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ANTHRAQUINONES PRODUCTION IN AERIAL PARTS-DERIVED CALLUS OF *RUBIA TINCTORUM* L.

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تم فى هذا البحث زراعة خلايا الجزء الخضرى لنبات الروبيا تنكتورم إل فى وسط إم إس مزود بمادتين إضافيتين هما بنزيل أمينوبيورين ، ١ نفثالين حمض الخليك. وقد تم تتبع التغير فى وزن الخلايا ومحتوىالأنثراكينونات على مدى ستة أسابيع ووجد أن الزيادة فى وزن الخلايا يقابلها زيادة موازية فى إنتاج الأنثراكينونات. وقد تمت دراسة تأثير الأس الهيدروجينى ، درجة حرارة الحضانة ، منظمات النمو على نمو الخلايا ومحتوىالأنثراكينونات ووجد أن الأس الهيدروجينى ٧,٥ وعند درجة حرارة ٣٠ درجة مئوية هى أفضل الظروف لنمو الخلايا.

ولقد أمكن فصل والتعرف على أربعة مركبات هى أليزارين (١) ، ليوثيدين إيثيل الإيثر (٢) ، ١ هيدروكسى ٢ هيدروكسىميثيل أنثراكينون ٣ أوبيتا جلوكوزيد (٣)، ليوثيدين بريمفيروزيد (٤). وقد تم التعرف على المركبات المفصولة بإستخدام الأشعة فوق البنفسجية ودون الحمراء ومطياف الكتلة والمقارنة ببيانات المركبات المرجعية. كما أمكن تقدير كمية الأنثراكينونات فى كل من الجزء الخضرى ، الجذور ، الخلايا المنزرعة بطريقة منشورة. وقد أظهرت النتائج أن الخلايا المنزرعة إحتوت على أنثراكينونات مماثلة للجذوروتصل إلى حوالى ثمانية عشرة أضعاف الكميات الموجودة فى الجزء الخضرى.

Callus cultures were developed from the aerial parts of Rubia tinctorum L. on MS medium containing 6-benzylaminopurine and 1-naphthaleneacetic acid. The changes in callus weight and anthraquinones content were followed between week 1 and 6. Anthraquinones content was monitored by HPLC. The linear increase in fresh weight was found to be parallel to the anthraquinones production. Effect of medium pH, incubation temperature and growth regulators on callus growth and anthraquinones content was studied and optimised. pH 7.5 and temperature of 30° were found optimum for the callus growth. 6-Benzylaminopurine (0.5 mg/L) and 1-naphthaleneacetic acid (2.0 mg/L) were found the most appropriate growth regulators.

*The callus ethanolic extract showed the same picture on TLC as that of the roots. The anthraquinones alizarin (**1**), lucidin-ω-ethyl ether (**2**), 1-hydroxy-2-hydroxymethyl-antraquinone-3-O-β-glucoside (**3**) and lucidin primeveroside (**4**) in addition to other four minor anthraquinone spots were found to be produced by these cultures. The four isolated compounds (**1-4**) were identified by spectral analysis (UV, IR and MS) as well as comparison with authentic samples. Aerial parts, roots and the callus were analysed for their anthraquinones content. The anthraquinones content of callus extract was about 91% and eighteen fold of the roots and aerial parts extract respectively.*

INTRODUCTION

Rubia tinctorum L. (dyer's madder),[1] Family Rubiaceae, is a perennial herbaceous plant having long fleshy roots which furnish a dye. It was used as diuretic, astringent, as remedy in liver disease and spleen complaints.[2] The roots extract was used for the treatment of kidney, bladder stones[3] and contains substantial amounts of anthraquinones.[4-6] The presence of minor biologically active anthraquinones in the aerial parts of this plant[7] has served as an incentive for the development of cell cultures capable of long-term cultivation and synthesis of secondary metabolities. In the present work, we study the effect of growth regulators on the developing callus cultures from the aerial parts of *R. tinctorum* L. as an alternative source of anthraquinones.

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EXPERIMENTAL

Materials

Apparatus

1- Uvidec-320 spectrophotmeter with matched 1 cm quartz cells, (Jasco, Tokyo, Japan).
2- Schimadzu Infra red-470 Spectrometer (Tokyo, Japan).
3- EI^+- and FAB-MS spectra were recorded by JEOL, JMS 600 H, Japan.
4- HPLC, Knauer, Germany.
5- UV-Lamp (Marine Lavalee-Cedex, France).
6- Incubator, Gallenkamp, England.

Reagents

- 5% alcoholic potassium hydroxide.
- ammonia vapours.

Solvent systems

I- n-Hexane - ethyl acetate (90:10).
II- n-Hexane - ethyl acetate (80:20).
III- Chloroform - methanol (80:20).
IV- Chloroform - methanol (70:30).

Chemicals

Silica gel for column, precoated silica gel plates 60 F_{254} (E. Merck, Germany). 6-Benzyl-aminopurine, 1-naphthaleneacetic acid (NAA), 6-furfurylaminopurine, 3,6-dichloro-2-methoxy-benzoic acid and 2,4-dichloro-phenoxyacetic acid were obtained from E. Merck, Darmstadt, Germany.

Plant material

Aerial parts of *R. tinctorum* L. were collected in January 2002 (before flowering) from the plant cultivated in the Experimental Station, Faculty of Pharmacy, Assiut University, were used as explant sources.

Method

Callus culture induction and development

Leaves and young stems of *R. tinctorum* L. were used as explant sources. After rinsing in water, explants were sterilized with 70% ethanol for few seconds, immersed in a 0.1% solution of mercuric chloride[8] for 15 minutes and washed three times with sterile distilled water. They were then cultured in the dark at 30° in 300-ml flasks containing 50 ml of MS medium[9] containing both 0.5 mg/L 6-benzylaminopurine and 2.0 mg/L 1-naphthaleneacetic acid.[10] Callus emerging on the explants (after 3 weeks) was excised and cultivated on the same medium at 30° for 3-weeks periods.

To select high-producing callus cultures, the deeper coloured small cell aggregates were isolated from the stock yellow callus and further subcultured on the same medium for another 3-weeks periods. Callus tissues of these selected strains were subcultured for a further 6-weeks before they were characterized quantitatively for pigment content.

Growth curves and anthraquinones content

The changes in fresh callus weight and anthraquinones content of cell cultures were followed by using ten flasks with 100 ml fresh MS medium and inoculated with 1 g callus cells (week zero) grown in the dark. Fresh callus weight and anthraquinones content were determined from week zero to week 6 at one week intervals by HPLC method, Figure 1.

HPLC method

It is used for the determination of anthraquinones in the study of each of the effect of pH, incubation temperature as well as the effect of growth regulators on the anthraquinones production.

The callus was dried under hot air flow and powdered, 5 g quantity was extracted exhaustively on cold with 50 ml of 70% ethanol for 3 hours. Extracts were filtered through filter paper and the filtrate was evaporated under reduced pressure at 40°. The residue was dissolved in 1 ml methanol and subjected to HPLC analysis. Elution was carried out with water-methanol (20-70%) in a Nucleosil 100-5 C_{18} column (Macherey-Nagel) at a flow rate of 1 ml/minute, and the detection wavelength set to 246 nm. Identification of anthraquinones was achieved from the retention time values. The area under the curves corresponding for the major compounds were used to calculate the anthraquinones content.[8,11] The results were calculated as alizarin.

Study of the effect of medium pH and incubation temperature on callus growth and anthraquinones content

The optimum pH and temperature for callus growth and anthraquinones content were studied by using a series of 100-ml flasks

inoculated with 1 g cultured cells grown in MS medium at different pH values between 6.0 and 9.0 in the dark for a period of 4 weeks. At the optimum pH value another series of inoculations were performed at different temperatures between 20 and 45°. The results are shown in Tables 1 and 2.

Effect of growth regulators on callus growth and anthraquinones content

At the optimum pH and incubation temperature, series of experiments were performed using different types of growth regulators at different concentrations (ranging from 0.3-4.0 mg/L) in the dark for a period of 4 weeks. Each two growth regulators were used together as follows: 6-Benzylaminopurine with 1-naphthaleneacetic acid, 6-furfurylamino-purine with 1-naphthaleneacetic acid, 6-furfurylaminopurine with 3,6-dichloro-2-methoxy-benzoic acid and 6-Benzyl-aminopurine with 2,4-dichloro-phenoxyacetic acid.

Determination of anthraquinones in the aerial parts, roots and callus by the reported colorimetric procedure[7,12]

Five grams of aerial parts, roots and callus were accurately weighed and separately transferred to 250-ml flasks, each sample was extracted by refluxing five times with 30 ml of 70% ethanol containing 1% hydrochloric acid on a boiling water bath until the alcohol gave no red colour with potassium hydroxide (T.S.). The combined alcohol extracts were filtered and transferred to a volumetric flask (250 ml). The volumes were then adjusted with ethanol 70%. To 20 ml of each solution, 10 ml of concentrated hydrochloric acid was added, the mixture was heated on a boiling water bath under reflux for 15 minutes, cooled and extracted with several successive portions of ether. The combined ether extracts were washed with 20 ml of water and the washing was rejected. The ether containing free anthraquinones was completed to 100 ml in a volumetric flask, 10 ml of the ether solution was extracted with known volume of 1 N sodium hydroxide and the developed colour in each case was measured colorimetrically within one hour. The concentration of total anthraquinones in each sample was calculated as alizarine from the calibration curve. Results were listed in Table 3.

Extraction and isolation of anthraquinones

Fifty grams of dried, ground callus of *R. tinctorum* L. were exhaustively extracted with 70% ethanol, concentrated under reduced pressure and the concentrate (4.5 g) was subjected to solvent fractionation using n-hexane (2x150 ml), ethyl acetate (2x150 ml) and n-butanol (2x200 ml) respectively. The obtained fractions were separately concentrated and screened by TLC for different constituents.

A- n-Hexane fraction

The n-hexane soluble fraction was chromatographed on silica gel column, elution was started with n-hexane followed by n-hexane/ethyl acetate gradient. Fractions eluted with n-hexane/ethyl acetate (90:10) afforded compounds **1** and **2**.

B- Ethyl acetate fraction

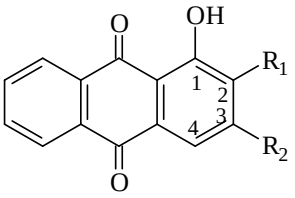
The ethyl acetate soluble fraction was chromatographed on silica gel column. Elution was started with chloroform followed by chloroform/ methanol gradient, similar fractions were combined and concentrated. Fractions eluted with chloroform/methanol (85:15) afforded compound (**3**) and fractions eluted with chloroform-methanol (80:20) gave mixture of two minor anthraquinones spots.

C- n-Butanol fraction

The n-butanol soluble fraction was chromatographed on silica gel column. Elution was started with ethyl acetate followed by ethyl acetate/methanol gradient, similar fractions were combined and concentrated. Fractions eluted with ethyl acetate/methanol (85:15) yielded compound (**4**) and fractions eluted with ethyl acetate-methanol (80:20) gave two minor anthraquinones spots.

Compound 1

Red purple crystals (10 mg), IR (KBr, υ, cm^{-1}): 3340, 1585, 1660 and 1625, UV (λ_{max}, nm, EtOH): 248, 263, 275 and 430. EI^+-MS at *m/z*= 240 (100%) $[M]^+$, other diagnostic peaks at *m/z*= 241 (21.5%)$[M + 1]^+$, 212 (32.4%), 184 (23.5%), 138 (33.1%), 128 (31.5%) and 77 (22.3%).



Compd.	R_1	R_2
1	OH	H
2	$CH_2OCH_2CH_3$	OH
3	CH_2OH	O-glucose
4	CH_2OH	O-glucose(6-1)xylose

Compound 2

Yellow crystalline needles (7 mg), IR (KBr, υ, cm^{-1}): 3210, 1675, 1630, 1590 and 1280, UV (λ_{max}, nm, EtOH): 246, 281 and 418. EI^+-MS at *m/z*= 298 (7.4%) $[M]^+$, 252 (100%) $[M - CH_3CH_2OH]^+$, other diagnostic peaks at *m/z*= 253 (19.2%), 255 (3.1%), 224 (11.2%), 196 (30.3%), 139 (31.4%), and 77 (7.0%).

Compound 3

Yellow powder (10 mg), IR (KBr, υ, cm^{-1}): 3415, 1655, 1621, 1578, 1474 and 1287, UV (λ_{max}, nm, MeOH): 244, 266, 333 and 404. FAB-MS at *m/z*= 433 $[M + 1^+]$ (8.8%), 271 (20.4%), 255 (25.5%), 254 (12.3%), 253 (40.4%), 225 (33%), 197 (37.7%), 169 (32.9%) and 139 (48.1%).

Compound 4

Yellow powder (30 mg), IR (KBr, υ, cm^{-1}): 3390, 1665, 1622, 1585, 1366 and 1286, UV (λ_{max}, nm, MeOH): 244, 266, 333 and 404. FAB-MS at *m/z*= 565 $[M + 1^+]$ (1.1%), 434 (7.6%), 271 (9.2%), 255 (24%), 254 (51.9%), 225 (15%), 197 (10.6%), 169 (9.6%) and 139 (20.7%).

RESULTS AND DISCUSSION

The leaves and young stems of *R. tinctorum* L. were used to establish callus cultures. After the third subculture, callus appeared as a yellow stock with brown coloured small cell aggregates which were isolated and further subcultured. The production of anthraquinones in callus extract was evidenced by their red coloured spots on TLC with 5% alcoholic potassuium hydroxide or ammonia vapours.

The growth of the callus in MS medium was characterized in the dark.[10] Figure 1 showed an increase in fresh weight between week 1 and week 6 after inoculation of cells into fresh medium. The increase in fresh weight was found to be parallel to anthraquinones accumulation.

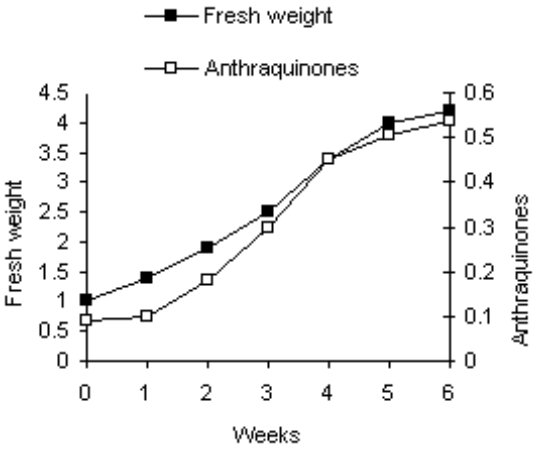


Fig. 1: Changes in fresh weight and anthraquinones content of *R. tinctorum* L. cell cultures.
* The data are mean values of three independent experiments.

Study of the effect of medium pH and incubation temperature on callus growth and anthraquinones accumulation revealed that the highest callus growth and anthraquinones production was obtained at pH 7.5 and an incubation temperature of 30°. The results were listed in Tables 1 and 2.

The anthraquinones content in root, callus (4 weeks old) and aerial parts extracts were compared using the reported colorimetric procedure which depends on the reaction with sodium hydroxide.[7,12] The results showed that, the aerial parts extract contain low amount of anthraquinones. The callus extract was found to contain about 91% of the anthraquinones content of the roots extract and about eighteen fold of aerial parts extract. Results were listed in Table 3.

The influence of some growth regulators on biomass accumulation and anthraquinones production by *R. tinctorum* L. callus cultures was tested. 6-Benzylaminopurine and 1-naphthaleneacetic acid, which have been reported to be appropriate regulators, supporting high level of anthraquinones accumulation in *R. cordifolia* L. callus

Table 1: Effect of medium pH on callus fresh weight and anthraquinones content.

Medium pH	Callus fresh weight(g)*, ± SD	Anthraquinones (%)*, ± SD
6.0	1.60 ± 0.041	1.45 ± 0.034
6.5	2.10 ± 0.046	1.60 ± 0.039
7.0	2.70 ± 0.052	1.85 ± 0.040
7.5	3.12 ± 0.064	2.16 ± 0.045
8.0	2.85 ± 0.059	1.90 ± 0.040
8.5	2.40 ± 0.053	1.65 ± 0.035
9.0	1.90 ± 0.039	1.33 ± 0.030

* The data are mean values of three independent experiments.

Table 2: Effect of incubation temperature on callus fresh weight and anthraquinones content.

Incubation Temperature (°C)	Callus fresh weight (g)*, ± SD	Anthraquinones content (%)* , ± SD
20	2.40 ± 0.050	1.30 ± 0.034
25	3.00 ± 0.058	1.90 ± 0.041
30	3.40 ± 0.075	2.20 ± 0.043
35	2.90 ± 0.059	1.80 ± 0.017
40	2.30 ± 0.047	1.40 ± 0.035
45	1.70 ± 0.033	0.90 ± 0.019

*The data are mean values of three independent experiments.

Table 3: Determination of anthraquinones in the aerial parts, roots and callus extracts by the reported colorimetric procedure[7,12].

Extract	Anthraquinones (%)	
	Found*, ± SD	Reported[7]
Aerial parts	0.13 ± 0.002	0.16
Roots	2.53 ± 0.056	2.90
Callus	2.30 ± 0.043	-----

* The data are mean values of three independent experiments.

Table 4: The effect of growth regulators on anthraquinones content in *R. tinctorum* L. callus cultures.

Growth regulators		Anthraquinones (%)*, ± SD
Name	Concentration, mg/L	
6-Benzylaminopurine and 1-Naphthaleneacetic acid	0.5 2.0	2.13 ± 0.048
6-Furfurylaminopurine and 1-Naphthaleneacetic acid	0.5 2.0	2.09 ± 0.044
6-Furfurylaminopurine and 3,6-Dichloro-2-methoxy- benzoic acid	0.5 2.0	2.11 ± 0.050
6-Benzylaminopurine and 2,4-Dichlorophenoxyacetic acid	0.5 2.0	1.10 ± 0.021

* The data are mean values of three independent experiments.

cultures,[10] were found to be the best effective regulators for the stimulation of anthraquinones production in our cultures.

Results of the effect of plant growth regulators on anthraquinones production from *R. tinctorum* L. callus were listed in Table 4.

The callus extract showed the same picture on TLC as that of the roots. The isolated anthraquinones were identified as alizarin (**1**), lucidin-ω-ethyl ether (**2**), 1-hydroxy-2-hydroxymethylanthraquinone-3-O-β-glucoside (**3**) and lucidin primeveroside (**4**) from their UV, IR, MS spectral data, co-chromatography with authentic samples and comparison with reported data.[4,5]

In conclusion, The callus extract of the aerial parts of *R. tinctorum* L. was found to contain about 91% of the anthraquinones content of the roots extract and about eighteen fold of aerial parts extract.

They were found to be of the same type as the roots, which can be offered as an alternative source of anthraquinones.

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