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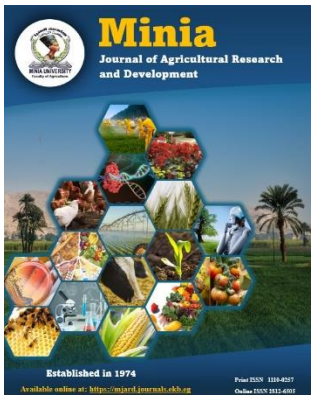

The application of Chlorocholine chloride to stimulate the secondary metabolites in the calli cultures of *Gardenia jasminoides* (Variegata and Ellis).

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ABSTRACT

Biotechnology means maximizing the benefit of the living cell to improve human life. One of the most successful applications of biotechnology is using plant biotechnology to produce valuable compounds in plants. *Gardenia jasminoides* is one of the Rubiaceae family's most important ornamental and medicinal plants. It is rich in various active compounds which qualifies it to possess a variety of biological activities. This research aims to apply Chlorocholine chloride (CCC) to the calli cultures of *Gardenia jasminoides* (Variegata and Ellis) to stimulate various secondary metabolites, and to study its impact on the growth of *Gardenia jasminoides* (Variegata and Ellis). Various concentrations (0, 100, 200, and 300 mg/ l) of CCC were added to the control medium. Samples were collected after 10, 20, and 30 days of treatment. Fresh weight was recorded and samples were dried using a freeze dryer and samples were kept at - 20°C for the determination of phenolic compounds via High-Performance Liquid Chromatography (HPLC). It was noted that the fresh weight gradually declined as the CCC concentration increased in both sub-species of *G. jasminoides* (Variegata and Ellis), even though the fresh weight showed an increase over time for both sub-species.

The control treatment, when supplemented with 200 mg/l CCC, shows the most promise because, after 10 days, the Calli cultures of *G. jasminoides* Variegata have been shown to produce various active compounds in appropriate amounts, including 702.24 ± 2.1 µg/GDW ferulic acid, 303.38± 1.3 µg/GDW chlorogenic acid, 158.16± 1.6 µg/GDW cinnamic acid, and 17.06± 0.2 µg/GDW caffeic acid. The Calli cultures of *G. jasminoides* Ellis accumulate the P-coumaric acid with its maximum levels (222.17±1.2 µg/GDW) after 20 days of treatment with the addition of 100 mg/l CCC.

Keywords: Calli cultures, Chlorocholine chloride (CCC), *G. jasminoides*, Secondary metabolites,

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INTRODUCTION

Biotechnology means maximizing the benefit of the living cell to improve human life. It utilizes living cells or their components to create products with particular objectives through various techniques. One of the most successful applications of biotechnology is using plant biotechnology to produce valuable compounds in plants, which are considered raw materials for human food and animal feed. Numerous top medications have been initially sourced from phytochemicals, such as aspirin or taxol, or continue to be derived from them**. (Murphy, 2004).**

Gardenia jasminoides is one of the Rubiaceae family's most important ornamental and medicinal plants. It is rich in various active compounds such as different phenolic compounds, flavonoids, iridoids, and natural pigments, which qualifies it to possess a variety of biological activities like anticancer, anti-inflammatory, antidiabetic, antithrombotic and anti-depression activities **(Chen *et al.*, 2020, Wang et al., 2020, Yin and Liu, 2018).**

Cinnamic acid, an aromatic carboxylic acid that occurs naturally, is a prominent compound found in Cinnamomum cassia (Chinese cinnamon), as well as in various fruits, whole grains, and vegetables **(Chandra *et al.*, 2019).** Cinnamic acid is frequently utilized as a flavoring agent in various food and beverage products and for its fragrance in perfumes and cosmetics. It is recognized for its potent broad-spectrum antimicrobial properties, anticancer, and antidiabetic characteristics **(Guo *et al.*, 2019).**

P-Coumaric acid is a hydroxy derivative of cinnamic acid, also known as 4-hydroxycinnamic acid. It is a popular phenolic compound that naturally occurs in various plants, grains, fruits, and vegetables. It exhibits a variety of bioactive characteristics, including antioxidant, antibacterial, anti-cancer, antiarthritic, anti-inflammatory, gout prevention, anti-diabetic, skin regeneration, gastroprotective, anti-ulcer, cardioprotective, hepatoprotective, reno-protective, bone formation promotion. According to this wide range of bioactive properties, there is potential for p-coumaric acid to be integrated into pharmaceutical products **(Kaur and Kaur, 2022).** According to its anti-melanogenic properties, it could be considered an active ingredient in cosmetics. **(Boo, 2019).**

Chlorogenic acid (CGA) is a phenolic compound believed to be a derivative of cinnamic acid, known for its biological advantages primarily linked to its anti-inflammatory effects. Due to its various antibacterial and anti-inflammatory characteristics, chlorogenic acid has recently been shown to offer multiple health benefits, such as lowering the risk of cardiovascular diseases, alleviating symptoms of Alzheimer's, and managing type 2 diabetes **(Ranheim, and Halvorsen, 2005, Salazar-Martinez *et al.*, 2004, Lindsay, *et al.*, 2002, Almeida, *et al.*, 2006, and Santos, *et al.*, 2006).**

Caffeic acid is a type of polyphenol, categorized as a hydroxycinnamic acid and as a secondary metabolite involved in lignin biosynthesis, which takes place in nearly all plants, particularly in coffee. It serves as a primary component of coffee's characteristic aroma and possesses antitumor, antioxidative, and anti-inflammatory properties **(Sakai *et al.*, 2022).**

Ferulic acid exhibits low toxicity and has numerous physiological functions, including anti-inflammatory, antioxidant, antimicrobial, anticancer, and antidiabetic effects. It is extensively utilized in the pharmaceutical, food, and cosmetic sectors. It protects key skin elements, such as keratinocytes, fibroblasts, collagen, and elastin. Additionally, it suppresses melanogenesis, promotes angiogenesis, and speeds up the healing process of wounds. It is commonly incorporated into skincare products as a protective agent against UV rays, a reducer of skin aging effects from sunlight, and a brightening agent. However, its application is often restricted due to its

quick oxidation tendency **(Zduńska, *et al.*, 2018).**

Chlorocholine chloride (CCC), a substance that slower growth by blocking gibberellin production in plant tissues acts as an anti-gibberellin compound and may be considered as abiotic stress **(Wang and Xiao, 2009).**

In our earlier research, the CCC was applied to shoot cultures of *G. jasminoides* (Variegata and Ellis) to evaluate the ability of both sub-species to accumulate different secondary metabolites as a response to this abiotic elicitor. **(El Ashry *et al.*, 2024).** The shoot cultures of both sub-species accumulate different phenolic compounds in different quantities. This allowed us to follow the different pathways of secondary metabolites in the shoot cultures of *G. jasminoides* (Variegata and Ellis).

This research aims to apply the CCC to the calli cultures to stimulate various secondary metabolites since the starting point of an effective cell suspension for the accumulation of metabolites is a callus culture that is optimized and grows quickly.

MATERIALS AND METHODS

Plant material

In vitro, growing *Gardenia jasminoides* (Variegata and Ellis) shoot cultures were used as plant material.

Establishment of calli cultures:

Leaves segments about 0.5 cm in length were placed on Murashige and Skoog (MS) medium **(Murashige, and Skoog,1962)** enhanced with 0.5 mg/l picloram + 0.5 mg/l naphthalene acetic acid and incubated in the dark for four weeks (one subculture) for callus initiation. The initiated callus was kept on the same medium for three subcultures.

Impact of Chlorocholine chloride (CCC) in various concentrations (0,100, 200, and 300 mg/l) on calli cultures of *Gardenia jasminoides* (Variegata and Ellis) fresh weight:

For studying the impact of CCC various concentrations on the *Gardenia jasminoides* (Variegata and Ellis) calli cultures fresh weight, various concentrations (0,100, 200, and 300 mg/l) of CCC were added to the control medium as follows: Table (1)

Table (1): medium composition

Treatment	Medium composition
1	MS medium +0.5 mg/l picloram + 0.5 mg/l NAA (Control)
2	Control +100 mg/l CCC
3	Control + 200 mg/l CCC
4	Control+ 300 mg/l CCC

Samples were collected after 10, 20, and 30 days of treatment. Fresh weight was recorded and samples were dried using a freeze dryer and samples were kept at -20°C for further use.

Sample extraction:

Following **Gabr *et al.* (2017**), the extraction process was performed in total darkness. For every treatment, 100 mg of ground-dried callus samples were subjected to extraction using 1.5 ml of 80% methanol for 24 hours. Subsequently, the extracts were subjected to sonication for 20 minutes in an ultrasonic water bath (Grant, United Kingdom). The samples were then centrifuged for 5 minutes at 6000 rpm (Sigma, 2–16 PK, Germany). The obtained extracts were collected, and the pellets were re-extracted using 500 µl of the same solvent. The resulting extracts were stored at -20 °C for future use.

Determination of phenolic compound content via High-Performance Liquid Chromatography (HPLC):

The methanol solution underwent evaporation and was concentrated to yield a

dry residue. The extract was reconstituted in 1 ml of methanol and stored at 4°C in the dark. The quantification of phenolic compounds was performed using HPLC on a UNICAM CRYSTAL 200 Liquid Chromatograph (Column: Kromasil C18 5um250*4.66 mm). The mobile phase comprised methanol and water, both of which were acidified with 0.3% orthophosphoric acid p.a. (w/v). Phenolic compounds were eluted with a linear gradient that transitioned from water to 50% methanol over 5 minutes, followed by a 20-minute isocratic elution with 50% methanol. The flow rate was maintained at 1.4 ml/min. Detection of the substances was accomplished by measuring absorption at λ = 288 nm, and their identification was performed by comparing retention times and absorption spectra with a complex of standard phenolic compounds: chlorogenic acid, gallic acid, rutin (quercetin-3-rutinoside), quercetin, dihydrokaempferol, 2,5-dihydroxy benzoic acid, 3,4-dihydroxy benzoic acid, vanillic acid, syringic acid, p-coumaric acid, cinnamic acid, rosmarinic acid, caffeic acid, and ferulic acid. The content of the sample was reported as µg/g dry weight and calculated using the known concentration of the standard and the peak areas of both the standard and sample.

Where Concentration of sample = [Area sample / Area of the standard] * Concentration of standard.

Statistical analysis:

All analyses were carried out in triplicate. The data is presented as means ± standard deviations. A two-way ANOVA was executed using GraphPad Prism version 5.01 to assess the p-value and significance.

RESULTS AND DISCUSSION

Impact of Chlorocholine chloride (CCC) various concentrations (100, 200, and 300 mg/l) on calli cultures of *Gardenia jasminoides* (Variegata and Ellis) fresh weight:

To study the impact of CCC on the fresh weight of *G. jasminoides* (Variegata and Ellis) callus cultures, the control medium was enhanced with various concentrations (0, 100, 200, and 300 mg/l) of CCC. Explants were harvested after 10, 20, and 30 days of treatments, and fresh weight was recorded (Fig. 1,2).

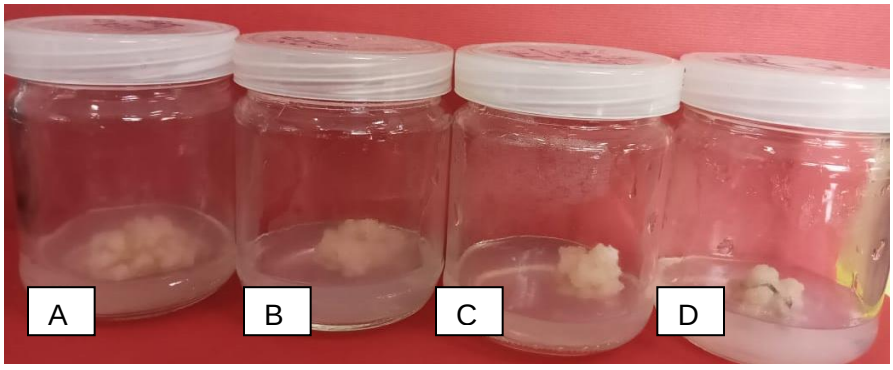

Figure (1): Effect of CCC on fresh weight of *G. jasminoides* Variegata after 30 days of treatment

A. Control
B. Control+ 100 mg/l CCC
C. Control+ 200 mg/l CCC
D. Control+ 300 mg/l CCC

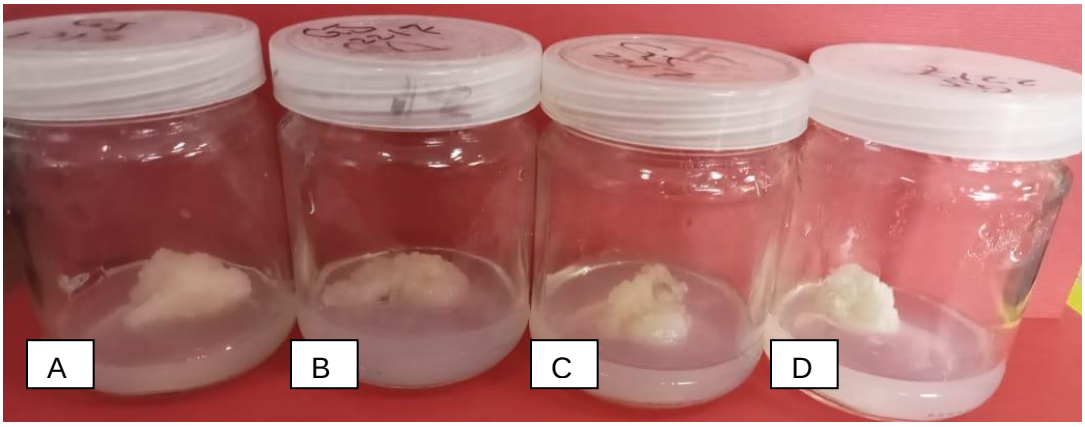

Figure (2): Effect of CCC on fresh weight of *G. jasminoides* Ellis after 30 days of treatment

A. Control
B. Control+ 100 mg/l CCC
C. Control+ 200 mg/l CCC
D. Control+ 300 mg/l CCC

By taking a glum at Fig. (3, 4) it could be observed that the fresh weight decreased gradually with increasing the CCC concentration in both sub-species of *G. jasminoides* (Variegata and Ellis), although the fresh weight increased with time with both sub-species. In comparing the fresh weight of *G. jasminoides* (Variegata and Ellis), it was found that the fresh weight of *G. jasminoides* Variegata is higher than the fresh weight of *G. jasminoides* Ellis after 10, and 20 days of treatment but after 30 days of treatment the fresh weight of *G. jasminoides* Ellis callus cultures recorded higher values of callus cultures compared to that of the *G. jasminoides* Variegata. As for *G. jasminoides* Variegata the highest fresh weight of calli cultures (4.88±1.39 g) was recorded with the control treatment after 30 days of treatment while the lowest fresh weight (3.269±0.89 g) was recorded by adding 300 mg/ l CCC for 10 days. Regarding the fresh weight of *G. jasminoides* Ellis calli cultures, it was found that the highest fresh weight (7.00±2.01 g) was recorded with the control treatment after 30 days of treatment while the lowest fresh weight (3.256±0.88 g) was recorded with adding 300 mg/l CCC to the control medium and after 10 days of treatment.

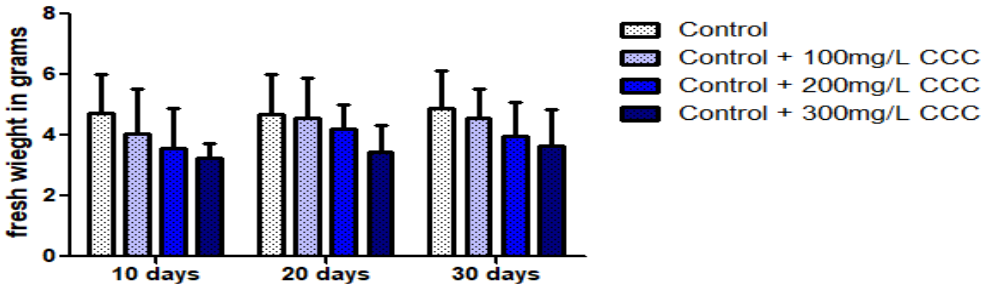

Figure (3): Effect of CCC on fresh weight of *G. jasminoides* Variegata after (10, 20, and 30 days) of treatment.

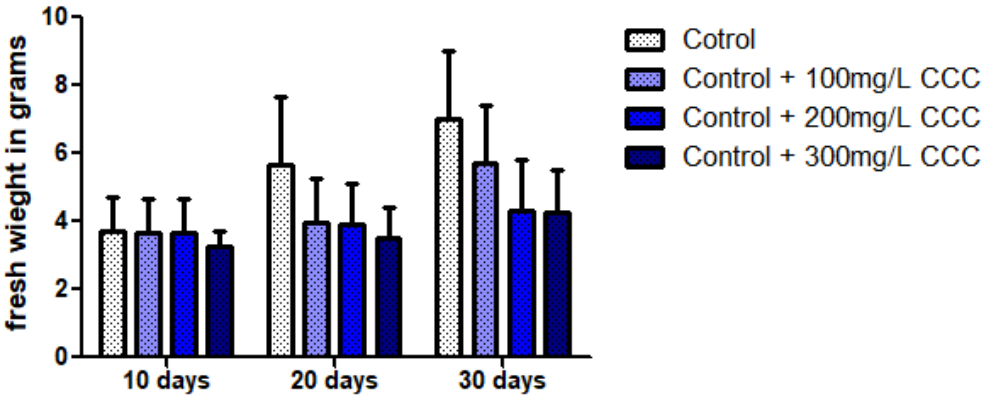

Figure (4): Effect of CCC on fresh weight of *G. jasminoides* Ellis after (10, 20, and 30 days) of treatment.

According to the two-way ANOVA, for G. *jasminoides* Variegata there is no significant difference between treatments and no significant difference among the three harvests also there is no significant difference in the interaction, since the P value > 0.05. Regarding the fresh weight of calli cultures of *G. jasminoides* Ellis, there is a significant difference between treatments and a significant difference among the three harvests since the P value < 0.05 but there is no significant difference in the interaction, since the P value > 0.05.

These results align to a great extent with what was found in our earlier work on the impact of CCC on the growth and secondary metabolites accumulation in *G. jasminoides* (Variegata and Ellis) shoot cultures **(El Ashry *et al.*, 2024),** since it was reported that the fresh weights of *G. jasminoides* Variegata are generally higher than that of *G. jasminoides* Ellis. This was attributed to the possibility that *G. jasminoides* Variegata may resist the effect of CCC than *G. jasminoides* Ellis. CCC is a synthetic regulator of plant growth that restricts the biosynthesis of gibberellins (GA), leading to the shortening and strengthening of stems in plants, along with diminished branching and foliage in specific shrub and tree species as reported **by Mobli, and Baninasab (2008).** This observation aligns with the results, as the fresh weight diminished as the concentration of CCC increased for both subspecies. Furthermore, our findings align with those reported by **Soliman *et al.* (2022),** who indicated that applying CCC treatments at various dosages to *G. jasminoides* Ellis plants led to a reduction in both fresh and dry weights (g/plant) when compared to the control group. They also noted that growth retardants hinder stem elongation, influence overall growth, and postpone the development of shoot biomass, which explains the decrease in fresh and dry weights following the CCC treatment.

Determination of phenolic compound content via High-Performance Liquid Chromatography (HPLC):

To investigate the impact of CCC on the phenolic compound concentrations in *G. jasminoides* (Ellis and Variegata), varying quantities of CCC (0, 100, 200, and 300 mg/l) were introduced to the control medium. Calli were harvested after (10, 20, and 30 days) of treatments, and samples were extracted for further analysis. To evaluate the impact of CCC on the phenolic compound content HPLC was used. Regarding the data presented in Table (2) which represents the phenolic compounds detected in the calli extracts of different treatments of *G. jasminoides* Variegata, it

was found that the abundant phenolic compound found in different treatments is cinnamic acid. This is present with all treatments even though with control treatment with varying contents since the highest cinnamic acid content (204.21±1.4 µg/ GDW) was reported with adding 100 mg/l CCC to the control medium and for 30 days of treatment while its lowest content (31.02±0.5 µg/ GDW) was reported with adding 100 mg/l CCC to the control medium and leave the calli cultures for only 10 days and then harvest it.

Table (2): Impact of adding various concentrations (0,100, 200, 300 mg / l CCC) on the phenolic compounds content in the calli cultures of *G. jasminoides* Variegata after (10, 20, and 30 days) of treatment.

Treatment	Period of treatment	Phenolic compound in µg/ GDW				
		Cinnamic acid	Caffeic acid	Chlorogenic acid	Ferulic acid	P-coumaric acid
Control	10 days	115.29±1.3	ND	ND	ND	ND
Control+ 100 mg/ l CCC		31.02±0.5	ND	ND	ND	ND
Control + 200 mg/l CCC		158.16± 1.6	17.06± 0.2	303.38± 1.3	702.24± 2.1	ND
Control + 300mg/l CCC		45.26±0.6	ND	ND	ND	ND
Control	20 days	89.90±0.8	ND	ND	ND	ND
Control + 100mg/ l CCC		133.72±1	ND	ND	ND	154.84±1.1
Control + 200mg/l CCC		107.91±0.9	ND	ND	ND	ND
Control + 300mg/l CCC		52.04±0.5	ND	ND	ND	215.03±1.5
Control	30 days	79.45±0.3	ND	ND	ND	
Control + 100mg/l CCC		204.21±1.4	ND	ND	ND	13.07±0.2
Control + 200 mg/l CCC		73.38±0.8	ND	ND	ND	44.34±0.4
Control + 300mg/l CCC		48.43±0.4	ND	ND	ND	130.33±1.1

Data represent Mean ± SD.

The P coumaric acid is the second phenolic compound detected with different treatments, starting from 20 days from the treatment, it did not appear after 10 days in any treatment. It did not appear in the control treatment after the different treatment periods. The highest P coumaric acid content (215.03±1.5 µg/ GDW) was noticed by adding 300 mg/l CCC to the control medium for 20 days. However, the lowest P coumaric content (13.07±0.2 µg/ GDW) was observed by adding 100 mg/l CCC to the control medium for 30 days. The most promising treatment is the control treatment when supplemented with 200 mg/ l CCC since the calli cultures *G. jasminoides* Variegata when left on this treatment for 10 days, has been directed to give different active compounds in reasonable quantities, as it has given 702.24 ± 2.1 µg/ GDW ferulic acid, 303.38± 1.3 µg/ GDW chlorogenic acid, 158.16± 1.6 µg/ GDW cinnamic acid and 17.06± 0.2 µg/ GDW caffeic acid. Then adding 200 mg/l CCC to the control medium is considered the most suitable treatment to stimulate calli cultures of *G. jasminoides* Variegata to produce several secondary metabolites.

As for the data presented in Table (3) which represents the phenolic compounds detected in the calli extracts of different treatments of *G. jasminoides* Ellis, it was found that generally the phenolic compound content detected in various extracts from different treatments of *G. jasminoides* Ellis calli cultures is lower in quantity and variety except the highest P coumaric acid content (222.17±1.2 µg/ GDW) which is higher than that detected in *G. jasminoides* Variegata, compared to what was detected in the extracts of different treatments of *G.*

jasminoides Variegata calli cultures. The most abundant phenolic compound found in different treatments is cinnamic acid. This occurs in all treatments, including the control treatment, with varying levels of cinnamic acid content, with the highest content (90.47±0.8 µg/ GDW) being noted by adding 200 mg /l CCC for 30 days. P-coumaric acid is the second phenolic compound identified under various treatments, and it was first detected starting 20 days after treatment; it was absent after 10 days across all treatments. In the control treatment, it was not observed at any of the treatment intervals. The highest levels of P-coumaric acid content (222.17±1.2 µg/ GDW) were found by adding 100mg/l CCC and after 20 days of treatment. Then adding 100 mg/l CCC for 20 days is more suitable for stimulating the calli cultures of *G. jasminoides* Ellis to produce P coumaric acid in reasonable content.

Table (3): Impact of adding various concentrations (0,100, 200, 300 mg / l CCC) on the phenolic compounds content in the calli cultures of *G. jasminoides* Ellis after (10, 20, and 30 days) of treatment.

Treatment	Period of treatment	Phenolic compound in µg/ GDW				
		Cinnamic acid	Caffeic acid	Chlorogenic acid	Ferulic acid	P- coumaric acid
Control	10 days	27.88±0.33	ND	ND	ND	ND
Control+ 100 mg/ l CCC		72.58±0.67	ND	ND	ND	ND
Control + 200 mg/l CCC		27.68±0.32	ND	ND	ND	ND
Control + 300 mg/l CCC		32.65±0.4	ND	ND	ND	ND
Control	20 days	29.15±0.44	ND	ND	ND	ND
Control + 100 mg/l CCC		82.57±0.87	ND	ND	ND	222.17±1.2
Control + 200 mg/l CCC		44.02±0.45	ND	ND	ND	24.38±0.12
Control + 300 mg/l CCC		62.17±0.53	ND	ND	ND	32.88±0.21
Control	30 days	72.67±0.6	ND	ND	ND	ND
Control + 100 mg/l CCC		37.04±0.23	ND	ND	ND	29.19±0.19
Control + 200 mg/l CCC		90.47±0.8	ND	ND	ND	113.00±0.91
Control + 300 mg/l CCC		46.18±0.3	ND	ND	ND	32.67±0.23

Data represent Mean ± SD.

To begin with, complex phenolic compounds such as flavonoids, tannins, lignin, and anthocyanins are derived from simpler phenolic acids, including trans-cinnamic and P-coumaric acids, which serve as their precursors **(Winkel-Shirley, 2002).** As previously noted, P-coumaric acid is a hydroxy derivative of cinnamic acid, commonly referred to as 4-hydroxycinnamic acid **(Kaur and Kaur, 2022).** This explains why P-coumaric acid is not present in the extracts from the initial periods of treatment but subsequently appears in both subspecies of G. jasminoides; Also, this explains why the cinnamic acid is present in all treatments even the control treatment, with the different periods of treatments while the P coumaric acid began to appear after 20 days of treatment in the different treatments except control.

Members of the chlorogenic acid (CGA) group, characterized by the combination of the hydroxyl group from quinic acid and the carboxyl group from caffeic acid as the foundational structure, are prevalent phenolic acid compounds present in various plants. The group includes 1L-(−)-quinic acid, caffeic acid (CA), ferulic acid, and the p-coumaric acid (P-CoQA) subgroup, which encompasses P-CoQAs, caffeoylquinic acids (CQAs), and feruloylquinic acids (FQAs) **(Clifford, et**

al., 2017, Li, et al., 2020, Stalmach et al., 2010, and Xue, et al., 2023). This may explain why adding 200 mg/l CCC to the control medium and keeping the calli culture of *G. jasminoides* Variegata for 10 days, members of chlorogenic acid (CGA) were stimulated in the calli cultures with high quantities while the P coumaric acid was not detected. Finally, it could be concluded that the most suitable treatment for stimulating the calli cultures of *G. jasminoides* Variegata to produce different secondary metabolites (ferulic acid, chlorogenic acid, and caffeic acid) is enhancing the control medium with 200 mg/l CCC and leave the calli on this treatment for 10 days. As for the *G. jasminoides* Ellis it could accumulate reasonable content of P coumaric acid by adding 100 mg/l CCC for 20 days as a period of treatment. While the cinnamic acid is present in both sub-species of *G. jasminoides* calli cultures. With different treatments even with the control treatment, it accumulated in high content by adding 100 mg/ l CCC to the control medium and keeping the calli cultures of *G. jasminoides* Variegata on this treatment for 30 days.

CONCLUSION

Then finally, it could be reported that Calli cultures of *G. jasminoides* Variegata could be stimulated by adding different CCC concentrations to produce cinnamic acid, ferulic acid, chlorogenic acid, and caffeic acid. While the Calli cultures of *G. jasminoides* Ellis could be stimulated by using CCC to produce P coumaric acid.

Ethics approval and consent to participate

Not applicable

Availability of data and material

All data supporting the conclusions of this article are provided with the article

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Conflicts of interest

The authors declare there are no conflicts of interest.

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الملخص العربي

استخدام كلوريد الكلوروكولين لتحفيز المركبات الثانوية في مزارع الكالس لنبات الجاردينيا الياسمينية (المبرقشة و الخضراء).

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التكنولوجيا الحيوية تعني تعظيم فائدة الخلية الحية لتحسين حياة الإنسان. أحد أنجح تطبيقات التكنولوجيا الحيوية هو استخدام التكنولوجيا الحيوية النباتية لإنتاج مركبات ذات قيمة في النباتات. الغردينيا الياسمينيةهي واحدة من أهم نباتات الزينة والطبية التابعة لعائلة Rubiaceae. وهي غنية بالمركبات النشطة المختلفة التي تؤهلها لعمل انشطة بيولوجية متنوعة. يهدف هذا البحث إلى تطبيق كلوريد الكلوروكولين (CCC) على مزارع الكالس من لنباتات الجاردينيا الياسمينة (المبرقشة و الخضراء) لتحفيز المركبات الثانوية المختلفة ، ودراسة تأثير كلوريد الكلوروكولين (CCC) علي نمو مزارع الكالس من لنباتات الجاردينيا الياسمينة (المبرقشة و الخضراء). تمت إضافة تركيزات مختلفة (٠ ، ١٠٠ ، ٢٠٠ ، و ٣٠٠ مجم / لتر) من CCC إلى بيئة المقارنة . تم جمع العينات بعد ١٠ و ٢٠ و ٣٠ يوما من المعاملة. تم تسجيل الوزن الطازج وتجفيف العينات باستخدام مجفف التجميد وتم الاحتفاظ بالعينات عند – ٢٠ درجة مئوية لتحديد المركبات الفينولية عبر الكروماتوغرافيا السائلة عالية الأداء .(HPLC) لوحظ أن الوزن الطازج انخفض تدريجيا مع زيادة تركيز CCC في كلا النوعين الفرعيين من الجاردينيا الياسمينة (المبرقشة و الخضراء) ، على الرغم من أن الوزن الطازج أظهر زيادة بمرور الوقت لكلا النوعين الفرعيين .

تعتبرالمعاملة باضافة ٢٠٠ مجم / لتر CCC لبيئة المقارنة تعتبر معاملة واعدة لانها تدفع مزارع كالس الجاردينيا الياسمينية المبرقشة لانتاج مركبات ثانوية متعددة بكميات مناسبة و ذلك بعد ١٠ ايام من المعاملة حيث انتجت هذه المعاملة ٧٠٢.٢٤ ± ٢.١ ميكروغرام / جرام مادة جافة حمض الفيروليك ، ٣٠٣.٣٨ ± ١.٣ ميكروغرام / جرام مادة جافة حمض الكلوروجينيك ، ١٥٨.١٦ ± ١.٦ ميكروغرام / جرام مادة جافة حمض سيناميك ، و ١٧.٠٦± ٠.٢ ميكروغرام / جرام مادة جافة حمض الكافيين. في حين ان مزارع كالس الجاردينيا الياسمينية الخضراء تتراكم بها حمض بيتا كيوماريك بكميات كبيرة (٢٢٢.١٧±١.٢ ميكروغرام / جرام مادة جافة) بعد ٢٠ يوما من المعاملة بإضافة ١٠٠ ملغ / لتر CCC.