

A new coumarin and total phenolic and flavonoids content of Bulgarian celeriac

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Dedicated to Acad. Dimiter Ivanov on the occasion of his 120th birth anniversary

Apium graveolens is a popular vegetable and also a well known medicinal plant. Different extracts have demonstrated diverse useful properties: antioxidant, hipolipidemic, antibacterial, anticancer, etc. In Bulgaria, the most often consumed parts are the leaves (as spice) and the thickened roots of *A. graveolens* var. *rapaceum*, celeriac. We studied the chemical composition of unpolar extract of Bulgarian celeriac. In petroleum ether fraction of the methanol root extract we identified by GC-MS several compounds: neocnidilide, isocnidilide, 3-*n*-butyl phthalide, allyl phenoxyacetate, fatty acids and hydrocarbons; the coumarins xanthotoxin, isopimpinellin, psoralene. A new natural compound 6-(3'-methyl-1'-oxobutyl)-7-hydroxy coumarin was isolated and its structure proved by spectral data. Total phenolic and total flavonoids content of plant material from different locations in Bulgaria was also analyzed spectrophotometrically. We studied methanol extract of leaves and roots from 19 different locations in Bulgaria, including some wild-growing *Apium nodiflorum*. The total phenolics varied between 15-67.2 mg/g GAE in leaves and 8.2-13.6 mg/g GAE in roots, ant total flavonoids were between 6-26.5 mg/g in leaves and 1-4.8 mg/g in roots (as quercetin). Our study demonstrated that the roots and leaves of Bulgarian celery are a rich source of biologically active constituents.

Key words: *Apium graveolens* var. *rapaceum*, celeriac, total phenolics, total flavonoids, 6-(3'-methyl-1'-oxobutyl)-7-hydroxy coumarin

INTRODUCTION

Apium graveolens L., belonging to the Apiaceae family, is a plant cultivated and consumed worldwide. In Bulgaria, the most often consumed parts are the leaves (as spice) and the thickened roots of *A. graveolens* var. *rapaceum* known as celeriac. There are abundant literature data reporting a wide spectrum of biological activity of *A. graveolens* [1,2]. Different authors reported that its extracts possessed antimicrobial [3], antifungal [4] and anti-inflammatory activity [5-7], as well as positive effects in treatment of rheumatoid arthritis and osteoarthritis [8]. Further, alcohol extracts of *A. graveolens* have demonstrated significant hepatoprotective [9,10], anticancerogenic, antiproliferative [11], cytotoxic [12] and hypolipidemic action [13]. Leave and root extracts of *A. graveolens* have potential to scavenge free OH[•] and DPPH[•] radicals and to inhibit lipid peroxidation [14-16].

Phytochemical studies revealed the presence of secondary metabolites belonging to different structural groups: flavonoids (major constituents glycosides of apigenin, kaempferol and quercetin),

phenolic acids (caffeic, ferulic, and their derivatives), phthalides, tannins, steroids, terpenoids, saponins, essential oils [16-24]. Among these, the most important biologically active constituents are phenolics, mainly flavonoids, phenolic acids, and coumarins. It is known that natural products rich in polyphenols are potently effective against dyslipidemia, cardiovascular diseases, inflammation, and underlying mechanisms of other disease processes, mainly because of their antioxidant activity [25].

The aim of the present work was to study the content of bioactive constituents of Bulgarian celeriac roots and leaves from different regions of the country. The quantification of groups of active constituents instead of individual compounds is reasonable because of the well recognized fact that the activity of medicinal plant extracts is mostly due to the simultaneous action of many different constituents [26-29].

EXPERIMENTAL

Extraction of celeriac roots

Commercial Bulgarian celeriac fresh roots (1.4 kg) were cut into small pieces and extracted with

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MeOH three times successively (1.5 l each) at room temperature for 24h. The extracts were filtered and the combined extracts after concentration *in vacuo* were mixed with water and extracted successively with petroleum ether (PE) (3x, 1:1 v/v). The extract obtained was evaporated to give 880 mg dry residue.

Fractionation of methanol extract of celeriac roots and GC-MS analysis

A part of the PE fraction (20 mg) was subjected to PTLC with mobile phase PE:EtOAc (10:1). Two sub-fractions were gathered and analyzed by GC-MS. The second, more polar fraction was silylated prior to the analysis.

The GC-MS analysis was performed with a Hewlett Packard gas chromatograph 5890 Series II Plus linked to a Hewlett Packard 5972 mass spectrometer system, equipped with a 23m long, 0.25mm i.d. and 0.5 mm film thickness HP-5 capillary column. The temperature was programmed from 100°C to 310°C at a rate of 5°Cmin⁻¹. Helium was used as a carrier gas, at flow rate 0.7mLmin⁻¹, split ratio 1:80, injector temperature 280°C and ionisation voltage 70 eV. For silylation, about 5 mg of the sample was mixed with 75 mL bis(trimethylsilyl)trifluoro-acetamide and 50 mL of dry pyridine, heated at 80°C for 20 min and analysed by GC-MS.

Isolation of individual compounds

Column chromatography was performed on silica gel 60 (Merck, 63-200 mm), normal phase. Analytical TLC was performed on silica gel 60 F254 plates (Merck). Preparative TLC (PTLC) was performed on silica gel 60 F254 glass plates (Merck, 20x20 cm and 0.25 mm). Detection of the spots was achieved under UV light (254 and 366 nm) and by spraying with vanillin-sulphuric acid in methanol, followed by heating at 100°C.

NMR spectra were recorded on a Bruker AV 600 spectrometer (600MHz for ¹H and 150 MHz for ¹³C) in CDCl₃.

A part of the PE extract (750 mg) was subjected to column chromatography on silica gel using a gradient system of PE-EtOAc (1:0-0:1). Nineteen fractions were obtained. Fraction 6 (19 mg, eluted with PE-EtOAc (95:5)) was separated by PTLC with mobile phase CHCl₃-EtOAc (20:1) to yield mixture of neo- and isocnidilide (**3a** and **3b**, 7 mg). Fraction 15 (10 mg, eluted with PE-EtOAc (75:25)) was subjected to PTLC with mobile phase CHCl₃ to yield two compounds: xanthotoxin (**5**, 1 mg) and 6-

(3'-methyl-1'-oxobutyl)-7-hydroxy coumarin (**7**, 1 mg).

Identification of isolated compounds

Compounds **3a** and **3b** (mixture of neo- and isocnidilide) were identified based on comparison of ¹H-NMR spectrum with literature data [30,31].

Xanthotoxin **5** was identified based on comparison of its ¹H-NMR and MS spectra with literature data [32].

6-(3'-methyl-1'-oxobutyl)-7-hydroxy coumarin **7**: ¹H NMR (CDCl₃, 600 MHz) δ: 0.97 (6H, d, J = 6.7 Hz, CH₃-4', CH₃-5'), 2.25 (1H, m, H-3'), 2.81 (2H, d, J = 6.9 Hz, H₂-2'), 6.23 (1H, d, J = 9.5 Hz, H-3), 6.79 (1H, s, H-8), 7.57 (1H, d, J = 9.5 Hz, H-4), 7.83 (1H, s, H-5), 12.81 (1H, s, OH); ¹³C NMR (CDCl₃, 150 MHz) δ: 22.7 (q, C-4', C-5'), 25.5 (d, C-3'), 47.1 (t, C-2'), 105.6 (d, C-8), 111.6 (s, C-4a), 114.1 (d, C-3), 117.3 (s, C-6), 130.9 (d, C-5), 142.9 (d, C-4), 159.0 (s, C-8a), 159.9 (s, C-2), 165.8 (s, C-7), 105.5 (s, C-1'). GC/MS (6-(3'-methyl-1'-oxobutyl)-7-hydroxy coumarin TMS), MS (EI, 70 eV), m/z (relative intensity %): 318 M⁺ (5), 303 [M-CH₃]⁺ (100), 261 [M-C₄H₉]⁺ (75), 233 [M-C₄H₉-CO]⁺ (15), 73 (20).

Plant material. The plant species, the geographic origin and the type of plant material are listed in Table 1.

Extraction of roots and leaves of samples from different regions in Bulgaria. Fresh plant material was extracted twice with methanol, 1:3 (w/v) for leaves and 1:2 (w/v) for roots. The extracts were evaporated to dryness *in vacuo* and 400 mg of the dry extract were dissolved in 70% methanol in a 50 ml volumetric flask (solution **a**) and subjected to spectrophotometric analyses. For every extract, the procedure was performed in triplicate.

Total flavonoids quantification. Methanol solutions of quercetin (0.0095, 0.019, 0.038, 0.076 mg/ml and 0.19 mg/ml) were used to generate the standard curve. In a volumetric flask, 20 ml MeOH and 2 ml of the standard solution were added, and 1 ml 5% AlCl₃ solution in MeOH (w/v) and the volume was made up to 50 ml. The obtained solution was allowed to stay 30 min. Absorbance was measured at 425 nm (blank prepared in the same way, 2 ml of MeOH instead of standard solution). Every analysis was performed in triplicate. For analysis of the plant extracts, 2 ml of solution **a** was applied in the same procedure. Every analysis was performed in triplicate.

Total phenolics quantification. Methanol solutions of gallic acid (0.009; 0.019; 0.037; 0.075 and 0.15 mg/ml) were used to generate the standard

curve. To 1 ml of the standard solution, 10 ml distilled water were added, after that 4 ml of Folin-Ciocalteu reagent and 6 ml of 20% Na_2CO_3 were added and the volume made up to 50 ml (volumetric flask). The solution was allowed to stay for 2 h (± 3 min). Absorbance was measured at 760 nm (blank prepared in the same way, 1 ml of MeOH instead of standard solution). Every analysis was performed in triplicate. For analysis of the plant extracts, 1 ml of solution **a** was applied in the same procedure. Every analysis was performed in triplicate.

RESULTS AND DISCUSSION

The major constituents of the celeriac roots methanol extract are phenolic compounds, including predominantly polar flavonoids and their glycosides, and phenolic acids. However, it contains also other bioactive compounds of lesser polarity and in order to study their chemical profile we used GC-MS. We obtained the petroleum ether fraction of the methanol extracts and divided it into two sub-fractions by preparative TLC. The less polar fraction from the PTLC was analyzed by GC-MS and aliphatic hydrocarbons, fatty acids, allyl phenoxyacetate **1**, and several phtalides were identified, using computer libraries. The phtalides detected were n-butyl phtalide **2** and two isomers of cnidilide (Fig. 1). For complete identification of the cnidilides, they were isolated by PTLC as an inseparable mixture and by comparison with literature NMR data [30,31] they were identified as neocnidilide **3a** and isocnidilide **3b**. N-butyl-phtalide and similar compounds are responsible for the flavor and aroma of celery and extracts thereof [32]. A number of phtalides with diverse biological activity have been found in *A. graveolens* [18,33].

The second, more polar fraction from the PTLC of the petroleum ether fraction, was silylated prior to GC/MS analysis. The data indicated the presence of fatty acids (major constituents), one diterpenic acid (dehydroabiatic acid) and the furocoumarines psoralen **4**, xanthotoxin **5**, and isopimpinellin **6**. Two major compounds of this fraction were isolated by PTLC and one of them was identified as xanthotoxin **5** by comparison of its mass and NMR spectra with literature data [34]. Compound **5** has been isolated from *A. graveolens* [35]. The second compound was identified as 6-(3'-methyl-1'-oxobutyl)-7-hydroxy coumarin **7** based on different NMR experiments (^1H , ^{13}C , DEPT, HSQC, HMBC) and GC/MS after silylation.

The ^1H NMR spectrum of compound **7** showed two one proton doublets at δ 7.57 ($J = 9.5$ Hz) and

6.23 ($J = 9.5$ Hz), typical for olefin protons H-4 and H-3 of α -pyrone ring in coumarins. The HMBC correlation (Fig. 2) between H-4 and quaternary C-2 (δ 159.9, C=O) and C-8a (δ 159.0), H-3 and C-4a (δ 111.6) confirm the base structure.

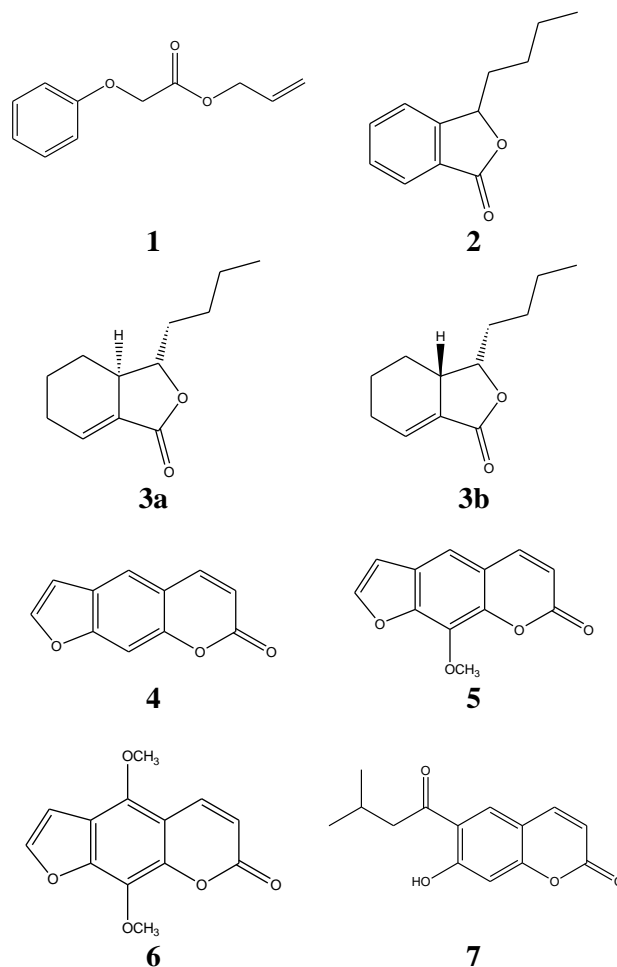


Fig. 1. Compounds, identified in celeriac roots.

Two singlets for one proton each, at δ 7.83 and 6.79 were assigned to *para* aromatic protons H-5 and H-8, respectively. In up field part of ^1H NMR spectrum appeared the signals for the side chain: doublet for two methyl groups at δ 0.97 ($J = 6.7$ Hz), multiplet for one proton at δ 2.25 and doublet for two protons at δ 2.81 ($J = 6.9$ Hz). The corresponding carbon atoms have resonances at δ 22.7 (CH_3 -4', 5'), 25.5 (C-3') and 47.1 (C-2'), respectively. The downfield shifted signal for methylene group at δ_c 47.1 (C-2') and HMBC correlation (Fig. 2) between H_2 -2' and carbon signal at δ 205.5 indicate the side chain as an oxoisopentyl group. The carbon atom C-1' (δ 205.5) was bonded to C-6 (δ 117.3) based on HMBC correlation between H-5 and C-1', and H-8 and C-6. The singlet for one proton at δ 12.81 was assigned to the proton from hydroxy group at C-7

using HMBC data. Correlation between H-5 and C-7 was seen in the same spectrum. Based on these data, the structure of **7** was concluded to be 6-(3'-methyl-1'-oxobutyl)-7-hydroxy-coumarin **7**. Compound **7** has not yet been isolated from a natural source, it has been synthesized back in 1955 [36].

Further, in order to characterize Bulgarian celeriac with respect to the content of potential antioxidants, total phenolics and total flavonoids content was measured spectrophotometrically in methanol extract of leaves and roots of *A. graveolens* var. *rapaceum* from 19 different locations in Bulgaria. The results are represented in Table 1.

The total phenolics varied between 15 - 67.2 mg/g GAE in leaves (mean value 29±13) and 8.2-13.6 mg/g GAE in roots (mean value 11±2), and total flavonoids were between 6 - 26.5 mg/g in leaves (mean value 15±6) and 1 - 4.8 mg/g in roots (as quercetin) (mean value 2±1). The mean values for both compound groups are higher in leaves, the differences being statistically significant ($p < 0.001$).

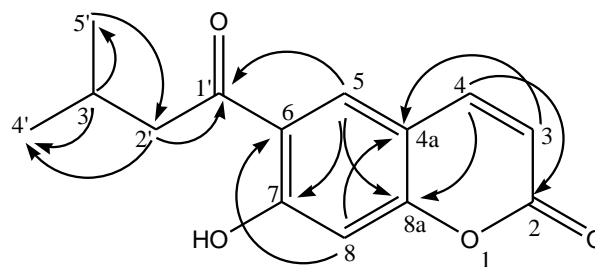


Fig. 2. HMBC (H → C) correlation in **7**.

The samples analyzed originated from regions with different climatic conditions. They demonstrated more or less similar content of phenolics and flavonoids; this fact suggests a possible dominance of genetic rather than ecological factors as determinant for the biosynthesis of these compounds.

Some samples of wild-growing *Apium nodiflorum* were also analyzed. The two leaf samples of *A. nodiflorum* demonstrated total phenolic content and total flavonoids in the range of the samples of celeriac from the same region (Varna).

Table 1. Total phenolics and total flavonoids in extracts of Bulgarian celeriac.

No.	Origin	Organ	Total Phenolics (GAE ^a , mg·g ⁻¹)	Total Flavonoids (QE ^b , mg·g ⁻¹)
1	Varna Region, Oborishte A	leaves	26.9±0.9	19.3±0.5
2	Varna Region, Oborishte B	leaves	15.7±1.1	6.9±0.1
3	Varna Region, Oborishte C	leaves	36±3	11.02±0.03
4	Varna Region, Beloslav A	leaves	20.2±0.1	15.3±0.3
5	Varna Region, Beloslav B	leaves	28.0±1.4	25.6±0.7
6	Varna region, Strashimirovo	leaves	67.2±1.9	26.5±1.6
7	Shabla	leaves	23.3±0.3	21.46±0.02
8	Balgarevo	leaves	38.05±0.05	13.5±1.1
		roots	10.7±0.5	1.45±0.02
9	Aitos Region, Bosilkovo	leaves	33.4±1.1	18.2±0.7
10	Aitos Region, Devetak	leaves	26.9±1.1	16.8±0.3
11	Aitos	leaves	45.8±2.5	18.1±0.1
12	Sofia Region, German	roots	8.2±0.3	1.15±0.1
13	Petrich Region, Drangovo	leaves	18.7±1.3	6.0±0.4
		roots	9.3±0.7	1.6±0.1
14	Petrich Region, Mihievo	roots	13.6±0.8	2.0±0.2
15	Karnobat Region, Gluche	leaves	41.2±0.9	16.9±1.3
16	Karnobat Region, Sokolovo	leaves	20.8±1.0	10.3±0.09
		roots	13.3±0.8	1.31±0.03
17	Karnobat Region, Tserkovsko	leaves	15.0±1.0	8.4±0.1
		roots	8.4±0.7	1.00±0.07
18	Karnobat Region, Dobrilovo	leaves	23.3±0.8	17.2±1.5
		roots	11.1±0.4	1.03±0.05
19	Kardzhali	leaves	16.7±0.8	9.3±0.6
20	Sofia	leaves	38.7±1.4	10.4±0.5
		roots	11.7±0.3	4.8±0.2
21	Bulgaria, commercial sample	roots	8.9±1.1	2.3±0.6
22	Varna Region, Devnya ^c	leaves	24±2	8.1±0.5
		roots	16.1±0.4	7.4±0.1
23	Varna Region, Beloslav ^c	leaves	14.9±0.5	10.7±0.2

^a GAE – gallic acid equivalents; ^b QE – quercetin equivalents; ^c *Apium nodiflorum*.

The only root sample of *A. nodiflorum* had total phenolics and total flavonoids higher than that of the highest values for *A. graveolens* var. *rapaceum*.

CONCLUSION

Our results demonstrate that the roots and leaves of Bulgarian celeriac are a rich source of biologically active constituents. Obviously, Fool's-water-cress roots deserve further, more detailed studies.

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НОВ КУМАРИН И СЪДЪРЖАНИЕ НА ТОТАЛНИ ФЛАВОНОИДИ И ТОТАЛНИ ФЕНОЛИ В БЪЛГАРСКА ЦЕЛИНА

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(Резюме)

Целината *Apium graveolens* е популярен зеленчук, но също така и добре познато лечебно растение. Различни екстракти от целина са показали разнообразни ценни свойства: антиоксидантни, антибактериални, противоракови, и др. В България най-често се използват листата (като подправка) и удебелените корени на *A. graveolens* var. *rapaceum*. Изследван беше химичният състав на неполярни екстракти от българска целина. В петролевоетерната фракция на метанолния екстракт от корени посредством ГХ-МС идентифицирахме мастни киселини и въглеводороди, алилфеноксиацетат, фталидите неокнидилид, изокнидилид, 3-*n*-бутилфталид и кумарините ксантотоксин, изопимпинелин и псорален. Беше изолирано и едно ново природно съединение, 6-(3'-метил-1'-оксобутил)-7-хидроксикумарин, като структурата му беше доказана с помощта на спектрални данни. Съдържанието на тотални феноли и тотални флаваноиди в растителен материал от различни райони на България беше определено спектрофотометрично. Анализирахме метанолните екстракти от листа и корени от 19 местонаходища в България, както и няколко проби от диворастящ *Apium nodiflorum*. Тоталните феноли варираха в границите 15 - 67,2 mg/g еквиваленти галова киселина (ЕГК) в листа и 8.2 - 13.6 ЕГК в корени, а тоталните флаваноиди – в границите 6 – 26,5 mg/g еквиваленти кверцетин в листа и 1 - 4.8 mg/g еквиваленти кверцетин в корени. Резултатите показват, че корените на българската целина са ценен източник на биологично активни компоненти.