Original Research Article

RESPONSE OF FLAVONOIDS PRODUCTION TO SOME ELICITORS IN CALLUS AND CELL SUSPENSION CULTURE OF GINKGO (*Ginkgo biloba* L.)

ABSTRACT

Aims: This study was conducted to investigate how to maximize the production of flavonoids from ginkgo leaves callus or cell suspension cultures.
Study design: The experiment was implemented in a completely randomized design with 3 replicates.
Place and Duration of Study: The study was carried out at the Research Laboratory of Tissue Culture and Preservation of Germplasm, Horticulture Research Institute, Agric. Res. Center, Egypt from 2020 to 2022,
Methodology: Adenine sulfate and pyruvic acid were added at concentrations of 0.1, 0.2, and 0.3 mg/l, for each. Cadmium and lead acetate were added (2 and 4 mg/l for each) to the cell suspension. Jasmonic acid (15, 50, and 100 µM), $AgNO_3$ (25, 50, and 75 µM) and tryptophan (50, 100, and 200 mg/l) were used in the hard callus culture.
Results: Results indicated that the maximum cell suspension fresh weight (6.50 g) was obtained from (0.5 g) cell suspension grown on MS medium supplemented with adenine 0.2 mg/l while lead acetate at 2.0 mg/l increased dry weight (0.89g) after the second subculture. The maximum packed cell volume (PCV) (24.33%) was obtained from lead acetate at 2.0 mg/l in the second cycle of growth. The best flavonoid production (91.29 mg/100 g DW) was produced in the second cycle of growth from callus treated with $AgNO_3$ at 25 µM. Treating Ginkgo biloba callus cultured with jasmonic acid at 100 µM increased the inhibition of AChE in the serum of AD patients from 235.2 U/l (untreated serum) to 76.08 U/l at 38.71 ug/ml compared with the *Ginkgo biloba* ® at 260 mg which inhibit the activity from 235.2 U/l to 55.73 U/l at 26.00 ug/ml at amount 25.00 ul for all.
Conclusion: Ginkgo biloba is a valuable source of anticholinesterase agents and could be used in pharmaceutical preparations by using callus culture technique treated with jasmonic acid and $AgNO_3$.

Keywords: Ginkgo biloba, callus, acetyl choline esterase, flavonoids, jasmonic acid, $AgNO_3$

1. INTRODUCTION

Ginkgo biloba L. is one of the most popular functional plants, especially medicinal plants. Extracts of *G. biloba* leaves contain active compounds such as flavonoids and terpene lactones (ginkgolides and bilobalide) and can therefore be used to increase peripheral and cerebral blood flow [1 and 2]. So far, about 38 kinds of flavonoids have been isolated from *G. biloba*, and among them were 28, 6, and 4 kinds of mono, di- and tri-glycosides of flavanols, respectively [3, 4, and 5]. *G. biloba* products' global annual sales are estimated to be $10 billion [6]. However, traditional methods to isolate and purify active compounds are usually low and subject to environmental factors which make it difficult

to meet the huge demand of international markets. Hence developing new strategies for the preparation of these remarkable compounds from *G. biloba* is needed [6]. In recent years, there have been some documents on the changes in flavonoids content (FC) and their affecting factors, the mechanism of flavonoids formation (FF), the external chemical synthesis of flavonoids, and tissue culture-producing flavonoids among others [7, 8, 9, 10, 11, 12, and 13]. Jasmonic acid (JA) is an important signaling molecule involved in elicitor-induced secondary metabolite synthesis such as terpenoids, flavonoids, and alkaloids [14]. Rare earth elements as exogenous elicitors, may stimulate the defense system of plant cells and promote the accumulation of secondary metabolites [15]. However, we are aware that no published studies confirmed that JA mediates (Rare earth elements) REE-induced secondary metabolite biosynthesis in medicinal plants. Metal ion (cadmium) influenced secondary metabolite production [16]. It has been shown to induce higher yields of secondary metabolites such as shikonin [17] and also on the production of digitalin [18]. $CdCl_2$ elicited the overproduction of two tropane alkaloids, scopolamine, and hyoscyamine, by hairy root cultures of *Brugmansia candida* [19].

2. MATERIAL AND METHODS

2.1 Plant source

Leave explants of *Ginkgo bioloba* L. were collected from the unique tree grown in Orman botanical garden, Giza, Egypt from the middle of April until the end of June 2020.

2.2 Callus induction and maintenance

Leaves were washed under running tap water for 30 min followed by rinsing in septol soap for 30 min as a primary cleaning step. Explants were surface-sterilized with 70% (v/v) ethanol for 60 sec., then with 10 % (v/v) Clorox (5.5% sodium hypochlorite) solution for 10 min., finally rinsed 3 times in sterile distilled water. Leave explants were then cut into small pieces (1.0 cm^2) and cultured on autoclaved MS medium [20] supplemented with 3 mg/l 2,4-D + 3 mg/l NAA and solidified with 7 g/l agar and a maintained in a growth chamber at 25 ± 2 ˚C with a 16-h photoperiod using cool white fluorescent light (1500 lux) for 8 weeks. At the end of the incubation period, induced callus from leaf segments was sub-cultured three times with four weeks intervals to get an efficient amount of callus for following investigations.

2.3 Suspension culture

2.3.1 Cell aggregate separation

The cells were separated from the balk of the obtained callus by using the tissue sieve instrument (Selector) which contains fine mesh screen (100-200 mesh). This instrument was produced by E-C Apparatus Corporation belonging to a Thermo Electron Company, U.S.A.

2.3.2 Select sieve size

To select the appropriate size screen (200 mesh screen) for eliminating the smaller sizes of the aggregate cells (from 3- 4 cells aggregate) by separating all calli formed from the explants and put these aggregates in a liquid MS medium then, use the pestle for distributing the cells inside MS solution then, passing through the 200-mesh screen [21]. Cell suspension cultures were initiated with 0.5 g of callus derived from leave explants. The suspension was placed in 100 ml of MS liquid medium (modified indicated) in a 250 ml – Erlenmeyer flask and kept on a reciprocal shaker (100-110 rpm) at 25 ˚C in light as mentioned before. For kinetic studies of flavonoids production by the cultures, samples (replicates) were extracted and assayed twice every 12 days during a culture period of 24 days.

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2.4 Elicitation treatments

2.4.1 Suspension

Cell suspension culture was exposed to trace elements of lead and cadmium in the form of acetate salts at 2.0 and 4.0 mg/l for each. The cell suspension also was exposed to adenine sulfate and pyruvic acid at three concentrations 0.1, 0.2, and 0.3 mg/l.

2.4.2 Callus

The callus cultured on MS medium was augmented with one of the following: $AgNO_3$ at 15, 25, and 50 uM; Jasmonic acid ((+/-)-1a,2B-3-Oxo-2-(cis-2-pentenyl) cyclo-pentane-acetic acid) Sigma) at 10, 50 and 100 uM with preparing a stock solution in ethanol (100 $mg.ml^{-1}$), diluted to the desired quantity using sterile distilled water; and tryptophan at 50, 100 and 200 ppm. After the first subculture (4 weeks later) FW, DW, and MC were measured and after another 4 weeks (second subculture) and the same parameters were assayed

2.5 Measurement of cell suspension culture growth

Cell growth parameter such as packed cell volume (PCV) was measured every 12 days until constant growth was achieved. Fresh cell weight (FCW) and dry cell weight (DCW) were determined at the stationary phase of growth (at the end of the experiment). The packed cell volume (PCV) was determined by centrifuging 10 ml of the suspension culture in a 15-ml graduated centrifuge tube at 2000 rpm for 5 min. Volume of backed cells was measured as a % of the tube volume.

To determine fresh and dry weights, cells were collected by filtration through a Whatman No. 1 filter paper under a vacuum, washed with 3 ml of distilled water, and dehydrated under a vacuum. The filtered cells were transferred to pre-weighed aluminum foil and the fresh cell weight (FCW) was measured. The cells were then dried in a drying oven at 60 °C for 12 h and dry cell weight (DCW) was determined [22]. The 100 ml flasks containing cell suspension were kept on the shaker in the first 12 days after the cell inoculation. All cultures were maintained under a 16 h photoperiod, provided by white fluorescent lamps (20 Watt) and a temperature of 25±2ºC. After 24 days of the culture of each experiment, the frequency of F.W, D.W with callus and cell suspension induction were assessed.

2.5 Total flavonoids

Determination: Total flavonoids were estimated using the method of [23]. To 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ methanol solution was added. After 1 hour at room temperature, the absorbance was measured at 420 nm. Total flavonoid contents were calculated as ginkgolic acid from a calibration curve.

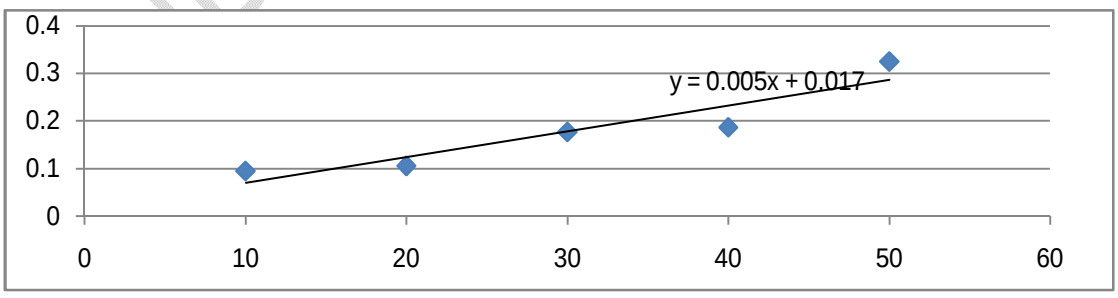

Fig. 1. Standard curve of ginkgolide

*Standard: Ginkgolide was received from Holland & Barrett. Ltd. (USA) ® HB7652, at 60 mg and diluted with distilled water.

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2.7 Acetylcholine esterase inhibition

2.7.1 Reagents and Chemicals

Tris-HCl Tris (hydroxymethyl) aminomethane hydrochloride, β-naphthyl acetate, bovine serum albumin (BSA), 3,3'-dimethoxybiphenyl-4,4'-di(diazonium) zinc chloride (fast blue B salt), acetylthiocholine iodide (ATCI), 5,5´-dithiobis [2- nitrobenzoic acid] (DTNB), galanthamine hydrobromide, ascorbic acid was purchased from Sigma-Aldrich.

2.7.2 Determination inhibition

The callus was grounded to fine pieces using a mortar and extracted with 60 % methanol (2 ml/g) at room temperature for 24 hours. The activity of AChE was determined according to Ellman's Method by using tris buffer 30 mM Tris HCl, 3 mM EDTA pH 8.2 and stored at 4 C. DTNB reagent prepared by dissolved 29.7 mg in 25 ml methanol. The reaction mixture of 20 ul of the sample (Serum), 75 ul substrate, 25 ul DTNB reagent, and 500 ul Tris HCl buffer and reading the absorbance at 412 nm in UV-VIS Spectrophotometer BYER® [24]**.** The callus extraction was using as an inhibition material (IC_{50}). To calculate the IC_{50} values, each sample was assayed at four concentrations (100, 75, 50, and 25 mg/ml). IC_{50} values were obtained from dose-effect curves by linear regression.

2.8 Statistical analysis

The experiment carried out for the callus induction was conducted in a completely randomized design with 3 replicates (glass jars) per treatment with one jar per replicate and three pieces in the jar. Analysis of variance and Fisher's LSD (0.05 significance level) [25] were performed using CO- Stat view 5.1 statistical package [26]**.**

3. RESULTS AND DISCUSSION

3.1 Cell suspension

3.1.1 Effect of adenine and pyruvic acid

Ginkgo bioloba cell suspension cultures were established and culture media turned turbidity with increasing cell density Fig. (2a). The results of the analysis of variance indicated that the growth of *Ginkgo bioloba* cell suspension cultures as measured by PCV, fresh and dry weights of cells were significantly influenced by the concentration of adenine and pyruvic acid. However, the effect of adenine at 0.1 mg/l on the fresh weight of cell suspension was significantly high (0.63 g) compared with control (0.288g). Among the adenine concentrations, 0.1 mg/l showed a good stimulatory effect on cell growth (fresh and dry weights) after two weeks of culture (6.50 and 0.51 g, respectively), while PCV gave 21 % for 0.3 mg/l adenine of cells, which were significantly higher than those of hormone-free MS medium (10.3 %) and media containing 0.1 and 0.2 mg/l pyruvic acid or 0.1 mg/l adenine sulfate (11.0, 10.33 and 12.33 %, respectively).

Table 1. Effect of adenine sulfate and pyruvic acid on fresh and dry weight of *Ginkgo biloba* cell suspension during two subcultures

Treatments	start (g)	Subculture I		Subculture II		PCV %
		FW (g)	DW (g)	FW (g)	DW (g)	
Control	0.12	0.29	0.009	1.08	0.15	10.33
Adenine sulfate 0.1 mg/l	**0.27**	**0.63**	0.032	4.33	0.29	12.33
Adenine sulfate 0.2 mg/l	0.18	0.24	0.014	6.50	0.51	14.67
Adenine sulfate 0.3 mg/l	0.15	0.17	0.01	3.93	0.26	21.00
Pyruvic acid 0.1 mg/l	0.14	0.31	0.01	1.08	0.11	11.00
Pyruvic acid 0.2 mg/l	0.22	0.58	0.03	5.29	0.29	10.33
Pyruvic acid 0.3 mg/l	0.15	0.37	0.02	0.67	0.04	20.33
LSD 5%	**0.081**	**0.30**	**0.018**	**0.87**	**0.2387**	**3.565**

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Packed cell volume is the relative quantity of cells, cell fragments, and some participant materials with culture medium these theoretic explain increased PCV to the maximum value 21% with low weight of fresh and dry (3.93 and 0.26 g, respectively). In the same trend, pyruvic acid at 0.2 mg/l gave significantly increasing of fresh weight at the first 12 days of growth from 0.22 g (start culture) to 0.58 g at the end of first subculture. These fresh materials scored 0.3 g D.W. at the same period, this effect similarly to adenine sulfate treatment at 0.1 mg/l. Also, this treatment (pyruvic acid 0.2 mg/l) following adenine at 0.1 mg/l in their effect of the second subculture. It was recorded 5.29 g F.W., 0.29 g D.W. and 10.33 % PCV. Although, PCV of pyruvic acid at 0.3 mg/l was 20.33 % and FW was 0.37 g from 0.15 g reached to 0.02 but the growth was more slowing. The highest percent of PCV may be return to many fragment cells, broken cells and elongation of cells with imbibition of liquid media (Fig. 2c).

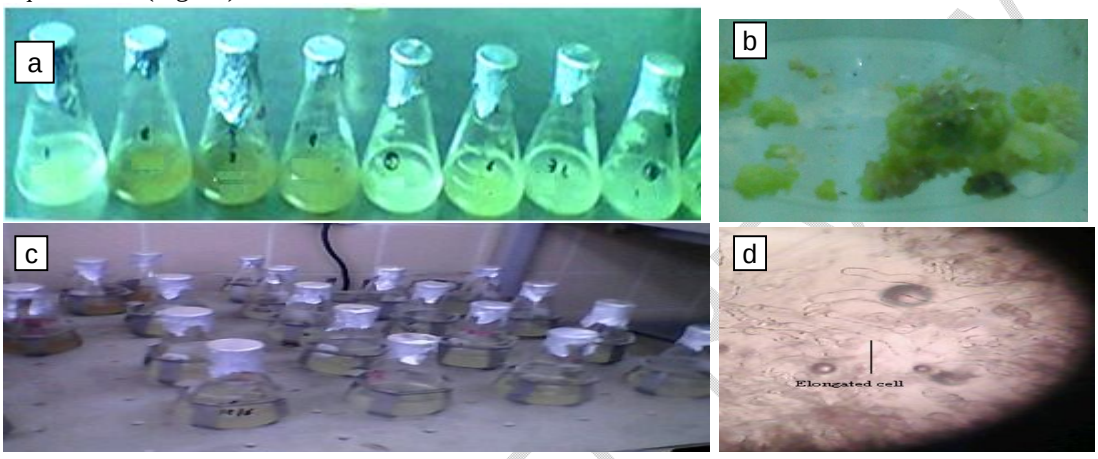

Fig. 2. a. cell suspension culture shows turbidity and color transfer two brown after 14 days culture, b callus of *G. biloba*, c cell suspension shaker and d elongated cells.

3.1.2 Effect of trace elements

Growth of *Ginkgo biloba* cell suspension cultures also varied significantly depending on the trace elements (lead and cadmium), (Table 2). The maximum cell fresh and dry weights of cells (6.35 g and 0.89 g, respectively) were recorded for MS medium supplemented with 2.0 mg/l Pb after the second subculture. On the other hand, in the first subculture, there are no significant effects among FWs or DWs in all treatments, but the highest record scored in the first subculture of FW and DW were 2.15 g and 0.03 g, respectively. As shown in Table 2 and Fig. 3, the lower concentration of Pb (2.0 mg/l) resulted in better cell suspension growth responses at PCV (24.33%) compared to other levels of Pb and Cd. In this regard, [27] revealed that exposed sugar cane (*Saccharum officinarum* L.) *in vitro* callus cultures to $CdCl_2$ (cadmium chloride) at lower concentrations such as 0.01 and 0.1 mM caused a significant increase in the growth of the callus cultures, whereas 0.5 and 1 mM $CdCl_2$ strongly inhibited it, but only after 9 days of $CdCl_2$ treatment. Red-brown patches were also observed in calluses exposed to 0.5 and 1 mM $CdCl_2$. Calluses grown in 0.01 and 0.1 mM $CdCl_2$ did not exhibit any changes in CAT (catalase) activity even after 15 days of growth in the presence of $CdCl_2$. Cadmium exposure led to the inhibition of callus growth in a concentration-dependent manner. Moreover, the toxic effect of the Cd was evident from the reduced biomass seen at higher Cd concentrations. The reduction of callus growth at high levels of Cd may be correlated with high Cd accumulations by the calli [28]. These results were in agreement with previous studies that have observed that, in *Sesbania drummondii* [29, 30] and *Cuscuta reflexa* [31], callus growth was significantly inhibited at higher concentrations of Cd. In contrast, a Cd-induced decrease in growth has been observed in soybean cells at lower concentrations [32].

Table 2. Effect of lead acetate and cadmium on *Ginkgo biloba* cell suspension growth

Treatments	Start g	Subculture I		Subculture II		PCV %
		FW (g)	DW (g)	FW (g)	DW (g)	
Control	0.5	1.72	0.017	3.10	0.79	14.67
Lead acetate 2.0 mg/l	0.5	1.82	0.010	6.35	0.89	24.33
Lead acetate 4.0 mg/l	0.5	2.10	0.023	4.07	0.84	12.00
Cadmium 2.0 mg/l	0.5	2.15	0.030	2.97	0.73	12.33
Cadmium 4.0 mg/l	0.5	1.71	0.013	3.95	0.48	13.67
LSD 5%		**NS**	**NS**	**1.906**	**0.146**	**9.017**

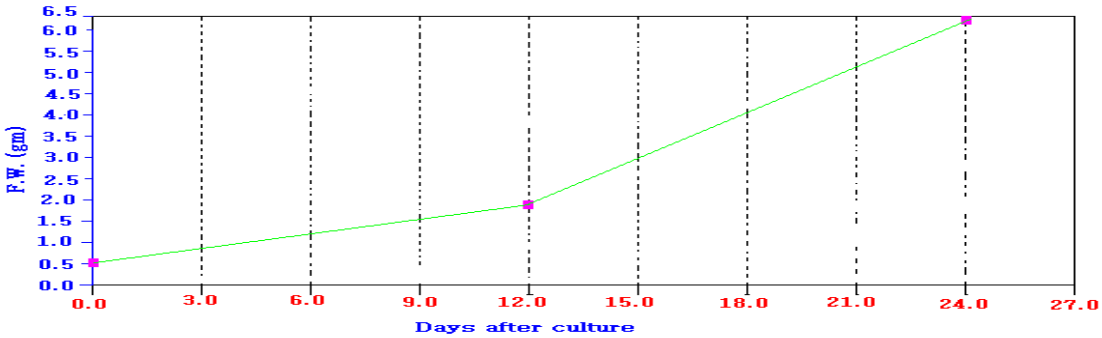

Fig. 3. Growth curve of suspension cultures of *Ginkgo biloba* grown in the MS medium supplemented with 2.0 mg/l Pb for 24 days.

3.2 Callus culture

Callus initiation was observed on the leave disc explants within 8 weeks in MS medium containing 2,4-D and NAA at 3 mg/l each. (Fig.2b). The effect of elicitation treatments on callus growth was recorded. Fresh and dry weights of callus were significantly affected by elicitation treatments.

The 6.21g and 0.312g (representing the highest significant values) of fresh and dry weights, respectively of callus were obtained in MS medium with 50 mg/l tryptophan at the first subculture with moisture content of 95.04%.

Moisture contents increased with increasing tryptophan concentration (from 50 to 100 mg/l) to the highest ratio (96.96 %) (Table 3). In the second subculture, 50 mg/l tryptophan scored fresh weight 6.0g as the first subculture but callus without treatments (control) increased the dry material (1.009g).

Comparing water contents in all treatments during the second subculture we found that jasmonic acid (JA) at 100 mg/l had the highest amount of water contents 97.17% compared with the other treatments. In this concern, the effects of organic nitrogen on callus production indicated that callus growth and production increased by supplementation of basal media E, G (MS modified medium with sources of nitrogen) with 100 mg/l glutamine high callus induction percentage treatments and growth callus was observed in media containing half-strength MS salts and ½ MS (4750 mg/l KNO_3, 4125 mg/l NH_4NO_3) alone or in combination with 100 mg/l glutamine [33].

According to these reports, the reduction of nitrates and application of glutamine proliferate callus [34]. Conifers and other forest tree species require a combination of both nitrate and ammonium for good growth stimulation. Moreover, glutamine is required for cell division and callus proliferation from protoplasts of Douglas-fir and maritime pine (*Pinus pinaster*) [35]**.**

Table 3. Effect of elicitation treatments on *Ginkgo biloba* callus growth during first and second subculture

Source / weight / Treatments	First subculture			Second subculture		
	FCW (g)	DCW (g)	M.C. (%)	FCW (g)	DCW (g)	M.C. (%)

Control	4.63	0.218	95.27	4.59	1.009	77.78
$AgNO_3$ 25µM	0.97	0.075	92.28	3.76	0.568	84.60
$AgNO_3$ 50 µM	0.52	0.074	85.77	4.88	0.461	90.26
$AgNO_3$ 75 µM	4.64	0.301	93.23	5.81	0.344	94.10
Jas. acid 15 µM	3.84	0.240	93.49	4.50	0.297	93.16
Jas. Acid 50 µM	2.37	0.177	92.63	3.15	0.213	93.24
Jas. acid 100 µM	2.37	0.108	95.52	4.01	0.113	97.17
Tryp. 50 mgl^{-1}	**6.21**	**0.312**	95.04	6.00	0.300	95.00
Tryp. 100 mgl^{-1}	3.41	0.104	96.96	4.86	0.310	93.52
Tryp. 200 mgl^{-1}	0.99	0.167	82.87	1.03	0.223	78.42
LSD 5 %	**1.167**	**0.0767**	**4.056**	**1.139**	**0.1085**	**5.528**

3.2 Flavonoid contents

3.3.1 In cell suspension

Data in Table (4) showed that the highest amounts of flavonoids compounds based on the dry weight, were 35.33 and 32.91 mg/100g DW, respectively for the *G. biloba* cell suspension during the first cycle of culture on MS medium containing pyruvic acid at 0.1 and 0.2 mg/l, respectively. These amounts increased to 42.38 and 33.97 mg/100 g DW, respectively during the second subculture after cultured on the same media. Applying cadmium at 4.0 mg/l increased amounts of flavonoids from 51.97 to 55.35 mg/100g DW during the first and second subcultures, respectively. While as, applying led in the suspension media at 4.0 mg/l decreased the amounts of total flavonoids from 36.22 to 15.19 mg/100 g DW during the first and second subculture, respectively. Using cadmium at 2.0 mg/l during second subculture gave 35.68 mg/100 g DW compared with the first subculture (24.64 mg/100 g DW). This result is agreed with this reported by [36] who reported that in many cell and organ cultures of many plants, Me Ja increased the accumulation of secondary metabolites. [37] cleared that the elicitation of antioxidant secondary metabolites was accomplished with Jasmonates and gibberellic acid in *Artemisia absinthium* L. plant. Moreover, the radical scavenging activity (RSA) of suspension cultures has been substantially improved. The application of Jasmonic acid and triacontanol on *Lycopersicon esculentum* plant culture improved the mechanism of root induction [38]. The synthesis of low-molecular-weight antioxidants, such as α-tocopherol, has been reported in a number of stressed plants [39, 40, and 41]. Oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in plants [41 and 42]. Based on the above trends, this study suggests that α tocopherol also plays an important role in Cd detoxification.

Table 4. Effect of adenine sulfate, pyruvic acid, trace elements, $AgNO_3$, Jasmonic acid, and tryptophan on flavonoids contents in *Ginkgo biloba* cell suspension and callus culture during first and second subculture

Treatments	Flavonoids mg/100 g DW SI	Flavonoids mg/100g DW SII
Control	10.89	9.99
Adenine sulfate 0.1 mg/l	10.56	11.55
Adenine sulfate 0.2 mg/l	9.07	13.20
Adenine sulfate 0.3 mg/l	12.73	13.13
Pyruvic acid 0.1 mg/l	32.91	33.97
Pyruvic acid 0.2 mg/l	35.33	42.38
Pyruvic acid 0.3 mg/l	25.28	29.82
LSD 5% for cell suspension	**4.098**	**5.174**
Control	10.89	9.99
Lead acetate 2.0 mg/l	28.04	16.46
Lead acetate 4.0 mg/l	36.22	15.19
Cadmium 2.0 mg/l	24.64	35.68
Cadmium 4.0 mg/l	51.97	55.35

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LSD 5 % for cell suspension	5.102	6.137
Control	41.71	12.35
AgNO3 25 µM	63.96	29.22
AgNO3 50 µM	21.25	21.25
AgNO3 75 µM	26.09	49.84
Jasmonic acid 15 µM	27.14	35.66
Jasmonic acid 50 µM	35.44	91.29
Jasmonic acid 100 µM	38.71	45.39
Tryptophan 50 mg/l	19.25	38.80
Tryptophan 100 mg/l	52.44	30.97
Tryptophan 200 mg/l	26.14	77.00
LSD 5% for callus	**6.211**	**9.245**

3.3.2 In callus culture

According to data in Table (4) and Fig. (2b), the MS culture medium added 25 µM $AgNO_3$ in the first 12 days of culture has a greater effect on the accumulation of flavonoids 63.96 mg/100 g DW in the callus from ginkgo compared with the other enhancer treatments followed by those treated with tryptophan 100 mg/l which scored 52.44 mg/100 g DW.

On the hand, in the second subculture, the amounts of flavonoids increased to the maximum level of 91.29 mg/100 g DW for callus cultured on MS medium containing 50 µM jasmonic acid (JA) following by 77.00 mg/100 g DW for callus cultured on media containing tryptophan 200 mg/l. In this concern, levels of α-tocopherol also revealed a remarkable increase in calli grown under Cd stress 100 uM [43].

The application of Cd resulted in a significant enhancement of α-tocopherol levels in the safflower calli. Cadmium caused increases in α-tocopherol levels by 46% and 68% at 75 and 100 µM Cd, respectively, in comparison to the control.

3.4 Acetylcholine esterase activity

AChE inhibition was represented in Table 5. Flavonoids imply the multipotent agents in combating Alzheimer's disease (AD) by enhancing acetylcholine levels. Among the 4 different concentrations 25, 50, 75, and 100 ul, flavonoids were found to inhibit AChE with IC50 26 µg/ml in the inhibitory assay. According to the data in Table (5), the inhibition effect of Ginkgo biloba extraction obtained from callus treated with jasmonic acid was the most effective than other treatments.In our results, jasmonic acid at 100 μM increased inhibition from 76.08 to 69.5 and 32.3 U/l by using amounts 25, 50, and 75 ul, respectively. Moreover, the extraction of Ginkgo biloba callus treated with jasmonic acid at 100 μM increased the inhibition of AChE in serum of AD patients from 235.2 U/l (untreated serum) to 76.08 U/l at 38.71 ug/ml compared with the traditional drug *Ginkgo biloba* ® at 260 mg which inhibit the activity from 235.2 U/l to 55.73 U/l at 26 ug/ml. On the other hand, applying jasmonic acid at 50 μM increased inhibition from 101.34 to 57.7, 41.9, and 28.7 U/l by using amounts 25, 50, 75, and 100 ul of callus extraction, respectively compared with the untreated serum of AD (control) which scored an average of AChE activity (235.2 U/l), this value was higher than normal range of AChE (8 – 18 U/l).The AChE inhibitory activity of test compounds revealed that flavonoids were found to inhibit AChE with IC50 values ranging from 57.8 to 133.1 µg/ml [44 and 45]. Thus, a variety of mechanisms of neuronal degeneration in Alzheimer's disease has been suggested, including improvement of free radicals, oxidative stress, mitochondrial dysfunction, inflammatory processes, genetic factors, environmental impact factors, and apoptosis, among others. Earlier studies conveyed that neuronal earliest changes and pathological alterations of this disease are interrelated to oxidative damage (with a very high input of oxidative stress), mainly in the development of neurogenetic abnormalities [46]. Previous reports possess the results of AChEI, antioxidant and cytotoxicity activity of tested plants, can conclude that the ethanol extract of G. max which has been used widely as a dietary plant, was able to inhibit AChE significantly in vitro. Ginkgolides B and C were found to be inactive in the AChE inhibitory test, while their effect target seemed to be GABA receptors [47].

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Table. 5. Inhibition effect of *Ginkgo biloba* callus extract treated with various elicitors on Acetylcholine esterase activity in serum of AD patient

Extraction of callus treat.	25 ul	ug/ml	50 ul	ug/ml	75 ul	ug/ml	100 ul	ug/ml
***Ginkgo biloba* 260 mg cap.**	55.73	26.00	24.65	52.00	17.44	78.00	8.99	104.0
Control	137.66	41.71	95.22	83.42	92.07	125.13	97.66	166.84
$AgNO_3$ 25 µM	209.27	63.96	176.19	127.92	305.7	191.87	334.2	255.83
$AgNO_3$ 50 µM	150.03	21.25	110.7	42.50	221.8	63.75	243.7	85.00
$AgNO_3$ 100 µM	121.12	26.09	99.80	52.19	188.8	78.28	200.6	104.37
Jasmonic acid 15 µM	117.82	27.14	67.80	54.29	73.70	81.43	112.8	108.58
Jasmonic acid 50 µM	101.34	35.44	57.70	70.87	41.90	106.31	28.70	141.75
Jasmonic acid 100 µM	76.08	38.71	69.50	77.41	32.30	116.12	181.6	154.83
Tryptophan 50 mg/l	233.33	19.25	95.00	38.50	291.0	57.75	277.0	77.01
Tryptophan 100	222.19	52.44	177.5	104.87	263.7	157.31	133.6	209.75
Tryptophan 200 mg/l	135.32	26.14	278.0	52.29	181.0	78.43	222.6	104.57
LSD 5% for callus	**1.2147**		**1.837**		**2.017**		**1.932**	

Patients AChE n=3 (average of activity without inhibition): 235.2 U/L; Reference value of AChE 8 – 18 U/l, PSD patients with post-stroke dementia

4. CONCLUSION

The ginkgo trees need various environments to grow but did not grow under Egyptian conditions. So, we concluded at the end of this work protocol of callus production of ginkgo trees from leaves and cell suspension protocol from callus obtained from leaves. After callus induction, we produce an elicitation protocol of callus and suspension cells which have more facility to produce flavonoids.

Elicitation is one of the promising strategies to induce secondary metabolite production in callus cultures. JA is considered as one of the major elicitor-induced signaling in the biosynthesis of secondary metabolites in plants. The elicitation by JA supplementation is therefore a potent solution that stimulates bioactive compound production in the culture medium to meet the large-scale ever-expanding industrial requirements of pharmaceuticals.

JA modulates MYC2/3/4- and ANAC019/055/072, JAV1 and JAM1 gene expression, and biosynthesis of the key enzymes chalcone synthase (CS), PAL, fumarate hydratase (FH) succinate dehydrogenase (SDH). Hence these elicitors are biodegradable, cost-effective, and quick in action [48]. Using jasmonic acid, $AgNO_3$, and tryptophan in callus was more effective on flavonoid production than applying trace elements, pyruvic acid, and adenine sulfate as stimulants in cell suspensions. Finally, the results of our work reveal that *Ginkgo biloba* would be a valuable source of anticholinesterase agents, with potential use in pharmaceutical preparations by using tissue culture technique especially callus culture treated with jasmonic acid and $AgNO_3$.

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