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STIMULATION OF THE PRODUCTION OF SOME MEDICINALLY ACTIVE COMPOUNDS FROM CALLUS TISSUE INDUCED FROM THE SHOOT TIP OF PARACRESS SEEDLINGS *IN VITRO* THROUGH ADDING DIFFERENT CONCENTRATIONS OF GLUTAMINE

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Abstract

The research was carried out in the objective of increasing the growth and multiplication of callus tissue involving increasing the pharmaceutically active compounds obtained from Paracress plant tissue culture. The secondary metabolic compounds were estimated by quantitative and qualitative analysis with High-Performance Liquid Chromatography (HPLC) device. Having antisepticised with 4.0 % of sodium hypochlorite for 20 minutes, the seeds of the Paracress plant were germinated and cultured in MS media of full salt power. Callus was induced from the shoot tips taken from Paracress seedlings and cultured in media contained 0.5 ng.l^{-1} BA and 2.0 mg.l^{-1} 2, 4-D. The initiators involving four concentrations of glutamine (0, 250, 300, and 350 mg.l^{-1}) were added to probe their effect on the induced callus four weeks after the culture. Results illustrate significant differences between the studied treatments in the fresh and dry of the callus induced from the shoot tips four weeks after culturing. The glutamine concentration of 300 mg.l^{-1} was significantly superior producing 6.260 and 0.140 g of the fresh and dry callus weight respectively. However, 350 mg.l^{-1} of glutamine gave 5.029 and 0.123 g respectively while the control treatment recorded the lowest amount fresh and dry callus weighed 2.340 and 0.057 respectively. On the other hand, producing the secondary compounds influenced by the initiators demonstrated significant differences between treatments. The best two concentrations of glutamine were 300 and 350 mg.l^{-1} producing the highest concentrations of Scopoletin and Spilanthol reached 429.03, 415.789, 89.399, and 62.329 µg.g^{-1} dry weight respectively, while the control treatment gave the highest concentration of stigmasterol averaged 117.507 µg.g^{-1} dry weight. The highest values of β-sitosterol and β-amyrin were 243.67 and 72.800 µg.g^{-1} dry weight obtained from the glutamine concentration of 250mg.l^{-1} while 300 mg.l^{-1} of glutamine increased α-amyrins to 8.27 µg.g^{-1} dry weight. The control treatment greatly affected the compounds Scopoletin, Spilanthol, β-sitosterol, α-amyrins, and β-amyrins recording the lowest values.

Introduction

Paracress *Spilanthes acmella* Murri (L) also known as toothache plant is one of the medically important ornamental plants. It belongs to the family *Asteraceae*. Sothern parts of Brazil is considered the original habitat of the plant. It spread widely in the tropics and subtropics of the world including Malaysia, South America, North Australia, Africa, and the Republic of India. The plant has important utilizations in many fields including manufacturing pharmaceutical materials, nutrition, and manufacturing cosmetics and body care as well as in the popular medicine for treating dental pain, moreover, the flowers and leaves of the plants are used as spices added to food. Several scientific studies documented the use of the plant as an insecticide, anti-inflammatory, anti-bacterial, anti-fungal and a stimulant for the immune system. It used to treat hemorrhoids, malaria, and severe anemia (Hossan *et al.*, 2010; Dubey *et al.*, 2013) for it has active compounds such as Scopoletin and Spilanthol as well as the phytosterols, including stigmasterol, β-sitosterol, α-amyrins, and β-amyrins all have important biological effects. This encouraged many researchers to produce some medicinally active compounds involving alkaloids, carbohydrates, glycosides, flavonoids, tannins, anthraquinone, saponins, cardiacglycosides, Antibiotics, amino acids, and many other else of the secondary metabolic compounds through the plant tissue culture technique (Rao *et al.*, 2002). Since the plant tissue culture technique in vitro is typical for producing pharmaceutically active materials within a short time, as it abbreviates many steps through callus culture and extracting the highly pure active substances directly regardless season restriction, this study was conducted aiming to increasing the production of pharmaceutically active materials from callus induced from the shoot tip of Paracress plants through shortening some constructing series stages by adding various levels of glutamine as a leading factor help to inducing the callus tissue for producing active compounds as a results of secondary metabolism processes.

Materials and Methods

The experiment was conducted at the plant tissue culture laboratory in the building of postgraduate studies laboratories, College of Agriculture - University of Baghdad during the period from 30/9/2018 to 15/5/2019 aiming to study the accumulation of secondary metabolism compounds in the callus culture under the influence of the additive initiator (Glutamine) and the possibility of producing these active materials using the plant tissue culture technique as well as to determine the best glutamine concentration for producing higher amount of the target active compounds.

Seeds surface-antisepsis and culture

Paracress seeds were antisepticised with 4.0 % of sodium hypochlorite for 20 minutes. Next, they were rinsed with sterile distilled water three times to ensure removing the antiseptic from the seeds. Having the seeds antisepticised, they were cultured in MS media of full-power salt and free of growth regulators in order to produce seedlings. The cultures were incubated in the growth chamber equipped with the light of 1000 lux and temperature of 25± 2 ºC for 16 hours. Four weeks later, Shoot tips of 1.5 cm length were cut from each seedling (explant) to be used for callus induction and differentiation.

MS media preparation

MS media was prepared in the laboratory from several micro and macro elements in addition to vitamins, sucrose, and growth regulators according to the required

concentrations. The pH was adjusted to 5.7 by adding sodium hydroxide 0.1N hydraulic acid 0.1N. Then, ager was added according to the required concentration. Finally, 10 ml of media were poured in each of the test tubes, Universal, dimensioned 8x2.5 cm.

Table 1 : Media ingredients of the organic compounds devoted to inducing callus from the Paracress plant

Full power	Chemical formula	Material (Salts)
0.5	$C_8H_{11}NO_3.HCl$	Pyrodoxine- Hcl
2.0	$C_2H_5NO_2$	Glycine
0.5	$C_6H_5NO_2$	Nicotinc acid
0.1	$C_{12}H_{17}C_1N_4OS.HCl$	Thiamine- Hcl
0.1	$C_6H_{12}O_6$	Myo-insitol
3.0	$C_{12}H_{14}N_2O_2S$	Biotin (Vitamin H)
0.5	$C_{14}H_{19}N_2O_4$	Folic acid (Vitamin B, M)
According to the concentrations used in the experiment	$C_8H_6CI_2O_3$	2,4-D
According to the concentrations used in the experiment	$(C_{12}H_{11}N_5)$	BA
30000	$C_{12}H_{22}O_{11}$	Sucrose
7000	$C_{24}H_{38}O_{19}$	Agar

Callus induction experiment

Callus was induced by culturing the shoot tips taken from the Paracress seedlings in the MS media supplied with 0.5 $mg.l^{-1}$ BA, 2.0 $mg.l^{-1}$ 2, 4-D, and the four concentrations of L-glutamine (Glu) (0, 250, 300, and 350 $mg.l^{-1}$). Data were recorded four weeks later.

Callus fresh weight measurement

The fresh and dry callus weight was measured five weeks after the culturing using a sensitive scale after getting rid of the media adherent to the callus using a surgical scalpel. Having the fresh weight recorded, the callus pieces were dried with an electric oven on temperature 70 °C, then, after the weight stabilized, they were weighted again.

Statistical analysis

The experiment was carried out relying upon the Completely Randomized Design (RCD) using the ASA software as a factorial experiment and the means were compared depending upon Duncan Multiple Range Test under the probability level of 5%. Every treatment comprised 10 replicates each of them included a part of the explant.

Glutamine effect test

The effect of glutamine was tested after it was prepared as a stock solution by dissolving 1.0 g of glutamine in 1000 ml of distilled water. The effect of three concentrations (0, 250, 300, and 350 $mg.l^{-1}$) on the plant growth and active material stimulation was tested by adding it to the media. An equal weight of the callus (100mg) was taken and cultured in the MS media containing 2 $mg.l^{-1}$ 2, 4-D and BA in addition to the glutamine concentrations (0, 250, 300, and 350 $mg.l^{-1}$). Each concentration treatment comprised 10 replicates. The cultures were incubated in the dark chamber of 25±2 C°. Four weeks later, the fresh and dry weight was recorded and extraction processes were performed.

Qualitative and quantitative assessment of medically active compounds

The medically active compounds were estimated using a High-Performance Liquid Chromatography (HPLC) according to Karen-Heide *et al.* (1986) involving the following steps:

- **Extraction and separation**

Having the period of incubation involving inducers and initiators ended, callus was sampled for analyzing. The samples were dried in an oven of 70 C°. Next, they were ground and preserved in opaque plastic containers under freezing conditions until the time of analysis. The samples were mixed with liquid nitrogen and treated by 50 ml of 25% ethanol with ammonia (NH_3) of ratio 20:1 at the room temperature for 30 minutes and then, the extract was centrifuged and evaporated on temperature 45C°. After that, the precipitate was treated twice with HC1 0.1 N at a concentration of 0.7 ml then, the mixture was filtered with a Millipore filter of porous with a diameter of 0.45 µm. After adding 0.4 ml of sodium carbonate Na_2CO_3 of pH 9.8, the filtrate was extracted twice by adding 3 ml of methyl chloride $CHCl_3$, then the filtrate extracted was evaporated with methyl chloride and dissolved in 100 ml of 50% aqueous ethanol. 200 µm was taken from the last extract and transferred to the HPLC device to estimate a standard solution of Scopoletin and Spilanthol.

- **Device conditions**

Scopoletin in *Spilanthes acmella Murr* L. was estimated in the laboratory of the soil and water department- Ministry of sciences and technology using the HPLC device type S 2100 Quaternary, Germany- SYKAM, S 3240 UV / Vis Detector Gradient Pump model to determine the retention time (Rt) and the area of each of the sample and standard solutions (Figures 1,2,3,4). Separation column type C18 (25cm×4.6mm × 5µ) was used and the mobile phase containing methanol (80: 20: 10 mL) was pushed with a flow rate of 10 ml/min. Data were recorded at a wavelength of 254 nm and a temperature of 45 C°. The area of the sample was compared to the area of the standard. The process was repeated with all samples under the same separation conditions to recognize the Scopoletin compound according to the following equation:

$$\text{Sample concentration} = \frac{\text{standard concentration x sample area}}{\text{tandard area}} \times \text{dilution times}$$

- **Estimating Spilanthol in the callus**

It was estimating with the High-Performance Liquid Chromatography (HPLC) according to Chaturvedi and Singh (2012) including the following steps:

- **Spilanthol extraction and separation**

Spilanthol compounds were extracted and separated as the same as the method formerly mentioned, nevertheless, the device conditions differed accordingly except for the Retention time that was constant. Separation column type C-Fast liquid chromatography column (FLC) 18, DB (deactivated base) (25cm×4.6mm×5um) was used where the mobile phase was pushed (acetonitrile: deionized water) with a ratio (V: V) of 10: 90 at a flow rate of 1.0 ml/ min. The data were recorded at a UV Detection wavelength of 237 nm and temperature 35C°. A standard solution and the sample were measured as the same method mentioned previously for Scopoletin.

Amyrines concentration estimation.

High-Performance Liquid Chromatography (HPLC) was used to estimate amyris according to Kothari *et al.* (2013) involving the following steps:

- **Amyris extraction and separation:**

Having the samples dried with an oven of 35 C°, they were ground to turn to powder where 250 mg of it was placed in a flask and 100 ml of methanol was added to. Then, the mixture was placed in a shaker for two hours. Next, the flask was kept under room temperature overnight. After that, the mixture was filtered with filter paper (Whatman No.1). This step was followed by filtration using a micro filter comprised of 0.45μm- diameter holes to prevent methanol loss and volatilization from the filtrate.

Device conditions

Compounds including α-amyrins and β-amyrins were separated with a separation column type C18- ODS (25cm×4.6mm × 5um) where the mobile phase (Methanol OH: deionized water) (80: 20 mL) was pushed at a flow rate of 1.0 ml/min and the results were measured on the UV wavelength 206 nm and temperature 35C°. The standard solutions for amyrins-α and β-amyrins were measured.

Phytosterol concentration estimation

It was estimated with a High-Performance Liquid Chromatography (HPLC) according to Maji *et al.* (2014) including the following steps:

Extraction and separation

A powder sample of 250 mg was extracted with ethanol 70% at a temperature of 37±2 C° relying on a cooling process for two days. After that, the samples were placed in an ultraviolet device for 30 minutes and 2 ml of Phosphoric acid was added for extracting the active material. Then the solvent was evaporated with a rotary evaporator leading at the end to obtaining 2.36% (w/w) of raw extract. Next, from the dried raw extract, 10 mg was dissolved in 5 ml of ethanol for preparing 2 $mg.l^{-1}$. Finally, the produced mixture was filtered a membrane filter of 0.45μm in diameter prior to injection into the device.

Device conditions

Steroid compounds including stigmasterol and β-sitosterol were extracted and separated using separation column type C18-ODS (25cm×4.6mm×5um). The mobile phase (acetonitrile: DW: Acetic Acid) (60: 25:5ml) was pushed at the flow rate of 1.0 ml/min. The data were measured at the UV wavelength of 280 nm and temperature of 35C°. Standard solutions of stigmasterol and β-sitosterol were measured as well as the samples as the same method mentioned in the paragraph devoted to the Scopoletin extraction.

Results and Discussion

Effect of glutamine on the fresh and dry callus weight induced from shoot tips taken from Paracress plant

Table 2 showed a significant increase in the fresh weight resulting from adding 300 $mg.l^{-1}$ of Glutamine producing the highest fresh callus weight of 6.260 g. differing significantly from the callus induced by 350 $mg.l^{-1}$ that produced 5.029 g while the control treatment produced the lowest induced callus not exceeding 2.340 g. The table also referred to the significant superiority of the media containing 300 $mg.l^{-1}$ of glutamine giving the highest callus dry weight reached 0.142g which differed significantly from the media supplied with 350 $mg.l^{-1}$ that gave 0.123g, whereas the control treatment gave the lowest callus dry weight of 0.057g. These results are consistent with those obtained by Sharabasy *et al.* (2012) that adding various glutamine concentrations increased the callus growth significantly compared to the untreated media. In an experiment conducted by Al-Memary (2014), he found that adding 0.75 $mg.l^{-1}$ of 2, 4-D and 0.4 $mg.l^{-1}$ of glutamine to MS media achieved the best response for callus induced from periwinkle leaf parts producing 0.697 g. Glutamine is one of the amino acids participating in protein constitution help to constitute enzymes that play a leading role in most biological processes in addition to constituting the DNA and RNA (دلالي, 1994).

Amino acids including glutamine help plants to tolerate drought through affecting the osmotic pressure of the plant cells where they accumulated making the plant absorb water and the dissolved nutrients from the media thus leading to increasing the plant cell growth (Abo Dhahi and Al-Yonis, 1998); moreover, it has a role in encouraging all cell bioactivities especially the processes of cell division and enlargement as well as increasing the activities of the enzymes which decompose the organic compounds and releasing the elements to be available and consequently enhancing the cell growth (Claussen, 2004; Nur *et al.*, 2006).

Table 2 : Effect of Glutamine on the fresh and dry weight (g) of the callus induced from the shoot tip of the Paracress plant cultured in MS media supplied with 2.0 mg.l-1 of 2,4-D and 0.5 $mg.l^{-1}$ four weeks after culturing

Dry weight	Fresh weight	Glutamine ($mg.l^{-1}$)
0.057B	2.340C	0.0
0.108AB	3.443BC	250
0.142 A	6.260A	300
0.123 A	5.029B	350

- Number carrying the same letter are not differ significantly according to the Duncan Multiple Range Test under the probability level of 5%

Effect of different levels of the chemical initiators on producing secondary compounds from Paracress callus propagated from in vitro tissue culture.

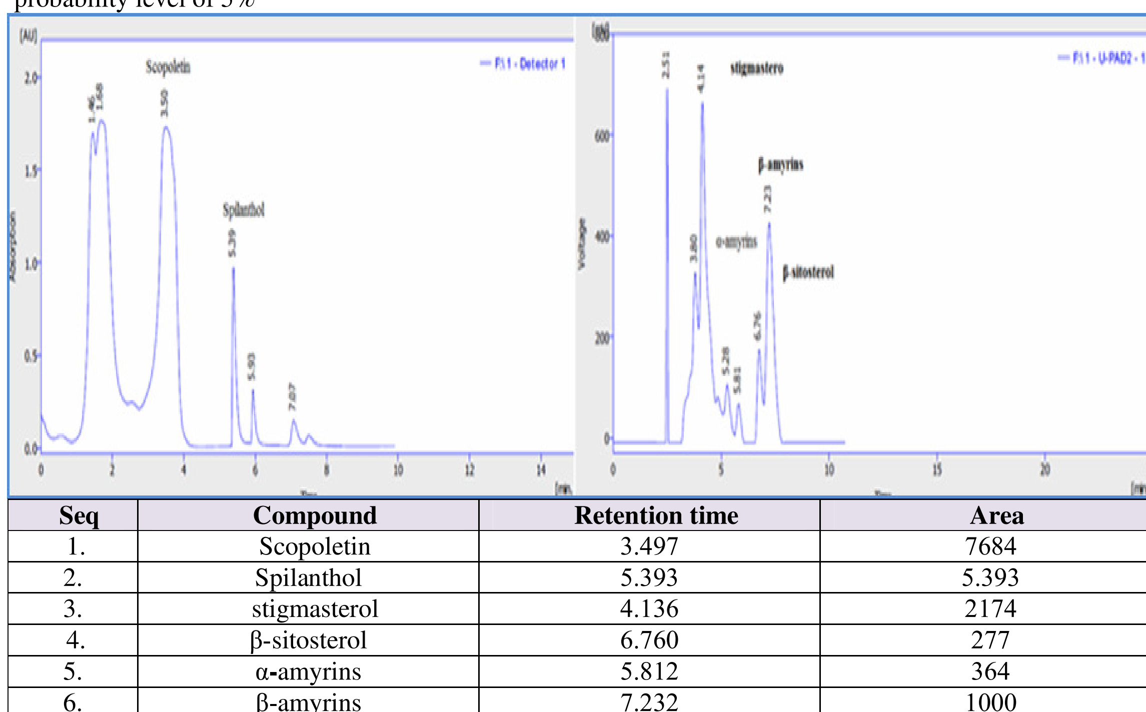
Results in Table 3 estimated with Mass HPLC refer to the superiority of the two treatments of 300 and 350 $mg.l^{-1}$ of glutamine in producing the highest concentrations of Scopoletin and Spilanthol reaching 429.03, 415.78, 89.399, and 62.249 $\mu g.g^{-1}$ dry weight respectively, however, they did

not differ significantly from the concentration of 250 mg.l^{-1} producing 241.70 of Scopoletin µg.g^{-1} dry weight whereas, they differed significantly from Spilanthol at the same concentration giving the lowest value did no exceed 152.07 µg.g^{-1} dry weight (Appendix 1). On the other hand, the stigmasterol value increased in the control treatment with no significant difference from the concentration 350 mg.l^{-1} of glutamine since the produced 117.507 and 116.367 µg.g^{-1} dry weight respectively, yet the concentration of 250 mg.l^{-1} of glutamine gave 89.355 µg.g^{-1} dry weight, the lowest value of stigmasterol. Increasing the initiator compared to the control treatment increased the β-sitosterol and β-amyrins compounds in callus tissue where the highest amount of them was obtained from adding 250 and 300 mg.l^{-1} which was the most effective on callus induction producing the highest amounts of these compounds reaching 243.67, 176.34, 72.800, and 61.807 µg.g^{-1} dry weight respectively compared to the control treatment that produced only 52.410 and 30.140 µg.g^{-1} dry weight of the compounds respectively. The α-amyrins amount at the glutamine concentrations of 300 and 250 mg.l^{-1} was increased to 8.327 and 7.393 µg.g^{-1} dry weight, nevertheless, higher glutamine concentration of 350 mg.l^{-1} gave 3.590 µg.g-1 dry weight, the lowest amount of α-amyrins. The obtained results may be explained that media involving plant growth regulator greatly affect the synthesis and production of the secondary metabolic products through tissue culture, in particular, the glutamine concentration within the media composition that encourage and enhance the pharmaceutically active compound accumulation. العكيدي (2017) concluded that using MS comprised salts and vitamins in addition to 3.0 g.l^{-1} sucrose, 1.0mg.$^{-1}$ 2, 4-D, and 0.5 mg.l-1 AB and supplied with 0.25 mg.l^{-1} glutamine for inducing callus cotyledon leaves of *Atropa belladonna* L. gave the highest concentrations of Sulfate and Hyoscyamine reached 0.29 mg.g^{-1} fresh weight.

Table 3 : Effect of Glutamine on producing secondary compounds (µg.g-1 dry weight) from Paracress callus cultured in MS media four weeks after culturing

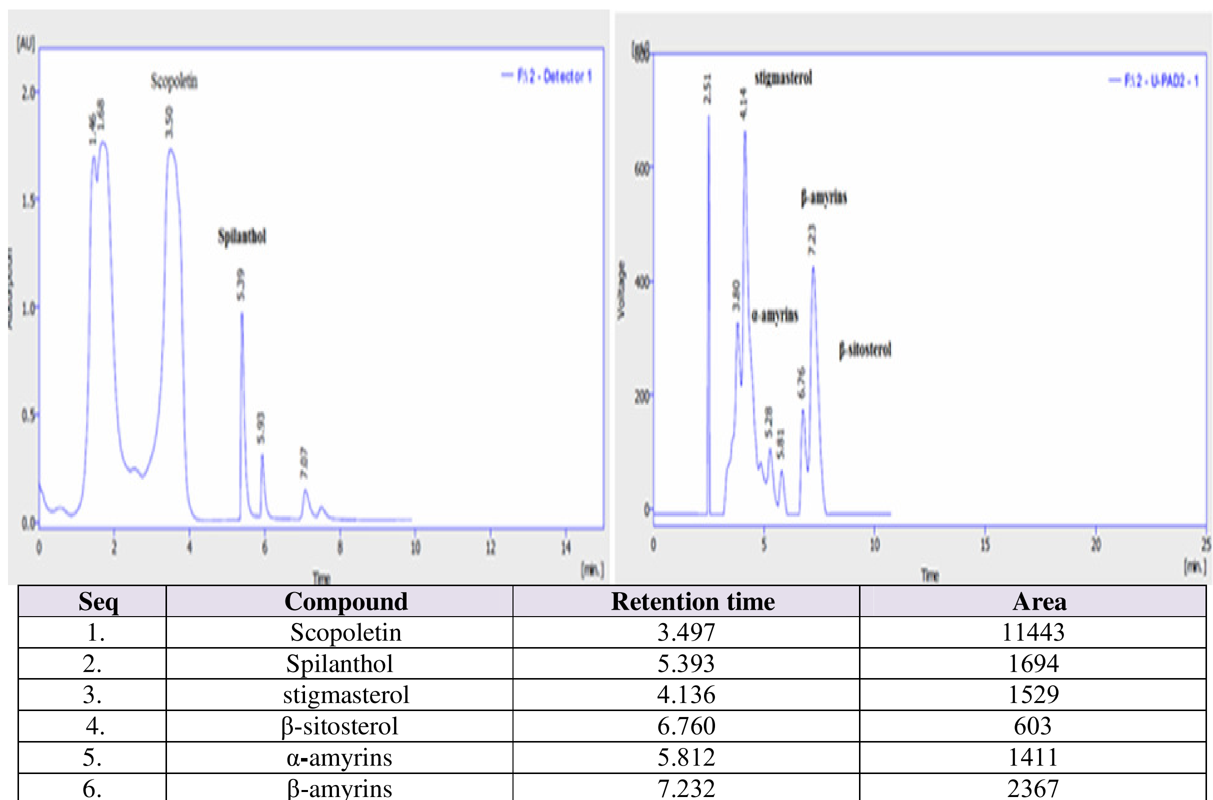
secondary compounds (µg.g-1 dry weight)						Glu (mg.l^{-1})
β-amyrins	**amyrins-α**	**β-sitosterol**	**stigmasterol**	**Spilanthol**	**Scopoletin**	
30.140 CC	4.467AA	52.410 CC	117.507A	50.270C	152.07BB	0.0
72.800A	7.303AA	243.67A	89.355C	40.613D	242.70B	250
61.807B	8.327A	176.34B	103.250B	62.249B	429.03A	300
29.410C	3.590AA	55.41C	116.367AA	89.399A	415.78AA	350

- Number carrying the same letter are not differ significantly according to the Duncan Multiple Range Test under the probability level of 5%



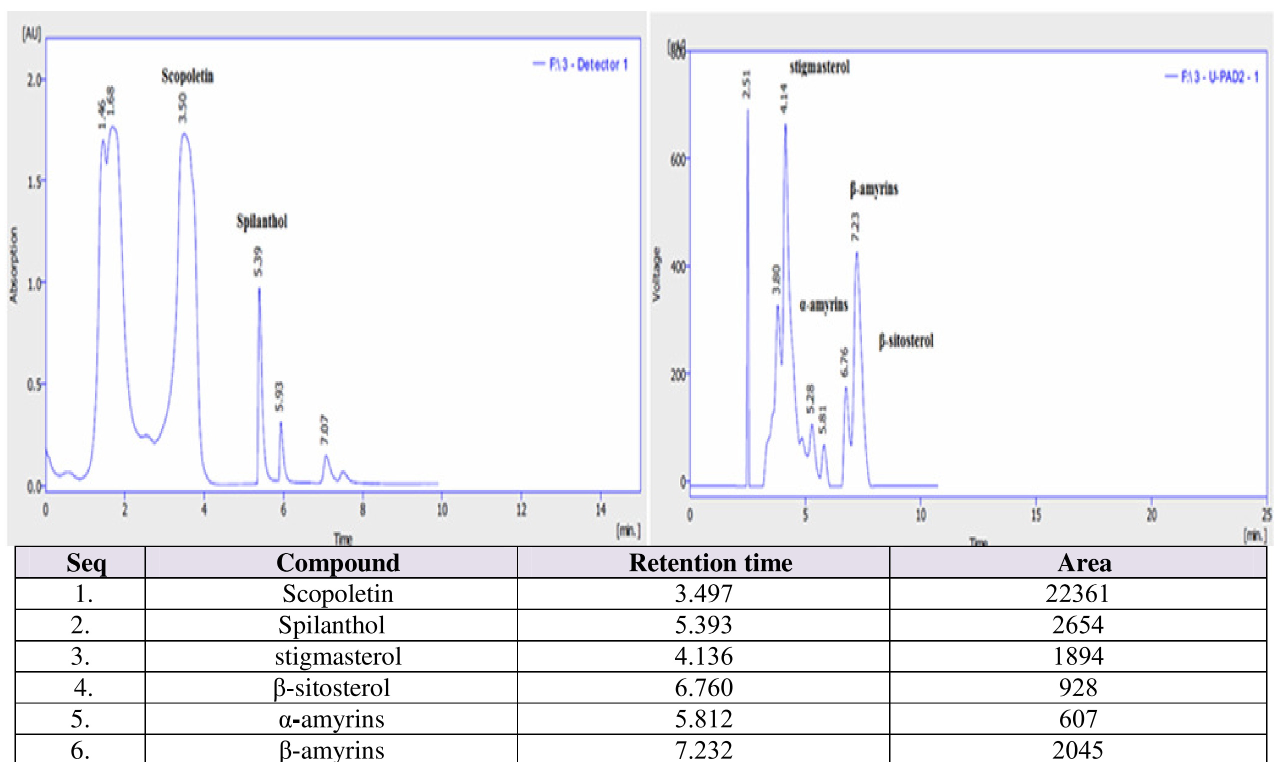
Seq	Compound	Retention time	Area
1.	Scopoletin	3.497	7684
2.	Spilanthol	5.393	5.393
3.	stigmasterol	4.136	2174
4.	β-sitosterol	6.760	277
5.	α-amyrins	5.812	364
6.	β-amyrins	7.232	1000

Fig. 1 : Effect of the no adding treatment on producing secondary compounds (µg.g-1 dry weight) from Paracress shoot tip in MS media four weeks after culturing in the MS media



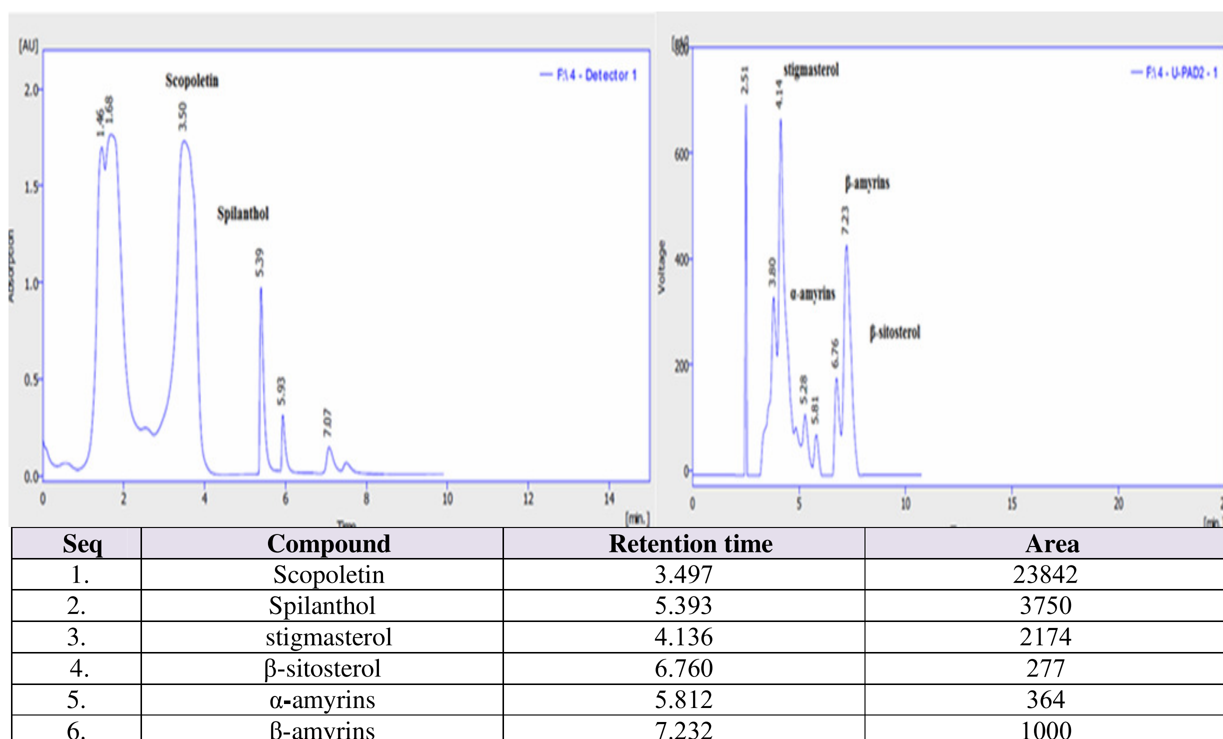
Seq	Compound	Retention time	Area
1.	Scopoletin	3.497	11443
2.	Spilanthol	5.393	1694
3.	stigmasterol	4.136	1529
4.	β-sitosterol	6.760	603
5.	α-amyrins	5.812	1411
6.	β-amyrins	7.232	2367

Fig. 2 : Effect of 250 mg.l^{-1} of glutamine on producing secondary compounds (µg.g-1 dry weight) from Paracress shoot tip in MS media four weeks after culturing in the MS media



Seq	Compound	Retention time	Area
1.	Scopoletin	3.497	22361
2.	Spilanthol	5.393	2654
3.	stigmasterol	4.136	1894
4.	β-sitosterol	6.760	928
5.	α-amyrins	5.812	607
6.	β-amyrins	7.232	2045

Fig. 3 : Effect of 300 mg.l^{-1} of glutamine on producing secondary compounds (µg.g^{-1} dry weight) from Paracress shoot tip in MS media four weeks after culturing in the MS media



Seq	Compound	Retention time	Area
1.	Scopoletin	3.497	23842
2.	Spilanthol	5.393	3750
3.	stigmasterol	4.136	2174
4.	β-sitosterol	6.760	277
5.	α-amyrins	5.812	364
6.	β-amyrins	7.232	1000

Fig. 4 : Effect of 350 mg.l^{-1} of glutamine on producing secondary compounds (µg.g-1 dry weight) from Paracress shoot tip in MS media four weeks after culturing in the MS media

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