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HPLC ANALYSIS OF THE TRIGONELLINE AND DIOSGENIN IN THE CALLUS OF FENUGREEK (*TRIGONELLA FOENUM GRAECUM* L.) SEEDS

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Abstract

Medicinal plants form an essential part of indigenous pharmaceutical system. *Trigonella foenum-graecum* (L.) (Fabaceae, Fenugreek) is employed in many traditional systems of medicine. The aim of this study is to increase some alkaloids and steroidal saponins. Callus initiation was carried out culturing seeds of the fenugreek on MS medium achieved by 1.0 mg/l^{-1} 2, 4-D and BA. Callus formation was allowed to take place for 45 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 seeds. For increasing, the concentration of secondary metabolites, magnetic water treatments' were added at three guesses 1000, 2000 and 3000 guess. The best medium to maintain callus was MS medium accomplished by magnetic water, at 3000 guess led to significant increase in diosgenin and trigonelline reaching 218.201 and 29.401µg/ ml^{-1} per 1 gm. dry weight of callus respectively. Statistical significant differences were observed between the results obtained by the means were compared using the Least Significant Difference test (L.S.D) at P≤ 0.05 level.

***Keywords*:** Fenugreek (*Trigonella*), Diosgenin, Trigonelline, cell cultures, high-performance liquid chromatography, quantitative determination.

Introduction

Plant cells naturally produce secondary metabolites that are the end products of primary metabolism. Fenugreek *Trigonella foenum-graecum* (L.) is an herbal plant belong to the leguminosae family (AL-Shehat, 2000). It is commonly used as a food and medicine and is a rich source of steroidal sapogenins, including diosgenin (25R-spirost-5-en-3β-ol) as a secondary metabolite produced by this plant (Chaudhary *et al*., 2018). Diosgenin deserves particular attention due to its biological activities and pharmaceutical applications. It is mainly used as a precursor for the production of many steroidal drugs, oral contraceptives and sex hormones such as testosterone, glucocorticoids and progesterone (Raghuram *et al.,* 1994). In addition, fenugreek is a source of some active ingredients such as pyridine alkaloids mainly trigonelline (Khorshidian *et al.*, 2016). It is a hormone finding naturally in plant products and is a vitamin B6 derivative. Moorthy *et al*. (2010) mentioned that the trigonelline was isolated from fenugreek seeds and proved to be beneficial in diabetes and has several therapeutic properties, including treatment against carcinogens and used as antifungal and anti-cholesterol agent. Plenty of the stress induced compounds are useful secondary metabolites in response to various abiotic stress factors. For example, in vitro cultured plant cells were noted to synthesize extra amounts of trigonelline in callus of *Trigonella foenum graecum* L. (Alalwani and Alrubaie, 2016). 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 magnetic water was added. Magnetic water is obtained by passing water through permanent magnets or through the electromagnets installed in or on a feed pipeline.

Materials and Methods

Collect and sterilize seeds: The fenugreek plant seeds obtained from local markets in the Babylon. A suitable amount of seeds takes in a flask and sterilized in 70% ethanol for 25 sec with hand shaking and 2% hypochloride sodium solution for 10 minute. Seeds were rinsed several times in sterile water.

Induction and maintenance of callus: After surface sterilization, the seeds were directly cultured in the germination basal medium MS (Murashige and Skoog's, 1962) supplemented with 1mg/l of 2,4-D and BA and incubated in growth room at 25±.1 ºC.

Stimulation of the production of secondary compounds in callus: A weight of 150 mg from callus was taken and cultured on MS medium, it is prepared using magnetized water instead of distilled water with three guesses including (1000, 2000 and 3000 guess). Distilled water has been considered a control.

Quantitative analysis of Diosgenin and Trigonelline compounds by High Performance Liquid Chromatography (HPLC) according of (Li *et al.,* 2012)

Parameter of HPLC: For HPLC analysis, diosgenin was conducted at detectors (UV – Vis, 210 nm) on a reversed-phase Agilent TC-C18 column (ODS 25 cm * 4.6 mm) was used for the separation. The mobile phases were composed of acetonitrile-water (90 : 10, v/v) as well as a diluent for preparing the sample extract. Column temperature was maintained at 30°C and the flow rate of the mobile phase was kept at 1 ml per min. The changes in absorbance at wavelength 210 nm were recorded with UV detector, and spectra from 190 to 400 nm were recorded on-line for peak identification. The peak area was calibrated to diosgenin content with a standard. In the same technique, the content of trigonelline was determined using the HPLC analysis, which is based on the union method and education of the standard material concentration.

Preparation of a sample extract: 1.5 g of crushed dry callus was weighed and placed in a 10-ml volumetric flask. Acetonitrile (about 5 ml) was added to ensure complete dissolving with ultrasonic treatment (10 to 15 minutes). The solution was diluted to a fixed volume 10 ml with phosphoric acid (5M) / methanol and then returned to the ultrasound device for 20 minutes, the sample subjected to centrifugation at 6000 rpm for 5 min for isolate the upper layer which transferred to the rotary evaporator for reduce the

dehydration and add 3 ml from acetonitrile, then filtered through a filter (pore size : 0.22 µm) before analysis.

Standard curve Solutions: The standards for each diosgenin and trigonelline were dissolved in chloroform and 100% methanol (respectively) to prepare a stock solution of 1000 ppm. Working standard solutions were prepared by serial dilution of the stock standard. Aliquots of standard solution containing 1 mg per L^{-1} of diosgenin or trigonelline were diluted with acetonitrile : water (90:10) solution to different concentrations. A calibration curve was constructed by plotting the peak area versus the respective diosgenin or trigonelline quantity [µg] and the obtained data were subjected to regression analysis. After Optimization of HPLC conditions as a standard procedure was carried out prior to analysis, The reliance on the following equation: *sample con.= standard con.*(sample area/standard area)*dilution factor*

Table : shows the effect of magnetic water on quantitative analysis for secondary compounds using by (HPLC).

Water quality		Secondary Compounds (means) µg/ml⁻¹ per 1g dry weight callus	
		Trigonelline	Diosgenin
Distilled water (control)		6.301	49.401
Magnetic water (guess)	1000	13.601	100.901
	2000	22.301	158.501
	3000	29.401	218.201
L.S.D.(0.05) =		7.211	48.673

Results

HPLC is more sensitive for diosgenin analysis and this method was characterized by compliance accuracy, which were then employed to detect the diosgenin content in the callus culture of the fenugreek seed. In the same technique, the content of trigonelline was determined using the HPLC analysis, which is based on the union method and education of the standard material concentration. Figure 1 and 2 shows the standard curve of secondary metabolites compared with the curves of compounds extracted from seeds callus of fenugreek. The concentration of each compound was quantitatively determined by comparison the peak area of the standard with that of the samples, figures (4, 5, 6) refers to the HPLC curves resulted using different guesses of magnetic water, which showed the presence of secondary metabolites. In above **table** callus gave the highest concentration of diosgenin and trigonelline reached 218.201 and 29.401 µg/ml⁻¹ respectively at the guess 3000 magnetic water, compared with control treatment which recorded of 49.401 and 6.301 µg/ml⁻¹ per 1 g dry weight of callus respectively while this metabolites (diosgenin and trigonelline) significantly increased reached 100.901, 13.601 and 158.501, 22.301 µg/ml⁻¹at the guesses 1000 and 2000.

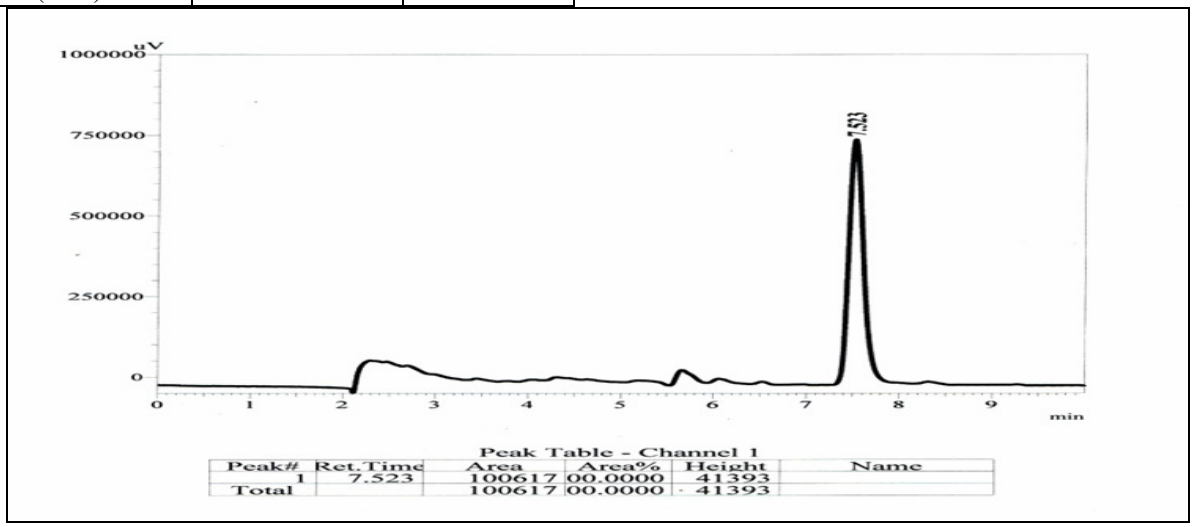


Fig. 1 : The stander curve of Diosgenine by using HPLC.

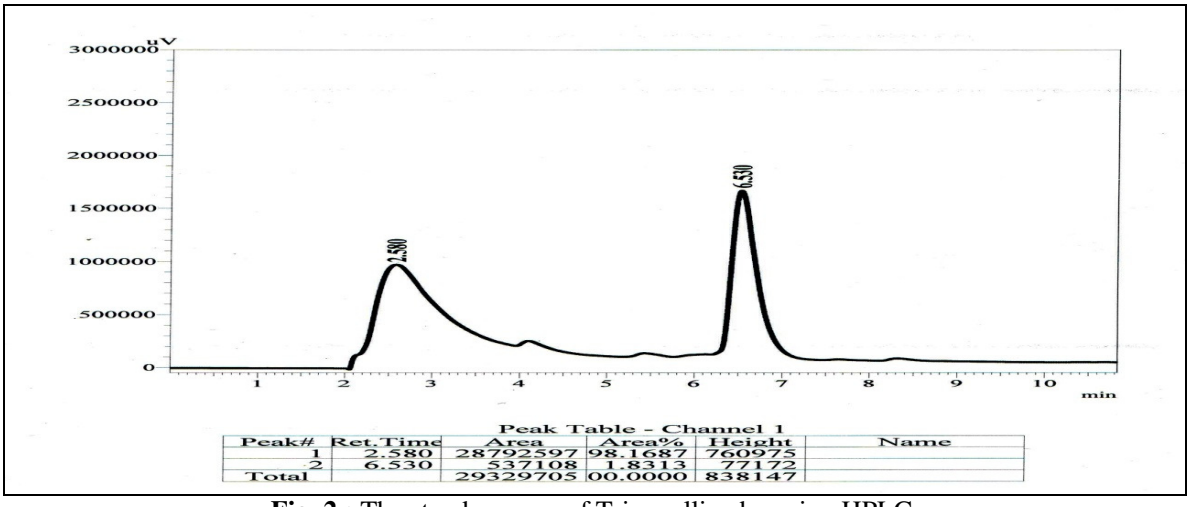
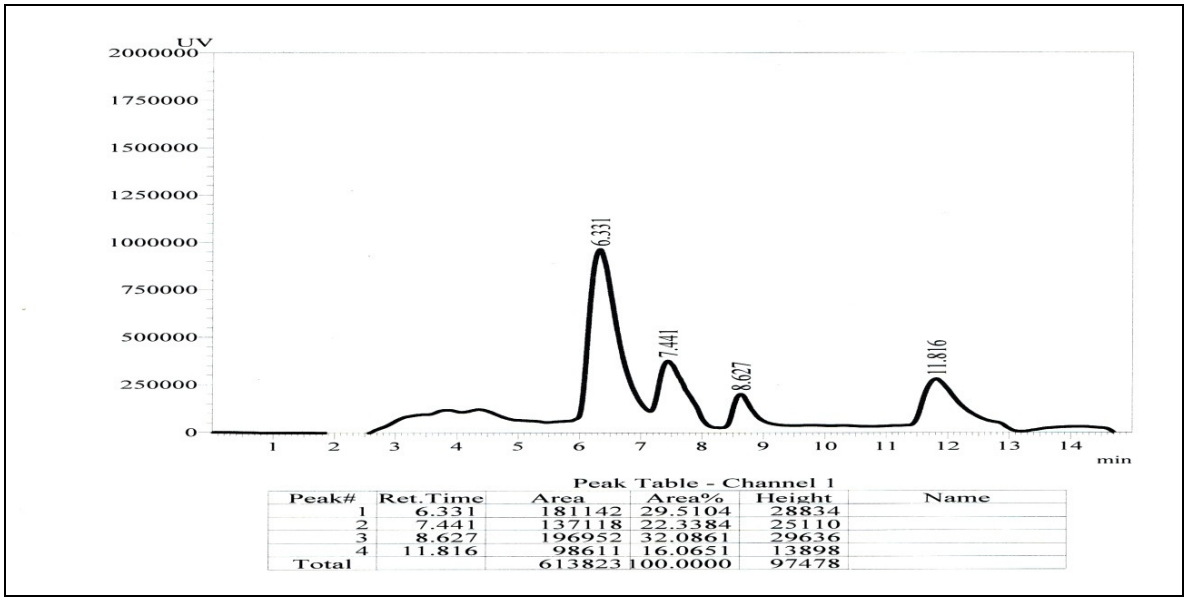


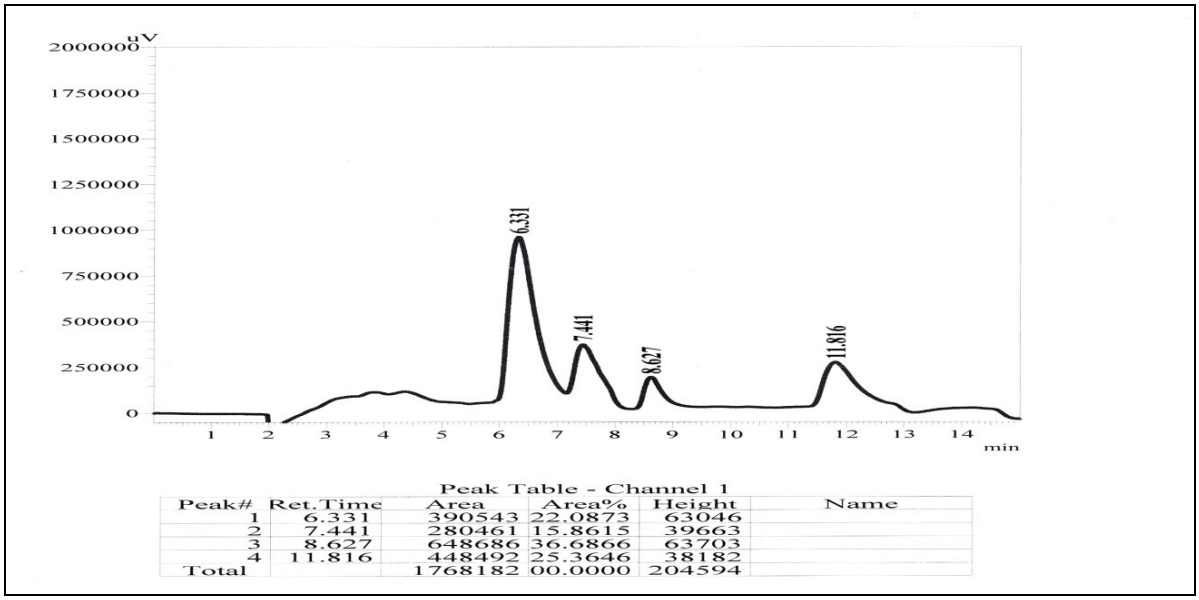
Fig. 2 : The stander curve of Trigonelline by using HPLC.



Peak Table - Channel 1

Peak#	Ret.Time	Area	Area%	Height	Name
1	6.331	181142	29.5104	28834	
2	7.441	137118	22.3384	25110	
3	8.627	196952	32.0861	29636	
4	11.816	98611	16.0651	13898	
Total		613823	100.0000	97478	

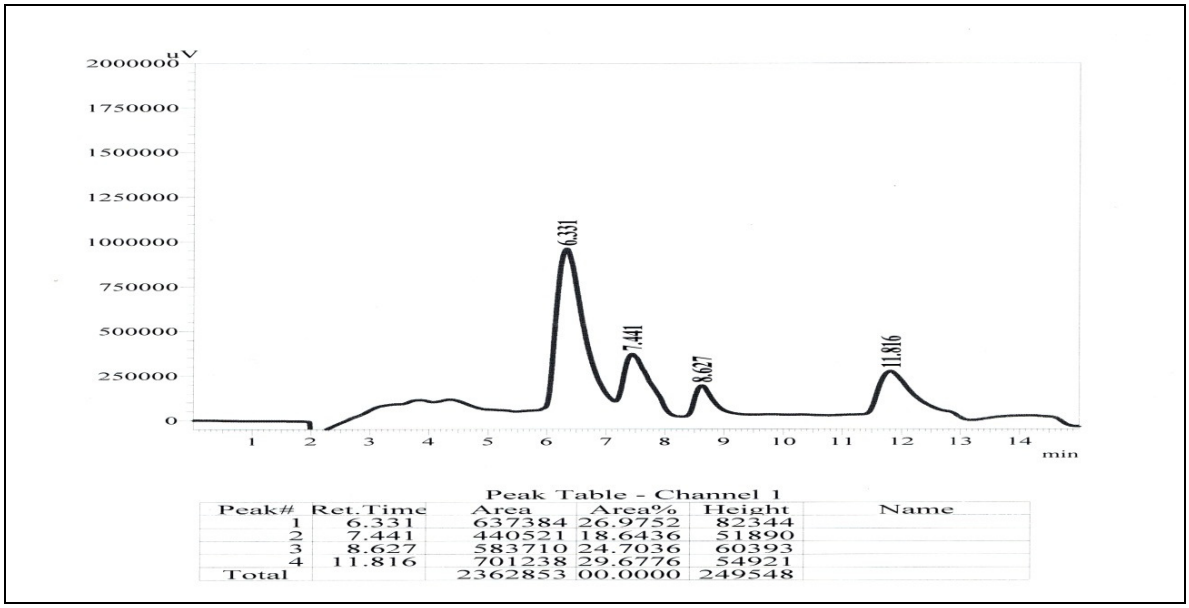
Fig. 3 : HPLC analysis of some secondary metabolites in the callus (used distilled water as control).



Peak Table - Channel 1

Peak#	Ret.Time	Area	Area%	Height	Name
1	6.331	390543	22.0873	63046	
2	7.441	280461	15.8615	39663	
3	8.627	648686	36.6866	63703	
4	11.816	448492	25.3646	38182	
Total		1768182	00.0000	204594	

Fig. 4 : Secondary metabolites that detected with HPLC at 1000 guess.



Peak Table - Channel 1

Peak#	Ret.Time	Area	Area%	Height	Name
1	6.331	637384	26.9752	82344	
2	7.441	440521	18.6436	51890	
3	8.627	583710	24.7036	60393	
4	11.816	701238	29.6776	54921	
Total		2362853	00.0000	249548	

Fig. 5 : Secondary metabolites that detected with HPLC at 2000 guess.

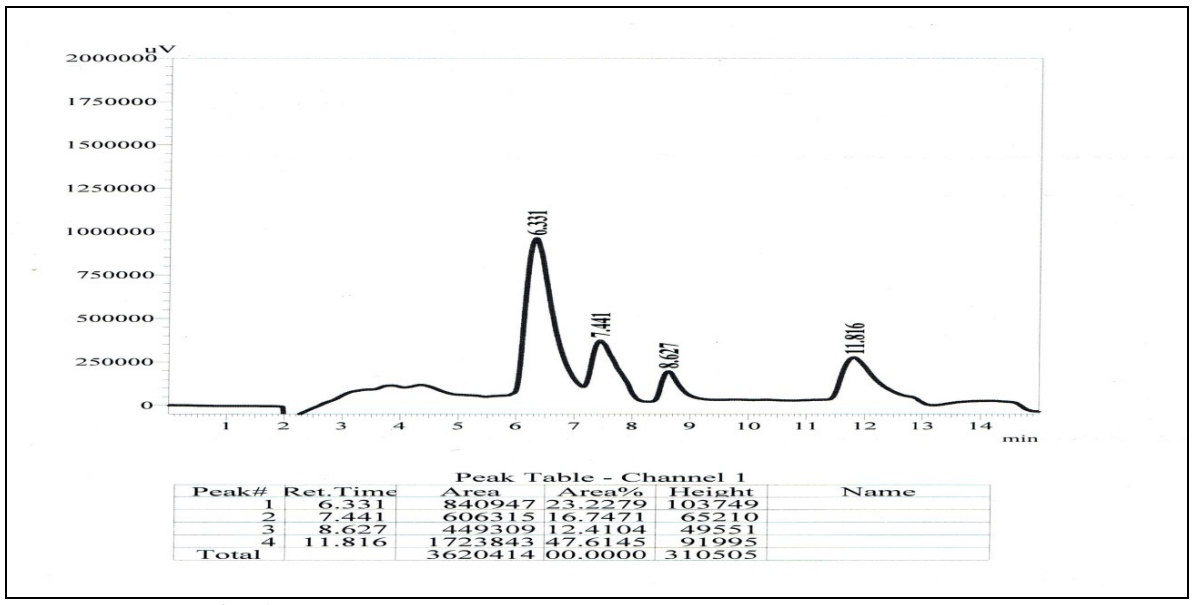


Fig. 6 : Secondary metabolites that detected with HPLC at 3000 guess.

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. The results indicated that insertion of magnetic water in the MS medium was found to be more active for the production of secondary metabolites compounds in *Trigonella foenum-graecum* L. Magnetic fields (MFs) can alter plant growth and development, by improve the plants in quantitatively and qualitatively. One way of applying a MF is by magnetizing water, thus creating magnetic water (MW). Teixeira da Silva and Dobranszki (2014) refer to improve the germination, early development and can also alter the mineral content by using MW, which depends on the quality and ion content of the water and on the type of magnetization and is very strongly species and genotype dependent. MW used as an abiotic stress agent, or as a growth-inducing or growth inhibiting factor, preferably against the use of permanent magnets with sustained magnetism, since MW can lose its magnetism over time and distance. An externally applied MF causes changes in atomic and molecular and electronic structure of the treated water, this lead to increasing polarization effects of water molecules, all summarized in (Pang and Deng, 2008). Based on the experiments of Pang and Deng, the surface tension force of MW decreases compared to untreated water, as does its hydrophobicity, due to the clustering structure and increased polarized effect of treated water. In the same research also showed MW has some important features, including effects and changes in the surface tension force.

The beneficial effects of magnetic treatment illustrated in the present study may be due to some alterations within plant systematic biochemical level and their possible effects at cell level and mainly due to increase the several byproducts such as results in above table by encouraged the precursor to produce secondary metabolites. Babaloo *et al.* (2018) found significance increase in the rate of water absorption accompanied with an increase in the magnetic force, These may be the result of bioenergetics structural excitement causing cell pumping and enzymatic stimulation.

Effect of Magnetic water on chemical composition and nutrients in some plants is perhaps the reason for increasing alkaloids and saponins in callus, is the added magnetic water to the medium of callus induction and maintaining callus led to induce and increase the production of some alkaloids and saponins in callus, or because of continuous subculture led to the emergence of somatic variation in cells, led to increasing production of secondary metabolites (Hassan and Jassim, 2018, Chen *et al.,* 2010 and Zaho *et al.,* 2002).

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