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Authors: Antoaneta Trendafilova, Gulmira Ozek, Suleyman Yur, Fatih Goger, Temel Ozek, Boban Andjelkovic, Dejan Godjevac, Ivana Sofrenic, Ina Aneva, and Milka Todorova

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Furanocoumarin content, antioxidant activity and inhibitory potential of *Heracleum verticillatum, H. sibiricum, H. angustisectum* and *H. ternatum* extracts against enzymes involved in Alzheimer's disease and type II diabetes

Gulmira Ozek[a], Suleyman Yur[a,b], Fatih Goger[a,b], Temel Ozek[a,b], Boban Andjelkovic[c], Dejan Godjevac[d], Ivana Sofrenic[c], Ina Aneva[e], Milka Todorova[f], Antoaneta Trendafilova[*,f]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir, Turkey

[b] Medicinal Plant, Drug and Scientific Research Center (AUBIBAM), Anadolu University, 26470 Eskişehir, Turkey

[c] Faculty of Chemistry, University of Belgrade, 11100 Belgrade, Serbia

[d] Institute of Chemistry, Technology and Metallurgy, National Institute, University of Belgrade, 11000 Belgrade, Serbia

[e] Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

[f] Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria, e-mail: trendaf@orgchm.bas.bg

Hexane extracts of *Heracleum verticillatum, H. sibiricum, H. angustisectum* and *H. ternatum* were studied for their furanocoumarin content antioxidant potential and acetylcholinesterase and α-amylase inhibitory activities. Quantification of the furanocoumarins was performed by ^{1}H NMR. Pimpinellin was found to be the main component in the roots of all studied species. Bergapten and imperatorin were the major compounds in the fruits of *H. sibiricum* and *H. verticillatum*, respectively, while byakangelicol dominated in *H. angustisectum* and *H. ternatum* fruits. The leaf and fruit extracts of *H. angustisectum* demonstrated the highest DPPH radical scavenging activity and TEAC (IC_{50} 0.58 mg/mL and 1.83 mM, respectively). The root extracts of *H. verticillatum* and *H. angustisectum* were found to be the most effective against acetylcholinesterase (IC_{50} 0.30 and 0.34 mg/mL, respectively). The studied extracts were not active or demonstrated a weak inhibitory effect (% Inh. up to 29.7) towards α-amylase.

Keywords: *Heracleum* species • furanocoumarins • antioxidant activity • acetylcholinesterase inhibition • antidiabetic activity

Introduction

The genus *Heracleum* is one of the largest genera of the family Apiaceae, with 125 species spread throughout the world and is represented in Bulgarian flora by four species: *H. sibiricum* L. (syn. *H. sphondylium* subsp. *sibiricum* (L.) Simonk.), *H. ternatum* Velen. (syn. *H. sphondylium* subsp. *ternatum* (Velen.) Briq.), *H. verticillatum* Pančić (syn. *H. sphondylium* subsp. *verticillatum* (Pančić) Brummitt), *H. angustisectum* (Stoj. & Acht.) Peev (syn. *H. ternatum* var. *angustisectum* Stoj. & Acht.)[1, 2]. Three of them are also recognized as subspecies of *H. sphondylium*. *Heracleum verticillatum* and *H. angustisectum* are Balkan and Bulgarian endemic species, respectively. *Heracleum sibiricum, H. ternatum* and *H. verticillatum* are medicinal plants. Decoctions of their roots are used as diaphoretic and antiepileptic remedies in Bulgarian folk medicine [3]. Many ethnobotanical uses have been also reported for hogweed (*H. sphondylium* sensu lato): against diarrhea and dysentery, as stomachic, digestive, aphrodisiac, sedative, against depression, etc. [4, 5].

It was shown that the genus *Heracleum* has a broad spectrum of biological activities. Extracts, essential oils, and constituents isolated from different parts of the plant displayed activities such as antibacterial, antioxidant, cytotoxic, anticandidal, antmicrobial and apoptotic activity on human leukaemia cell lines [4, 6]. Literature data concerning inhibitory effects of *Heracleum* extracts against enzymes involved in Alzheimer disease and type II diabetes are scarce and limiting to several reports on α-amylase and α-glucosidase inhibitory activity of *H. persicum* [7-9], and acetylcholinesterase inhibitory activity of *H. platytaenium* [10, 11] and *H. crenatifolium* [12].

Literature survey showed that only 1/6 of the species of genus *Heracleum* has been studied phytochemically so far [4]. The genus is rich in furanocoumarins and essential oils. Various furanocoumarins were found in the fruits and roots of *H. sibiricum* [4, 13-22], *H. verticillatum* [15, 22], and *H. sphondylium* [4, 6, 21-24]. To the best of our knowledge, *H. angustisectum* as well as leaves of *H. sibiricum*, *H. verticillatum* and *H. ternatum* have not been studied for the presence of furanocoumarins. Furthermore, the literature data on the antioxidant and enzyme inhibitory potentials of these species showed only one report on antioxidant activity of essential oil obtained from fruits of *H. sphondylium* subsp. *ternatum* [5].

In the present work, the hexane extracts obtained from the leaves, fruit and roots of *Heracleum verticillatum*, *H. sibiricum*, *H. angustisectum* and *H. ternatum* were evaluated for their furanocoumarin content, antioxidant potential, acetylcholinesterase and antidiabetic inhibitory activities.

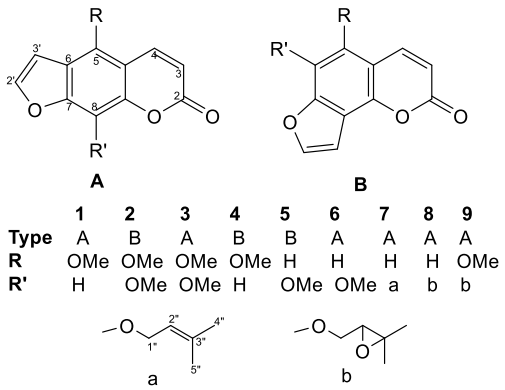

Figure 1. Structures of identified furanocoumarins in the investigated *Heracleum* species

Results and Discussion

Determination, identification and quantification of furanocoumarins by ^{1}H NMR

NMR spectroscopy has a long history in the qualitative and quantitative assessment of secondary plant metabolites [25]. NMR techniques are reproducible with rich structure information. The only essential requirement for compound detection in ^{1}H NMR experiments is the availability of observable protons in a molecule, thus resulting in the applicability of ^{1}H NMR to a wide range of plant metabolites. NMR spectroscopy is also an invaluable technique for the structural determination of coumarins and furanocoumarins [26-28]. We were focused on simultaneous determination and identification of furanocoumarins by ^{1}H NMR in the hexane extracts obtained from fruits, roots and leaves of *H. verticillatum*, *H. sibiricum*, *H. angustisectum* and *H. ternatum*. Assignment of the signals for each individual compound was performed using a combination of 1D and 2D NMR techniques, including COSY, HSQC and HMBC spectra. Thus, nine furanocoumarins (**1** - **9**) were identified in the hexane extracts of the studied *Heracleum* species and their structures are shown in Fig. 1. Among them, bergapten (**1**), pimpinellin (**2**), isopimpinellin (**3**), sphondin (**5**), xanthotoxin (**6**), imperatorin (**7**) and heraclenin (**8**) were unambiguously assigned by comparison of their spectral data with those of authentic standards (Table 1). The structures of isobergapten (**4**) and byakangelicol (**9**) were tentatively determined by comparison with data reported in the literature [9, 19].

Table 1. ^{1}H NMR data the compounds detected in the hexane extracts of *Heracleum* species in $CDCl_3$

H	1	2	3	4[a]	5	6	7	8	9[a]
H-3	6.27 d[b]	6.37 d	6.29 d	6.31 d	6.40 d	6.37 d	6.37 d	**6.38 d**[c]	6.24 d
H-4	8.15 d	**8.08 d**	8.12 d	8.16 d	7.76 d	7.77 d	7.76 d	7.77 d	8.14 d
H-5					**6.78 s**	**7.35 s**	**7.36 s**	**7.40 s**	
H-6				**6.89 s**					
H-8	**7.14 s**								
H-2′	7.59 d	**7.66 d**	**7.63 d**	**7.57 d**	**7.71 d**	7.69 d	7.69 d	7.70 d	**7.62 d**
H-3′	7.02 d	7.08 d	7.00 d	7.02 d	7.14 d	6.82 d	6.81 d	6.83 d	7.01 d
OCH_3	**4.27 s**	**4.04 s** **4.15 s**	**4.17 s** **4.17 s**	**3.97 s**	**4.05 s**	**4.30 s**			**4.19 s**
H_R							**5.59-5.64** m, **5.00** d (2H), 1.73 s ($2CH_3$)	**4.57 dd**, 3.31 t, 1.28 s, 1.34 s	**4.44 dd**, 3.31 t, 1.24 s, 1.32 s

[a] Tentatively identified by comparison with literature data [9, 19] . [b] For compounds **1-9**: $J_{3,4}$ = 9.8 Hz, $J_{2',3'}$ = 2.4 Hz, for compound **7**: $J_{1a'',2''}$= $J_{1b'',2''}$6.5 Hz; for compounds **8** and **9**: $J_{1a'',2''}$ = $J_{1b'',2''}$= 5.6 Hz; $J_{1a'',1b''}$= 11.5 Hz . [c] Key signals of each compound used for quantitative determination

As can be seen, furanocoumarins were characterized by two pairs of doublets in the downfield region, one at δ_H 6.24-6.40 and 7.76 - 8.16 (J = 9.8 Hz) was attributed to the C-3 and C-4 protons of the coumarin nucleus, while a second pair of signals at δ_H 7.57-7.71 and 6.81-7.14 (J = 2.4 Hz) confirmed the presence of the benzofuran moiety. The singlets at δ_H 6.78 -7.40 were assigned to the single aromatic protons at C-5, C-6 or C-8 positions. In

monosubstituted psoralenes (Type A), the upfield position of H-8 in **1**, in contrast to the H-5 (**6 - 8**), is due to the diamagnetic shift of an aromatic proton adjacent to an oxygen atom. The presence of OMe substituent at C-5 (**1 - 4**, **9**) shifted H-4 signal at lower field and H-4 appeared at δ 8.08-8.16 ppm. The isoprenyl ether group in the C-8 position (**7**) can be easy distinguished since the gem-dimethyl groups of the former showed a singlet at δ 1.73 indicating a similar magnetic environment for both methyls [26]. The presence of epoxyisopentenyloxy moiety in the structure of compounds **8** and **9** followed by chemical shifts and coupling constants of the protons at C-1″, C-2″, C-4″ and C-5″. Thus, protons at C-1″ appeared as doublet of doublets at δ 4.57/4.44 with *J* values of 11.5 and 5.6 Hz, H-2″ was triplet at δ 3.31with *J* values of 5.6 Hz and methyl groups at C-4″ and C -5″ were singlets at δ 1.24/1.28 and 1.34/1.34.

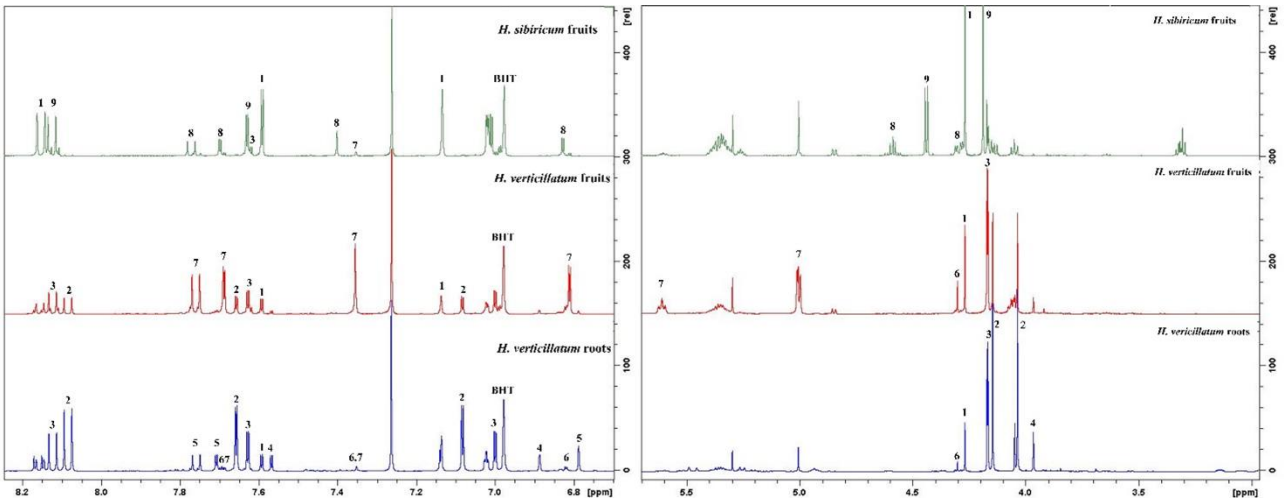

Figure 2. Expanded parts of the ^{1}H NMR spectra of the hexane extracts *H. verticillatum* roots, *H. verticillatum* fruits and *H. sibiricum* fruits with assignment of selected key signals used for the quantification.

Further, ^{1}H NMR spectroscopy was used to determine the content of the main furanocoumarins in the hexane extracts from *H. sibiricum, H. verticillatum, H. angustisectum* and *H. ternatum*. For this purpose an accurately weighted amount of 2,6-*bis*(1,1-dimethylethyl)-4-methylphenol, BHT (MW 220.35) was added to the studied samples as an internal standard [29]. The strong singlet at 6.98 ppm, corresponding to two protons was used as reference signal. The expanded parts of the spectrum of the hexane extracts *H. verticillatum* roots, *H. verticillatum* fruits and *H. sibiricum* fruits with assignment of selected key signals used for the analysis are given in Fig. 2. The quantities of individual compounds were determined from the integral areas of the corresponding key signals (Table 1). Among the quantified compounds (Table 2), pimpinellin (**2**) was found to be the main component in the roots of all studied species. Bergapten (**1**) and imperatorin (**7**) were the major components in the fruits of *H. sibiricum* and *H. verticillatum*, respectively, while byakangelicol (**9**) dominated in *H. angustisectum* and *H. ternatum* fruits.

H. verticillatum was found to contain furanocoumarins **1** - **7** in fruits, roots and leaves, differing in their amounts. Thus, pimpinellin (**2**) was the main compound in the roots and leaves, while imperatorin (**7**) was the major one in the fruits. Isopimpinellin (**3**) was detected in substantial amounts in the roots and leaves. The presence of compounds **1** - **7** in *H. verticillatum* is in accordance of previous findings for this species [15, 22]. *H. angustisectum* and *H. ternatum* showed similar pattern with pimpinellin (**2**) as the main component in their roots and byakangelicol (**9**) in the fruits. The leaves of *H. angustisectum* and *H. ternatum* were found to be poor in furanocoumarins and none of the nine compounds was detected in the studied extracts at concentration of 20 mg/mL. It is worth to note, that all furanocoumarins identified in the roots and fruits of *H. ternatum* have been recently reported as constituents of the species collected in Montenegro [22]. *Heracleun angustisectum* has not been studied previously and all identified compounds are now described for the first time. Similarly to the above-mentioned species, some qualitative differences in different plant parts of *H. sibiricum* were observed. Thus, bergapten (**1**) and isopimpinellin (**3**) were the only furanocoumarins present in all plant parts. Pimpinellin (**2**) was the main component in roots and leaves, while fruits were characterized with significant amounts of bergapten (**1**) and byakangelicol (**9**). Xanthotoxin (**6**) was detected in the leaves only. Comparison of the obtained results with those published for *H. sibiricum* displayed both similarities and differences in the chemical content. Thus, furanocoumarins **1, 3**, **7 - 9** were occurring in the fruits and bergapten (**1**) was the principal one in *H. sibiricum* fruits of other origins [15, 19, 22]. On the other hand, xanthotoxin (**6**), phelopterin and byakangelicin reported for the fruits of Polish [19] and Serbian [22] samples were not detected in our sample. Further, all furanocoumarins identified in the roots of the studied sample have been previously found in roots of the species from different origins [14, 15, 17, 18, 20, 22]. It should be mention that the results obtained in this study correlated well with the chemotaxonomic classification of the species in genus *Heracleum* proposed recently by Ušjak et al. [22]. According to this classification, *H. verticillatum* should be separated from *H. sphondylium* group due to the very

different furanocoumarin content of its fruits. The chemical profile of the newly studied *Heracleun angustisectum* was similar to that of *H. sibiricum* and *H. ternatum* and therefore could be placed it in the same group.

Determination of antioxidant activity

The free radical scavenging potential of the extracts was evaluated against DPPH radicals and the results are presented in Table 3. As can be seen, the leaf extracts of *H. angustisectum* (IC_{50} 0.58 ± 0.1 mg/mL), *H. sibiricum* (IC_{50} 0.68 ± 0.04 mg/mL) demonstrated the highest free radical scavenging activity unlike those obtained from the roots and fruits. In *H. ternatum* the roots showed noteworthy inhibitory effect (IC_{50} 0.73 ± 0.03 mg/mL). The weak DPPH radical scavenging activity found in this study is consistent with the results for other *Heracleum* species. Thus, they were comparable with those found for methanol extract from edible parts of *H. persicum* [30] (IC_{50} 0.43 ± 0.1 mg/mL), lower from those reported for 70% ethanolic extracts obtained from leaves and flowers of *H. sphondylium* from Romania [6], and higher from those recorded for various polar extracts (ethyl acetate, acetone, methanol and water) from aerial parts of *H. sphondylium* from Serbia [31], n-hexane extract from roots of *H. persicum* [9] and methanol extracts from aerial parts of *H. platytaenium* [11].

TEAC (Trolox equivalents of antioxidant capacity) indicated the relative ability of hydrogen or electron-donating antioxidants to scavenge the ABTS radical cation compared with that of Trolox. As can be seen, the studied extracts (Table 3) exhibited low to moderate radical scavenging activity between 0.05 and 1.83 mM. The highest TEAC value was obtained for the root extract of *H. angustisecum* (1.83 ± 0.002 mM). The present work is the first contribution about the free radical scavenging potential of the hexane extracts from *Heracleum* species against $ABTS^{+\cdot}$ radicals.

Table 2. Furanocoumarins in the hexane extracts from *Heracleum* species

Species	Plant part	Furanocoumarins* [mg/g extract]								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
H. verticillatum	roots	40.6±2.0	193.9±1.9	118.2±1.5	45.3±1.5^{a}	48.5±1.9^{a}	11.1±0.5	10.5±0.9^{a}		
	fruits	47.9±1.2^{a}	61.5±1.0^{a}	90.9±2.1	10.6±1.2^{b}	7.2±1.0	19.8±1.2^{a}	220.2±1.6		
	leaves	23.6±1.2	58.2±2.5^{a}	23.9±1.7^{a}	12.3±1.5^{b}	18.6±1.5	22.9±1.9^{a}	45.5±1.5		
H. sibiricum	roots	13.3±1.4	161.6±1.9	11.8±0.5	43.6±2.8^{a}	62.9±2.3^{b}				
	fruits	190.2±1.0		31.9±1.2				12.5±1.1^{a}	69.6±2.1	175.7±1.8
	leaves	7.7±0.9^{b}	14.8±1.1	5.2±0.6	5.5±0.6	2.6±0.6	4.52±0.6			
H. angustisectum	roots		256.9±1.9	105.0±1.5	53.5±1.1^{c}	59.1±1.0^{b}				
	fruits	100.4±1.9						26.6±0.6	100.7±1.5	130.3±1.5
	leaves	nd	nd	nd	nd	nd	nd	nd	nd	nd
H. ternatum	root	29.7±1.7	218.2±3.0	39.3±1.5	54.1±1.5^{c}	45.5±1.0^{a}				
	fruits	48.8±1.5^{a}	39.9±1.5	24.7±1.0^{a}	10.2±0.6^{b}			34.7±1.0	38.6±1.0	84.2±1.0
	leaves	nd	nd	Nd	nd	nd	nd	nd	nd	nd

*Means in the columns with the same letter are not significantly different from each other (p>0.05); nd – not detected

Determination of antiacetylcholinesterase and antidiabetic activities

The hexane extracts were further investigated for their antiacetylcholinesterase (AChE) potential using Ellman's method [32] (Table 3). The root extracts of *H. verticillatum* and *H. angustisectum* were found to be the most effective against AChE with IC_{50} values of 0.30 ± 0.07 and 0.34 ± 0.03 mg/mL, respectively. There are only three reports in the literature concerning antiacetylcholinesterase activity of *Heracleum* species [10-12]. Thus, for the furanocoumarin mixture obtained from *H. crenatifolium* was reported to exert 68.8 ± 0.76 % [12] against AChE, while the extracts of *H. platytaenium* [10, 11] exhibited 32.52 ± 3.27 and 49.28 ± 1.28 % at concentrations of 100 and 200 µg/mL, respectively. In the same study, eight furanocoumarins, isolated from *H. platytaenium* were investigated for their acetylcholinesterase inhibition and pimpinellin was found to be the most effective one, causing 78.57 ± 2.86% inhibition of AChE [10]. Therefore, better activity of the root extracts of *H. verticillatum* and *H. angustisectum* when compared with that of their fruit extracts could be explained with the presence of pimpinellin as a principal component [10]. In addition, fruit extracts of *H. verticillatum* and *H. sibiricum* were found to have noteworthy inhibitory effect with IC_{50} values of 0.49 ± 0.02 and 0.48 ± 0.02 mg/mL, respectively. This activity might be due to the presence of a notable amount of imperatorin, bergapten, and byakangelicol for which such activity has been already reported [12, 33, 34].

The hexane extracts obtained from the leaf, fruit and roots of *H. verticillatum*, *H. sibiricum*, *H. angustisectum* and *H. ternatum* were also evaluated for their antidiabetic inhibitory activity using the iodine/potassium iodide (IKI) method [35] (Table 3). The studied extracts were not active or demonstrated a weak inhibitory effect (% Inh. up to 29.7 ± 3.1) towards α-amylase (from porcine pancreas) at a concentration of 5 mg/mL. Our results are not in accordance with those published in the literature for antidiabetic activity of *Heracleum* species. In fact, there are only two reports on the α-amylase inhibition effect of *Heracleum* species. Thus, Afrisham et al. [7] reported about significant activity of the methanol extract of *H. persicum* fruits with IC_{50} value of 307 μg/mL. In another study, hexane, ethyl acetate, and methanol extracts obtained from the different plant parts of *H. persicum* at a concentration of 238.1 mg/mL were evaluated for their antidiabetic activity [8]. It has been reported that the hexane extract from aerial parts exhibited significant antidiabetic activities in α-amylase assay (78.5 ± 3.9 %) unlike the same extract from roots, which reached only 41.9 ± 2.7 % of inhibition.

Table 3. Antioxidant, antiacetylcholinesterase and antidiabetic activities of the hexane extracts of different *Heracleum* species

	Plant part	DPPH [IC_{50}, mg/mL]	TEAC [mM]	AChE [IC_{50}, mg/mL]	α -Amylase [Inh %][a]
	roots	2.92±0.15	0.42±0.05	0.30±0.07	N/A
H. verticillatum	fruits	1.69±0.09	0.34±0.01	0.49±0.02	N/A
	leaves	17.0±0.76 %[a]	0.52±0.03	0.63±0.47	N/A
	roots	0.93±0.07	0.87±0.05	0.94±0.64	15.9±2.0
H. sibiricum	fruits	45.76±0.75 %[a]	-	0.48 ±0.02	23.9±3.6
	leaves	0.68±0.04	0.57±0.06	26.7±5.7 %[a]	14.0±4.0
	roots	2.34±0.43	1.05±0.02	0.34±0.03	N/A
H. angustisectum	fruits	4.26±0.40	1.83±0.002	0.66±0.0	20.9±1.2
	leaves	0.58±0.10	0.64±0.005	28.6±0.0 %[a]	29.7±3.20
	roots	0.73±0.03	1.03±0.4	42.4±1.5%[a]	22.45±1.7
H. ternatum	fruits	30.0±2.4 %[a]	0.05±0.01	16.7±3.0 %[a]	16.17±2.4
	leaves	NA	0.16±0.06	12.1±1.3%[a]	24.28±0.9
Gallic acid	-	0.002	-	-	-
BHT	-	0.5	-	-	-
Ascorbic acid	-	0.03	-	-	-
Acarbose	-	-	-	-	90
Galanthamin	-	-	-	0.01	-

[a]The inhibition effect was expressed as percentage value for the extracts (5 mg/mL) with activity lower than 50 %. IC_{50} values were calculated for the samples with inhibition >50 %; N/A – not active

Conclusions

In the present work, four *Heracleum* species collected in Bulgaria: *H. verticillatum*, *H. sibiricum, H. ternatum* and *H. angustisectum* were investigated for their furanocoumarin content. The comparative ^{1}H NMR study of the hexane extracts of roots, leaves and fruits showed qualitative and quantitative differences between the organs of the plants as well as between the species. The fruits and roots extracts were rich in furanocoumarins. The roots of all studied species were characterized by the presence of pimpinellin as the main component, while fruit extracts were differing in the principal furanocoumarin. Leaves of *H. ternatum* and *H. angustisectum* did not contain this class of secondary metabolites. In addition, the hexane extracts were screened for their antioxidant, antiacetylcholinesterase and antidiabetic activities. Among studied species, *H. angustisectum* and *H. sibiricum* leaf extracts were found to be the best scavengers of DPPH radicals, while *H. angustisectum* fruit extract possessed the highest TEAC. The root extracts of *H. verticillatum* and *H. angustisectum* showed some notable inhibition against AChE. The studied extracts were not active or demonstrated a weak inhibitory effect towards α-amylase.

Experimental Section

Chemicals

Hydrochloric acid, *n*-hexane, dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany), anhydrous sodium sulfate (Fluka, Germany), magnesium chloride hydrate, sodium chloride, iodine (ACS reagent), potassium iodide (Saint Louis, USA), methanol (Sigma-Aldrich, Poland), potassium persulfate (Sigma-Aldrich, Saint Louis, USA), sodium phosphate, disodium phosphate and (2,6-bis(1,1-dimethylethyl)-4-methylphenol, BHT) were of analytical grade. Gallic

acid (GA), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), soluble starch, acarbose, α-amylase from porcine pancreas (Type VI-B, EC 3.2.1.1), tris(hydroxymethyl) aminomethane (ACS reagent), acetylcholinesterase (AChE) from *Electrophorus electricus* (Type VI-S), bovine serum albumin (BSA), acetylthiocholine iodide (ATCI), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), galanthamine from *Lycoris* sp. were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Instrumentation

NMR (500 MHz) spectra (in $CDCl_3$) were recorded on a Bruker Avance III 500 NMR spectrometer (Bruker, Karlsruhe, Germany) equipment. Microtiter plate assays were performed with Biotek Powerwave XS microplate reader. Ultrapure water (0.05 μS/cm) was obtained from a Direct-Q® Water Purification System (Germany). Pipetting in microtiter plate assays was performed with multichannel automatic pipette (Eppendorf Research® plus, Germany).

Plant Material

Plant material was collected from native populations in Bulgaria in 2016 (Table 4). The plant material was separated in leaves, fruits and roots, air-dried and kept in a dark and cool place until extraction. Voucher specimens have been deposited with the Herbarium of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences.

Table 4. Collection site and voucher specimen of *Heracleum* species

Species	Collection site	GPS coordinates	Voucher specimen
H. angustisectum	Pirin Mts, Yavorov hut	41°49'35.19"/ 23°22'31.32"	SOM 1368
H. sibiricum	Rila Mts, Bistritza Village	42° 3'43.02"/ 23°12'13.54"	SOM 1369
H. ternatum	Rodopi Mts, Gela Village	41°38'27.92"/ 24°34'1.99"	SOM 1370
H. vertcillatum	Rila Mts, Macedonia hut	42° 2'53.25"/ 23°26'55.89"	SOM 1371

Extraction

The air-dried samples (2-10 g) were extracted with 40-200 mL of *n*-hexane in a Soxhlet apparatus for 4 hrs. The extracts were obtained after evaporation of the solvent under reduced pressure at 40°C by using a rotary evaporator and stored at +4°C in dark until use. Dry hexane extracts from leaves and roots and semisolid extracts from fruits were further used for quantitative determination and biological assays.

Identification and quantification of the compounds in plant extracts

The obtained extracts were dissolved in $CDCl_3$. and analyzed by ^1H NMR. All compounds were identified by comparison of their spectral data with those in the literature and/or by comparison with authentic standards. For quantitative analysis, the extract (10 mg) was dissolved in 0.5 mL $CDCl_3$ containing the standard (2,6-*bis*(1,1-dimethylethyl)-4-methylphenol, BHT) in concentration 2 mg/mL. Peak area was used and the start and end points of the integration of each peak were selected manually. The quantity of furanocoumarin components in the studied mixture was based on the integrals of the respective signals of the individual compounds not overlapped with other signals, and the two-proton singlet (δ 6.98) of BHT, used as the internal standard and was determined using the following general Equation 1:

$$m_X = m_S \times [(I_X \times M_X \times N_S)/(I_S \times M_S \times N_X)], \quad (1)$$

where m_X is the unknown mass of the furanocoumarin component, m_S is the weighted mass of the standard; M_S and M_X are the molar masses (in Da) of the standard and the furanocoumarin component, respectively; I_S and I_X represent the integrated signal area of standard and the furanocoumarin component, respectively; N_S and N_X correspond to the number of protons from the respective integrated signal for the standard and the furanocoumarin component.

Free radical scavenging assay (DPPH test)

The scavenging effect of the samples on DPPH free radical was determined using a modified method of Brand-Williams [36]. The free radical scavenging activity of the samples was expressed as percentage of inhibition calculated according to Equation 2:

$$\%\ \text{Inh} = [\frac{A_{control} - A_{sample}}{A_{control}}] \times 100, \quad (2)$$

where $A_{control}$ is the absorbance of the control (containing all reagents except the test compound), A_{sample} is the absorbance of the sample with added DPPH. The IC_{50} values were obtained by plotting the DPPH scavenging percentage of each sample against the sample concentration. Data were analyzed using the SigmaPlot software (Version 12.0).

ABTS radical cation scavenging activity (TEAC assay)

Trolox equivalent antioxidant capacity of the samples was estimated towards to $ABTS^{\cdot+}$ according to the procedure described by Re et al. [37] with slight modification. $ABTS^{\cdot+}$ scavenging activity of the samples was expressed as TEAC and calculated using linear equation obtained for Trolox.

α-Amylase inhibition assay

The effect of the samples on α-amylase was evaluated using the iodine/potassium iodide (IKI) method [35] with slight modification. In the experiment, the substrate solution (0.05 %) was prepared by dissolving of soluble potato starch (10 mg) in 20 mL ultrapure water then boiling for 5 min and cooling to room temperature before use. Acarbose (0.01-0.1 mg/mL in buffer) solution was used as a positive control experiment. 20 mM sodium phosphate buffer (pH 6.9) was pipetted in the 96-well flat bottom plates with 8-multichannel automatic pipette, then 25 μL sample solution and 50 μL α-amylase (0.8 U/mL in buffer) were added and incubated for 10 min at 37 °C. After incubation, 50 μL substrate solution was added to the mixture. The mixture was incubated again for 10 min at 37 °C. The reaction was stopped by addition of 25 μL HCl (1 M). Finally, 100 μL of I_2/KI reagent was added to the wells. The sample blanks contained all reaction reagents and 50 μL buffer instead of enzyme. The control wells contained all reaction reagents and 25 μL solvent (instead of the sample solution). The absorbance values were recorded for the sample and blank at 630 nm. The percentage inhibition was calculated according to Equation 3:

$$\% \text{ Inh} = \left[\frac{(A_{control\ blank} - A_{control}) - (A_{sample\ blank} - A_{sample})}{A_{control\ blank} - A_{control}}\right] \times 100, \quad (3)$$

where $A_{control}$ and $A_{control\ blank}$ are the absorbance values of the control and its blank, A_{sample} and $A_{sample\ blank}$ are the absorbance values of the sample and its blank.

Acetylcholinesterase inhibition assay

The effect of the samples on acetylcholinesterase (AChE) was evaluated using Ellman's method [32] with slight modification. Three buffers were used: (A) 50 mM Tris-HCl (pH=8.0, in ultrapure water); (B) 0.1 % BSA in buffer A; (C) 0.1 M NaCl and 0.02 M $MgCl_2 \cdot 6H_2O$ in buffer A. The extracts were previously solved in DMSO (20% in buffer). In the experiment 25 μL sample (extract or standard), 50 μL buffer B and 25 μL AChE (0.22 U/mL in buffer A) solution were pipetted into wells of 96-well flat bottom plates using 12-channel automatic pipette and incubated for 15 min at 25 °C. Then, 125 μL of Ellman's reagent DTNB (3.0 mM in buffer C) and 25 μL substrate ATCI (15 mM, in ultrapure water) were added. Hydrolysis of ATCI was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader. The mixture allowed to stand 15 min at 25 °C and the absorbance was recorded at 412 nm. Similarly, a blank (for eliminating the colors of the samples) was prepared by adding sample solution to all reaction reagents and 25 μL buffer instead of enzyme. The control wells contained all the reagents without the sample (the solvents of the samples instead were added). Galanthamine hydrobromide (0.1 mg/mL) was used as the positive control. The percentage inhibition was calculated according to Equation 4:

$$\% \text{ Inh} = \left[\frac{(A_{control} - A_{control\ blank}) - (A_{sample} - A_{sample\ blank})}{A_{control} - A_{control\ blank}}\right] \times 100, \quad (4)$$

where $A_{control}$ and $A_{control\ blank}$ are the absorbance values of the control and its blank, A_{sample} and $A_{sample\ blank}$ are the absorbance values of the sample and its blank.

Statistical analysis

All experiments were carried out in triplicate. The results were expressed as mean values and standard deviation (SD). The differences between the different extracts were analyzed using one-way analysis of variance (ANOVA).

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Author Contribution Statement

G. O., S. Y., F. G. and T. O. – antioxidant, acetylcholinesterase inhibitory and antidiabetic activity; discussion of the results, preparation and discussion on the manuscript; B. A., D. G. and I. S. – NMR analysis, interpretation of NMR data, discussion on the manuscript; I. A. – collection and identification of plant material, discussion on the manuscript; M. T. – supervising all experiments on extraction and identification of compounds, discussion on the manuscript; A. T. – extraction of plant material, interpretation of NMR data and identification of compounds, discussion, preparation and discussion on the manuscript.

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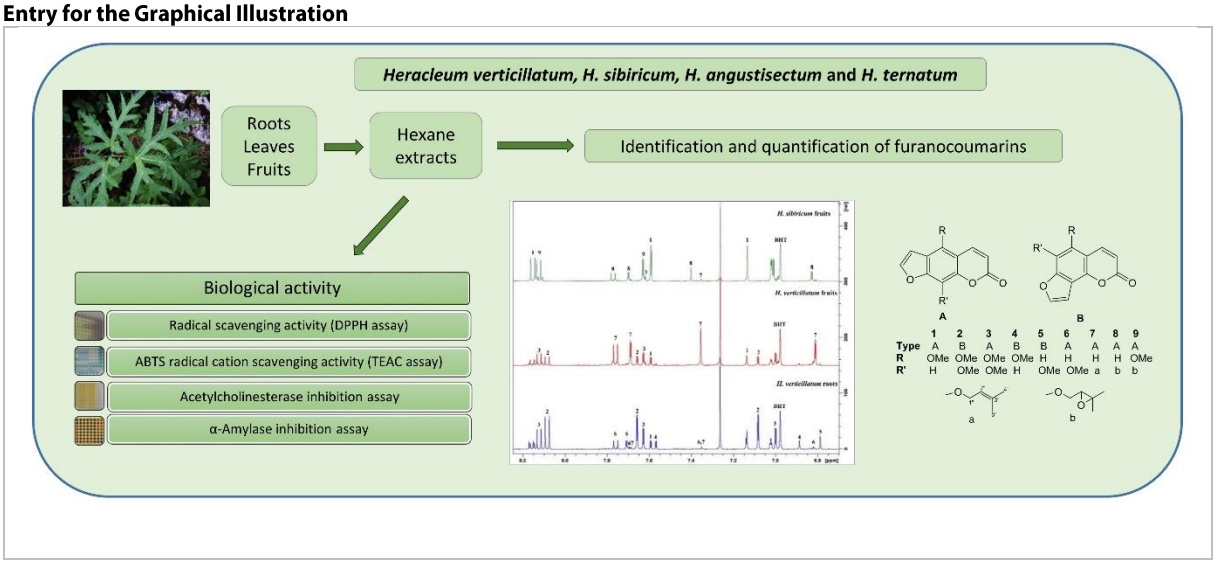

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