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Impact of Chlorocholine Chloride on Phenolic Metabolism in *In Vitro* Cultures of *Gardenia Jasminoides* (Variegata and Ellis)

Amal A. El Ashry[1, *], Hanan S. Ebrahim[1], Mohamed K. El-Bahr[1] and Ahmed M. M. Gabr[1, 2]

[1]*Department of Plant Biotechnology, Biotechnology Research Institute, National Research Centre (NRC), Cairo 12622, Egypt*

[2]*Academy of Scientific Research and Technology, 101 Kasr El Ainy St. Kasr El Ainy, Cairo 11516, Egypt*

1. Abstract

The impact of different doses (0, 100, 200, and 300 mg/l) of chlorocholine chloride (CCC) on the phenolic compounds content and the fresh weight of *Gardenia jasminoides* (Variegata and Ellis) shoot cultures throughout three harvests (10, 20 and 30 days) were studied. The analysis revealed that the chlorogenic acid, vanillic acid, dihydrokampferol, rutin, and rosmarinic are the dominant phenolic compounds in the shoot cultures of both sub-species. These bioactive compounds were discovered in significant quantities across various treatments. The presence of 300 mg/l CCC clearly resulted in the significant accumulation of phenolic compounds in the shoot cultures of *G. jasminoides* Variegata. The second harvest of this treatment yielded the highest amount of chlorogenic acid 59.1±0.04 mg/g dry weight. However, in the third harvest, the chlorogenic acid disappeared and was replaced by other phenolic compounds such as rutin, dihydrokampferol, and vanillic acid which suggest that the phenolic acid pathway was shifted to produce another phenolic compound with time, the quantities of these compounds recorded 54.8±0.06, 43.9±0.05, 25.5±0.1, and 1.2±0.1 mg/g dry weight, respectively, under the same treatment. The bioactive phenolic compounds content of *G. jasminoides* Ellis shoots are higher than that of G. jasminoides Variegata. The addition of 200mg/l CCC to the control medium resulted in the presence of significant levels of phenolic compounds. Specifically, the extracts of G. jasminoides Ellis shoots cultured on the control medium supplemented with 200mg/l CCC showed the highest concentrations of chlorogenic acid, dihydrokampferol, and rutin at the second harvest (255.7±.0.3, 110.3±0.2, and 98.5±0.5 mg/g dry weight, respectively). The phenolic compounds pathway in *Gardenia jasminoides* (Variegata and Ellis) shoots cultures was investigated in order to declare the results obtained. Also, it could be noticed that, the shoot cultures of *G. jasminoides* Variegata which produce smaller amounts of chlorogenic acid and other secondary metabolites in their extracts compared to the extracts from shoot cultures of *G. jasminnoides* Ellis. Simultaneously, the *G. jasminoides* Variegata typically exhibits greater fresh weights compared to the *G. jasminoides* Ellis. This suggests that the shoot cultures of *G. jasminoides* Variegata are more resilient to the stress induced by CCC treatments than the shoot cultures of *G. jasminoides* Ellis.

Keywords: Chlorocholine chloride (CCC), *Gardenia jasminoides*; phenolic compounds, secondary metabolites

Introduction

Plants have unique methods to remain cellular and physiologically unaffected during extreme environmental conditions. To adapt to changing environmental conditions, plants have several metabolic processes that produce secondary metabolites [1 - 2]. Secondary metabolites in plants are bio-active, non-nutritive molecules produced through the metabolic pathway in response to both biotic and/or abiotic stress [3]. Plants can produce a wide range of secondary metabolites, such as alkaloids, anthocyanins, flavonoid, and phenolic compounds. These metabolites have been shown to have significant health advantages for consumers. These substances may have anticoagulant, anti-

*Corresponding author e-mail: *aaelashry@gmail.com.;(Amal A. El Ashry).
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diabetic, antibacterial, and antioxidant properties [3-4].

Plant cell and tissue culture techniques are currently considered to be a promising tool for producing bioactive compounds; elicitors could be used to increase these compounds in tissue culture [5]. Recent studies focused on the development of novel *in vitro* culture techniques to elevate the bioactive metabolites in plant material are required to meet the growing demand for plant metabolites utilized in the pharmaceutical industry [6]. Enhancing the synthesis of metabolites reduces damage and increases resistance to pests, disease and environmental stress by using elicitors, which are foreign molecules frequently linked to plant diseases or pests [7].

Boerjan *et al.* [8] reported that the ester link between caffeic acid and quinic acid produced chlorogenic acid (CGA), a phenolic compound. It is thought to be a derivative of cinnamic acid with biological benefits mostly associated with its anti-inflammatory properties. Because of its numerous antibacterial and anti-inflammatory properties, chlorogenic acid has recently been demonstrated to have a number of medicinal benefits, including a reduction in cardiovascular disease, Alzheimer's symptoms, and diabetes type 2 [9 -13]. Vanillic acid can be found in a variety of dietary and medicinal plants. In addition to being extracted from these biological sources, it is chemically produced. It has antioxidant, anti-diabetic, antibacterial, anti-inflammatory, anticancer, and anti-obesity properties. Although it has a high safety profile and therapeutic promise, it has not received enough attention as a nutraceutical or therapeutic compound [14].

Another polyphenol antioxidant that is frequently found in fruits and vegetables is kaempferol. Studies in the field of epidemiology have demonstrated a negative correlation between kaempferol implementation and cancer. By boosting the body's antioxidant defense against free radicals, which stimulate the growth of cancer. Furthermore, kaempferol causes cancer cells to go through apoptosis and limits their proliferation and angiogenesis; however, it also seems to maintain normal cell viability [15].

Furthurmore, phenolic compound with several pharmacological properties that have been recognized is rutin. Rutin is one of the active compounds in buckwheat, most citrus fruits, figs, apples, green and black tea, and many other plants. Powerful antioxidant effects can be recognized in rutin. It additionally helps in the body's usage of vitamin C and collagen production. Antioxidant, anti-carcinogenic, vasoprotective, neuroprotective, and cardioprotective are only a few of its numerous pharmacological properties [16- 18]. According to Rothwell *et al*. [19]., Buckwheat is ranked as the third primary plant source of rutin. Gardenia (*Gardenia jasmonides* Ellis) may be ranked fourth for rutin accumulation, according to Gabr *et al*. [20].

An ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid called rosmarinic acid (RA) is found in nature as phenolic compound. Antiviral, antibacterial, anticancer, antioxidant, anti-aging, anti-diabetic, cardioprotective, hepatoprotective, nephroprotective, antidepressant, antiallergic, and anti-inflammatory properties are only a few of the extraordinary biological benefits of rosmarinic acid [21].

Chlorocholine chloride (CCC), a growth retardant that inhibits gibberellin synthesis in plant tissues, functions as an anti-gibberellin agent [22]. CCC has a well-established impact on the growth rate of grasses and the development of potato plants cultivated *in vitro*. CCC has gained popularity in the potato industry as it has proven effective in enhancing both quality and yield. Applying CCC to sweet potato tubers enhances the production of dry matter [23]. Furthermore, it was found that CCC stimulated the production of anthocyanins in the foliage of spiderwort and red cabbage [24]. Investigations have shown that applying exogenous CCC therapy improves the growth and productivity of crops in unfavorable growing conditions. In addition, Zhang *et al.* [25] suggested that the CCC treatment was a viable strategy for improving flavonoid production in *Ginkgo biloba* plants. Although PGRs might hinder plant growth, they have been found to have beneficial effects on plants, especially when it comes to dealing with abiotic stressors. For instance, research has shown that the application of CCC to potatoes increased the activity of antioxidant enzymes [26]. The methanolic extracts of stevia calli and leaves treated with CCC shown high levels of antioxidant activity [27].

The woody ornamental and medicinal gardenia are a member of the *Rubiaceae* family, which includes coffee plants. The genus *Gardenia* comprises around one hundred species, two of which are subspecies of the main species, *jasminoides* (Ellis and Variegata). Its abundance of anti-inflammatory flavonoid and phenolic compounds makes it suitable for use as a pain reliever or in the management of inflammatory conditions [28]. So, in traditional Chinese medicine, gardenia is highly prized. [29].

Consequently, the purpose of this study is to assess how different CCC concentrations affect the development and synthesis of secondary metabolites in both *Gardenia jasminoides,* Variegata and Ellis shoot cultures**.**

Experimental:

In vitro growing of both *Gardenia jasmonides* Variegata and Ellis plantlets were used as a source of

plant material. The plantlets were sub-cultured for three times on Murashige and Skoog (MS) medium [30] supplemented with 2 mg/l benzyl adenine (BA) for shoot multiplication.

Effect of different Chlorocholine chloride (CCC) concentrations on shoots cultures of *Gardenia jasminoides* (Variegata and Ellis) fresh weight:

For studying the effect of different CCC concentrations on Gardenia shoot cultures. The shoots were sub cultured on MS medium supplemented with 1mg /l BA +0.5 mg/l naphthalene acetic acid (NAA) (control), the treatments were as follow:

1. Control
2. Control +100 mg/l CCC
3. Control +200 mg/l CCC
4. Control +300 mg/l CCC

Plantlets were harvested after 10, 20, 30 days, which represent as the first, second and third harvest, and fresh weight was recorded. Thereafter, samples were dried using freeze dryer and storage at -20°C for further analysis.

Sample extraction:

The extraction was performed according to Gabr *et al*., [20] ,100 mg of grounded dried samples was extracted with 1.5 ml 80 % methanol for 24 h. Then the extracts sonicated in an ultrasonic water bath (Grant, United Kingdom) for 20 min. Samples were centrifuged for 5 min at 6000 rpm (Sigma, 2- 16 PK, and Germany). The supernatants were collected, and the pellets were re-extracted twice with 500 µl of the solvent. The extracts were stored at - 20 ◦ C until further use.

Determination of phenolic compounds content by High Performance Liquid Chromatography (HPLC)

The methanol solution was evaporated and concentrated to a dry residue. The extract was dissolved in 1 ml of methanol and kept at 4°C in darkness. The content of phenolic compounds was determined by HPLC on a UNICAM CRYSTAL 200 Liquid Chromatograph (Column: Kromasil C18 5um250*4.66 mm). The mobile phase consisted of methanol and water (both acidified with 0.3% orthophosphoric acid p.a. - w/v). phenolic compounds were eluted with linear gradient from water to 50% methanol in 5 min, following by isocratic elution with 50% methanol for 20 min. The flow-rate was 1.4 ml/min. Substances were detected by absorption at λ = 288 nm and their identification were carried out by the comparison of retention times and absorption spectra with standards complex of phenolic compounds: chlorogenic acid, gallic acid, rutin (quercetin-3-rutinosid), querectin, dihydrokaempferol 2,5_dihydroxy benzoic,3,4_dihydroxy benzoic,vanilic acid,synirigic acid, p-coumaric ,cinammic acid, rosmarinic acid, caffeic acid and ferulic acid. Sample's content was expressed as mg/g dry weight and derived using a known concentration of standard and sample peak areas.

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

Statistical analysis:

All analyses were conducted in three replicates. The means ± standard deviations of the data are displayed. A two - way ANOVA was performed using GraphPad Prism version 5.01 to determine the p-value and significance.

Results and Discussion

Effect of different Chlorocholine chloride (CCC) concentrations on shoot cultures of both *Gardenia jasminoides* (Variegata and Ellis)

To study the effect of CCC on shoots cultures of *G. jasminoides* (Ellis and Variegata), different concentrations of CCC (0, 100, 200 and 300 mg/ l) were added to the control medium for each sub-species.Plantlets were harvested after 10, 20, 30 days, which represent as the first, second and third harvest, and fresh weight was recorded.

By taking a glum on the data in figs (1,2), it could be concluded that as for the fresh weight of shoots, the fresh weights of *G. jasminoides* Variegata are generally higher than that of *G. jasminoides* Ellis fig (3,4). It could be reported that, *G. jasminoides* Variegata shoot cultures may resist the effect of CCC than *G. jasminoides* Ellis. It could be noticed that, the fresh weight decreased with increasing the CCC concentration with both sub – species while, it increased during the three harvests. Then, the highest fresh weight was recorded with the control in the third harvest with the two sub – species, since the control treatment recorded (4.92±1.45, 4.37±1.34) with *G. jasminoides* Variegata and Ellis shoots respectively.

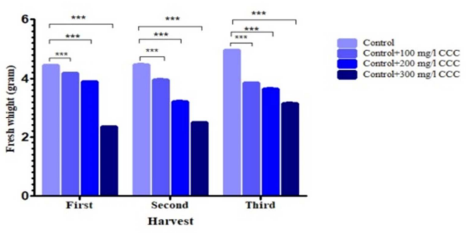

Figure 1: Effect of CCC on fresh weight of *G. jasminoides* Variegata during three harvests.

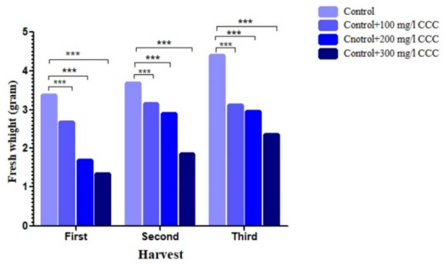

Figure 2: Effect of CCC on fresh weight of *G. jasminoides* Ellis during three harvests.

The lowest fresh weight was recorded with adding 300 mg/l CCC to the control treatment at the end of the first harvest with both sub - species since it recorded (2.3±1.10, 1.31±0.65) with *G. jasminoides* Variegata and Ellis shoots respectively (Fig.3,4).

Figure 3: *G. jasminoides* Variegata shoots after 30 days of culturing on:
1. Control
2. Control+ 100 mg/l CCC
3. Control+ 200 mg/l CCC
4. Control+ 200 mg/l CCC

Figure 4: *G. jasminoides* Ellis shoots after 30 days of culturing on:
1. Control
2. Control+ 100 mg/l CCC
3. Control+ 200 mg/l CCC
4. Control+ 200 mg/l CCC

Mobli *et al.,* [31] stated that, CCC is a synthetic plant growth regulator that inhibits gibberellin (GA) biosynthesis, resulting in shortening and strengthening of stems in plants and reduced A little quantity of 2,5 dihydroxy benzoate, synergic and tiny quantities of 3 ,4 dihydroxy benzoate, gallic acid and cinnamic acid can be observed. P-coumaric which is found in the branching and foliage in certain species of shrubs and trees. This can explain the results found since the fresh weight decreased with increasing the CCC concentration with both sub – species. Our results are on line with that was reported by Wawrzyniak *et al.,* [32], who reported that, the Jerusalem Artichoke plants sprayed with CCC had notably decreased in heights from the control plants. After the initial three weeks of spraying the retardant, the growth of the plants sprayed with CCC was noticeably slower each week. Also, our results are in accordance with what was reported by Soliman *et al.,* [33], who stated that the application of CCC treatments at different doses on *G. jasminoides* Ellis plant resulted in a decrease in fresh and dry weights (g/plant) as compared to the control. However, the lowest fresh weight value was recorded with cycocel at a rate of 4500 ppm (41.83 g/plant). In contrast, the highest fresh weight value which was recorded in the first season with the untreated plants (94.47 g/plant). They added, that growth retardants inhibit stem elongation, affect overall growth, and delay the development of shoot biomass, which is why fresh and dry weights decreased as a result of the treatment by CCC, according to Alem [34].

Determination of phenolic compounds content by High Performance Liquid Chromatography (HPLC)

To study the effect of CCC on the phenolic compounds' contents of *G. jasminoides* (Ellis and Variegata) different concentrations of CCC (0,100, 200 and 300 mg/ l) were added to the control medium. Plantlets were harvested after 10, 20, 30 days, which represent as the first, second and third harvest. Then the samples were dried using freeze dryer, extracted and undergo HPLC determination for phenolic compounds.

To evaluate the influence of CCC on phenolic compounds content HPLC was used. Among the different phenolic compounds which were examined in the samples extracted from shoot cultures of both sub species grown on different treatments it was declared that the chlorogenic acid, vanillic acid, dihydrokampferol, rutin, rosmarinic are the most abundant phenolic compounds that were detected with considerable amounts in the different treatments in the shoot's cultures of both sub species with varying quantities table 1 (*G. jasminoides* Variegata) and table 2 (*G. jasminoides* Ellis). A little quantity of 2,5 dihydroxy benzoate,

extracts of the first harvest then it disappeared in both sub – species. By looking generally at the contents of the tables (1 and 2) it could be observed that *G. jasminoides* Ellis shoots content

of phenolic compound are higher than that found in *G. jasminoides* Variegata ones. By taking a deep look on data in table (1), it could be obviously noted that using 300 mg/l CCC led to the appearance of high amounts of phenolic compounds, since at the second harvest the extracts of *G. jasminoides* Variegata shoots cultured on the control medium supplemented with 300 mg/l CCC recorded the highest amount of chlorogenic acid (59.1±0.04 mg/ g dry weight) followed by the same treatment at the first harvest which gave (26.4±0.02 mg/ g dry weight) of chlorogenic acid which is not detected in any other treatment. It disappeared with the third harvest with the appearance of other phenolic compounds such as: rutin, dihydro kampferol and vanillic acid which recorded (54.8± 0.06, 43.9 ± 0.05 ,25.5±0.1 and 1.2±0.1 mg/ g dry weight, respectively) which is considered the highest amounts of these phenolic compounds with the extracts of *G. jasminoides* Variegata shoots cultured on the control medium supplemented with 300 mg/l CCC at the third harvest. So, it could be concluded that adding 300 mg/l CCC to the control medium is more suitable for *G. jasminoides* Variegata shoots to release high quantities of phenolic compounds. As for *G. jasminoides* Ellis shoots, data presented in (table 2) declared that phenolic compounds content of *G. jasminoides* Ellis shoots are higher in quantity than that found in *G. jasminoides* Variegata. Adding 200mg/l CCC to the control medium led to the appearance of high amounts of phenolic compounds, since at the second harvest the extracts of *G. jasminoides* Ellis shoots cultured on the control medium supplemented with 200mg/l CCC recorded the highest amount of chlorogenic acid, dihydro kampferol and rutin (255.7±.0.3 , 110.3±0.2 and 98.5±0.5 mg/ g dry weight, respectively) while the vanillic acid was observed with its highest amount (137.1±0.8 mg/ g dry weigh) with adding 300mg/l CCC at the first harvest. All the phenolic compounds released from *G. jasminoides* Ellis shoots cultured on different concentrations of CCC declined in the third harvest.

Table 1: Effect of different Chlorocholine chloride (CCC) concentrations on shoot cultures of *Gardenia jasminoides* Variegata phenolic compounds contents

harvest	treatment	Bioactive phenolic compounds found in *Gardenia jasminoides* Variegata shoots in mg/ g dry weight during three harvests													
		Gallic acid	3.4_DiHydroxy Benzoate	2.5_Dihydroxy Benzoic	Chlorogenic acid	Vanilic acid	Synirigic acid	P-coumaric	Dihydro Kampferol	Rutin	Rosmarinic acid	Cinamic acid	Quercetin	Caffiec acid	Ferulic acid
First harvest	Control	0 ±0	0.77±0.02	2.8±0.013	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	0 ±0	52.1±0.1	0.1± 0.01	0.75±0.02	nd	nd
	100mg/l CCC	0 ±0	0.7±0.004	1.6±0.013	0 ±0	0 ±0	0 ±0	0.14±0.04	18.9±0.02	0 ±0	69.5±0.03	0 ±0	0.3±0.02	0.364 ±0.01	nd
	200mg/l CCC	0 ±0	0.41±0.004	0.5±0.004	0 ±0	0 ±0	0.86±0.02	0.12±0.03	5.07±0.04	0 ±0	17.2±0.05	0.01± 0.02	0.6±0.04	nd	nd
	300mg/l CCC	0 ±0	0 ±0	3.9±0.01	26.4±0.02	0 ±0	1.67±0.01	0 ±0	32.6±0.1	28.8± 0.1	37.8±0.06	0 ±0	1.2±0.1	nd	0.257 ±0.01
Second harvest	Control	0.5±0.01	0.7±0.02	8.13±0.02	0 ±0	13.3±0.1	1.49±0.05	0 ±0	11.0±0.01	12.2± 0.04	5.0±0.05	0 ±0	0 ±0	nd	nd
	100mg/l CCC	0.4±002	0 ±0	0 ±0	0 ±0	7.03±0.03	0.77±0.02	0 ±0	10.5±0.06	9.4± 0.05	3.1±0.03	0 ±0	0 ±0	nd	nd
	200mg/l CCC	0.3±0.04	0.6±0.04	3.65±0.05	0 ±0	4.46±0.02	0.64±0.05	0 ±0	7.4±0.05	10.9± 0.03	11.2±0.03	0 ±0	0 ±0	nd	nd
	300mg/l CCC	0.6±0.04	0 ±0	2.45±0.1	59.1±0.04	0 ±0	3.07±0.07	0 ±0	41.9±0.03	0 ±0	18.2±0.02	0 ±0	0 ±0	nd	nd
Third harvest	Control	0.49±0.04	2±0.06	0.54±0.04	0 ±0	4.92±0.05	0.6 ± 0.05	0 ±0	8.17±0.07	10.8± 0.08	4 ± 08	0 ±0	0 ±0	nd	nd
	100mg/l CCC	0.96±0.04	0.49±0.04	0 ±0	0 ±0	8.81±0.07	0.79±0.04	0 ±0	13.7± 0.05	20.4± 0.04	10.6± 0.05	0 ±0	0 ±0	nd	nd
	200mg/l CCC	0.94±0.05	0.65±0.05	0 ±0	0 ±0	11.1±0.05	1.02± 0.02	0 ±0	14.3±0.04	16.6± 0.06	5.6± 0.03	0 ±0	0 ±0	nd	nd
	300mg/l CCC	1.2±0.1	0.62±0.03	0 ±0	0 ±0	25.5±0.1	2.7±0.02	0 ±0	43.9± 0.05	54.8± 0.06	18.4± 0.03	0 ±0	0 ±0		nd

Data represent mean± SD

Table 2: Effect of different Chlorocholine chloride (CCC) concentrations on shoot cultures of *Gardenia jasminoides* Ellis phenolic compounds content

harvest	treatment	phenolic compounds found in *Gardenia jasminoides* Ellis shoots in mg/ g dry weight during three harvests													
		Gallic acid	3.4_DiHydroxy Benzoate	2.5_Dihydroxy Benzoic	Chlorogenic acid	Vanilic acid	Synirigic acid	P-coumaric	Dihydro Kampferol	Rutin	Rosmarinic acid	Cinamic acid	Quercetin	Caffiec acid	Ferulic acid
First harvest	Control	0 ±0	0 ±0	0 ±0	3.7±0.1	54±0.5	1.59± 0.09	0.05±0.03	0.84± 0.06	25.2± 0.2	12.5±0.5	0.03±0.02	0.37±0.03	nd	1.39± 0.01
	100mg/l CCC	0 ±0	0.56±0.02	42.6±0.6	0 ±0	33.4±0.6	0.88±0.1	0.55±0.05	31±0.5	0 ±0	17.7±0.3	0.23±0.3	3.3±0.2	nd	nd
	200mg/l CCC	0.78 ±0.05	0 ±0	26.8±0.7	0 ±0	100±0.7	1.95±0.05	2.9±0.1	69.3±0.7	60.7± 0.3	20.4±0.6	0.13±0.06	1.6±0.07	nd	nd
	300mg/l CCC	0 ±0	0 ±0	4.12±0.07	0 ±0	137.1± 0.8	0 ±0	0 ±0	74.4±0.04	88.5± 0.5	22.1±0.7	0.018 ±0.01.	0.56±0.03	nd	nd
Second harvest	Control	0 ±0	0 ±0	1.85±0.02	182.2± 0.7	0 ±0	0 ±0	0 ±0	91.9±0.4	70.5± 0.5	55.6±0.4	0 ±0	0 ±0	5.879±0.05	nd
	100mg/l CCC	0.649	0 ±0	2.82±±0.02	90.5±0.5	0 ±0	8.6±0.07	0 ±0	46.9±0.3	37.6± 0.3	16.8±0.2	0 ±0	0 ±0	nd	nd
	200mg/l CCC	0 ±0	0 ±0	0 ±0	255.7± 0.3	0 ±0	20.9±0.6	0 ±0	110.3±0.2	98.5± 0.5	47.0±0.5	0 ±0	0 ±0	nd	nd
	300mg/l CCC	0 ±0	0 ±0	4.23±0.04	0 ±0	74.3±0.05	15.09±0.09	0 ±0	66.6±0.4	53.9± 0.5	31.8±0.4	0 ±0	0 ±0	nd	nd
Third harvest	Control	0.45 ±0.05	0 ±0	0 ±0	0 ±0	20.6±0.4	4.5±0.3	0 ±0	19.7±0.2	22.0± 0.2	8.2±0.06	0 ±0	0 ±0	nd	nd
	100mg/l CCC	0.45 ±0.02	0 ±0	0 ±0	0 ±0	20.6±0.3	4.5±0.05	0 ±0	19.7±0.4	0 ±0	8.2±0.1	0 ±0	0 ±0	nd	nd
	200mg/l CCC	0.91 ±0.05	0 ±0	0 ±0	0 ±0	21.9±0.4	5.4±0.04	0 ±0	23.6±0.4	17.3± 0.3	15.6±0.7	0 ±0	0 ±0	nd	nd
	300mg/l CCC	0.12 ±0.04	0.21±0.04	0 ±0	0 ±0	1.71±0.09	0.3±0.06	0 ±0	2.8±0.05	1.3±0.2	0.52±0.05	0 ±0	0 ±0	nd	Nd

Data represent mean± SD

In order to validate the results reported in tables 1 and 2, it is essential to have a thorough understanding of the phenolic compounds route. According to Guan *et al.,* [35] who reported that, the secondary metabolites in plants are the phenolic compounds, which are mostly produced via the phenyl alanine metabolism, and according to Wang *et al.,* [36] who reported that the flavanol synthesis in cherry tomato varieties is regulated by SlMYB12. The chemical structures of the hydroxybenzoic analogs according to Kinugawa *et al.,* [37]. According to [35- 37] the phenolic compounds pathway could be summarized as in Fig (5). By taking a glum on Fig (5), the data in tables (1 and 2) could be discussed. First of all, complex phenolic compounds like flavonoid, tannin, lignin, and anthocyanins originate from simple phenolic acids like trans cinnamic and P-coumaric acids which acts as precursors [38]. This can justify why the P-coumaric is found in the extracts of the first harvest then it disappeared in both *G. jasminoides* sub -species.

Members of the chlorogenic acid (CGA) family, which conjugates the hydroxyl group of quinic acid and the carboxyl group of caffeic acid as the parent structure, are common dietary phenolic acid compounds found in plants. 1L-(−)-quinic acid, caffeic acid (CA), ferulic acid, and the p-coumaric acid (P-CoQA) group, which consists of P-CoQAs, caffeoylquinic acids (CQAs), and feruloylquinic acids (FQAs), are the members of the CGA family [39- 42]. This can justify, why the extracts of *G. jasminoides* Variegata shoot cultures recorded 59.1±0.04 mg / g dry weight of chlorogenic acid in the second harvest with adding 300 mg/l CCC and the vanillic acid was not detected with this treatment while in the third harvest there was no chlorogenic acid detected with the different treatments, with the appearance of vanillic acid to reach its highest content (25.5±0.1 mg/ g dry weight) with the addition of 300 mg/L CCC in the third harvest. This is because vanillic acid is produced from the oxidation of ferulic acid fig (5). The caffeate O-methyltransferase is the enzyme responsible for the transformation of caffeic acid into the ferulic acid fig (5).

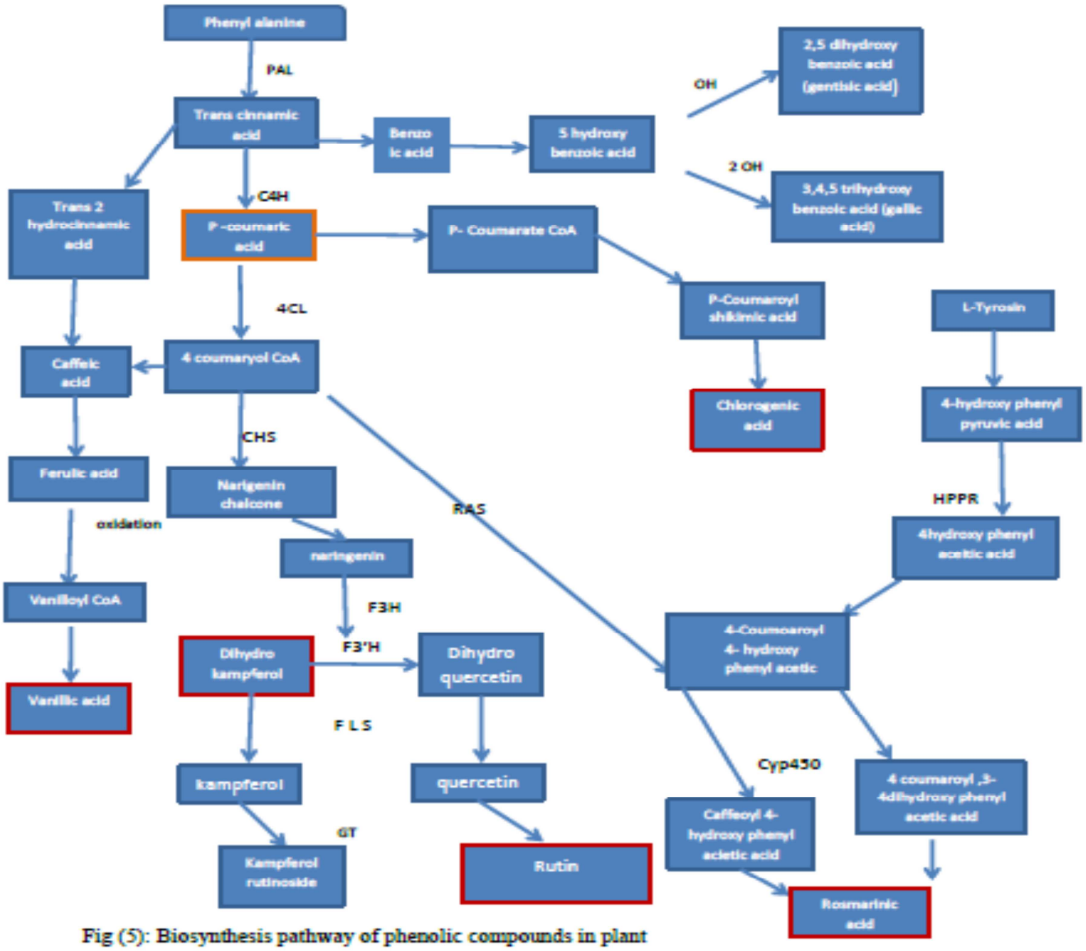

Fig (5): Biosynthesis pathway of phenolic compounds in plant

Caffeic acid can be produced if hydrolysis occurred to chlorogenic acid, which turns into caffeic or ferulic acid and quinic acid fig (6) [43]. As for the extracts *G. jasminoides* Ellis shoot cultures it takes the same trend since it recorded the highest chlorogenic acid 255.7±.0.3 mg / g dry weight with adding 200 mg/ l CCC in the second harvest with no vanillic acid detected. while the highest vanillic acid content (137.1±0.8) was detected with adding 300 mg/ l CCC in the first harvest with no chlorogenic acid detected table (2).

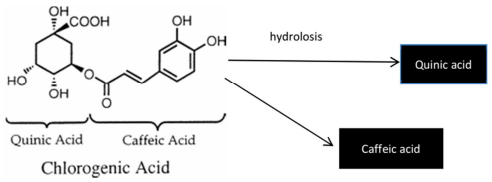

Figure 6: Chlorogenic acid structure.

As for the dihydro kampferol and rutin, the naringenin converted to dihydrokampferol or dihydro quercetin which is converted to quercetin then to rutin as a results for flavonoid metabolism Fig (5), then the pathway of the dihydrokampferol and rutin is the same and from naringenin this explains the results that show that the highest dihydrokampferol and rutin content (43.9± 0.05, 54.8± 0.06 mg / g dry weight, respectively) was reported with adding 300 mg/L CCC in the third harvest with the extracts of *G. jasminoides* Variegata shoot cultures. However, adding 200 mg/ l CCC in the second harvest recorded the highest dihydrokampferol and rutin content (110.3±0.2 and 98.5±0.5mg / g dry weight, respectively) in the extracts of *G. jasminoides* Ellis shoot cultures.

As for rosmarinic acid which is considered an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid [44]. Then as represented in fig (5) it could be noticed that the caffeic acid either turned to ferulic

acid which may be oxidated to give vanillic acid or it can give rosmarinic acid as a result for esterification with the 3,4- dihydroxyphenyl lactic acid. This can justify why with the treatment which reported the highest rosmarinic content (69.5±0.03 mg / g dry weight), the vanillic acid was not detected with this treatment which is adding 100 mg / l CCC at the first subculture with the extracts of *G. jasminoides* Variegata shoot cultures. Also, the extracts of *G. jasminoides* Ellis shoot cultures on the control treatment in the second harvest give the highest rosmarinic content (55.6±0.4mg / g dry weight) with no vanillic acid detected with this treatment.

Our results are on line with what reported with Niggeweg *et al.,* [45]. who reported that, the buckwheat plants' elevated levels of chlorogenic acid are considered as a response to the effects of CCC. Since chlorogenic acid is a significant antioxidant in plants that can protect against lipid peroxidation, it can provide evidence regarding the stress response of buckwheat plants subsequent to CCC foliar treatment. Also, according to the findings of Weiwei *et al.,* [46], who reported that the ginkgo leaves treated with 0.5, 1.0, and 2.0 g /l CCC considerably enhanced the photosynthetic rates of the leaves as well as their levels of soluble sugar, chlorophyll, total amino acids, and phenylalnine. 1.0 and 2.0 g /l CCC treatments resulted in significant increases in total polyphenols, flavonoids, anthocyanins content, phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and chalcone isomerase (CHI) activities. Therefore, foliar CCC treatment may be an effective method to enhance the pharmacological properties of *Ginkgo biloba* leaves. As mentioned by Niggeweg *et al*., [45], the chlorogenic acid elevated levels can be considered evidence that the buckwheat plants elevated the chlorogenic acid as a response to the stress caused by CCC foliar treatment. Also, our result match what was reported by Qin et al., [47] who reported that the use of chlormequat (CCC) changed the proportions of several active ingredients in *Astragali Radix*. Higher amounts of chlormequat resulted in more active ingredients as well as more chlormequat residues in *Astragali Radix*

Conclusion:

This the first work to apply CCC on shoot cultures of *G. jasminoides* (Variegata and Ellis). In order to evaluate the ability of both sub-species to accumulate different secondary metabolites. Data clearly indicates that *G. jasminoides* Variegata shoot cultures exhibit lower levels of chlorogenic acid and other secondary metabolites in their extracts compared to the extracts obtained from *G. jasminoides* Ellis shoot cultures. Simultaneously, the fresh weights of *G. jasminoides* Variegata are typically greater than those of *G. jasminoides* Ellis. This suggests that *G. jasminoides* Variegata shoot cultures are more resilient to the stress induced by CCC treatments compared to *G. jasminoides* Ellis shoot cultures.

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