} of BA recorded the lowest mean value to reduce SCI of 5.4 days. While the same media with 1.0 mg l^{-1} of 2,4-D and the control treatment or 0.5 mg l^{-1} of BA showed the highest mean value of SCI of 16.8 days.

Regarding the CCF, the combination (LS×3.0 mg l^{-1} of 2,4-D×1.0 mg l^{-1} of BA) recorded the lowest mean value of 21.6 days. While it achieved the same media with 1.0 mg l^{-1} of 2,4-D and 0.5 mg l^{-1} of BA, the highest CCF was 32.2 days. The mixture of B5 media with of 2.0 mg l^{-1} of 2,4-D and 1.5 mg l^{-1} of BA recorded the highest mean value of FW of callus cultures, which was 587 g. At the same time, the same media with 1.0 mg l^{-1} of 2,4-D and the comparison treatment of BA achieved the lowest mean value of 177 g. The mixture between LS media with 2.0 mg l^{-1} of 2,4-D and 1.5 mg l^{-1} of BA achieved the highest mean value DW of 46.8 g. It reached the same media with 1.0 mg l^{-1} of 2,4-D, and the comparison treatment of BA with the lowest mean value of 5.3 g. As for the RWC, the mixture between LS media with 2.0 mg l^{-1} of 2,4-D and 1.5 mg l^{-1} of BA achieved the highest mean value of 92.82%. B5 media with 1.0 mg l^{-1} of 2,4-D and comparison treatment of BA showed the lowest mean value of 77.61%. The difference in culture media in their effect on the biomass induction of calli cultures and their growth could be due to the differences in their chemical components (6, 13). Thus, the extent to which each culture medium interacts with the different combinations of auxin and cytokinin growth regulators. These results also indicate the importance of auxins, including 2,4-D, in stimulating the elongation of plant cells and increasing biomass through their influential role in increasing the elasticity of the cell wall, as it works to break the bonds of the cell wall and restore them to new sites, which contributes to increasing the size of the plant cell. In addition to the role of auxins in disengaging the calcium and magnesium ions from the carboxyl group attached to the pectinic substances that make up the cell wall, which facilitates cell expansion, in addition to the role of auxins stimulates the construction of m-RNA, which has an essential role in which has an essential role in activating the structure of enzymes and proteins, and thus contributes to increasing the biomass of plant cells (26). The results also showed the importance of growth regulators, cytokinins, represented by BA, in improving plant cell growth by stimulating the process of parenchymal cell division and increasing tissue biomass after transplantation (3).

Production of alkaloid compounds

The HPLC analysis that there is an improvement in the production of some alkaloid compounds from the *T foenum-graecum* L. in vitro (Figure 6), as we note the effect of the type of culture medium and the concentration of nanoparticles on the production of trigoneline. The callus grown in B5 medium treated with 2.0 mg l^{-1} of Ag-NPs achieved the highest value of compound production, amounting to 59.85 ppm. In comparison, the 1.5 mg l^{-1} of Ag-NPs reached a lower value of 54.98 ppm when grown in the same media. In contrast, the lowest production values were 36.58 ppm produced by callus grown in LS medium that did not contain nanomaterial (Figure 6A). As for diosgenin, the results showed that its highest production level reached 66.58 ppm when growing the callus in a B5 medium containing 2.0 mg l^{-1} of Ag-NPs. In comparison, the concentration of 1.5 mg l^{-1} achieved a value of 63.59 ppm when it was included in LS. The lowest production value was 44.15 ppm produced by callus grown in LS culture media not treated with nanomaterial (Figure 6B).

Table 1. Effect of the culture media, 2,4-D and BA on some induction parameters of *T. foenum-graecum* callus culture from the cotyledons after 35 days

Media	2,4-D mg l^{-1}	BA mg l^{-1}	CFR (%)	SCI (day)	CCF (day)	FW (mg)	DW (mg)	RWC (%)
		0	10	12.2	30.2	118	5.3	79.78
		0.5	80	16.8	32.2	251	24.8	81.80
	1.0	1.0	70	16.0	30.2	295	28.4	83.65
		1.5	70	16.0	25.0	386	41.7	83.78
		2.0	60	10.2	24.4	353	36.3	84.21
		0	30	13.0	27.0	346	23.9	83.20
		0.5	90	9.4	23.4	466	42.4	85.11
LS	2.0	1.0	60	11.4	22.8	391	37.0	87.85
		1.5	70	14.6	25.2	545	46.8	92.82
		2.0	70	8.6	26.0	372	39.9	90.89
		0	90	13.8	28.4	388	33.2	84.24
		0.5	100	10.2	28.8	433	42.0	84.89
	3.0	1.0	100	8.2	21.6	514	38.4	86.12
		1.5	90	10.2	30.4	413	39.0	82.93
		2.0	90	10.6	30.2	259	27.8	83.52
		0	30	12.4	29.4	177	10.2	77.16
		0.5	70	9.4	27.0	215	13.4	78.70
	1.0	1.0	60	12.0	25.0	386	23.0	81.54
		1.5	80	8.2	26.6	384	23.0	83.78
		2.0	80	8.6	28.6	323	18.3	84.21
		0	70	12.2	25.4	338	17.1	81.65
		0.5	80	11.4	26.4	446	35.4	83.50
B5	2.0	1.0	70	12.6	25.6	359	29.1	86.79
		1.5	60	15.2	22.4	587	38.3	89.72
		2.0	70	5.4	28.4	362	15.6	90.58
		0	60	8.0	23.8	321	17.6	82.88
		0.5	60	8.4	24.2	421	18.6	84.94
	3.0	1.0	40	12.0	22.0	232	12.7	86.36
		1.5	80	13.6	27.0	291	21.3	87.68
		2.0	40	9.6	29.4	292	24.3	84.86
L.S.D p< 0.05			34.45	4.849	3.464	166.1	12.310	2.498

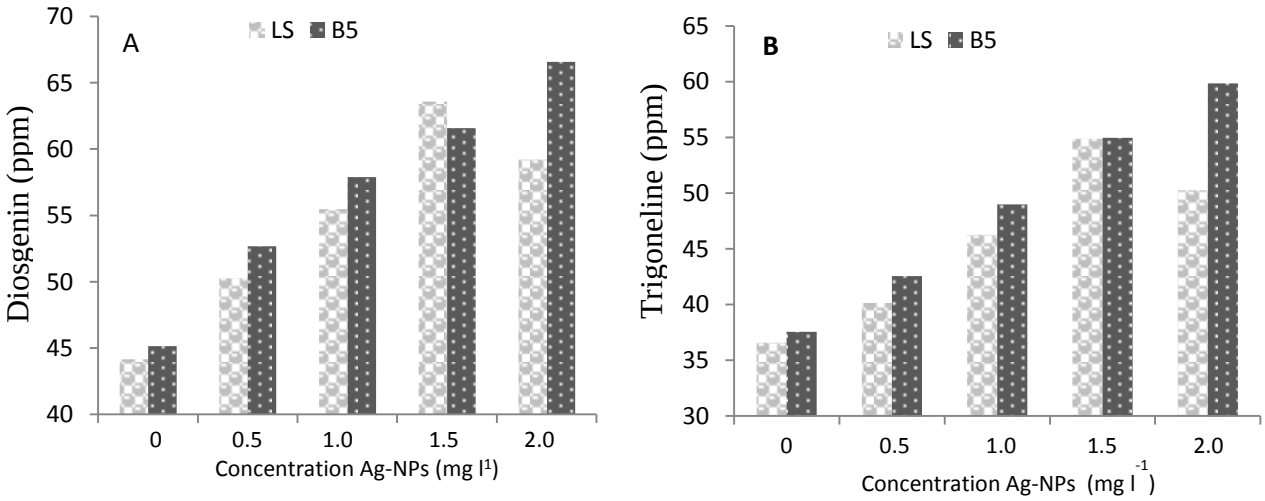

Figure 6. Diosgenin (A) Trigoneline (B) under different medium and Ag-NPs from callus culture of *T. foenum-graecum* L. after 30 days

Production of flavonoid compounds

The results of the quantitative and qualitative analysis of some flavonoids in Figure (7) showed an effect on the production of these compounds according to the type of medium and the concentration of Ag-NPs. In the B5 medium when it was included with 2.0 mg l^{-1} of Ag-NPs, while 1.5 mg l^{-1} recorded a value of 20.46 ppm when grown in the same medium. While the lowest value of the compound yield, 8.12 ppm, appeared when growing callus tissue in an LS medium not treated with Ag-NPs (Figure 7A). The results also showed that there was an effect of the medium and different concentrations of Ag-NPs on the production of vitxein compound from *T. foenum- graecum* L. callus, as the results showed that the highest level of production of the compound reached 16.99 ppm when growing callus of *T foenum-graecum* L. in B5 medium that was included at a concentration of 2.0 mg l^{-1} of Ag-NPs, while the concentration of 1.5 mg l^{-1} recorded a value of 14.89 ppm for the same compound when grown in the same medium. The treatments showed that the lowest value for the production of the compound was 6.55 ppm when the comparison of LS (Figure 7B). As for the rutin compound, the results showed that the combination of the type of medium and the different concentrations of Ag-NPs affected the extent of its production from callus cultures, as it was found that the highest level of production of the compound reached 25.88 ppm when growing callus in B5 medium containing of 2.0. mg l^{-1} of Ag-NPs, while the concentration was 1.5 mg l^{-1}, had a lowest value for the compounds production, reaching 23.65 ppm when grown in the same medium. In contrast, the lowest value for the production of the compound was 12.05 ppm, the comparison treatment of LS (Figure 7C). The results of the compound apigenin in Figure (7D) showed that the type of medium and the concentration of Ag-NPs played a role in stimulating its production in the callus cultures, as the results showed that the highest level of production of the compound reached 25.44 ppm when growing callus in B5 medium treated by 2.0 mg l^{-1} of Ag-NPs, while the concentration of 1.5 mg l^{-1} recorded a minimum production value of 23.98 ppm when growing callus in the same medium mentioned above, it was found that the lowest value for the production of the compound amounted to 14.58 ppm when the comparison treatment of LS media. The results also showed in Figure (7E) that there was a variation in the extent of isovitexin production according to the change of the type of medium and the concentration of Ag-NPs prepared for the development of calli cultures, as the concentration of 2.0 mg l^{-1} achieved the highest production value of 15.88 ppm when growing callus in B5 medium when treated with 2.0 mg l^{-1} of Ag-NPs. A concentration of 1.5 mg l^{-1} achieved a value of 13.56 ppm when grown in the same medium. The lowest value of compound yield (5.48 ppm) was shown in callus grown in LS medium and did not treated with nanomaterial .The results showed in Figure (7F) that kaempfrol production was positively affected by the difference in the medium and the concentration of Ag-NPs in the callus cultures. It included 2.0 mg l^{-1} of Ag-NPs, while the concentration of 1.5 mg l^{-1} recorded a lower value of 29.89 ppm when grown in the same medium. In contrast, the lowest value of the compound yield was 16.55 ppm when the comparison treatment of LS medium**.** The superiority of the nanomaterial could be attributed to its direct adhesion with the plant tissue it enjoys, which allows it to have a higher effectiveness than the traditional element (11, 20). The main reason for the increase in Ag-NPs can also be attributed to the non-activity of Ag-NPs. antioxidant enzymes are considered signalling compounds that stimulate genes known to activate vital pathways responsible for building secondary compounds (14, 36). These results are similar to previous findings, including Shakeran et al. (21) in *Datura metel*, Fazal et al. (5) in *Prunella vulgaris* L., Ali et al. (2) in *Caralluma tuberculata*, and Salih et al. (19) in *Juniperus procera*.

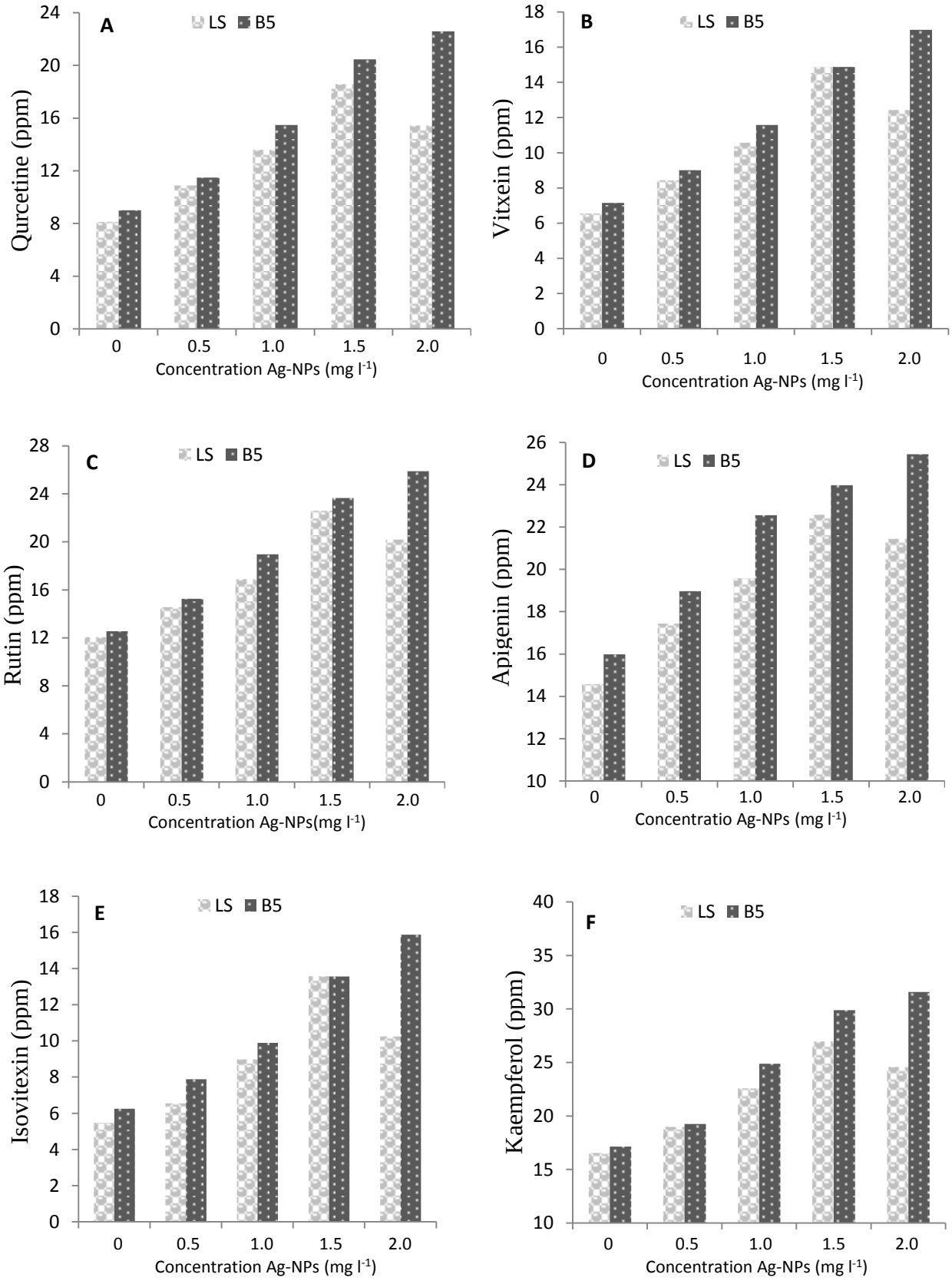

Figure 7. Qurcetine (A) Vitxein (B) Rutin (C) Apigenin (D) Isovitexin (E) Kaempferol (F) under different medium and Ag-NPs from callus culture of *T. foenum-graecum* L. after 30 days

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