Tissue culture and evaluation of some active constituents of *Ruta graveolens* L. II: Effect of plant growth regulators, explant type and precursor on coumarin content of *Ruta graveolens* L. callus cultures

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ABSTRACT

The effect of plant growth regulators and explant type on the coumarin constituent and yield in Ruta graveolens L. callus cultures was studied. Identification of coumarins in different callus cultures by thin layer chromatography showed variation in number of coumarin spots and revealed the presence of bergapten, xanthotoxin, herniarin, isopimpinellin, isoimperatorin, umbelliferone, scopoletin, psoralen and marmesin. The coumarin contents were estimated spectrophotomerically. The stem callus cultures on medium III (MS +0.5mg/l 2,4-D+ 0.5mg/l kinetin) produced yield (0.43 mg%) higher than that of the aerial parts (0.32 mg%) of the plant.

The effect of different concentrations of cinnamic acid (as a precursor) on coumarin contents was investigated. The highest percentage was produced in the stem explant cultures grown on medium I (MS+ 1mg/l 2,4-D) supplemented with 25 mg/l cinnamic acid (0.79mg%). It is about 8 times of stem explant cultures on the same medium without cinnamic acid (0.1mg%), and more than double the coumarin percent of the aerial parts of the cultivated plant (0.32mg%). Isolation and identification by spectroscopic methods of some coumarins from stem and root callus cultures were performed.

Key words: *Coumarin, Ruta graveolens, callus cultures.*

INTRODUCTION

Coumarins afforded great interest due to the wide range of their physiological activity (Gray and waterman, 1978). Increasing attention has been paid to their antibacterial, vasodilator, diuretic, anticoagulant, and respiratory stimulant effects (Murray *et al.*, 1982).

Family *Rutaceae* contains about 200 coumarins most of them appear to be cinnamic acid-derived (Murray *et al.*, 1982). Coumarin

constituents of *Ruta graveolens* L. are responsible for some of its pharmacological activities as spasmolytic, antihistaminic, abortifaciant, bactericide and fungicide (Bajaj, 1989).

Several authors studied the *in vitro* production of some coumarins by tissue culture (Baumert *et al.*, 1992, Bohlmann and Eilert, 1994, Bohlmann *et al.*, 1995, Sharma and Brown, 1978. Steck, *et al.*, 1971, Wolters and Eilert, 1981). However, no results were reported concerning the effect of the precursors on the coumarin contents of *Ruta graveolens* L. Therefore, the present study deals with the effect of plant growth regulators and explant type in the presence of cinnamic acid as a precursor in a trial to increase coumarin contents in callus cultures of *Ruta graveolens* L.

MATERIALS AND METHODS

Plant materials

The herb *Ruta graveolens* L. was obtained from the Experimental Farm of the Ministry of Agriculture and authenticated by Prof. Dr. Nabil El-Hadidy, Professor of Taxonomy and Flora, Faculty of Science, Cairo University. Voucher samples of the herb were kept in the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Cairo University. The plant material was collected during the non-flowering season.

Reference authentic materials

Xanthotoxin (Ammodin) was obtained from the Reference Standard Department, National Organization for Drug Control and Research (NODCAR). Bergapten, coumarin, hyhroxycoumarin, umbelliferone were kindly provided by Prof. Dr. M. Shabana. Scopoletin, herniarin, psoralen, isopimpinellin, imperatorin, isoimperatorin, and marmesin were obtained from the National Research Center. Sugars were obtained from Merck, Darmstadt, Germany.

Materials for chromatographic studies

- TLC plates: Precoated silica gel plates G60 F 254 0.2 mm, 20x20 (Reide- de Haen, Germany).
- All solvents used in this study are of analytical grade chemicals.
- Solvent systems used in planner chromatography:

  A1.Benzene- Ethyl acetate (80: 20 V/V) [TLC for coumarin]

  A2.Toluene- Ether (1:1 V/V) saturated with 10% glacial acetic acid [TLC for coumarin].

  A3.Ethyl acetate-acetic acid-methanol-water (60: 15: 15: 10 V/V/V/V) [TLC for sugars].
- Spray reagent: Thymol sulphuric acid reagent (for sugars) (Adachi, 1965).

Apparatus

1. UV-VIS spectrophotometer (Unicam, UK).
2. Rotatory evaporator (Buchi, Germany).
3. UV lamp (Vilber Laurmat CV-6T, France) for localization of spots on chromatoplates.
4. Koffler's heating stage microscope.

Techniques

1- Tissue cullure techniques

Preparation of Murashige and Skoog (1962) basal medium (MS), cultivation of seeds on the medium, induction of callus cultures, and establishment of callus cultures with different concentrations of auxins and cytokinins were done as previously reported in the communication part I (Shabana *et al.*, under publication). Stem, leaf, shoot tip and root callus cultures were used for extraction,

identification, estimation, and isolation of coumarins.

Cultures of stem explants on (MS+1mg/l 2,4-D) were used to study the effect of cinnamic acid as a precursor for coumarins. Different concentrations from 12.5 mg/l to 75 mg/l were added to the medium I. Fifty jars were used for culture, incubated for 8 weeks at 16 hour/ day photoperiod. Then, the produced calli were used for the assay of total coumarin content.

2- Quantitative estimation of coumarin contents in different organs and callus cultures of *Ruta graveolens* L.

A- Calibration curve of xanthotoxin solution

About 10 mg of xanthotoxin were dissolved in 90 ml absolute ethanol, then completed with ethanol to 100 ml, then 10 of this solution were diluted to 100 ml. Different aliquots of 2.5-20 ml equivalent to 25-20 g/ml of xanthotoxin solution were evaporated. The residue was dissolved in 10ml absolute ethanol. For each concentration, the sample was measured at 300 nm (Egyptian Pharmacopoeia, 1984), compared to a blank of absolute ethanol using spectrophotometer. For each concentration, three determinations were carried out then the absorbances were plotted versus concentrations.

B- Extraction and assay of coumarin content

Five grams of each of fresh aerial parts, underground parts or calli extracted previously with hexane, were extracted with 80% boiling methanol till exhaustion (Steck *et al.*, 1971). The extracts were filtered and the filtrates were con

remove the solvent. The aqueous layer was treated with about 20 ml conc. HCl and extracted with 20 ml / 5 times of dichloromethane to obtain a coumarin-rich fraction (Steck *et al.*, 1971). Each extract was completed to 100 ml with dichloromethane and 10 ml of the solution were evaporated to dryness. The residue was dissolved in 10 ml absolute ethanol, then measuring the absorbance at 300 nm using absolute ethanol as a blank (Egyptian Pharmacopoeia, 1984; Murray *et al.*, 1982; Mousa *et al.*, 1997). The coumarin content, calculated as xanthotoin, was deduced from the pre-established standard calibration curve.

3- TLC examination of the plant aerial parts, underground organs and the callus cultures

Examination was performed in the coumarin-rich fraction extracted with dichloromethane, using solvent system (A_1) and ammonia on UV 365 nm for visualization.

4- Isolation of non-identified coumarin from stem and root callus cultures by preparative TLC

TLC plates were spotted with dichloromethane extracts previously prepared and developed in solvent system (A_1) and the plates were visualized under ultraviolet light (365 nm). The main fluorescent zones were marked, scraped off, collected and extracted with methanol, fillered and kept for crystallization.

RESULTS AND DISCUSSION

TLC investigation of the coumarin content of aerial parts, and underground organs parallel with authentics (Tables 1 and 2) revealed the presence of marmesin, scopoletin, isopimpinellin, hydroxy coumarin, xanthotoxin, umbelliferone, isoimperatorin, psoralen, bergapten and herniarin in the aerial parts and the absence of hydroxy coumarin and isopimpinellin from the underground organs.

TLC investigation of coumarin content of the calli (Tables 3, 4, 5 and 6) showed variation in number of main spots in the different explant type and media according to the concentration of auxins and cytokinens. It is noticed that medium III (MS+ 0.5mg/l 2, 4-D+ 0.5 mg/l kinetin) produced a large number of main spots in leaf and root callus cultures, but less than those present in the plant organs. At the same time, non-identified coumarins appeared in callus cultures of stem and root.

Table (1): Thin layer chromatography of the extract of aerial parts (benzene:ethyl acetate 80:20 v/v).

Spot no.	R_f	Fluorescence in UV (365 nm)	UV/NH_3	Run parallel with
1	0.02	blue	++	-
2	0.05	blue	++	-
3	0.07	blue	++	-
4	0.09	bluish-violet	++	-
5	0.16	violet	++	marmesin
6	0.31	blue	++	scopoletin
7	0.35	yellow	++	isopimpinellin
8	0.38	blue	++	
9	0.40	violet	++	hydroxycoumarin
10	0.42	yellow	++	-
11	0.45	blue	++	xanthotoxin
12	0.51	blue	++	-
13	0.54	yellow	++	umbelliferone
14	0.61	violet	++	isoimperatorin
15	0.72	yellow	++	-
16	0.78	yellow	++	psoralen
17	0.80	blue	++	-
18	0.86	bluish-violet	++	bergapten herniarin

Table (2): Thin layer chromatography of the extract of underground parts (benzene:ethyl acetate 80:20 v/v).

Spot no.	R_f	Fluorescence in UV (365 nm)	UV/NH_3	Run parallel with
1	0.01	bluish-violet	++	-
2	0.03	blue	++	-
3	0.04	yellowish	++	-
4	0.05	bluish-violet	++	-
5	0.07	bluish-violet	++	-
6	0.16	violet	++	marmesin
7	0.25	yellowish	++	-
8	0.34	yellowish	++	-
9	0.31	blue	++	scopoletin
10	0.40	violet	++	-
11	0.42	yellow	++	xanthotoxin
12	0.51	blue	++	umbelliferone
13	0.54	yellow	++	isoimperatorin
14	0.61	violet	++	-
15	0.72	yellow	++	psoralen
16	0.78	yellow	++	-
17	0.80	blue	++	bergapten
18	0.86	bluish-violet	++	herniarin
19	0.91	blue	++	-
20	0.93	blue	++	-

++ Fluorescence is intensified by exposing spot to NH3 vapour.

Table (3): TLC examination of the coumarin fractions of Ruta graveolens L. stem cultures (benzene: ethyl acetate 8:2 v/v).

Spot no.	R_f	Fluorescence in UV (365nm)	UV/NH_3	Run parallel with	Stem cultures on I	Stem cultures on II	Stem cultures on III
1	0.04	blue	++	_	-	+	-
2	0.16	violet	++	Marmesin	-	-	+
3	0.33	yellow	++	_	+	+	-
4	0.40	violet	++	_	+	+	+
5	0.42	yellow	++	Xanthotoxin	+	+	+
6	0.57	yellow	++	Isoimperatorin	-	-	+
7	0.72	blue	++	_	+	-	-
8	0.78	yellow	++	Psoralen	+	-	-
9	0.80	blue	++	Bergapten	+	-	-
10	0.89	green	++	_	+	-	-

I	MS + 1mg/l 2,4-D.	+	Spot is present in the TLC.
II	MS + 1mg/l 2,4-D + 1mg/l kinetin.	_	unidentified component.
III	MS + 0.5mg/l 2,4-D + 0.5mg/l kinetin.	-	Spot is absent.
IV	MS + 1mg/l 2,4-D + 5mg/l kinetin.	++	Fluorescence is intensified by exposing spot to NH3 vapour.

Table (4): TLC examination of the coumarin fractions of Ruta graveolens L. leaf cultures (benzene: ethyl acetate 8:2 v/v).

Spot no.	R_f	Fluorescence in UV (365nm)	UV/NH_3	Run parallel with	Leaf cultures on I	Leaf cultures on II	Leaf cultures on III	Leaf cultures on IV
1	0.04	blue	++	_	-	-	-	+
2	0.30	yellow	++	_	+	-	-	+
3	0.33	blue	++	Scopoletin	-	+	+	-
4	0.40	violet	++	_	+	-	+	+
5	0.42	yellow	++	Xanthotoxin	+	+	-	+
6	0.51	blue	++	Umbelliferone	-	+	+	-
7	0.66	blue	++	_	+	-	+	-
8	0.72	blue	++	_	-	+	+	-
9	0.78	yellow	++	Isopimpinellin	-	-	+	-
10	0.80	blue	++	Bergapten	-	-	-	+
11	0.90	violet	++	Herniarin	-	-	+	-
12	0.93	blue	++	_	-	-	+	-
13	0.94	yellow	++	_	+	-	-	-

I	MS + 1mg/l 2,4-D.	+	Spot is present in the TLC.
II	MS + 1mg/l 2,4-D + 1mg/l kinetin.	_	unidentified component.
III	MS + 0.5mg/l 2,4-D + 0.5mg/l kinetin.	-	Spot is absent.
IV	MS + 1mg/l 2,4-D + 5mg/l kinetin.	++	Fluorescence is intensified by exposing spot to NH3 vapour.

Table (5): TLC examination of the coumarin fraction of Ruta graveolens L. shoot tip cultures (benzene: ethyl acetate 8:2 v/v).

Spot no.	R_f	Fluorescence in UV (365nm)	UV/NH_3	Run parallel with	Shoot tip cultures on I	Shoot tip cultures on II	Shoot tip cultures on III	Shoot tip cultures on IV
1	0.04	blue	++	_	-	-	-	+
2	0.06	bluish-violet	++	_	-	-	+	-
3	0.16	violet	++	Marmesin	-	+	+	-
4	0.18	yellow	++	_	-	-	+	-
5	0.30	yellow	++	_	-	-	-	+
6	0.30	blue	++	Scopoletin	-	+	+	-
7	0.40	violet	++	_	+	+	+	+
8	0.42	yellow	++	Xanthotoxin	+	+	-	-
9	0.51	blue	++	Umbelliferone	-	-	+	-
10	0.54	yellow	++	Isoimperatorin	+	+	+	-
11	0.64	blue	++	_	+	-	+	-
12	0.72	blue	++	_	-	+	-	+
13	0.78	yellow	++	_	-	+	-	-
14	0.80	blue	++	Bergapten	-	+	-	-
15	0.89	green	++	_	+	-	-	-
16	0.91	blue	++	_	-	-	-	+

I	MS + 1mg/l 2,4-D.	+	Spot is present in the TLC.
II	MS + 1mg/l 2,4-D + 1mg/l kinetin.	_	unidentified component.
III	MS + 0.5mg/l 2,4-D + 0.5mg/l kinetin.	-	Spot is absent.
IV	MS + 1mg/l 2,4-D + 5mg/l kinetin.	++	Fluorescence is intensified by exposing spot to NH3 vapour.

Table (6): TLC examination of the coumarin fraction of Ruta graveolens L root callus (benzene : ethyl acetate 8:2 v/v).

Spot no.	R_f	Fluorescence in UV (365nm)	UV/NH3	Run parallel with	Root cultures on I	Root cultures on II	Root cultures on III	Root cultures on IV
1	0.04	blue	++	_	-	+	-	-
2	0.30	yellow	++	_	+	-	-	-
3	0.34	blue	++	Scopoletin	-	-	+	+
4	0.40	violet	++	_	+	-	+	+
5	0.50	blue	++	_	-	-	+	-
6	0.51	blue	++	Umbelliferone	-	-	+	-
7	0.54	yellow	++	Isoimperatorin	-	+	+	-
8	0.66	blue	++	_	+	-	-	+
9	0.72	blue	++	_	-	+	+	+
10	0.78	yellow	++	_	-	-	+	-
11	0.80	blue	++	Bergapten	+	+	-	-
12	0.89	violet	++	_	+	-	-	+
13	0.95	blue	++	_	-	+	-	-
14	0.97	yellow	++	_	-	+	-	-

I	MS + 1mg/l 2,4-D.	+	Spot is present in the TLC.
II	MS + 1mg/l 2,4-D + 1mg/l kinetin.	_	unidentified component.
III	MS + 0.5mg/l 2,4-D + 0.5mg/l kinetin.	-	Spot is absent.
IV	MS + 1mg/l 2,4-D + 5mg/l kinetin.	++	Fluorescence is intensified by exposing spot to NH3 vapour.

Total coumarin content was calculated as xanthotoxin using its standard calibration curve (Table 7, Fig 1). Comparing the estimated coumarins of stem, leaf and shoot tip callus cultures with that of aerial parts, it is evident that no increase in coumarin content occurred except in case of stem cultures incubated on MS supplemented with 0.5 mg/l 2,4-D and 0.5 mg/l kinetin. The yield of coumarins in these cultures was much higher (0.43 mg%) (Table 9) compared to the coumarin yield in aerial parts of Ruta graveolens L. herb (0.32 mg%) (Table 8), i.e., about 75% of the callus weight of such culture was enough to produce the same yield as that of the aerial parts. Also, leaf cultures on MS + 1mg/l 2,4-D + 1mg/l kinetin produced equal coumarin yield (0.32mg%) to that of the aerial parts. In case of root cultures, no increase in the coumarin content was observed in comparison with that obtained from the underground parts of the herb (0.44mg%) (Table 8).

Table (7): Absorbances of different concentrations of xanthotoxin at 300 nm.

Concentration in µg/ml (mg%)		Absorbance
2.5	(0.25)	0.16
5	(0.50)	0.347
9	(0.90)	0.576
9.5	(0.95)	0.669
11	(1.10)	0.783
12.5	(1.25)	0.884
15	(1.50)	1.085
17	(1.70)	1.229
18	(1.80)	1.264

Table (8): Total coumarin content in extracts of aerial and underground parts.

Content in aerial parts in µg/ml (mg%)	Content in underground parts in µg/ml (mg%)
3.2 (0.32)	4.4 (0.44)

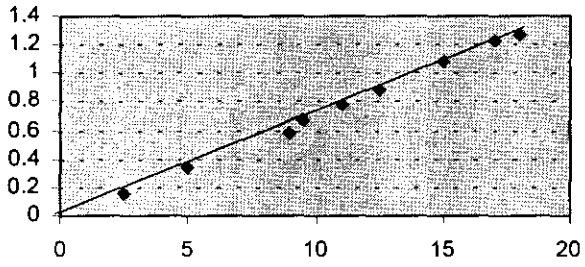


Fig. (1): Absorbance standard calibration curve of xanthotoxin.

On the other hand, data presented in table 9 showed that the stem cultures grown on medium MS+0.5mg/l 2,4-D + 0.5mg/l kinetin with minimal growth measurements, produced the highest coumarin content among all the cultures and even higher percentage than the aerial parts of the herb itself. The leaf cultures on MS + 1mg/l 2,4-D + 1mg/l kinetin produced the highest coumarin content were of the highest fresh weight and callus index values amongst leaf cultures while shoot tip cultures on media MS + 1mg/l 2,4-D + 5 mg/l kinetin were shown to produce the high growth measurements and also the highest coumarin content compared to all shoot tip cultures. This agreed with the fact that presence of coumarin in conjunction with auxin increased growth (George, 1993). Finally, root cultures on medium MS+ 1mg/l 2,4-D possessed minimal fresh and dry weights produced the highest coumarin content amongst all root cultures, showing that auxin only may be required in root cultures to develop coumarins in the culture in a moderate yield than other cultures grown on MS supplemented with both auxin and cytokinin.

As a general rule, undifferentiated tissues growing in synthetic media showed a greatly reduced capacity for secondary metabolism (George, 1993), this agreed with the previous results in which the highest yield of different cultures were of the differentiated.

Table (9): Total coumarin content in stem, leaf, shoot tip and root callus cultures.

µg/ml (mg%) PGR	Stem cultures	Leaf cultures	Shoot tip cultures	Root cultures
I	1.0 (0.10)	0.4 (0.04)	1.5 (0.15)	3.3 (0.33)
II	1.8 (0.18)	3.2 (0.32)	0.8 (0.18)	1.1 (0.11)
III	4.3 (0.43)	2.2 (0.22)	1.7 (0.17)	1.0 (0.10)
IV	-	2.1 (0.21)	2.2 (0.22)	2.9 (0.29)

I MS + 1mg/l 2,4-D. III MS + 0.5mg/l 2,4-D + 0.5mg/L kinetin
II MS + 1mg/l 2,4-D + 1mg/L kinetin IV MS + 1mg/l 2,4-D + 5 mg/L kinetin.

ones although the non-differentiated cultures on MS+ 1mg/l 2,4-D produced the highest coumarin content between all root explant cultures but still less than that of the herb. This was formerly emphasized by Ramawat et al (1985), who concluded that high alkaloid contents were recorded in differentiating strains of Ruta graveolens L. cultures.

It has been reported that 2,4-D and kinetin strongly inhibited and promoted the nicotine synthesis in tobacco cells, respectively. Similarly, in Lithospernum

cultures, fromation of shikonin was completely inhibited by synthetic auxins. Such inhibition was observed in stem and leaf cultures on MS+ 1mg/l 2,4-D, while shoot tip cultures produced moderate yield of coumarin but still less than present in the herb. Oppositely, root cultures on MS+1mg/l 2,4-D showed highest coumarin content compared to other root cultures grown on MS with different ratios of 2,4-D to kinetin. Therefore, it was clear that the equal ratios of 2,4-D and kinetin had increased the coumarin content in stem and leaf cultures than other cultures.

The cinnamic acid was added to stem cultures on MS medium supplemented with 1mg/l 2,4-D to study its effect as a percursor for coumarins (Dewick, 1998). It was found that 25mg/l cinnamic acid addition to the media was the concentration required for producing highest coumarin content in the cultures (Table 10), i.e., about 8 times as much coumarin was produced as that present in the same cultures grown in the absence of cinnamic acid, and more than double that of the aerial parts of the plant coumarin content. Also, addition of 50mg/l cinnamic acid in the medium produced about 5 times as much that produced by cultures without cinnamic acid. On the other hand, cinnamic acid in the medium did not affect the morphological characters of the non-differentiated state of the callus. The higher or lower concentrations than 25-50 mg/l caused inhibition for callus growth.

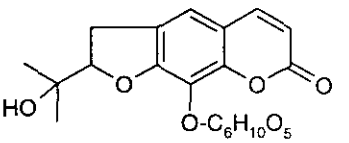
Table (10): Total coumarin content in extracts of stem callus cultures.

Content in cultures with 25 mg/l cinnamic acid in µg/ml (mg%)	Content in cultures with 50 mg/l cinnamic acid in µg/ml (mg%)	ontent in cultures without cinnamic acid in µg/ml (mg%)
7.9 (0.79)	4.8 (0.48)	1.0 (0.10)

Two coumarins were isolated: Compound C1 was obtained by preparative TLC from coumarin-rich fraction of stem callus cultures on medium I (MS+ 1mg/l 2,4-D). Rf 0.89 (A1), 0.95 (A2), yellowish small crystals (methanol) 10mg, m.p. 142oC, soluble in methanol, ethanol, gave greenish fluoresence in UV light (365 nm) that intensified with exposure to ammonia vapors. It gave positive Molisch's test which confirms its glycosidic nature UV max nm, MeOH: 248, 258 and 336 nm. Mass spectrum (EIMS), (M/Z, rel. Int.) M+ 426 (1%), 262 (2%), 219 (25%), 162 (3%), 149 (100%), 101 (5%), 97 (17%), 83 (18%), 69 (38%), 59 (2%), 57 (44%).

After acid hydrolysis, the sugar moiety was identified as glucose. From the present data and the published ones (Murray *et al.*, 1982; Schneider *et al.*, 1967) compound C_1 may be rutarin.

Compound C_1



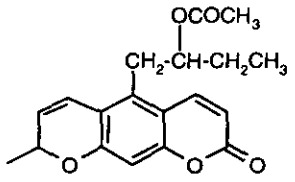
Compound C_2 was isolated by preparative TLC from coumarin-rich fraction of root callus culture on medium I (MS+1mg/l 2,4-D). R_f 0.89 (A_1), 0.78 (A_2) white crystals (methanol), sublimable, 5 mg, soluble in methanol, ether, gave violet fluorescence in UV light (365 nm) that intensified with

exposure to ammonia vapors. It gave negative Molisch's test.

UV λ_{max} nm, Me OH: 205, 220, 240 and 270. Mass spectrum (EI MS) (M/Z el. Int) M^+ 327 (12.7%), 299 (100%), 298 (29.6%), 284 (13.2%), 233 (5.4%), 169 (19.4%), 168 (44.7%), 155 (11.1%) 141 (12.1%), 128 (11.9%), 115 (11.6%), 101 (11.3%), 90 (5.7%), 85 (19.9%), 76 (12.4%), 62 (51%), 61 (10%), 59 (11.9%), 56 (19.1%).

The obtained data suggested that the isolated compound C_2 may be coumarin, and may possess the following structure

Compound C_2



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الملخص العربي

زراعة الأنسجة وتقييم المواد الفعالة لنبات السذب (روتا جرافيولنس)

الجزء الثانى- دراسة تأثير منظمات النمو ونوع الجزء النباتى

وحمض السينناميك على الكومارينات فى زراعات الكالوس لنبات السذب

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** شعبة زراعة الأنسجة النباتية الهيئة القومية للرقابة والبحوث الدوائية

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تمت دراسة تأثير منظمات النمو المختلفة والأجزاء النباتية على التركيب الكيميائى والنسبة الكلية للكومارينات. وقد تم تحضير الخلاصة الغنية بالكومارينات بالزراعات المختلفة للتعرف على المواد الكومارينية بواسطة كروماتوجرافيا الطبقة الرقيقة حيث أعطت نتائج متباينة فى كل حالة. تم التعرف على الكومارينات باستخدام هلام السيليكا وبنزين: خلات الإيثيل (٨: ٢) والمقارنة بالمواد المرجعية. وقد اتضح أن الكومارينات الموجودة فى الزراعات هى كالآتى: برجابتين، زانثوتوكسين، هرنيارين، ايزوبيمبنللين، ايزوايمبراتورين، سكوبولتين، سورالين، مارميزين وامبيليفرون وهيدروكسى كومارين.

تم تعيين النسبة الكلية للكومارينات فى الزراعات بالطريقة الطيفية واتضح إنها قد زادت فى زراعات الكالوسات الناتجة من الساق على بيئة (0.43 % III) عن تلك الموجودة فى الأجزاء الخضرية للنبات (٠,٣٢%).

تمت دراسة تأثير وجود تركيزات مختلفة من حمض السينناميك لأول مرة على نسبة الكومارينات وذلك بزراعة أجزاء من الساق على بيئة I مزودة بتركيزات مختلفة من حمض السينناميك هو ٢٥ مجم/ لتر حيث أعطى نسبة كومارينات (٠,٧٩ %) حوالى ثمانية أضعاف تلك الموجودة فى زراعات الساق بدون حمض السينناميك (٠,١%) وأكثر من ضعف تلك الموجودة فى الأجزاء الخضرية للنبات (٠,٣٢%).

تم فصل أثنين من الكومارينات اللذين لم يمكن التعرف عليه بكوروماتوجرافيا الطبقة الرقيقة من خلاصات الكالوس المتكون من زراعة أجزاء من الساق والجذور على بيئة I وذلك بواسطة كروماتوجرافيا الطبقة الرقيقة باستخدام بنزين: خلات الإيثيل (٨: ٢).

المادة الأولى: بلورات تميل إلى الاصفرار، درجة انصهارها ١٤٢–١٤٥م، تعطى إزهاراً أخضراً تحت الإشعة فوق البنفسجية وقد تم التعرف عليها بواسطة كروماتوجرافيا الطبقة الرقيقة مع استخدام مذيبات مختلفة والطريقة الطيفية وطيف الكتلة. وقد تبين أنه من المحتمل أن تكون مادة الروتارين.

المادة الثانية: بلورات بيضاء اللون، وهى مادة متسامية، تعطى إزهاراً بنفسجياً تحت الأشعة فوق البنفسجية وتم تحليلها بواسطة كروماتوجرافيا الطبقة الرقيقة مع استخدام ٢١ذيبات مختلفة والطريقة الطيفية وطيف الكتلة. وأعطت نتائج مقترحة لمادة كومارينية لم يتعرف عليها من قبل .