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PHENYLETHANOID GLYCOSIDES FROM *BARLERIA CRISTATA* L. CALLUS CULTURES

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تم فصل والتعرف على ثلاثة مركبات من نوع الفينيل إثانويد وهي: بيتا -[(٣و٤′ ثنائي هيدروكسي فينيل)-إثيل] (٤″ أ كافييويل) بيتا دي جلوكوزيد (ديس رامنوزايل اكتيوزايد) (1) وبيتا [(٣و٤′–ثنائي هيدروكسي فينيل) إثيل] (٣″ أ الفا إل رامنوزيل) (٤″ أ كافييويل) بيتا دي جلوكوزيد (اكتيوزايد) (2) وبيتا [(٣و٤′ ثنائي هيدروكسي فينيل) إثيل] (٣″، ٦″ أ الفا إل ثنائى رامنوزيل) (٤″ أ كافييويل) بيتا دي جليكوزيد (بوليموزايد) (3) من زراعة الأنسجة لنبات البارليريا كريستاتا وذلك باستخدام الوسائل الطيفية المختلفة وقد تم التأكد من ذلك بالمقارنة بعينات قياسية. وقد تم تعيين كميات هذه المركبات باستخدام كروماتوجرافيا السوائل عالية التقنية.

Three phenylethanoid glycosides viz; β-[(3′,4′-dihydroxyphenyl)-ethyl]-(4″-O-caffeoyl)- β-D-glucoside (desrhamnosylacteoside) ***(1)****, β-[(3′,4′-dihydroxyphenyl)-ethyl]-(3″-O-L-rhamnosyl)-(4″-O-caffeoyl)-β-D-glucoside (acteoside)* ***(2)*** *and β-[(3′,4′-dihydroxyphenyl)-ethyl]-(3″,6″-O-L-dirhamnosyl)-(4″-O-caffeoyl)-β-D-glucoside (poliumoside)* ***(3)*** *were isolated and identified from the callus cultures of Barleria cristata L.The structures of the isolated compounds were established by spectroscopic evidence (UV, 1D and 2D-NMR and ESIMS), further confirmation has been done by comparison with authentic samples. The amount of compounds 1-3 were determined in the callus culture using HPLC.*

INTRODUCTION

Barleria cristata L. is a perennial shrub, belonging to family Acanthaceae. The plant is used in skin diseases, bronchitis, blood diseases and asthma.[1]

The plants of the genus *Barleria* are used for boils, bee bite, tooth ache.[2] Recently the potential antidiabetic effect of *B.lupulina* Lindl. extract in rats was studied.[3]

The anti-inflammatory effect of the extract of *B. prionitis* L. was evaluated against acute and chronic animal test models, the neuropharmacological profile and anti-HSV-2 activities of *B. lupulina* Lindl. and the anti-infertility effect of the root extract of *B. prionitis* L. were studied.[4-7]

Many compounds of interested biological activities were isolated from different *Barleria* species as iridoids,[8-14] flavonoids,[15-19] and phenylethanoid derivatives,[20] in addition to other constituents belonging to other groups of chemical constituents.[12-21] This encourage us to study the production of these compounds from the callus cultures of *B. cristata* L. as a new source of these valuable compounds.

The previous phytochemical study of the aerial parts of *Barleria cristata* L. deals with isolation and structure elucidation of iridoid and flavonoid glycosides.[22] Nothing was mentioned about the presence of phenylethanoids in *Barleria cristata* L. in available literature.

Experimental

General Experimental Procedures

The UV spectra (λ_{max}) were recorded on Ultrospec 1000 UV/Visible spectrophotometer (Pharmacia, Biotech, Cambridge, England). The 1D and 2D-NMR spectra were obtained on (Brüker 400 FT spectrometer, Germany) operating at 400 MHz for ^{1}H-NMR and at 100 MHz for ^{13}C-NMR. The chemical shift values are reported as part per million (ppm) units relative to tetramethylsilane (TMS). For ^{13}C-NMR spectrum, multiplicities were determined

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by a distortionless enhancement by polarization transfer (DEPT) experiment. The coupling constant values are in hertz. ESIMS data were obtained using MAT 95 x L Trap hybrid tandem mass spectrometer (ThermoFinnigan MAT, Bremen, Germany) instrument. Medium pressure liquid chromatography (MPLC) separations were performed on CIG column system (22 mm. i.d.x30 cm, Kusano Scientific Co. Tokyo, Japan), using prepacked RP-18 glass column, flow rate 1ml/min. HPLC: Waters 2484, connected with Dual Absorbance Detector (waters 1525) Binary HPLC Pump using nucleosile 100-5 C-18 column (Macherey-Nagel), using flow rate at 1ml/min. Silica gel GF 60 (60-120 mesh) was used for column chromatography. Pre-coated silica gel 60 F_{254} and RP-18 aluminium sheets (Merck, Germany) were used for TLC. Plates were examined under UV lamp at (254 and 365 nm) and sprayed with 10% H_2SO_4, followed by heating at 110° for 1 min.

The following solvent systems are used:

I- $CHCl_3$-MeOH-H_2O (70:28:2).
II- MeOH-H_2O (20:80).
III- MeOH-H_2O (40:60).
IV- n-Butanol-HOAc-H_2O (40:10:20)
V- Toluene-MeOH (80:20)

Plant material

Barleria cristata L. aerial shoot was obtained from Botanical Garden, Faculty of Agriculture, Assiut University, Assiut, Egypt, and kindly identified by Prof. Dr. N.E. El-Keltawi, Prof. of Horticulture, Faculty of Agriculture, Assiut University.

Authentic compounds

The authentic samples; desrhamnosyl-acteoside, caffeic acid, cinnamic acid, *p*-coumaric acid, glucose, galactose, arabinose and rhamnose were provided by Prof. Dr. L. Beerhues, Institute for Pharmaceutical Biology, Technical University Braunschweig, Germany.

Callus Cultures

Callus cultures of *Barleria cristata* L. were obtained from young shoots cultivated in BDS medium (Table 1) supplemented with 1 mg/l kinetin [(6-furfurylamino)-purine] and 0.7 mg/l dicamba (3, 6-dichloro-2-methoxybenzoic acid) at pH 5.5 and 25°.[23] The resulting callus was periodically subcultured to new BDS medium at the end of exponential growth phase, at 3-week intervals (Fig. 1) and grown in dark.

Table 1: Composition of BDS medium.[23]

Ingredients	mg/l
$CaCl_2.2H_2O$	150
KNO_3	2530
NH_4NO_3	320
$(NH_4)H_2PO_4$	230
$(NH_4)_2SO_4$	134
$MgSO_4.7H_2O$	247
$MnSO_4.4H_2O$	13.2
$ZnSO_4.7H_2O$	0.2
$CuSO_4.5H_2O$	0.039
KI	0.75
$CoCl_2.6H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.25
$FeSO_4.7H_2O$	27.85
$Na_2EDTA.2H_2O$	37.25
H_3BO_3	3.0
$NaH_2PO_4.2H_2O$	172.0
Nicotinc acid	1.0
Pyridoxal hydrochloride	10.0
myo-Inositol	100.0

Procedure:

Mix the ingredients with about 600 ml distilled water in one litre glass beaker, add 30 g sucrose and dissolve. Bring the solution up to one litre by adding distilled water, then adjust the pH to 5.5 with KOH solution. Add 7 g agar, place the beaker on hot plate and frequently stir the solution until the agar is dissolved, divide the solution in 300-ml Erlenmeyer flasks each one contains 50 ml, keep the cap closed and sterilize at 120° for 20 min in autoclave.

Growth curve (Fig. 1)

Ten flasks with fresh BDS medium (50 ml) were prepared and inoculated with 1 g cultured cells of *Barleria cristata* L. (week zero) grown in BDS medium in the dark. Fresh weight was determined from week zero to week 6 at one week intervals.

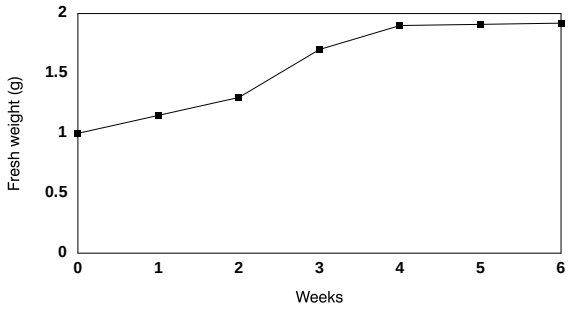


Fig. 1: Changes in fresh weight of *B. cristata* L. cell cultures. The data are mean values of two independant experiments.

EXTRACTION AND ISOLATION

Extraction

a- Extraction for isolation of major compounds

Callus constituents were extracted as mentioned in literature,[24] about 115 g fresh cells were ground in 800 ml ethanol till a homogenate was obtained. The homogenate was filtered and the residue was re-extracted twice with 500 ml ethanol, the ethanol extract was evaporated till dryness (12 g).

b- Extraction for quantitative determination of the isolated compounds using HPLC

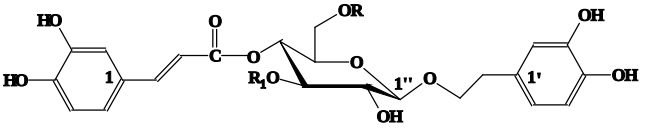
Fresh cells (1 g) were ground with 7 ml ethanol till a homogenate was obtained. The homogenate was filtered and the residue was re-extracted twice with 5 ml ethanol, the combined ethanolic extract was evaporated till dryness. The residue was re-dissolved in 1 ml methanol, filtered, a serial dilution was made from this solution. The prepared solution was used for quantitative determination of the isolated compounds using HPLC. This was performed on a nucleosile 100-5 C-18 column using water (A) and MeOH (B) as the solvents. The following gradient was employed: 10% B for 2 min., 15-40% B within 20 min., then isocratic elution at 60% B for 5 min. The flow rate was 1 ml/min and the detection wavelength set to 330 nm. Desrhamnosyl acteoside in a concentration of 1 ug/100ul was used as reference compound.

Isolation of the constituents

The residue of the ethanol extract was re-dissolved in 100 ml methanol for phytochemical study. The constituents of ethanol extract were screened by TLC on silica gel 60 F_{254}-precoated sheets using system I as a mobile phase and on RP-18 precoated sheets using systems II and III as mobile phases, where several spots were detected. The ethanol extract (10 g) was subjected to silica gel (40 g) column chromatography, eluted with chloroform-methanol gradiently to yield two fractions. Fraction 1 (150 mg) was further subjected to MPLC on RP-18 column eluted with MeOH-H_2O (3:7) at a flow rate of 1 ml/min, where two compounds were isolated (**1,2**) (3 and 15 mg, respectively). Fraction 2 (100 mg) was injected in MPLC on RP-18 column using MeOH-H_2O (2:8) to yield two sub-fractions (subfrs2_a & 2_b). Sub-fraction 2_b was further purified by MPLC on RP-18 column eluted with MeOH-H_2O mixtures (4:6) to give compound **3 (**25 mg**)**.

Acid hydrolysis of compound 3: Compound **3** (10 mg) was subjected to partial acid hydrolysis using 5% methanolic HCl for 3 hours. Small samples were withdrawn each 10 min from the reaction mixture and spotted on TLC and PC alongside with compounds (**1**) and (**2**) and reference sugars, using solvent systems **I** and **IV,** during the process of hydrolysis. The chromatograms were air dried and subjected to spraying with 10% H_2SO_4 in case of TLC and aniline hydrogen phthalate in case of PC.

Compound 1: was identified as desrhamnosylacteoside (Fig. 2) by comparison with authentic refrence compound on TLC plate and HPLC (R_f =0.64, System I)



	R	R_1
Compound 1	H	H
Compound 2	H	α-rhamnopyranose
Compound 3	α-rhamnopyranose	α-rhamnopyranose

Fig. 2: Compounds isolated from the callus culture of *Barleria cristata* L.

Compound 2 (Acteoside, R_f= 0.44, System I, Fig. 2): UV(MeOH) λ_{max} 205, 332 nm, ESIMS m/z 624 $[M]^+$, 461 [M-Caffeoyl] (calc. for $C_{29}H_{36}O_{15}$) ^{1}H-NMR (400 MHz, DMSO-*d6*): δ 7.46 (1H, d, *J*= 15.8 Hz, H-7), 7.02 (1H, d, *J*= 1.8 Hz, H-2), 6.97 (1H, dd, *J*= 1.8, 8.2 Hz, H-

6), 6.75 (1H, d, *J*= 8.2 Hz, H-5), 6.62 (2H, m, H-2′,5′), 6.50 (1H, dd, *J*= 1.9, 8.07 Hz, H-6′), 6.19 (1H, d, *J*= 15.8 Hz, H-8), 3.60 (2H, m, 2H -8′), 2.69 (2H, m, 2H-7′). Sugar protons: δ 5.01(1 H, brs, H-1‴ rham.), 4.7 (1H, m, H-4″glu), 4.33 (1H, d, *J*= 7.8 Hz, H-1″glu), 3.69 (1H, m, H-2‴rham.), 3.68 (1H, m, H-3″glu), 3.47 (1H, m, H-5″glu.), 3.38 (2H, m, H-6 ″glu), 3.34 (1H, m, H-5‴ rham), 3.27 (1H, m, H-3‴ rham), 3.20 (1H, m, H-2″ glu), 3.10 (1H, m, H-4‴rham.), 0.95 (3H, d, *J*= 6.1 Hz, CH_3-rham). ^{13}C-NMR (100 MHz, DMSO-d_6) data were reported in Table 1.

Compound 3 (Poliumoside, R_f= 0.32, System I, Fig. 2): UV (MeOH) λ_{max} 332 nm, ESIMS m/z 793 $[M+Na]^+$ (calc. for $C_{35}H_{46}O_{19}$) ^{1}H-NMR (400 MHz, DMSO-*d6*): δ 7.48 (1H, d, *J*= 15.8 Hz, H-7), 7.02 (1H, s, H-2), 6.97 (1H, d, *J*= 8.2 Hz, H-6), 6.75 (1H, d, *J*= 8.2 Hz, H-5), 6.62 (2H, m, H-2′, 5′), 6.45 (1H, d, *J*= 8.07 Hz, H-6′), 6.19 (1H, d, *J*= 15.8 Hz, H-8), 3.68 (2H, m, H-8′), 2.71 (2H, m, H-7′). Sugar protons: δ 5.01 (1H, brs, H-1‴ rham.),4.7 (1H, t, *J*= 9.0 Hz, H-4″glu), 4.53 (1H, br s, H-1‴′rham), 4.33 (1H, d, *J*= 7.8 Hz, H-1″ glu), 3.3-3.6 (other sugar protons), 1.02 (3H, d, *J*= 6.0 Hz, CH_3-rham.), 0.95 (3H, d, *J*= 6.2 Hz, CH_3 rham).

RESULTS AND DISCUSSION

From the callus cultures of *Barleria cristata* L., three phenylethanoid glycosides were isolated.

Compound **1** was identified as desrhamnosylacteoside by comparison with authentic sample on TLC plate (has the same R_f and color reaction with H_2SO_4) and by comparison of its retention time on HPLC with that of the authentic sample.

Compound **2** was obtained as a brownish amorphous residue. It has the molecular formula deduced as $C_{29}H_{36}O_{15}$ as determined by ESIMS, ^{1}H, ^{13}C-NMR and DEPT data (Table 2). ^{1}H and ^{13}C-NMR data indicated the presence of two sugar moieties, due to the presence of signals at δ_C 102.37 with δ_H 4.33 (1H, d, *J*= 7.8 Hz) for β-glucose moiety and at δ_C 101.32 with δ_H 5.01 (1H, br.s.) together with methyl group at δ_H 0.95 (3H, d, *J*= 6.1 Hz) with δ_C 18.18 for α-rhamnose moiety. The ^{1}H-NMR spectral data also revealed the presence of trans-olefinic protons attached to trisubstituted benzene ring along with the carbonyl carbon (trans-caffeoyl moiety). The presence of trans-caffeoyl moiety was indicated from the signals at δ_H 7.46 (H-7, *J*= 15.8 Hz) and δ_H 6.19 (H-8, *J*= 15.8 Hz) for trans double bond and δ_H 7.02 (H-2), 6.75 (H-5) and 6.97 (H-6) for the trisubstituted benzene ring.

Table 2: ^{13}C NMR (100 MHz, DMSO-d_6) data of compounds **1** and **2**.

Position	Compound **1**[32]	Compound **2**
Caffeic acid moiety		
1	126.15	125.6, s
2	115.43	114.7, d
3	146.16	148.5, s
4	149.00	145.7, s
5	116.39	115.9, d
6	121.99	121.6,d
7	146.00	145.6, d
8	114.61	113.6, d
C=O	166.54	165.8, s
Aglycone		
1′	129.81	129.2, s
2′	116.90	116.4, d
3′	145.55	144.9, s
4′	144.11	143.7, s
5′	116.06	115.6, d
6′	120.16	119.6, d
7′	35.66	35.1, t
8′	70.82	70.4, t
Glucose		
1′′	103.40	102.4, d
2′′	74.19	74.1, d
3′′	75.22	79.3, s
4′′	71.89	69.2, d
5′′	74.67	74.5, d
6′′	61.42	60.8, t
Rhamnose		
1′′′		101.3, d
2′′′		70.7, d
3′′′		70.4, d
4′′′		71.7, d
5′′′		68.8, d
6′′′		18.2, q

The ^{1}H-NMR spectral data suggested the presence of another trisubstituted benzene ring in form of phenylethanoid group, where three aromatic proton signals appeared at δ_H 6.62 (2H) and 6.50 (1H) for H-2′, H-5′ and H-6′, respectively together with two multiplet signals at δ_H 2.69 and 3.60 for 2H-7′′ and 2H-8′′ of the ethylene group, while ^{13}C-NMR showed six aromatic carbon signals for trisubstituted benzene ring at δ_C 129.2 (s, C-1′′), 116.4 (d, C-2′), 144.9 (s, C-3′), 143.7 (s, C-4′), 115.6 (d, C-5′) and 119.6 (d, C-6′) together with oxygenated ethylene group at δ_C 35.05 (t, C-7′) and 70.35 (t, C-8′).

The attachment of rhamnose unit to C-3′′ of glucose was indicated from the downfield shift of C-3′′′ (δ_C 79.26) and the upfield shift of C-2′′′ and 4′′′ (Table 2).

The complete assignment of most protons and carbons were based on the ^{1}H-^{1}H COSY, HMQC and HMBC experiments. In HMBC spectrum correlation was observed between H-4′′′ and C=O of caffeoyl moiety indicate the attachment of caffeoyl moiety to C-4′′′ of glucose unit. While the correlation between H-1′′′′of rhamnosyl moiety and C-3′′′of glucose unit confirm their connection. Thus compound **2** was characterized as acteoside. This compound was isolated for the first time from *Barleria cristata* L.

Compound **3** was isolated as brownish amorphous residue, with the molecular formula $C_{35}H_{46}O_{19}$ as determined from ESIMS and ^{1}H-NMR data. The ^{1}H-NMR is similar to that of compound **2**, but here the ^{1}H-NMR data exhibited an additional anomeric proton resonate at δ_H 4.53 (br.s) and methyl protons resonate at δ_H 1.02 (*J*= 6.0 Hz). The ESIMS showed that compound **3** differ from compound **2** by 146 mass unit, this indicate that compound **3** contain additional methylpentose (i.e. rhamnose). Therefore, compound **3** was assumed to have a trisaccharide structure. Partial acid hydrolysis of compound **3** followed by TLC and PC yielded rhamnose as the sugar unit in addition to mixture of glycosides, one of them is acteoside (**2**). Further hydrolysis yielded compound **1**. From all the above mentioned data (UV, ESIMS, ^{1}H-NMR and acid hydrolysis) and by comparing these data with the published data,[25] compound **3** was identified as poliumoside. This compound was isolated for the first time from genus *Barleria*.

TLC of the total extract alongside with authentic aromatic acids (caffeic acid, cinnamic acid and *p*-coumaric acid), using solvent system V, showed the presence of cinnamic acid and caffeic acid in minor amounts.

Quantitative determination of compounds

The prepared sample for quantitative determination was subjected to HPLC (30 ul) and the quantity of each glycoside was estimated on the basis of its area with respective to the area of desrhamnoacteoside (0.3 ug) as external standard. The results were recorded in Table (3).

Table 3: Concentrations of the isolated compounds (mg/g fresh callus).

Extract	Desrhamno-acteoside	Acteoside	Poliumoside
Callus	0.0081	0.0076	0.013

Compounds (**1-3**) are belonging to phenylethanoid group. The compounds belonging to this group have many biological activities as antifungal, antibacterial, antiviral, immunosuppressive, antineoplastic and analgesic, in addition to cardivascular effect.[26]

Acteoside (compound **2**) isolated previously from many plants showed a protective effect against the free radical induced oxidative stress[27] and on carbon tetrachloride-induced hepatotoxicity.[28] In addition to these actions, acteoside has also antioxidant and anti-inflammatory effects.[29]

Many iridoids and flavonoids were isolated from plants belonging to genus *Barleria*, in this study the iridoids and flavonoids were not detected and instead of these compounds, phenylethanoids were produced by tissue culture, according to the available literature, only one article concerned the occurrence of phenylethanoid glycosides in *Barleria strigosa* was published.[20]

Although tissue cultures are used for production of active constituents. The production of these active constituents depends upon the method of cultivation and the media used. In some studies conducted on plants of family Oleaceae, it showed that, the iridoids and lignans which are the main active constituents of this family were disappear in the tissue culture of these plants and

phenylethanoid were produced in a high concentration.[30, 31]

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