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EFFECT OF L-PHENYLALANINE ON THE PRODUCTION OF SOME ALKALOIDS AND STEROIDAL SAPONINS OF FENUGREEK COTYLEDONS DERIVED CALLUS

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ABSTRACT

The aim at this study is to increase some alkaloids and steroidal saponins in *Trigonella foenum-graecum* L. Callus initiation was carried out culturing cotyledons of the fenugreek on MS medium achieved by 1.0 mg/l^{-1} 2, 4-D and 0.4 mgl^{-1} kinetin. The best medium to maintain callus was MS medium accomplished by 0.5 mg/l^{-1} 2, 4-D and 0.5 mg/ l^{-1} kinetin. Callus formation was allowed to take place for thirty days in the dark at 25±2 °C. Callus was analyzed using high performance liquid chromatography (HPLC). Methanol callus extract showed high concentrations of some alkaloids and steroidal saponins compared to the methanol extract of cotyledons. For increasing, the concentration of secondary metabolites, L-phenylalanine was added at concentrations 0, 100, 200 or 300 mg/l^{-1}. L-phenylalanine at 200 mg/l^{-1} led to significant increase in diosgenin, choline and trigonelline reaching177.82, 415.12 and 1815.53 µg/ ml^{-1} per 100 mg fresh weight of callus respectively, L-phenylalanine at 100 mg/l^{-1} increased smilagenin, tigogenin and carpaine reached 259.52, 122.87 and756.72 µg/ ml^{-1} per 100 mg respectively.

Key words: *Trigonella foenum-graecum* L., steroidal saponins, alkaloids, L-phenylalanine.

INTRODUCTION

Fenugreek *Trigonella foenum-grae- cum* L.is an herbal plant belong to the leguminosae family (ALShehat, 2000). It is commonly used as a food and medicine and is a rich source of steroidal sapogenins, including (diosgenin, smilagenin, tigogenin) and diosgenin, which is one of the most important compounds discovered as strong anti-cancer agent (Lohvina *et al.*, 2012). In addition, fenugreek is a source of some alkaloids such as trigonelline, carpaine and choline (Khorshi-dian *et al.*, 2016). Moorthy *et al.*, (2010) mentioned that the trigonelline has several therapeutic properties, including treatment against carcinogens of the cervix and liver cancer and used as antifungal and anti-cholesterol agent. Plant cells naturally produce secondary metabolites that are the end products of primary metabolism. Studies have shown that it is possible to increase the production of active substances from plant tissues when adding some chemicals such as precursor's molecules to the nutrient medium. They are directed towards the biosynthetic pathways of secondary metabolites. The information available on accelerating the bio-production of alkaloids by including amino acids in the medium was encouraging and enhances (Ramawat, 2004). There is a truth say that the amino acid not only works a "precursor" but also catalysis agent. (Abyari *et al.*, 2016) showed in a study of *spilanthes acmella*, and used different concentrations of L-phenylalanine in MS medium to stimulation and production of secondary metabolites in plant cells cultures. It was observed that the presence of L-phenylalanine in the nutrient medium at a concentration of 100 µM/l^{-1} was more effective for the production of secondary metabolites and for the high accumulation of some alkaloids in the cell suspension. (Jassim, 2011) noted that when different concentrations of L-phenylalanine were added to the MS medium, there was an increase in the compounds alkaloids but, the addition of 10 mg/l^{-1} phenylalanine with sucrose at a concentration of 60 mg/l^{-1}resulted in the highest amount of some alkaloids from the induced callus of the shoot tip of belladonna. This study was achieved to stimulate the callus and increased the production of some alkaloids and steroidal saponins of economic interest from fenugreek *in vitro* and by L-phenylalanine was added.

MATERIALS AND METHODS

The seeds of the Indian fenugreek plant were used and obtained through an agricultural office in Baghdad. The seeds were planted in pots plant filled with peat moss and sand 1:1 after germination, seedlings were left to grow naturally until formation of the cotyledons. Samples of the cotyledons were taken for the chemical analysis.

Sterilization and culturing of seeds: Seeds were treated with 70% ethanol for 60 seconds and then were submerged with 4.5 % of NaOCl for 15 minutes. After that, the seeds were rinsed three times with sterile distilled water. The antiseptic seeds were transferred to MS media with half

strength of the salts inside the laminar air flow cabinet (figure 1a). The seeds were then placed in the incubator chamber with light intensity of 1000 lux for 16 hours at 25°C ± 2 (Jassim, 2011).

Induction and maintenance of callus: Cotyledons (figure1b) of the sprouts were cut under aseptic conditions into 5 mm long for callus induction. For initiation of callus from cotyledon, MS containing 30 g/l^{-1} sucrose supplemented with two plant growth regulator types were used to assess their effect on callus induction. Cotyledons explants were cultured in culture tubes containing MS medium supplemented with (1 mg/l^{-1}) 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.4 kinetin then distributed in to 10 replicates for 30 days (figure 1c). PH value of the culture medium was regulated to 5.7 prior to autoclaving with 7 g/l^{-1} (w/v) agar, autoclaving at (15 min., 121°C and a pressure of 1.04 kg/cm^{-1}) was achieved. In this study, the maintenance of callus was used 0.5 mg/l^{-1} (2, 4-D) and 0.5 mg/l^{-1} kinetin (figure 1d).

Figure 1a: Seeds on MS media with half strength of the salts b: Seedling plantlet after 7 days. c: Callus on MS plus 1 mg/l^{-1} 2,4-D with 0.4 mg/l^{-1}kin. After 30 days. d: Callus on MS plus 0.5 mg/l^{-1} 2,4-D with 0.5 mg/l^{-1}kin

Stimulation of the production of secondary compounds in callus: A weight of one hundred and fifty milligrams from callus was taken and was cultured on MS medium supplemented with 0.5 mg/l^{-1} of 2, 4-D and 0.5 mg/l^{-1} of kinetin and with different concentrations of L-phenylalanine (0,100, 200 or 300 mg/l^{-1}). The number of replicates was 10 per concentration of L-phenylalanine. All calli were incubated in the dark at 25±2 °C. After 30 days, callus was sampled for the extraction of secondary metabolites.

Extraction and assessment of active substances in Callus: The separation happened on liquid chromatography Shimadzu 10AV-LC supplied with binary delivery pump model LC-10A Shimadzu. The eluted peaks were monitored by UV-Vis 10A-SPD spectrophotometer.

Alkaloid extraction: A weight of 3 grams from the cotyledons of the plants grown in pots, and 100 mg of callus grown *in vitro*. Plant samples were homogenized, grinding, dissolved in 3% H2SO4 for 2 hours at room temperature. The supernatants were made alkaline with 25% NH4OH (pH 9.5) and applied to (Merck) columns. The alkaloids were eluted by CH2Cl2 (6 ml per 1 g extrelut) and the extracts were evaporated to dryness. Thus, obtained residues were resolved in CH3OH for the further analysis by HPLC according the optimum separation of authentic standard, then 20µl was injected on HPLC column. The concentration of each compound was quantitatively calculated by comparison the peak area of the standard with that of the samples (Zaho *et al.*, 2002). The alcoholic extract was separated on Fast Liquid Chromatographic (FLC) column, 3 µm particle size (50×4.5 mm, I.D) C18-DB (debased) col-umn. Mobile phase was 0.01M phosphate buffer PH 8.2: acetonitrile (55:45, V/V). The detection UV set at 220 nm and flow rate of 0.9 ml/min.

Separation of steroidal saponins: HPLC column; Lichrospher C18 (50x4.6mm, I.D) 3µm particle size was used. Mobile phase of: aceto-nitrile: water (25:75, V/V) was used. The detection: UV set at wavelength 203 nm and flow rate was 1.2 ml/min. Temperature was 30°C for (100 mg) of each sample was weighed, then dissolve in 10 ml HPLC methanol. The samples were shaking and agitated in ultrasonic bath for 10 minutes, then were concentrated by evaporating the solvent with a stream of liquid N2 until reach nearly 0.5 ml, then add some of the mobile phase reached 1 ml and then 20µl was injected on HPLC column. The concentration of each compound was quantitatively determined by comparison the peak area of the standard with that of the samples (Yang *et al.*, 2003).

Conc. of sample = area of sample/area of standard× conc. of standard × dilutions factor.

Note: that the concentration of the standard solution=25µg/ml and Number of dilutions=4

Statistical analysis: were used CRD and compared the averages by using the least significant difference L.S.D to show the statistical differences among coefficients and the probability level of 0.05 (SAS, 2012).

RESULTS

Figure 2 shows the standard curve of secondary metabolites compared with the curves of compounds extracted from cotyledons of fenugreek grown under field conditions and extracted callus (figures 3a, 3b, 3c and 3d) showed the HPLC curves resulted using different concentrations of L-phenylalanine, which showed the presence of secondary metabolites. (table1) Callus gave the highest concentration of diosgenin, choline and trigonelline reached 177.82, 415.12 and 1815.53 µg/ml⁻¹ respectively at the concentration 200 mg/l⁻¹ L-phenylalanine, compared with control treatment which recorded of 87.62,214.84 and 121.42 µg/ml⁻¹ per 100 mg fresh weight of callus respectively while smilagenin, tigogenin and carpaine significantly increased reached 259.52, 122.87 and 756.72 µg/ml⁻¹at the concentration 100 mg/l⁻¹ L-phenylalanine compared with control treatment which recorded of 172.58, 52.02 and 70.69 µg/ml⁻¹ per 100 mg fresh weight of callus respectively while diosgenin, choline , trigonelline, smilagenin, tigogenin and carpaine the lowest concentration recorded was in cotyledon 89.42, 59.35, 63.278, 68.616, 1.11 and 26.175 µg/ml⁻¹ per 3 g fresh weight of intact plant respectively (Table 2 and figure 4).

Table 1: Effect of L-phenylalanine concentrations on some secondary metabolites from callus.

L-phenylalanine µg.ml⁻¹	carpaine µg.ml⁻¹	trigonelline µg.ml⁻¹	choline µg.ml⁻¹	tigogenin µg.ml⁻¹	smilagenin µg.ml⁻¹	diosgenin µg.ml⁻¹
0	70.69	214.84	87.62	52.02	172.58	121.42
100	756.72	1480.16	17.45	122.87	259.52	1.13
200	8.17	1815.53	415.12	107.33	224.3	177.82
300	299	431.77	95.63	115.36	83.31	104.33
L.S.D 0.05	84.27*	163.75*	10.26*	13.07*	27.82*	27.91*

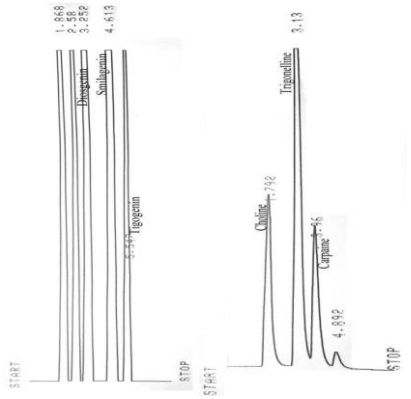


Figure 3a: HPLC analysis of some secondary metabolites in callus.

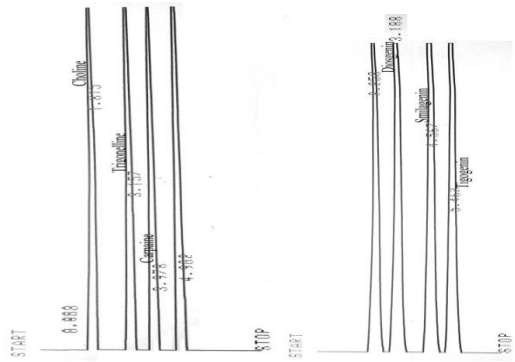


Figure 2: Standard curve of some secondary metabolites in fenugreek using HPLC.

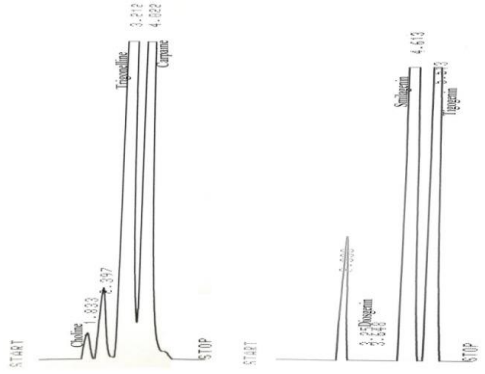
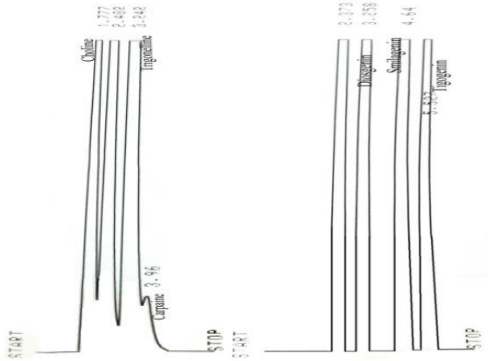


Figure 3b: Some secondary metabolites that detected with HPLC at 100 mg/l⁻¹ of L-phenylalanine.



Figre 3c: Some secondary metabolites that detected with HPLC at 200 mg/l⁻¹ of L-phenylalanine.

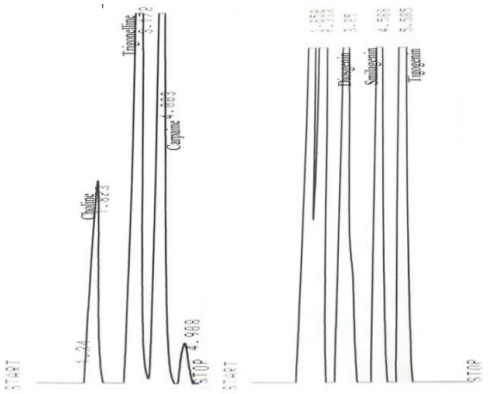


Figure 3d: Some secondary metabolites that detected with HPLC at 300 mg/l^{-1} of L-phenylalanine.

Table 2: Some secondary metabolites detected and quantified (µg.ml^{-1}) in the cotyledon extract and callus using HPLC.

Secondary metabolites (µg.ml^{-1})	per100 mg callus	per3000 mg cotyledon
Diosgenin	121.42	89.42
Smilagenin	172.58	68.616
Tigogenin	52.02	1.11
Choline	87.62	59.35
Trigonelline	214.84	63.278
Carpaine	70.69	26.175

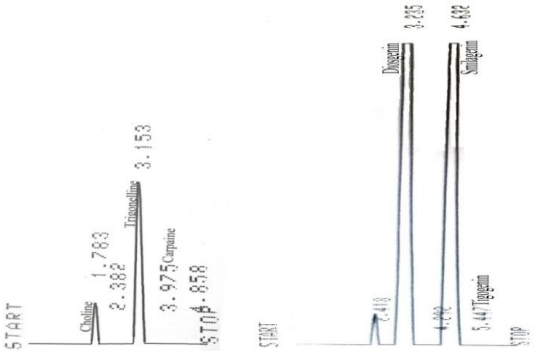


Figure 4: HPLC of some secondary metabolites in cotyledon extract.

DISCUSSION

Among the biotechnological ways, induction of metabolic pathways in the cultivated plant tissue cells toward production of a required compound most likely outcomes in a dramatic increase in secondary metabolites product (Wang *et al*., 2001). The results indicated that insertion of L-phenylalanine in the MS medium was found to be more active for the production of secondary metabolites in *Trigonella foenum-graecum* L. The results were deal with Abyari *et al.*, (2016) that used for L-phenylalanine to increase secondary metabolites of *Spilanthes acmella*. The success of the use of L-phenylalanine with Jassim, (2011) was associated with an increase in concentrations of this amino acid, leading to an increase in the alkaloids. This may be due to the synthetic origin of these compounds directly entered in the bio construction path of secondary metabolites (Ramawat, 2008). The precursor is encouraged to produce secondary metabolites are often either increased by the specified amounts of synthetic precursors or stimulated biochemistry enzymes or both (Demain, 1998). The increase in the production of secondary metabolites may be due to an increase in L-phenylalanine concentrations, which have added to the medium to represent the synthetic precursor to many of the secondary metabolites. The addition L-phenylalanine also stimulates enzymes that synthesize these alkaloids (Khanna *et al.*, 2005). The increased concentration of 300 mg /l^{-1} resulted in a decrease in alkaloids production as shown in table 1 and the increase in nitrogen compounds has a negative effect on plant content of secondary metabolites. The increase in amino acids will lead to the increase of dissolved

nitrogen compounds inside the cells, which leads to an increase in the pressure of the osmotic, which drives them to withdraw water from neighboring cells filled and the cell wall becomes soft and thin, and are easily torn apart, leading to the destruction of cells as showed (Coruzzi and Last, 2000). Increased phenylalanine concentrations may be due to increased stress, which has negatively impacted the cells, causing damage, thus reducing the efficiency of the enzymes responsible for the synthesis of secondary metabolism (Abdelkader, 1982). These results were agreed with a study conducted by Ortuno *et al*, (1998). He confirmed that the amount of diosgenin and tigogenin isolated from one of the plant species of the fenugreek was proportional to the growth of callus and reached the highest level in the callus by eight weeks as mentioned earlier. The amount of diosgenin in leaf callus may be due to its quantity in the leaves of the fenugreek *in vivo* because of age of the plant and the growth regulators used to support the nutrient medium or both (Al-Mallah and Zubaidi, 2007). The present study and previous pointed that the L-phenylalanine has active role for production of secondary metabolites in cell culture of different plant types. These results were not consistent with Ciura *et al*., (2015). The level diosgenin of in leaves were high compared with the content of this compound in callus cultures. This may be due to the use of one type of growth regulator. A rather than a combination of growth regulators with auxines supported in the nutrient medium as mentioned above. The results also agreed with Ahmed *et al*., (2000) that the trigonelline content of the leaves was 0.45 mg/g dry weight. The content of this compound was increased in the callus of leaves to 0.61 mg/g dry weight.

CONCLUSIONS

Some steroidal saponins and alkaloids in callus of fenugreek significantly increased after treatment with L-phenylalanine.

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