European Journal of Molecular & Clinical Medicine

ISSN 2515-8260 Volume 09, Issue 07, 2022

Enhancing Accumulation of Flavonoids in Callus of the Rare Medicinal *Iphiona scabra* Plant

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Abstract

Iphiona scabra (family Astraceae), is a medicinal endangered wild plant growing in Sinai Peninsula. It is rich in flavonoids and used in traditional medicine as an antispasmodic drug. Flavonoids have various biological activities like anti-inflammatory, antioxidant, Alzheimer's disease suppression and anti-cancer. The aim of this study was to use tissue culture technique for callus production of I. scarba and enhancing productivity of callus biomass and total flavonoids content (TFC) in solid culture medium. Shoot tips have been cultured on Murashige and Skoog (MS) medium fortified with 4-dichlorophenoxyacetic acid (2.4-D) at concentrations 0.25, 0.50 and 1.00 mg/l with kinetin (Kin) at 0.10 mg/l for the initiation of callus. For mass production of callus it was transferred to MS supplemented with thidiazuron (TDZ) 0.25, 0.50 and 0.75 mg/l combined with 0.5mg/l 1-naphthaleneacetic acid (NAA). Callus biomass was transferred to MS medium with different concentrations of either yeast extract as an elicitor or phenylalanine as a precursor at concentrations of 0.0, 25, 50, 100 and 200 mg/l. The best concentration that gave the highest accumulation of TFC was MS medium supplemented with 25 mg/l yeast extract. It gave 5.44 fold increase in callus biomass on the fourth week. The best treatment was MS medium fortified with 25 mg/l phenylalanine on the sixth week. That gave the highest fresh weight of callus 7.93 g/jar and accumulation of TFC 5.33 fold. Such potential method biotechnological strategies ensure better homogenous and stable production of sustainable flavonoids throughout the year under controlled environmental conditions.

Key words ; Precursor, Elicitor, in vitro, Biomass, Astraceae

INTRODUCTION

Iphiona scabra DC. belong to family Asteraceae **(Boulos, 2000)**. It is a medicinal endangered wild plant growing in Sinai Peninsula **(Abd El-Wahab *et al*., 2004)**. It is rich in coumarin and pyrrolizidine alkaloid and flavonoides. Thirteen flavonoides, quercetin, and pyrrolizidine alkaloid were isolated **(Ahmed and Tom, 1987)**. *I. scabra* is used in traditional medicine as an antispasmodic drug **(Font-Quer, 1990)**. *I. scabra* extract has anticoagulant, anti-platelet aggregation and anti-inflammatory effects. Moreover, the mean blood pressure significantly lowered by administration of the aqueous extracts of *I. scabra* when compared with nefidepine

treatment (hypotensive standard drug) in a dose dependant manner (**Nada *et al.*, 2006)**. According to **Panche et al., (2016**), flavonoids are now considered as an indispensable compound in a variety of pharmaceutical, nutraceutical, medicinal and cosmetic applications. This is attributed to their anti-inflammatory, anti-oxidative, anti-mutagenic, Alzheimer's disease suppression and anti-cancer properties coupled with their capacity to modulate key cellular enzyme function. However, it has been known for centuries that derivatives of plant tissue possess a broad spectrum of biological activity. Furthermore, flavonoids are polyphenolic compounds widely distributed in plants. The biosynthesis of flavonoids is a complex process involving two metabolic pathways: the malonic acid pathway, and the shikimic acid pathway through which shikimic acid is obtained and then the aromatic amino acids – phenylalanine or tyrosine which are precursors of flavonoids. The product of the reaction is an unstable 15-carbon chalcone acting as the skeleton of flavonoids (**Winkel, 2006**).

In this way, many researchers have concentrated on creating effective procedures for enhancing the accumulation and production of these important metabolites in plant without breeding or gene modification **(Kim, 2011)**. As numerous secondary metabolites biosynthesis in plants is normally a plant protection reaction to different stresses, biotic and abiotic elicitors could enhance secondary metabolites accumulation in plant tissue cultures (**Narayani and Srivastava, 2017**). Presently, the plant tissue culture plays an important role in the production of secondary metabolites, and ex suite conservation of valuable phytodiversity **(Anis et al., 2009)**. The biotechnological production of secondary metabolites in plant cell and organ cultures is an attractive alternative to the extraction of the whole plant material **(Skrzypczak–Pietraszek et al., 2014)**. In particular, plant–specific important compounds are obtained by using the plant cell and organ cultures **(Verpoorte et al., 2002)**. The faster proliferation rates and shorter biosynthetic cycle of cell and organ cultures leads to have a higher rate of metabolism when compared to field grown plants. Various strategies have been developed for use in biomass accumulation and the synthesis of secondary compounds, such as elicitation and precursor feeding (**Rao and Ravishankar, (2002) and Sarin, (2005)**). Biotic elicitors are the substances of biological origin that include polysaccharides originated from plant cell walls and micro–organisms. Elicitor may be defined as a substance for stress factors, when applied in small quantity to a living system, it induce or improve the biosynthesis of specific compound which do have an important role in the adaptation of plants to a stressful conditions (**Radman et al., 2003**). Elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amounts of elicitors (**Radman et al., 2003**). An elicitor can be divided as a compound natural or synthetic that initiates or improves the biosynthesis of specific metabolites when introduced in small concentrations to a living cells system (**Zhao et al., 2005 and Namdeo, 2007**). The application of an elicitor triggers stress or defence related responses in plant cells. leading to the enhanced synthesis and accumulation of secondary metabolites or the induction of novel secondary metabolites ((**Naik and Al Khayri, 2016** and **Narayani and Srivastava 2017**). Several biotechnological strategies have been hypothesized and applied for the productivity enhancement, and elicitation is recognized as the most practically feasible strategy for increasing the production of desirable secondary

compounds from cell, organ, and plant systems (**Poulev et al., 2003; Angelova et al., 2006 and Namdeo, 2007**). Elicitors are divided into two types depending on the origin including biotic and abiotic. Biotic elicitors are produced from microbial or plant sources. Yeast extract is one of the biotic elicitors which derived from microbial sources **(Ramirez- Estrada et al. 2016)**. Yeast extract is an important elicitor and is found to be richin vitamin B-complex. It also contains essential components likechitin, N-acetyl-glucosamine oligomers, -glucan, glycopeptidesand ergosterol **(Boller, 1995)**. Yeast extract contains various compounds such as chitin, β-glucan, glycopeptides, and ergosterol that are involved in plant defense responses **(Baenas et al., 2014)**. **Turgut-Kara and Ari (2011)** reported elicitation efficiency of yeast extract on the biosynthesis of isoflavonoids, and anthocyanin and phenolic acid, respectively. The addition of yeast extract has successfully been used in culture and overproduction of important phytocom-pound was observed in several studied plant genera **(Cai et al., 2012)**. One of the biotic elicitors that can result in the improvement of secondary metabolites content is yeast extract **(Loc et al 2014)**. Like other elicitors, yeast extract concentration is an important factor with significant impact on biosynthesis of valuable metabolites and its optimal level may be different for each plant species. The stimulating influence of yeast extract on secondary metabolites was confirmed in several studies **(Kochan et al., 2017)**. Yeast extract is a very interesting natural plant inducer due to its role in producing some growth regulators, as well as its ability to act as a biostimulant of plant growth or the biosynthesis of plant pigments and some other bioactive compounds **(Taha et al., 2016 and Złotek, 2017)**. For example, in a study conducted by **Złotek and Swieca (2016)** yeast extract elicitation increased the level of phenolic compounds in lettuce leaves. The foliar application of yeast extract was found to increase the contents of phenolic compounds in neem leaves **(Taha, et al., 2016)**. It is one of the most common natural elicitors used in in vitro culture to induce secondary metabolites production **(Cheng et al. 2013; Karalija et al. 2020)**.

In vitro cultivation of plant cells is a viable alternative to increase the growth rate of biomass and the stability during the continuous production of flavonoids and other metabolites. Additionally, in vitro cultivation allows for the manipulation of growth variables, as well as the use of precursors or elicitors. These variables might change the biosynthetic pathways of the compounds and optimizing its production (**Sajca et al., 2000)**. The present study aimed to use tissue culture technique for callus production of *I. scarba* and enhancing biosynthesis of total flavonoids content (TFC) in solid culture medium using a precursor and an elicitor. To the best of my knowledge, secondary metabolites from this medicinally important plant

MATERIALS AND METHODS

A. Plant Material and Sterilization

Seeds of I. scarba were collected from south Sinai and washed under running tap water for 30-60 min. and soaked in (1.5%) sodium hypochlorite solution for 5- 10 min, then rinsed 4-5 times with sterile distilled water under sterile conditions.

B. Culture Medium and Conditions

The culture medium used in this work was Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962) supplemented with 100 mg/l myo-inositol, 3% (w/v) sucrose. The medium was supplemented with different combinations of

plant growth regulators (PGRs), in addition to the control medium without PGRs. The pH of the medium was adjusted to 5.7-5.8 before the addition of 0.3% (w/v) phytagel.

Seeds were cultured on the media under complete aseptic conditions in the Laminar Air Flow Hood. Cultures were placed in an incubation room at 26 ± 2^{o}C under a photoperiod of 16 hours. Seedlings were used after two months as a source for shoot tips culture experiments.

C. Callus induction

Shoot tips were culture on MS medium supplemented with 4-dichlorophenoxyacetic acid (2.4-D) at concentrations of 0.25, 0.5 and 1.0mg/l with kinetin (Kin) at 0.1 mg/l for the initiation of callus. Calli were transferred to MS medium fortified with thidiazuron (TDZ) at concentration 0.25, 0.50 and 0.75mg/l combined with 0.5mg/l 1-naphthaleneacetic acid (NAA)for increasing callus biomass. The mean fresh weight, texture and color of callus were measured after 60 days of culture in complete darkness for each treatment.

D. Elicitor and precursor feeding

Yeast extract have been added to MS medium at concentration 0.0, 25, 50, 100 and 200 mg/l as an elicitor. Also, phenylalanine was use as a precursor of flavonoid. A stock solution of 1 mg/ml (w/v) was prepared by dissolving 10 mg of phenylalanine separately with 10 ml of water. Phenylalanine solution was microfilter sterilized using 0.2 μm of polyethersulfone membrane (Whatman, UK) because it is heat sensitive.

E. Determination of flavonoid content (FC)

Flavonoid compounds were determined by flavonoid-aluminum ($AlCl_3$) complexation method (**Marinova et al., 2005**) using quercetin as a standard. Briefly, a volume of 1 ml of samples were added in different test tubes and 0.3 ml of 5% $NaNO_2$ was added to each sample, followed by 5 min of incubation. Then, 0.5 ml of 2% $AlCl_3$ was added and the sample was softly shaken and neutralized 6 min later with 0.5 ml of 1 N NaOH. After 10 min, the absorbance was read at a wavelength of 425 nm. Samples without $AlCl_3$ were used as blank. FC was calculated using a standard calibration of quercetin alcoholic solution (200 – 100 – 25 – 12.5 and 6.25 mg/ml) and expressed as milligrams of quercentin equivalent per gram of fresh biomass weight, mg QE/g F.W.

F. **Chromatographic analysis of flavonoids**

Chromatographic of flavonoids was carried out in the callus obtained from the two samples that produced the highest of flavonoid content compared with the control. High Performance Liquid Chromatography (HPLC) analysis was carried out using an Agilent 1260 series. The separation was carried out using Eclipse C18 column (4.6 mm x 250 mm i.d., 5 μm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in a cetonitrile (B) at a flow rate of 0.9 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (82% A) ; 15-16 min (82% A) and 16-20 (82% A). The multi-wavelength detector was

monitored at 280 nm. The injection volume was 5 µl for each of the sample solutions. The column temperature was maintained at 40 °C.

RESULTS AND DISCUSSION

Seeds of *I. scarba* were germinated on MS medium free from PGRs (fig.1). Shoot tips of seedling were cultured on MS medium fortified with various concentrations of 2,4-D and 0.1 mg/l Kin to induction callus. To increase callus biomass, TDZ was used at various concentrations with NAA. Yeast extract or phenylalanine were added to improve total flavonoids content in callus.

G. Callus induction of *Iphoina scarba*

Data in table (1) show the effect of MS medium fortified with 2,4-D and 0.1 mg/l Kin on the callus initiation of *I. scarba* plant. It was observed increasing the concentration of auxin increased the fresh weight of callus. The highest value was obtained at concentration of 1.0 mg/l 2,4-D with 0.1 mg/l Kin and reached 4.77g/ jar. The texture of callus was friable and brown in color. This was similar as reported by **Tan et al., (2013)** who found that MS basal medium with B5 vitamin, 2,4-D with kinetin produced callus of *Centella asiatica* L.

The obtained results in Table (2) and fig.(2) reveal no significant differences among the treatment. The highest fresh weight of *I. scarba* callus was achieved at 0.5 mg/l TDZ with 0.5mg/l NAA. It gave 15.43 g/jar followed by 0.25mg/l and 0.75 mg/l TDZ with 0.5 mg/l NAA. They gave 9.43g callus per jar compared with the control without TDZ. The texture of callus was friable and green in color. These results are in harmony with **Liu et al., (2016)** who showed that optimum callus induction of *Orostachys fimbriata* was obtained on MS medium supplemented with 0.5 mg/L TDZ and 0.2 mg/l NAA. This was similar to that reported by **Abd El-kafie et al., (2019)**, who believed that the highest callus fresh weight of *Impatiens balsamina*was recorded by using TDZ. Callus culture has significant commercial potential, particularly with regard to the production of plant secondary metabolites with medicinal activity, as well as therapeutic antibodies and other recombinant proteins. Also, regeneration of agricultural or ornamental plants from calli. In addition, Active ingredients may be purified directly from callus culture to relieve the pressure on plants in their natural habitats (**Park and Wi, 2016 ; Top et al., 2019 and Efferth 2019**). Also, callus cultures may be easily converted into single cell cultures growing as suspension in bioreactors. Such culture may be subjected to scale-up and process optimization (**Georgiev et al., 2009, 2018**). Some commercially-available drugs are produced by large-scale callus cultures, such as Paclitaxel from yew (*Taxus.* sp.) or taliglucerase alpha (Elelyso) from carrot Daucus carota (**Tabata, 2004 and Grabowski et al., 2014**).

H. Effect of yeast extract on flavonoids content

Data in Table (3) show the effect of yeast extract as an elicitor on the accumulation of flavonoids in *I. scarba* callus. There were significant differences among the treatment. Flavonoids concentration it ranged between 0.27 to 1.47 ug/mg fresh weight. The best concentration that gave the highest accumulation of flavonoids was MS medium supplemented with 25 mg/l yeast extract, it was gave fresh callus weight of 3.41 g/jar and 5.44 fold on the fourth week. However, the lowest value was obtained on MS medium supplemented with 100 mg/l yeast extract and 0.96 fold on the fourth week comparing with MS medium without yeast extract. Generally, on the

fourth and sixth weeks, the concentration of yeast extract of 25, 50 and 200 mg/l was gave highly accumulation of flavonoid. **Elshahawy et al., (2022)** found that at *Echinacea purpurea* the highest content of total flavonoids was recorded with yeast extract, 3.11 folds increase compared to control. **Khlebnikova et al., (2022)** found that in *Satureja hortensis* flavonoids accumulated in significantly higher amounts in tissue retains the ability to synthesize flavonoids at deficient levels. Yeast extract as a biotic elicitor derived from fungal sources, modifies the enzyme effectiveness of phenylpropanoid metabolism that finally produces the phenolic compounds as recorded by **Ramachandra and Ravishankar,** (**2002**). The yeast elicitor is increased the production of berberine by 4-folds in *Thalictrum rugosum* as reported by **Rajendran et al. (1994)**. Yeast extract elicitor treatment during the exponential growth phase show a significant flavonoid induction than during stationary growth phase. Yeast extract at 1 g/l with culture harvested on day 12 were the best treatment affecting the production of flavonoid of *Medicago sativa* (**Alia, 2008 and Khalil et al., 2008**). One advantage of producing polysaccharides using yeast cell factories is that the production isn't affected by seasons, regions, and pests **(Schmid and Sieber, 2015)**. With the rapid development of yeast synthetic biology, yeast has been used as cell factories for the production of plant natural products (**Parapouli et al., 2020)**.

I. Effect of phenylalanine on flavonoids content

Data in Table (4) clearly show the effect of phenylalanine as a precursor on accumulation of flavonoids in *Iphoina scarba* callus. There were significant differences among the treatment. The best treatment was MS medium fortified with 25 mg/l phenylalanine on the sixth week followed by 200 mg/l on the fourth week. They were gave 5.33 fold comparing with the medium without phenylalanine. The concentration of 25 mg/l phenylalanine was the best because it gave the highest fresh weight of callus of 7.93g/jar in addition to the highest accumulation of flavonoids. The lowest value was shown at MS medium supplemented with 100 mg/l phenylalanine on the second weeks was 0.82 fold. The medium supplemented with 50 mg /l phenylalanine on the fourth and sixth week and gave 5.07 fold. Isoflavones and flavonoids originated from phenylalanine, an upstream metabolic precursor thorough phenylpropanoid pathway. Supplementation of phenylalanine is expected to increase elevated level of target compound (**Shinde et al., 2009**). In addition, **Meena et al., (2014)** studied the effect of phenylalanine on production of flavonoid as exogenous incorporation of phenylalanine in liquid culture increased the yield of total flavonoid about 2 to 3 fold in comparison to control on *Citrullis colocynthis*. However, the activities of phenylalanine ammonia lyase increased, which led to the enhancement of flavonoid production as reported by **(Wang et al., 2015**). However, **Tana and Mahmmod, (2013)** found that in suspension culture of pegaga the intracellular flavonoid content increased by a 3.2 fold with the feeding of 60 mg/L phenyalanine. The addition of 60 mg/L phenylalanine have successfully produced increased amount of flavonoid in the cell of pegaga. In addition, **Feduraev et al., (2020)** studied the effect of phenylalanine involved in plant metabolism through the phenylalanine ammonia-lyase (PAL) pathway, on the synthesis and accumulation of flavonoid compounds, its biosynthesis, based on the example of common wheat (*Triticum aestivum* L.) They can promote carbon fortified in culture media, as flavonoid glycosylation increases the number of carbon rings and available

carbohydrates (**Yang et al., 2017)**. Flavonoids are ubiquitous in plants and may be involved in several functions such as plant development, plant-microorganism/ animal interactions, and ultraviolet protection (**Mathesius, 2018**).

J. HPLC analysis of flavonoids

Analysis of HPLC gave sixteen compounds of flavonoid. The results obtained in table (5) show the analysis by HPLC of the best two sample
from precursor and elicitor feeding experiment that gave the highest accumulation of flavonoids in adding to the control. The highest accumulation compound was naringenin (27.82 µg/g) followed by ellagic acid (10.97 µg/g) and chlorogenic acid (9.09 µg/g) in yeast extract and in phenylalanine chlorogenic acid (10.32 µg/g) followed by rutine (7.22 µg/g). Phenylalanine gave the best accumulation of many compounds such as chlorogenic acid, catechin, coffeic acid, syringic acid, rutin, daidzein, cinnamic acid and only accumulated of coumaric acid than control and yeast extract fig. (3). However, yeast extract gave the highest accumulation of gallic acid, ellagic acid, ferulic acid, naringenin and apigenin compared with phenylalanine or control fig. (3). In addition, the control medium gave the highest accumulation of querectin and only produced vanillin and hesperetin compared with phenylalanine or yeast extract fig.(3). Phenylalanine as a precursor plays an important role in flavonoids biosyntheses. Phenylalanine is a substrate for many different secondary metabolites, including phenylpropanoids, flavonoids, anthocyanins, and the cell wall lignin. Mutations that inhibit PAL synthesis are usually associated with significant changes in the levels of many phenylpropanoids **(Shadle et al., 2003)**. In addition, **Maeda and Dudareva (2012)** reported that Phenylalanine is the common precursor of flavonoids. However, **Sajjalaguddam and Paladugu (2015)** tested the effect of phenylalanine in enhancing flavonoid content in in vitro studies it produced more than 3 fold increase in flavonoid content in elicitor induced callus of *Abutilon indicum* compared to control.

CONCLUSION

To the best of our knowledge, the developed protocol for the enhancement of important metabolites is the first attempt for *Iphiona scabra* plant . In our view, the callus culture of shoot tip derived callus will be a promising model for studying the impacts of elicitation on biomass production and important metabolites accumulation in the species of *I. scabra*. The results showed enhanced accumulation of flavonoids in *I. scabra* callus when treated with phenylalanine(5.33 fold) and fresh weight (7.93 g/jar) and yeast extract (5.44 fold) and fresh weight (3.41g/jar). However, in callus culture phenylalanine has been proven best for the precursor. The results also depict the importance of callus culture over the excised shoot tip culture. This protocol has the potential of further research for the industrial level production of the health promoting metabolites of shoot tip. Phenylalanine as a precursor was more effective in enhancing flavonoids production than yeast extract as an elicitor.

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Table (1): Effect of 2.4-D concentrations and 0.1 mg/l kin on callus initiation from *Iphiona scarba* shoot tip.

Type of PGRs and concentration (mg/l)		Fresh weight of callus (g/jar)	Texture	Color
2.4-D	Kin			
0.00	0.0	1.54 ± 0.17[b]	friable	creamy
0.25	0.1	1.74 ± 0.45ab	friable	brown
0.50	0.1	2.16 ± 0.09ab	friable	brown
1.00	0.1	4.77 ± 1.90a	friable	brown

Table (2) Effect of TDZ concentration and 0.5 mg/l NAA on increasing callus biomass of *Iphiona scarba*.

Type of PGRs and concentration (mg/l)		fresh weight of callus (g/jar)	Texture	color
TDZ	NAA			
0.00	0.5	6.51a	friable	creamy
0.25	0.5	9.43 a	friable	Green
0. 50	0.5	15.43 a	friable	Green
0.75	0.5	9.43 a	friable	Green

Table (3) Effect of yeast extract as an elicitor on fresh weight and accumulation of total flavonoids in *Iphoina scarba* callus

Duration (week)	Yeast extract concentration (mg/l)	Fresh weight of callus (g/jar)	Flavonoids content	
			concentration (ug/mg FW)	Increase compared control (Fold)
control	0.0	5.00	0.27 ± 0.01e	1.00
2	25	2.90	0.39 ± 0.01d	1.44
	50	3.12	0.67 ± 0.00c	2.48
	100	2.80	0.28 ± 0.00e	1.04
	200	2.80	0.39 ± 0.03d	1.44
4	25	3.41	1.47 ± 0.00a	5.44
	50	2.73	1.43 ± 0 .00ab	5.30
	100	2.90	0.26 ± 0.01e	0.96
	200	3.36	1.43 ± 0.00ab	5.30
6week	25	3.53	1.45 ± 0.00ab	5.37
	50	2.73	1.42 ±0.00b	5.26
	100	2.90	0.66 ± 0.03c	2.44
	200	4.01	1.46 ± 0.00ab	5.41

Table (4) Effect of phenylalanine as a precursor on fresh weight and accumulation of total flavonoids in *Iphoina scarba* callus

Duration (week)	Phenylalanine concentration (mg/l)	Fresh weight of callus (g)	Flavonoids content	
			concentration (ug/mg FW)	Increase compared to control (Fold)
Control	0.0	5.00	0.27±0.01 g	1.00
2	25	2.90	0.71 ± 0.03e	2.63
	50	3.56	0.94±0.00c	3.48
	100	2.80	0.22 ± 0.01e	0.81
	200	3.04	1.34± 0.00bc	4.96
4	25	2.90	0.65 ± 0.04 f	2.40
	50	4.61	1.37±0.00 b	5.07
	100	2.90	0.27 ± 0.03 g	1.00
	200	1.81	1.44± 0.00a	5.33
6	25	7.93	1.44± 0.00a	5.33
	50	4.61	1.37±0.00 b	5.07
	100	2.90	0.61 ± 0.01f	2.48
	200	3.89	1.31±0.00c	4.85

Table (5) HPLC analyses of flavonoids in *Iphiona scabra* callus

N.	Compounds Name	Control	phenylalanine	Yeast extract
		Conc. (µg/g)	Conc. (µg/g)	Conc. (µg/g)
1	Gallic acid	3.89	4.59	5.42
2	Chlorogenic acid	1.92	10.32	9.09
3	Catechin	0.00	2.58	2.35
4	Coffeic acid	0.69	3.05	1.99
5	Syringic acid	0.00	1.39	0.16
6	Rutin	4.44	7.22	6.79
7	Ellagic acid	0.68	2.66	10.97
8	Coumaric acid	0.00	0.10	0.00
9	Vanillin	0.33	0.00	0.00
10	Ferulic acid	0.38	0.00	3.79
11	Naringenin	0.56	1.43	27.82
12	Daidzein	0.29	0.70	0.00
13	Querectin	4.92	2.01	4.16
14	Cinnamic acid	0.10	0.12	0.00

15	Apigenin	0.00	0.74	3.03
16	Hesperetin	1.04	0.00	0.00

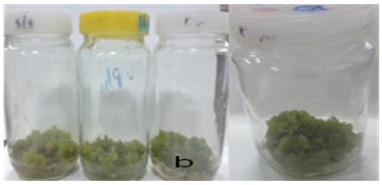

Figure (1) Germination of *Iphoina scarba* Figure (2) Callus formation of *Iphoina scarba*

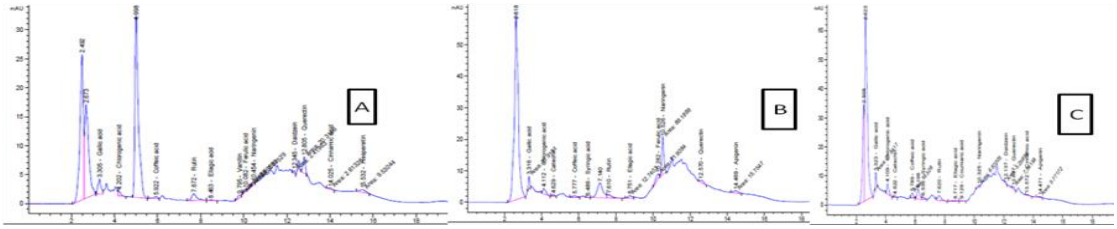

Figure (3) HPLC chromatography of flavonoid in callus biomass of *Iphiona scabra*; A) control, B) phenylalanine at 25mg/l on the six weeks C) yeast extract at 25mg/l of the four weeks