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PRODUCTION OF FLAVONOIDS IN CELL CULTURES OF *ASTRAGALUS SIEBERI* DC.

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تم فصل ٦ مركبات فلافونيدية من خلايا نبات استراجالاس سيبراى دى سى وذلك عن طريق زراعة أوراق حديثة النمو مستنبتة من بذور النبات فى وسط أم أس مزود بهرمونين إضافيين هما ١-نفثيل حامض الخليك والكينيتين وقد تم التعرف على التركيب الكيميائى للمركبات بإستخدام الطرق الطيفية المختلفة بالإضافة إلى مقارنة المركبات المفصولة بعينات قياسية وكان التركيب الكيميائى للمركبات هو استراجالين ، كامبيفيرول-٣-أ-روتينوزيد ، موريتيانين ، أبيجينين ، كامبيفيرول والكويرسيتين.

The flavonoid glycosides kaempferol-3-O-β-D-glucopyranoside (astragalin), kaempferol-3-O-rutinoside and kaempferol-3-O-(2-gal-rhamnosyl-robinobioside) (mauritianin) as well as aglycones apigenin, kaempferol and quercetin were isolated from the cell suspension cultures of Astragalus sieberi DC.; Family Leguminosae. The isolated compounds could be detected by TLC and HPLC in varying amounts and identified by spectral analysis and comparison with authentic samples.

INTRODUCTION

Astragalus sieberi DC. is a perennial dwarf, spiny shrublet growing wild in the Egyptian deserts. In those areas having a climate of moderate humidity.[1] Many species of the genus *Astragalus* had been reported to exhibit a variety of interesting pharmacological activities. Many *Astragalus* species have shown cytotoxic activities in animals[2] and have been used for treatment of patients with leukemia and uterine cancer.[3] In several chinese traditional medicines, *Astragalus* species are used as antiperspirants, diuretic or tonics.[4] Previously, we have isolated and identified triterpene and kaempferol glycosides from the ethanolic extract of *Astragalus sieberi* DC.; Family Leguminosae.[5] In our studies on the cell suspension cultures of *Astragalus sieberi* DC. we established a good culture medium for production of flavonoids.

EXPERIMENTAL

Melting points were determined on Koffler's heating stage microscope. ^{1}H-NMR (500 MHz) spectra were determined in DMSO-d_6 using TMS as internal standard. EI-MS spectra were carried out on Hitachi M-80 and on MAT 311A, 70 ev. spectrometer. UV spectra were determined in Unicam SP-1750 ultraviolet spectrometer. Column chromatography was performed on silica gel (70-230, E. Merck), while TLC using silica gel G_{60} (E. Merck) and PC using Whatmann No. 1 sheets. The following solvent systems were used:

I- $CHCl_3$-CH_3OH (95:5)
II- EtOAc-CH_3OH-H_2O (85:15:1)
III- $CHCl_3$-CH_3OH-H_2O (75:25:3)
IV- $CHCl_3$-CH_3OH-H_2O (65:35:5)
V- n-Butanol-HOAc-H_2O (4:1:2)

Plates were visulazide by spraying with 1% w/v Aluminum chloride.

Further purification of all isolated compounds were carried on HPLC (L-6200A intelligent pump, L-4000 UV detector).

Plant material

Astragalus sieberi DC. seeds used for calli cultures production, were obtained from Botany Department, Faculty of Science, Assiut University.

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Authentic reference substances

The following authentic samples were used in this work, viz; β-D-glucose, β-D-galactose, α-L-rhamnose, apigenin, kaempferol, quercetin, astragalin, kaempferol-3-O-rutinoside and mauritianin. These samples were obtained from Institute for Pharmaceutical Biology, University of Bonn.

Cultivation of cell suspension cultures

Solid MS medium,[6] containing 0.8% agar and 20 g/l sucrose, for calli, or liquid MS medium (without agar), for cell suspension was used as basal medium. Calli cultures of *Astragalus sieberi* DC. were established from the shoots of sterile germinated seeds, maintained on solid MS medium, supplemented with 4.5 μM indole-3-acetic acid (IAA) and 2.3 μM kinetin (KIN). Calli cultures were initiated and maintained on solid medium supplemented with 4.5 μM of auxin and 2.3 μM of cytokinin. Calli were maintained at 25±1° in the light. Cultures were subcultured, at the end of exponential growth phase, at 4-week intervals. Cell suspension cultures were established in liquid MS medium supplemented with 4.5 μM NAA and 2.3 μM KIN from calli grown on solid MS medium with the same hormone supplementation. Cultures shaken at 120 rpm, under the same environmental conditions referred for calli, were subcultured every three weeks, at the end of exponential growth phase, by transferring 25 ml of cell suspension containing 2-2.5 g biomass fresh weight to a 500 ml flask containing 150 ml of fresh medium. After the 4th subculture cell suspensions were stabilized. After the 8th subculture, at the end of exponential growth phase, suspended cells were collected for flavonoids isolation and analysis.

Cell harvest

Cultured cells were collected by suction filtration through Miracloth®-Filter Paper (Calbiochem, Hamburg) in a Büchner-funnel.

Extraction and isolation of flavonoids

Cells (40 g) were ground in about 100 ml EtOH and the homogenate was filtered. The residue was extracted twice with 50 ml EtOH. The EtOH phases were combined and evaporated to dryness.

The residue of EtOH extract was dissolved in a small amount of EtOH, adsorbed on about 20 g of silica gel (Merck) and the solvent was completely evaporated at room temperature. The mixture was inserted on the top of column (50 x 5) containing 200 g silica gel (Merck). Gradient elution was performed with $CHCl_3/CH_3OH$, collecting 20 ml fractions which were separately monitored by TLC. Fractions eluted with $CHCl_3$ revealed three spots, all of them attaining yellow colours with 1% w/v $AlCl_3$ and were separated by preparative TLC to compounds **1**, **2** and **3** (R_f 0.75, 0.69 and 0.64 respectively, system I). Fractions eluted with $CHCl_3/CH_3OH$ (95:5) revealed two spots, one of them attaining yellow colour with 1% w/v $AlCl_3$ and brown colour under UV light which was separated by preparative TLC to compound **4** (R_f 0.24, system II). Fractions eluted with $CHCl_3/CH_3OH$ (90:10) afforded another flavonoid compound **5** which appeared as a single spot, attaining a yellow colour with 1% w/v $AlCl_3$ and brown colour under UV light (R_f 0.16, system III). Fractions eluted with $CHCl_3/CH_3OH$ (70:30) afforded compound **6** which appeared as a single spot, having a yellow colour with 1% w/v $AlCl_3$ and brown colour under UV light (R_f 0.15, system IV). Further purification of all isolated compounds were carried on HPLC. Elution was carried out with aqueous CH_3OH (15 to 80%) in a Nucleosil 100-5 C_{18} column (Macherey-Nagel) at a flow rate of 1 ml/min., and the detection wavelength set to 350 nm.

Growth curves and flavonoid content

Flavonoids were quantified spectrophotometrically.[7-8] Solutions of authentic reference compounds served as standards. Ten flasks with fresh modified MS medium (50 ml) were prepared and inoculated with 4 g cultured cells of *Astragalus sieberi* DC. grown in modified MS medium in the light. Fresh weight and flavonoid content were determined from day zero to day 12 at 2-day-intervals.

Acid hydrolysis

The glycosides were dissolved in CH_3OH, mixed with an equal volume of N/2 H_2SO_4 and refluxed for 2 hours. Evaporate the CH_3OH and the aglycones were extracted with ether and subjected to TLC and PC. The acidic mother liquor of each hydrolysate containing the sugar

moiety was neutralized, concentrated and spotted alongside with authentic sugars on Whatmann No. 1 filter paper using system V as solvent system.

Identification of the isolated compounds

All isolated compounds were identified by UV, mass and ^{1}H-NMR spectroscopy, as well as co-chromatography (TLC and HPLC) with samples of authentic reference compounds as apigenin (Fig. 1; **1**), kaempferol (**2**), quercetin (**3**), kaempferol-3-O-β-D-glucopyranoside astragalin) (**4**), kaempferol-3-O-rutinoside (**5**), and kaempferol-3-O-(2 gal-rhamnosyl-robinobioside) (mauritianin) (**6**). All these flavonoids were known compounds, their spectroscopic properties agreed with published data.[9-15]

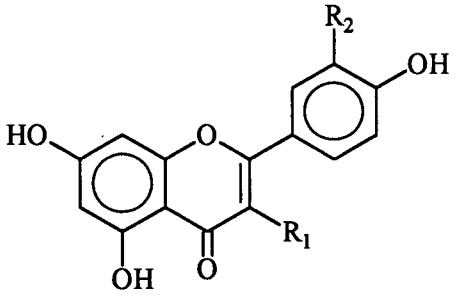

Fig. 1 Chemical structures of compounds **1-6**

Compds	R^1	R^2
1	H	H
2	OH	H
3	OH	OH
4	O-β-D-glucose	H
5	O-β-D-glucose (6→) a-L-rhamnose	H
6	O-β-D-galactose (6→) α-L-rhamnose; (2→) α-L-rhamnose	H

RESULTS AND DISCUSSION

Shoots of sterile germinated seeds of *Astragalus sieberi* DC. were used to establish callus cultures. After the third subculture,callus appeared completely de-differentiated with a pale yellow to green colour, friable and stabilized. Direct HPLC analysis of a freshly prepared CH_3OH extract from dry biomass of callus revealed the presence of flavonoids, when checked with a UV detector. the isolated flavonoids from our callus cultures are of the same type of those usually found in the whole plant,[5] except the two aglycones (apigenin and quercetin). All isolated flavonoids have been identified by co-chromatography (TLC and HPLC) and by comparing their spectroscopic data (UV, MS, ^{1}H-NMR) with published data.[9-15]

Apigenin (compound 1)

15 mg, yellow amorphous powder (CH_3OH). UV: λ_{max} (CH_3OH, nm) 261, 341; +CH_3ONa 269, 392; +$AlCl_3$ 269, 384; +$AlCl_3$/HCl 269, 384; +NaOAc 270, 348; +NaOAc/H_3BO_3 261, 339. MS (m/z, %): 270 (M^+), 269 (84), 240 (56), 153(83), 149 (38), 121 (70). ^{1}H-NMR (δ, ppm): 6.17 (1H, s, H-3), 6.31 (1H, d, J= 2.3 Hz, H-6), 6.55 (1H, d, J= 2.3, H-8), 7.07 (2H, dd, J= 8.5, 1.2 Hz, H-3′, H-5′), 8.0 (2H, dd, J= 8.5, 1.2 Hz, H-2′, H-6′).

Kaempferol (compound 2)

20 mg, yellow amorphous powder (CH_3OH), m.p 280-281° UV: λ_{max} (CH_3OH, nm) 265, 367; +CH_3ONa 275, 413; +$AlCl_3$ 269, 424; +$AlCl_3$/HCl 269, 424; +NaOAc 274, 378; +NaOAc/H_3BO_3 267, 368. MS (m/z, %): 286 (M^+), 258 (14), 153 (6), 152(3), 149 (28), 125 (6), 121 (4), 61 (100), 43 (39). ^{1}H-NMR (δ, ppm): 6.1 (1H, d, J= 2.2 Hz, H-6), 6.41 (1H, d, J= 2.2 Hz, H-8), 7.0 (2H, dd, J= 8.5, 1.2 Hz, H-3′, H-5′), 8.18 (2H, dd, J= 8.5, 1.2 Hz, H-2′, H-6′).

Quercetin (compound 3)

30 mg, yellow amorphous powder (CH_3OH), m.p 314-316°. UV: λ_{max} (CH_3OH, nm) 269, 371; +CH_3ONa 275, 413; +$AlCl_3$ 273, 439; +$AlCl_3$/HCl 269, 400; +NaOAc 275, 380; +NaOAc/H_3BO_3 265, 386. MS (m/z, %): 302 (M^+), 286 (74), 153 (10), 152 (16), 137 (28), 122 (14), 61. ^{1}H-NMR (δ, ppm): 6.09 (1H, d, J= 2.2 Hz, H-6), 6.38 (1H, d, J= 2.2 Hz, H-8), 7.0 (1H, d, J= 8.1 Hz, H-5′), 7.48 (1H, dd, J= 8.1, 1.4 Hz, H-6′), 7.76 (1H, d, J= 1.4, H-2′).

Kaempferol-3-O-β-D-glucopyranoside (compound 4)

18 mg, Yellow amorphous powder (CH_3OH). UV: λ_{max} (CH_3OH, nm) 267, 370;

+CH_3ONa 277, 417; +$AlCl_3$ 273, 375; +$AlCl_3$/HCl 273, 374; +NaOAc 277, 392; +NaOAc/H_3BO_3 270, 372. MS (m/z, %): 286 (M^+ agl.), 258 (14), 153 (6), 149 (28), 125 (6), 61 (100), 43 (39). ^{1}H-NMR (δ, ppm): 5.4 (1H, d, J= 7.21 Hz, H-1 glc.), 6.19 (1H, d, J= 2.12 Hz, H-6), 6.46 (1H, d, J= 2.12 Hz, H-8), 7.02 (2H, dd, J= 8.5, 1.4 Hz, H-3′, H-5′), 8.08 (2H, dd, J= 8.5, 1.4 Hz, H-2′, H-6′).

Kaempferol-3-O-rutinoside (compound 5)

16 mg, yellow needles (CH_3OH). m.p 156-158° UV: λ_{max} (CH_3OH, nm) 268, 369; +CH_3ONa 277, 418; +$AlCl_3$ 273, 372; +$AlCl_3$/HCl 274, 373; +NaOAc 277, 391; +NaOAc/H_3BO_3 268, 375. MS (m/z, %): 286 (M^+ agl.), 258 (14), 153 (6), 149 (28), 125 (6), 61 (100), 43 (39). ^{1}H-NMR (δ, ppm): 0.97 (3H, d, J= 6.1, CH_3-rha), 4.35 (1H, bs, H-1 rha.), 5.22 (1H, d, J = 7.44, H-1 glc.), 6.23 (1H, d, J= 2.06 Hz, H-6), 6.40 (1H, d, J= 2.06 Hz, H-8), 7.02 (2H, dd, J= 8.5, 1.4 Hz, H-3′, H-5′), 8.08 (2H, dd, J= 8.5, 1.4 Hz, H-2′, H-6′).

Kaempferol-3-O-(2-gal-rhamnosyl-robinobioside) (compound 6)

12 mg, Yellow amorphous powder (CH_3OH). UV: λ_{max} (CH_3OH, nm) 266, 368; +CH_3ONa 276, 415; +$AlCl_3$ 269, 373; +$AlCl_3$/HCl 270, 375; +NaOAc 274, 383; +NaOAc/H_3BO_3 264, 370. MS (m/z, %): 286 (M^+ agl.), 258 (14), 153 (6), 149 (28), 125 (6), 61 (100), 43 (39). ^{1}H-NMR (δ, ppm): 0.73 (3H, d, J= 6.07, CH_3-rha), 1.06 (3H, d, J= 6.17, CH_3-rha.), 4.35 (1H, bs, H-1 rha.), 5.12 (1H, bs, H-1 rha.) 5.54 (1H, d, J = 7.76, H-1 gal.), 6.23 (1H, d, J= 2.16 Hz, H-6), 6.40 (1H, d, J= 2.16 Hz, H-8), 6.95 (2H, dd, J= 8.3, 1.2 Hz, H-3′, H-5′), 8.08 (2H, dd, J= 8.3, 1.2 Hz, H-2′, H-6′).

In addition, the glycosides were submitted to acid hydrolysis and their aglycones were identified by TLC alongside with authentic reference samples, as well as, the sugar part of each glycoside was identified by PC, alongside with authentic reference of sugars.

The growth of the *Astragalus sieberi* DC. cell cultures in modified MS medium in the light was characterized (Fig. 2).

A linear increase in fresh weight was observed between day 1 and day 7 after inoculation of cells into fresh medium. Flavonoid accumulation paralleled cell growth, with flavonoid content increasing to approx. 0.1% relative to the fresh weight. No appreciable qualitative changes in the pattern of the constituents were observed during cell culture growth.

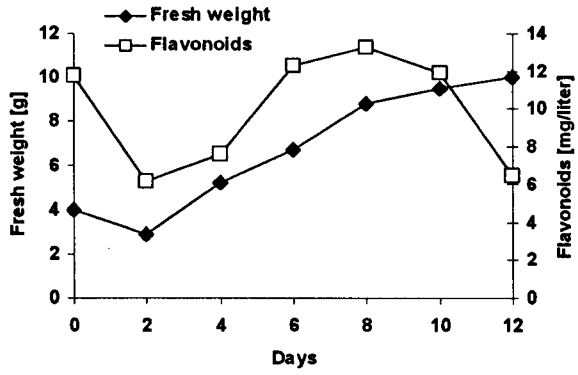

Fig. 2: Changes in fresh weight and flavonoid content of *Astragalus sieberi* cell cultures. The data are mean values of two independent experiments.

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