Effects of feeding tyrosine or phenylalanine on the accumulation of polyphenols in *Coleus Blumei* in *Vivo* and in *Vitro* .

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Abstract

Background: Polyphenols are considered an important compound with a wide range of medical activities. Several plant families are rich with phenolics. Attempts have been carried out to increase them at the intact and cellular levels.

Objective: The current study aimed to increase the production of polyphenols in *Coleus blumei* plant using two precursors at the whole and tissue culture levels.

Materials and methods: Two precursors namely Tyrosine and phenylalanine at the concentrations 0.20 , 0.25 or 0.30 g.l^{-1} were added either by spraying on the vegetative parts or supplemented to the tissue culture medium. Total phenols were estimated in the whole plant and compared with the total phenolic content in callus tissues. They were estimated by Folin-ciocalteu and spectrophotometric methods. Induction and maintenance of Coleus callus cultures were carried out on Gamborg medium (B5) for 21 days supplemented with the growth regulators 2,4 dichlorophenoxy acetic acid (2-4-D) at 0.8 mg.l^{-1} , 2 mg.l^{-1} Benzyl adenine (BA) and 0.5 mg.l^{-1} Naphthalene acetic acid (NAA). Stem explants were used as a source for callus induction.

Results: Results showed a significant increase in both fresh and dry weights of the fresh compared with control of intact plants in vivo after treatment with the two precursors. The fresh weight of shoots increased up to 15.9 g at 0.30 g.l^{-1} of Tyrosine treatment compared with the control which recorded 11.05 g. while the fresh weight of the shoots recorded 13.470 g at 0.30 g.l^{-1} after treatment with phenylalanine compared with the control which recorded 11.05 g. The dry weight of the shoots increased as well up to 3.127 g at 0.30 g.l^{-1} of Tyrosine treatment compared with control 1.837 g while the dry weight of shoot recorded 2.880 g at 0.30 g.l^{-1} phenylalanine treatment compared with the control 1.837g. Despite the slight increase in both root fresh and dry weights at 0.30 g.l^{-1} due to the treatment with Tyrosine or phenylalanine ,such increments were not significantly different with control. Total poly phenols in callus cultures accumulated more compared with mother plants after treatment with precursors. Phenols were also measured in both callus tissue and liquid medium then compared with the whole plant and control treatment. The highest value was recorded in callus cultures supplied with Tyrosine recording 348.36 µg.l^{-1} at 0.20 g.l^{-1} of Tyrosine while phenylalanine treatment recorded the highest weight 293.98 µg.l^{-1} when applied at 0.30 g.l^{-1} in both liquid medium and callus tissues. The intact plants however recorded the highest mean in total phenols in plants grown *in vivo* with mean values 68.58, 66.53 µg.l^{-1} for Tyrosine and phenylalanine respectively.

Conclusion: It is concluded that both precursors are good candidates for increasing total phenols in *Coleus blumei*. Plant tissue culture techniques can be utilized commercially for this purpose.

Keywords: *Coleus blumei* , Tyrosine , Phenylalanine , Precursors , fresh and dry weights , total phenols , *in vivo, in vitro.*

Introduction

Plants are considered as good source for important compounds such as phenolics, which have anti-oxidant activities. These phytochemicals play a significant role in improvement of new pharmaceuticals using raw substances to produce synthetic ones (1). Natural products are consumed as an alternative medicine with low side effects on human, therefore, there is a growing interest in health care and efforts are carried out to accomplish this through extracting plant material and evaluating their activity (2). Thus, it is crucial to produce valuable secondary products using plant cell culture, rather than intact plants. Production can be more reliable, simpler and more predictable in using such techniques. Moreover, phytochemicals can be rapidly and efficiently isolated as compared with extraction of complex whole plants particularly when using bioreactors. Coleus which has a medicinal value (3) and receives increasing attention nowadays. It has been used for the treatment of various diseases, high blood pressure, cut and wounds, bladder stones, diarrhea, cough (4). Plant extracts are rich in phenolic contents therefore; it can be used as free radical scavengers. Phenolic compounds are known to be synthesized by plants in response to environmental

stresses (5). Both plant cell suspension and callus cultures have been used to produce all the primary and secondary metabolites that are produced by the parent plant (6).
Meanwhile, precursors are considered to be an important strategy toward improved *in vitro* production of secondary metabolites.
Precursors are compounds supplemented to target the biosynthetic pathway and intermediates can be used as precursors then converted into down-stream compounds after specific enzyme catalysis. The concentration of precursors determines the reaction speed since the reaction speed is usually higher than that when a precursor concentration is low (7). The addition of a precursor is so advantageous when it is inexpensive. The role of amino acids in the biosynthesis of hyperforin was reported in *Hypericum. perforatum* shoot cultures. Isoleucin, when added as a precursor, stimulated a 2-fold increase in the accumulation of hyperforin (8). Another example, production of titerpenes in leaf derived callus and cell suspension cultures of *Centella asiatica* was enhanced after feeding the medium with amino acids (9). Through plant cell culture, these intermediates are maintained at very low concentration, and convert to downstream phytochemicals quickly. Therefore, for one specific plant species, the precursor concentration must be carefully adjusted and optimized (7). Thus, the aim of this study is to increase and accelerate the production of polyphenols and estimating their quantities in whole plants and cell cultures exposed to Tyrosine or Phenylalanine treatment and thereafter comparing polyphenols accumulation between intact plants and tissue cultures after treatment and estimating phenols that leaked to the liquid medium.

Materials and methods

1. Plant material

Coleus blumei (Lamiaceae) plants were purchased from local nurseries in pots. Some were grown directly in the soil as bedding plants for continuous supply of explants for tissue culture work.

2. Preparation of stocks solutions

Stock solutions for plant growth regulators were prepared at the concentration 100 mg.l-^{1}, stored in a refrigerator at 50 ^{0}C, and then replaced monthly. Callus initiation was conducted according to the method of (10). The auxin 2, 4-D was added at 0.8 mg.l-1 0.5 mg.l^{-1} of NAA and 2 mg.l^{-1} BA, then added to the culture medium as required before autoclaving.

3. Preparation of plant tissue culture medium

Gamborg medium (11) was prepared and used in tissue culture experiments. Sucrose at 20 g.l^{-1}, Myo inositol 0.1 g.l^{-1} and 2,4-D, NAA and BA at different concentrations were added. The pH was adjusted to 5.8 then 8 g.l^{-1} of the agar type Agar-Agar was added to the nutrient medium, placed on a hot plate magnetic stirrer till boiling. Afterwards, aliquots of 10 ml were dispensed into 10 × 5cm culture vessels, sterilized by autoclaving at 121 ^{0}C under 15 1b.in^{2} pressures, for 15 min. Glassware and other instruments were placed in electric oven at 200 ^{0}C for 2 hrs. (12). The medium was left at room temperature (25 ^{0}C) to cool then explants were inoculated.

4. Surface sterilization of explants

Stem explants (1.5) cm long were surface sterilized according to the procedure of (13). Briefly, explants were excised, rinsed with tap water for 10 min, then transferred to a laminar air flow-cabinet where submerged in 70% ethanol for 1 min., washed with sterilized double distilled water, then rinsed with sodium hypochlorite at 1.5% (v/v) for 10 min, finally rinsed with sterile distilled water for three times.

5. Incubation of cultures

Surface sterilized stem explants (1 cm) long were inoculated into the culture vessels under aseptic conditions, placed in an incubator at 22-25 ^{0}C in dark (14).

6. Initiation and maintenance of calli

Callus was induced following the method of (10) using B5 medium supplemented with 2,4-D at 0.8 mg.l^{-1}, NAA at 0.5 mg.l^{-1} and BA at 2 mg.l^{-1}. Culture vessels were placed in an incubator at 25 ^{0}C. The response to callus induction of these explants to auxin and cytokinin combinations was evaluated after 30 days. The initiated calli were removed from the explants, then pieces weighting 50 mg each were sub cultured onto a newly prepared fresh medium supplemented with the same combinations of 2,4-D, NAA and BA as described previously. Callus fresh weight was then determined.

7. Spraying tyrosine or phenylalanine on intact plants

Phenylalanine and Tyrosine were prepared at the concentrations 0.20, 0.25, 0.30 g.l^{-1} and sprayed on vegetative parts twice. The first spray was done when plants were at 2 weeks old and the second was before

10 days of harvest. Fresh and dry weights were determined in both. Total content of phenols was estimated in the dried leaves and compared with control plants according to the procedure described by (15).

8. Addition of Tyr or Phe to callus cultures

A quantity of 350 mg fresh weight of initiated callus was subcultured on the same medium that was used in callus maintenance but without agar to be used as a liquid medium and supplemented with different concentrations of Phe (0.20, 0.25 or 0.30) $g.l^{-1}$ or Tyr (0.20, 0.25 or 0.30) $g.l^{-1}$. Three replicates for each concentration of Phe and Tyr were used. Culture vessels were placed into a shaking incubator in dark at 25 ± 1 ^{0}C for 30 days (16), these were filtered by using 2 mm sieve to separate cells and cell aggregates which were placed inside an electric oven at a temperature of 40 ^{0}C for 24 hrs. to determine the dry weight. Both cell clumps and liquid medium were subjected to phenolics determination.

9. Preparation of galic acid standard curve

Gallic acid (0.01) g was dissolved in 100 ml ethanol using a 100 ml volumetric flask. Concentrations were prepared (1-100) $\mu g.ml^{-1}$. The total phenols were expressed as $\mu g.l^{-1}$, gallic acid equivalent depending on the plotted standard curve (Figure 1).

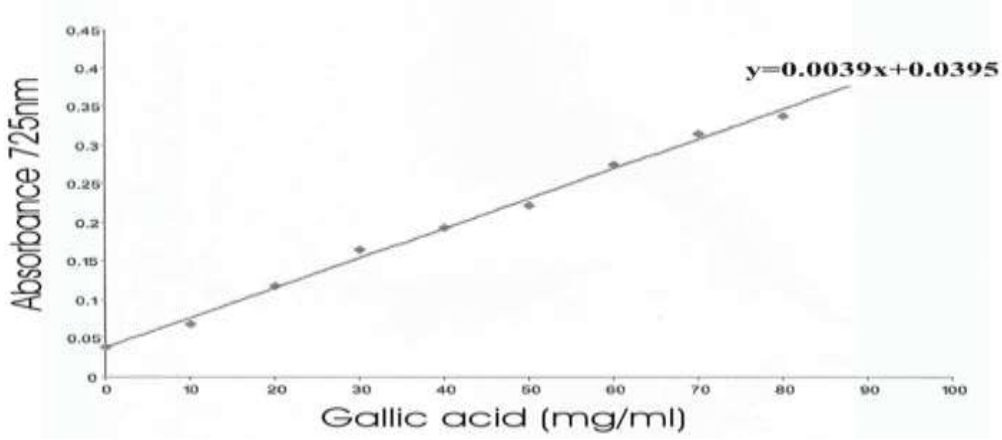

Figure (1): Galic acid standard curve

10. Total phenolic content in intact plants

Total phenolic content was determined by using Folin-Ciocalteu's method (15). Aliquot of 0.5 ml of the extract was oxidized with Folin-Ciocalteu's reagent (0.5 ml) and then neutralized with saturated 1 ml of 25% (w/v) of sodium carbonate solution. The volume was completed to 10 ml with distilled water, then vigorously agitated and allowed to stand for 45 min and then centrifuged for 5 min at 4000 g and the absorbance of the clear supernatants was measured at 725 nm by using spectrophotometer type Unico 3200.

11. Total phenols in cells aggregates

Phenolics were extracted from 50 mg of dried callus tissue with 2 ml of 70% ethanol in an ultrasonic bath at 70 ^{0}C for 20 min. The total phenol content was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method described by (17) using gallic acid (GA) as a standard and expressing the results as GA equivalents. The reaction was carried out by mixing 20 ml of ethanol extract, 1.58 ml of water, 0.1ml of sodium carbonate (200 $g.l^{-1}$). After 2 hrs at 37 ^{0}C, absorbance was measured at 725 nm and compared with the GA calibration curve. Data represented the mean of three independent measurements.

12. Total phenols in liquid medium

Aliquot of 1ml containing the liquid medium was transferred into dark universal vial then 2 ml of 70% ethanol was added, and the mixture was left for 24 hrs.

Results and discussion

1. Effect of Tyrosine spraying on fresh and dry weights

Spraying of vegetative parts by tyrosine exhibited a significant increase in both fresh and dry weights of shoots compared with control except at 0.2 $g.l^{-1}$ which did not differ from the control in relation to dry weight of shoots (Table 1). Concentrations at 0.2, 0.25, or 0.3 $g.l^{-1}$ gave mean values of shoot fresh weight 15.067, 15.583, and 15.90 g respectively compared with control treatment which recorded 11.05g, while the mean dry weight values for the vegetative parts at the same levels were 2.217, 3.073 and 3.127 g respectively compared with control which recorded 1.837 g.

Table (1): The effect of spraying tyrosine on shoot and root fresh and dry weight of *Coleus blumei* after 15 days of treatment

Tyrosine ($g.l^{-1}$)	Shoots		Roots	
	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
Control	11.050 b	1.837 b	4.183 a	0.643 a
0.2	15.067 a	2.217 ab	4.213 a	0.587 a
0.25	15.583 a	3.073 a	4.190 a	0.573 a
0.3	15.900 a	3.127 a	5.140 a	1.093 a
LSD P≤0.05	3.940	0.921	3.068	0.592

***similar letters refer to none significant differences between means.**

Although there was a slight increase in both root fresh and dry weight due to the treatment with tyrosine, but such increments were not significantly different with control. Data presented in table (1) showed that treatment with the amino acid tyrosine caused a significant increase in fresh and dry weight of shoots compared with control, the more pronounced effect on these growth criteria were obtained in the treatments 0.25 and 0.30 $g.l^{-1}$. Foliar application of 0.30 $g.l^{-1}$ tyrosine resulted in the greatest increments on the fresh and dry weight of shoot compared with other treatments. Similar findings were obtained in beet plants by {18} where tyrosine and proline proved to be successful agents in improving growth and yield characters of beet plants, especially at 100 and 200 $mg.l^{-1}$ respectively. Amino acids have been shown to improve growth and yield of plants,

Investigation revealed that tyrosine was more effective than proline in increasing vegetative growth of plants,especially at 100 $mg.l^{-1}$ (18). Tyrosine is an aromatic amino acid, not only used for the synthesis of proteins, but also serves as an important precursor for natural products, including pigments, alkaloids, and hormones (19).

Because of the preferable properties of tyrosine, and may be other amino acids in improving growth parameters, they can be used in arid and semi-arid countries where there is a crucial need to increase yield of important horticultural crops without using excessive fertilization. From the environmental issue, amino acids are cheap and biodegradable chemicals which is likely due to the N-content of these amino acids, even though their metabolic functions are different.

2. Effect of phenylalanine spraying on vegetative fresh and dry weights

Fresh weight of the vegetative parts revealed a significant increase compared with control after spraying with PhA except at 0.2 g.l^{-1}, recording 12.087 g, 12.383 g and 13.47 g at PhA concentrations 0.2 and 0.25 or 0.3 g.l^{-1} respectively compared with control treatment which recorded 11.05 g (Table 2). Results of shoots dry weight exhibited a significant increase at 0.25, 0.30 g.l^{-1} of Phe recorded 2.79, 2.88 g respectively. Data presented also showed that treatment with the amino acid Phe at 0.25 and 0.3 g.l^{-1} caused a significant increase in fresh and dry weight of aerial parts. Foliar application of 0.30 g.l^{-1} Phe resulted in the greatest augmentation on the fresh and dry weight compared with other treatments. This positive effect on growth could be explained through the influence of gibberellin biosynthesis and accumulation of amino acids which are the building blocks of proteins regulating metabolism, transport and act as nitrogen storage. Amino acids may protect plant from ammonia toxicity as they remove amide formation, serving as a source of energy as well as biosynthesis of other organic compounds such as amines, purines, pyrimidines, vitamins, enzymes and terpenoids (20).

Table (2): Effect of Phenylalanine spraying on the shoot and root fresh and dry weight of *coleus blumei* after 15 days of treatment

Phenylalanine g.l^{-1}	Shoots		Roots	
	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
Control	11.050 c	1.837 b	4.183 a	0.643 a
0.2	12.087 bc	2.543 ab	4.877 a	0.660 a
0.25	12.383 ab	2.790 a	4.433 a	0.667 a
0.3	13.470 a	2.880 a	4.857 a	0.840 a
LSD P≤0.05	1.269	0.811	1.899	0.503

***similar letters refer to no significant differences, different letters refer to significant differences between means**

3. Total phenols accumulation in plants: (spraying with tyrosine or phenylalanine)

Total phenols accumulated in *Coleus* leaves exhibited a significant increase when tyrosine was supplied to the plant shoots recording 62, 68.58 and 64.31 µg.l^{-1} at 0.20, 0.25 and 0.30 g.l^{-1} respectively compared with control treatment (44.48) µg.l^{-1}.

Table (3): Total phenols (µg.l^{-1}) accumulated in *C. blumei* leaves after spraying with different concentrations of tyrosine after 15 days of treatment

Tyrosine(g.l^{-1})	Total phenols(µg.l^{-1})
Control	44.48 b
0.20	62.00 a
0.25	68.58 a
0.30	64.31 a
L.S.D P≤0.05	14.78

Table (4) exhibits that the leaves of *C. blumei which were sprayed with* phenylalanine led to significant increase in total phenols at 0.20 and 0.25 g.l^{-1} phe while not at 0.30 g.l^{-1} , recording 66.530 , 61.463 and 54.227 µg.l^{-1} compared with control treatment (44.483) µg.l^{-1}.

The Total phenols accumulated in *Coleus* leaves exhibited a significant increases when Pha or Tyr supplied to the plant shoots compared with control. Both precursors revealed dissimilarities response in *Coleus* plant for enhancement of total phenols. Tyr increased total phenols at all concentrations used, 0.20, 0.25 or 0.30 g.l^{-1} while Phe gave significant differences in the concentrations 0.20 and 0.25 g.l^{-1} only. Foliar spray with the amino acids might induce high activity of key enzymes of the phenylpropanoid biosynthetic pathway. High activity of these enzymes in a precursor sprayed on different plants species was documented previously (20).

Table (4): Total phenols (µg.l^{-1}) accumulated in *C. blumei* leaves after spraying with different concentrations of Phenylalanine after 15 days of treatment

Phenylalanine (g.l^{-1})	Total phenols (µg.L^{-1})
Control	44.48 c
0.20	66.53 a
0.25	61.46 ab
0.30	54.23 bc
L.S.D P≤0.05	11.27

4. Tyrosine addition to callus cultures

Table (5) exhibits that Tyr addition to the medium led to a significant increase in all treatments compared with control. Total phenols mean values were 205.93, 218.46 and 215.94 µg.l^{-1} in callus tissues at tyr concentrations 0.20, 0.25 and 0.30 g.l^{-1} compared with control treatment (17.99) µg.l^{-1}. Total phenols which accumulated in calli approached 12 times than those accumulated in control treatment. In liquid medium, total phenols illustrated a significant increase recording 142.43 and 66.79 µg.l^{-1} at 0.2 and 0.25g.l^{-1} tyr respectively compared with control (14.65 µg.l^{-1}), while the treatment with 0.25 and 0.3 g.l^{-1} although decreased but still higher than control.

Table (5): Total phenols (µg.l^{-1}) accumulated in callus tissue and liquid medium after exposure to different concentrations of tyrosine

Tyrosine (g.l^{-1})		Mean
Callus	Control	17.99 b
	0.2	205.93 a
	0.25	218.46 a
	0.3	215.94 a
Liquid medium	Control	14.65 c
	0.2	142.43 a
	0.25	66.79 b
	0.3	42.09 bc
L.S.D P≤0.05		33.07
Callus		164.58 a
Liquid medium		66.49 b
L.S.D P≤0.05		16.54

Results exhibited that total phenols are almost doubled in callus culture (164.058) µg.l^{-1} than those accumulated in liquid medium (66.49) µg.l^{-1}.

6. Phenylealanine addition to callus cultures

Table (6) shows that total phenols displayed a significant differences when Phe was added to the callus cultures.

Total mean values in callus tissues were 199.87, 193.84 and 215.26 µg.l^{-1} Phe at concentrations 0.20, 0.25 and 0.30 g.l^{-1}, compared with control treatment which recorded (17.99) µg.l^{-1}. Total phenols which accumulated in callus tissues approached 10 times than those accumulated in control treatment. Treatment with 0.30 g.l^{-1} Phe recorded the highest mean value in callus tissues compared with other concentrations. In liquid medium, total phenols showed a proportional increments with the increase in Phe concentration recording 29.23, 31.03 and 78.72 µg.l^{-1} at Phe concentrations 0.20, 0.25 and 0.30 g.l^{-1} respectively compared with control treatment(14.65) µg.l^{-1}. Meanwhile treatment with 0.30 g.l^{-1} phe recorded the highest mean value in liquid medium which was significantly higher than other means. Total phenols highly accumulated in callus tissue compared with liquid medium recording 215.255 and 78.72 µg.l^{-1} respectively. All means were significantly higher than control.Total phenols accumulated in callus (156.74) µg.l^{-1} was significantly higher than those accumulated in liquid cultures (38.4) µg.l^{-1}.

Table (6): Total phenols (µg.l^{-1}) accumulated in callus tissue and liquid medium after exposure to different concentrations of Phenylalanine

Phenylalanine (g.l^{-1})		Mean
Callus	Control	17.99 c
	0.2	199.87 ab
	0.25	193.84 b
	0.3	215.26 a
Liquid medium	Control	14.65 b
	0.2	29.23 b
	0.25	31.03 b
	0.3	78.72 a
L.S.D P≤0.05		20.87
Callus		156.74 a
Liquid medium		38.40 b
L.S.D P≤0.05		10.43

In the present study, addition of tyrosine or Phe to the culture medium increased the synthesis of phenolic compounds (Table 5, 6). This presented in total phenols and which might be due to stimulating the key enzyme PAL activity which is the gate way of shikimik acid pathway. Pervious research supported the view, that addition of the precursors amino acids to the culture medium resulted in stimulating some intermediates at the beginning of secondary metabolic biosynthesis route. The additives might induce activity of key enzymes such as phenylpropanoid biosynthetic pathway. High-activity of these enzymes in a precursor fed culture of different plant species was observed by pervious workers. A positive correlation was observed in polyphenol synthesis and rosmarinic acid production in a study conducted by (21).

Among the two precursors tested, tyrosine was more efficient in total phenols in callus tissues and liquid medium since it accumulated more phenols than phenylalanine. Total phenol content augmented to 218.46 µg.l^{-1} in callus tissues after addition of 0.25 g.l^{-1} tyrosine reached 142.43 µg.l^{-1} in liquid medium after adding 0.20 g.l^{-1} tyrosine (Table 5). It is nearly twelve folds times greater than the control of callus tissues and nearly ten folds greater than control of liquid medium. Phe treatment augmented total phenols up to 215.26 µg.l^{-1} in callus tissues and 78.72 µg.l^{-1} in liquid medium after addition of 0.30 g.l^{-1} Phe (Table 6). High concentration of tyrosine did not favor increment in total phenol content. Results agree with studies applied excess amino acids exogenously which resulted in an increase in the synthesis of phenolic content in tissue cultures (22).

In the present investigation, total phenols were very high as compared with total phenols of the plant leaf *in vivo*. Callus tissues and liquid medium showed 348.36 µg.l^{-1} of total phenols in both after treatment with tyrosine at 0.20 g.l^{-1} (Table 5) while Phe applications showed 293.98 µg.l^{-1} total phenols in both callus tissues and liquid medium at 0.30g.l^{-1} (Table 6) compared with the highest amount of total phenols *in vivo* (68.58,66.53) µg.l^{-1} for Tyr and Phe treatments respectively (Table 3, 4). This may due to the controlled conditions *of vitro* cultures, so it is concluded that secondary products in plant cell culture can be generated on a continuous year-round basis, no seasonal constraints, production is independent of ambient weather, the yield per gram fresh weight may exceed that which is found in the field grown plants. Plant cell culture eliminates potential political boundaries or geographic barriers to the production of a crop or a compound. Extraction from *in vitro* cultures is much simpler than extraction from organized, complex tissues of a plant (23) and can be produced on a large scale using bioreactors.

Leakage of total phenols to the liquid medium after addition of precursors occurred and the highest percentage was at 0.20 g.l^{-1} in the liquid medium supplemented with tyrosine, and at 0.30 g.l^{-1} in the liquid medium supplemented with Phe Tables (5, 6).

Conclusion

The conclusion of the results are concluded that plant cell culture is effective tool for both studying and producing plant secondary metabolites under controlled *in vitro* conditions. Tyrosine and phenylalanine were effective in increasing the yield of polyphenols particularly at the cellular level. For industrial purposes, it is recommended to harvest both callus and liquid medium.

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