ORIGINAL ARTICLE

PRELIMINARY ASSESSMENT OF THE ANTIOXIDANT, ANTIFUNGAL AND GERMINATION INHIBITORY POTENTIAL OF *HERACLEUM SPHONDYLIUM* L. (*APIACEAE)*

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Abstract

The present work studies potential viable applications of the hydroalcoholic extract of a Romanian native *Heracleum* species, such as antifungal, antioxidant and germination inhibitory effect. The plants were collected in June 2014 and a voucher specimen was deposited at BUAG Herbarium, Bucharest, Romania. The hydroalcoholic extract obtained was characterized by analytical techniques and in terms of phytochemical assays. The analytical characterization of the extract revealed its chemical composition. The antioxidant potential of the extract was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The hydroalcoholic extract revealed an important *in vitro* antifungal activity on the studied fungal lines, as well as a very interesting germination inhibitory effect, the extract influencing the growth in length of embryonary roots, lateral roots formation and the embryonary stem growth for *Cucumis sativus*.

Rezumat

Studiul prezintă potențialele aplicații ale extractului hidroalcoolic obținut dintr-o plantă din genul *Heracleum* nativă în Romania, precum efectele antifungice, antioxidante și de inhibitor al germinării. Plantele au fost colectate în Iunie 2014, iar un specimen-martor a fost depus la herbarul BUAG, București, România. Extractul hidroalcoolic obținut a fost caracterizat prin tehnici analitice și prin analize fitochimice. Caracterizarea analitică a relevant compoziția sa chimică. Potențialul anti-oxidant a fost determinat prin testul cu DPPH (2,2-diphenyl-1-picrylhydrazyl). Extractul hidroalcoolic prezintă un efect antifungic important asupra liniilor studiate *in vitro,* precum și un foarte interesant efect de inhibare a germinației, influențând creșterea în lungime a rădăcinilor embrionare, formarea de rădăcini laterale și creșterea tulpinilor embrionare pentru *Cucumis sativus*.

Keywords: *Heracleum sphondylium* L. extract, antioxidant, antifungal, germination inhibition

Introduction

Since ancient times, plants have been used for various purposes in medicine, cosmetics or food preparation [2, 20]. In the last years, researchers have paid special attention to the development of drugs from natural origins and the screening of less known plants which can lead to the discovery of novel therapeutics [14].

Many plants are known only for certain therapeutic effects, while the rest of their effects remain unknown. Since not all the plants growing in our geographic area have been extensively studied for their biological activities, the plants with different species and subspecies represent an invaluable source of potentially useful and biologically active compounds. The chemical characterization of plant extracts is necessary in order to understand their biological and therapeutic properties.

Heracleum sphondylium (Hogweed) is a plant native to the forests of Europe. Its leaves resemble the paw of the bear (whence the Romanian popular name of the plant – Bear-paw – *Branca Ursului*). Since antiquity, it has been known that those who ate Hogweed were free from genital diseases. In traditional medicine, the aerial parts of this plant have been used for the treatment of sterility, both in female and male, hypertension, digestive diseases, epilepsy,

renal insufficiency and, in a lesser extent, as antifungal agent [10, 17].

Studies of different species of *Heracleum* have reported various biological properties, such as ascytotoxic activity for *H. sibiricum* [6], antioxidant and antimicrobial activity for *H. nepalense* [11], or immunostimulatory potential of *H. maximum* [21]. The present study represents a preliminary evaluation regarding the potential applications of a Romanian native *Heracleum* species, by determining the antioxidant, antifungal and germination inhibitory activity of its hydroalcoholic extract [5].

Materials and Methods

Plant material and hydroalcoholic extract

The *Heracleum sphondylium* subsp. *sphondylium* plants were collected in June 2014 from Leordeni area, Pitesti hills, Romania (N 44°47'30", E 25°8'4", 226 meters above sea level). A voucher specimen no. 40000 was deposited in BUAG Herbarium (Herbarium of University of Agronomical Science and Veterinary Medicine), Bucharest for further references (Figure 1).

Figure 1.
Voucher specimen deposited at BUAG herbarium: *Heracleum sphondylium* L. subsp. *sphondylium* (A) compared with *Heracleum sphondylium* 352.1 from the Linnean Collections (B)

In order to evaluate the composition and the antifungal, antioxidant and germination inhibitory properties, we used the hydroalcoholic extract obtained from the upper aerial part of *Heracleum sphondylium* L. subsp. *sphondylium*. The extract used for the study was obtained from shade dried plant (approx. 20 g.) in 1:1 mixture water-ethanol (100:100 mL), method previously demonstrated to be appropriate for obtaining hydroalcoholic extracts with good antifungal activity [12]. Also, this specific technique allows the extraction of both phenolic compounds, as well as the furanocoumarins. The ethanol used for all the experiments was of analytic grade, purchased from Merck KGaA (Germany). The water used was bi-distilled obtained using GFL 2102.

Chemical characterization methods

The extract was characterized by analytical techniques (Fourier transform infrared spectroscopy, gas chromatography–mass spectrometry, UV-Vis spectrometry) in order to evaluate the chemical composition responsible for the properties.

For the Fourier transform infrared spectroscopy (FTIR) studies, it was used a Varian 3100 Excalibur spectrometer equipped with a Harrick Praying Mantis diffuse reflectance (DRIFT) accessory. The IR spectrum was collected in the region 4000 - 500 cm^{-1} at a resolution of 2 cm^{-1}. For the UV-Vis evaluation, it was used an UV-Vis spectrometer Unicam Helios α Thermo Orion from 200 to 900 nm, at the resolution of 1 nm, with 1 nm slit width and automatic scan rate. The obtained results were processed using specific data analysis software (Origin Pro 8.0). The Extraction Factor (EF) was determined, considering the absorption values ($A_{\lambda max}$), multiplied with the dilution factor (DF) [7].

Gas chromatography–mass spectrometry (GC-MS) analyses were performed with a Varian model 3800 gas chromatograph coupled with a Varian Saturn Ion Trap 2000 MS. The gas chromatograph was equipped with a Factor Four capillary column. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Output files were analyzed using Varian MS workstation version 6 and the NIST98 Mass Spectral Database. Before injection, the extract was first evaporated using a rotary evaporator and afterwards diluted using a non-polar solvent (hexane, 1 g / 10 mL of solvent).

The phytochemical quantification procedures were used for the determination of total terpenoids, total flavonoids and total phenolics content. The procedures were exhaustively presented in a previously published work [15].

Antioxidant, antifungal and germination inhibitory effect

The antioxidant activity was determined following the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical method. The experimental procedure was previously described [12]. The antioxidant activity (AA%) percentage was calculated using the formula:

$$AA\ (\%) = [(A_{control} - A_{sample}) / (A_{control}] \times 100 \quad (1)$$

where: $A_{control}$ is the absorbance of the DPPH solution without sample, A_{sample} is the absorbance of the extract mixed with 0.02 mg/mL DPPH solution.

The half maximal effective concentration (EC_{50}), representing the concentration required to obtain a 50% antioxidant effect, was calculated using a specific data analysis software (Origin Pro 8.0) [9].

The antifungal susceptibility was evaluated using the disc diffusion or Kirby-Bauer method [3, 4, 18]**.** The antifungal activity was tested against two relevant fungal strains *Aspergillus niger* ATCC 15475 and *Penicillium hirsutum* ATCC 52323. The stock culture was maintained at 4°C.

The strains were cultivated on potato-dextrose agar from Sigma-Aldrich with the following composition: potato extract (PDA), 4 g/L., dextrose, 20 g/L, agar, 15 g/L. Sterile PDA plates were prepared by pouring the sterilized media in sterile Petri dishes under aseptic conditions. The test organism (1 mL) was spread on agar plates. Using a sterile Durham tube of 6 mm diameter, the wells were made according to the number of samples. The wells were inoculated with 50 µL of hydroalcoholic extract. Similarly, each plate carried a blank well by adding solvent (ethanol:H_2O = 1:1) alone to serve as a negative control. As positive control was used Miconazole nitrate (Sigma-Aldrich) at a concentration of 5.2 10^{-2} mM. All the plates containing fungal strains were incubated at 37°C for 144 h.

Antifungal activity of the hydroalcoholic extract was determined by measuring the sizes of inhibition zone (IZ, mm) as clear, distinct zones of inhibition surrounding agar wells, and values < 6 mm were considered as not active against microorganisms. The percent inhibition of the target fungi was calculated according to the following formula:

$$I\ (\%) = [(IZ - NC)/IZ] \times 100 \qquad (2)$$

where: IZ - inhibition zone diameter, NC - negative control.

All the experiments were performed in triplicate. The results were reported as the mean of three experiments and were presented as mean ± standard deviation (SD). Standard deviation was calculated as the square root of variance using STDEV function in Excel 2010.

The *germination inhibitory effect* was determined on certified seeds of *Cucumis sativus* Cornichon Wisconsin. The extract was diluted in bidistilled water (1:100 mL) and compared with a blank sample (1 mL of solvent diluted in bidistilled water).

The seeds of cucumber were hydrated in water for 30 minutes and were then immersed in the extract, according to the experimental variants for 30 minutes. The seeds thus treated were germinated in Petri dishes on filter paper moistened. In order to maintain moisture in Petri dishes, there were sealed with Parafilm M® laboratory film and then placed in the growth chamber EKO POL KK (25°C at day and 15°C at night), at constant humidity and light (photoperiod 16 hours of light, 8 hours dark). After 3 days, the germination percent of the seeds was determined and the increase in length of the embryonary roots was measured. After 6 days from the start of the experiment, we measured again the embryonary roots which increased in length, as well as the embryonary stems. Also, observations were conducted on lateral roots formations [13]. All the assays were carried out in triplicate and the results were expressed as means ± standard errors.

Results and Discussion

The UV-Vis analysis performed on the extracts identified the wavelengths specific to phenolic acids at 220 - 280 nm, to flavonoids, quinones and furanocoumarins at 290 - 420 nm and chlorophylls at 600 - 670 nm [7, 19]. Table I presents the specific absorption values for the plant extract, as well the extraction efficiency (EF factor) calculated according to the formula described in Materials and Methods.

Table I

Specific absorption values for the extract and EF calculated values

Dilution factor	$A_{220-280\ nm}$	$EF_{220-280\ nm}$	$A_{290-420\ nm}$	$EF_{290-420\ nm}$	$A_{600-670\ nm}$	$EF_{600-670\ nm}$
DF10	-	-	-	-	A_{665} = 0.11849	1.1849
	-	-	-	-	A_{605} = 0.08251	0.8251
DF100	-	-	A_{416} = 0.06048	6.048	-	-
			A_{365} = 0.29438	29.438		
	-	-	A_{330} = 0.61683	61.683	-	-
	-	-	A_{290} = 0.79266	79.266	-	-
DF1000	A_{228} = 0.63659	636.59	-	-	-	-
	A_{278} = 0.21477	214.77	-	-	-	-

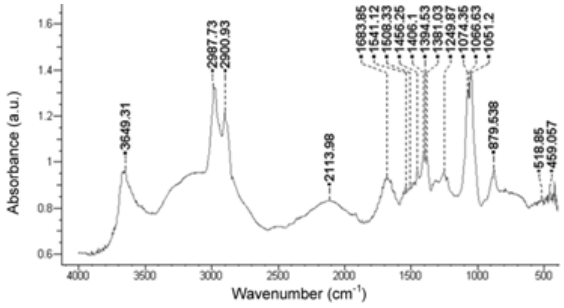

Figure 2.

FTIR Spectrum of the *H. sphondylium* extract

The peaks in FTIR spectrum (Figure 2) are attributed to the following type of compounds: hydroxy compounds (OH stretching) – 3649 cm^{-1}, carboxylic acid (2987 cm^{-1}, 2900 cm^{-1}), alkynes (2114 cm^{-1}), aldehydes and ketones (1684 cm^{-1}), amides (1540 cm^{-1}), aromatic compounds (1508 cm^{-1}, 880 cm^{-1}), aromatic alcohols (1381 cm^{-1}, 1249 cm^{-1}), alcohols (1406 cm^{-1}, 1394 cm^{-1}, 1074 cm^{-1}, 1066 cm^{-1} and 1051 cm^{-1}), alkanes (1456 cm^{-1}) and minerals (519 cm^{-1} and 460 cm^{-1}). The peaks at 1067 cm^{-1} and 1049 cm^{-1} are attributed to functional

groups of poly-flavonoids and, respectively, -C-O- groups of polyols as flavones and terpenoids [8, 22]. The *H. sphondylium* extract was further characterized by GC-MS. The identified components (based on comparison of the GC-MS spectra with the internal NIST library) are summarized in Table II.

Table II

Compounds identified by GC-MS and their corresponding PubChem CID (compound accession identifier)

Retention time (min.)	Compound	Class	CID
1.933	Amyl butyrate	Ester	10890
2.589	5-O-Methyl-d-gluconic acid dimethylamide	Aromatic acid	542382
6,140	2,5,5,8a-tetramethyl-6,7,8,8a-tetrahydro-5H-chromen-3-one	Phenolic compound	596376
9.473	Nonanol	Fatty alcohol	8914
14.451	α-Terpineol	Monoterpene alcohol	17100
15.105	Tetradecanol	Saturated fatty alcohol	8209
19.505	2-Pinen-4-one	Terpene	29025
19.538	3,5-Heptadienal, 2-ethylidene-6-methyl-	Monoterpene aldehyde	572127
20.807	β-Pinene	Monoterpene	14896
20.823	β-Ocimene	Monoterpene	5281553
22.693	2 Methyl-1-Undecanol	Fatty alcohol	66341
24.275	Bergamotenol	Sesquiterpenol	5368743
25.181	trans-β-Farnesene	Sesquiterpene	5281517
26.158	Germacrene D	Sesquiterpene	5373727
30.160	Beta caryophyllene oxide	Sesquiterpene	1742211
39.712	2,10-Dimethyl-9-undecenol	Alcohol	549711
40.302	Methyl hexadecadienoate	Ester	185713
40.887	6,7-Dihydrogeraniol	Acyclic monoterpenoid	5365836
41.811	1-Methyldodecilbenzene	Alkyl benzene	20636
43.967	Methyl pentadecanoate	Ester	23518
44.738	Methyl 2-methylhexadecanoate	Ester	94236
44.849	Hexadecanoic acid, ethyl ester	Ester	12366
45.851	Methoxsalen	Furanocoumarin	4114
45.898	Isobergapten	Furanocoumarin	68082
46.607	Bergaptene	Furanocoumarin	2355
46.655	Sphondin	Furanocoumarin	108104
48.083	gamma-Palmitolactone	Terpene hydrocarbon	97747
48.735	2-Methyl-Z,Z-3,13-octadecadienol	Terpenoid	5364412
48.849	Ethyl iso allocholate	Steroid	536919
49.174	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	Alcohol	5364768
49.823	Linoleic acid ethyl ester	Ester	5282184
50.027	6,9,12,15-Docosatetraenoic acid methyl ester	Ester	5362672
51.040	2-Methyl-1-hexadecanol	Alcohol	17218
51.756	Pimpinellin	Furanocoumarin	4825
51.818	Xanthinin	Sesquiterpene lactone	160533
53.516	2-Methylhexadecan-1-ol	Fatty alcohol	17218
54.344	Xanthotoxol	Furanocoumarin	65090
62.133	7,8-Epoxylanostan-11-ol, 3-acetoxy	Alcoholic compound	541562

Phytochemical evaluations were performed by spectrophotometric methods presented in the Experimental part. Standard curves for linalool [$y = 0.0016x + 00168$, $R^2 = 0.993$], gallic acid [$y = 0.01122x + 0.00804$, $R^2 = 0.9979$] and rutin [$y = 0.0067x - 0.0401$, $R^2 = 0.996$] were used to quantify the contents of total terpenoids (71.6 ± 3.52 mg LE/g dried weight), total phenolics (45.7 ± 2.14 mg GAE/100 g dried weight) and total flavonoids (21.909 ± 1.02 mg RE/g dried weight), respectively.

The antioxidant potential of the extract (determined by the DPPH method) ranged between 69.29 - 72.19%. The calculated EC50 (2.11 mg/mL) revealed a good antioxidant activity of the tested extract.

The antifungal activity investigation of the *H. sphondylium* extract was performed using the Kirby-Bauer diffusion method as antifungal susceptibility testing method. The diameters of inhibition zones against test strains are shown in Figure 3 compared with the negative and respectively, positive control. *H. sphondylium* extract strongly affected the growth of all target fungi. The strong antifungal activity of *H. sphondylium* alcoholic extract (the percent inhibition, $I \approx 85\%$, acc. eq. 2) is probably correlated with the content of furanocoumarins (Table II) which were also mentioned in previous studies [1].

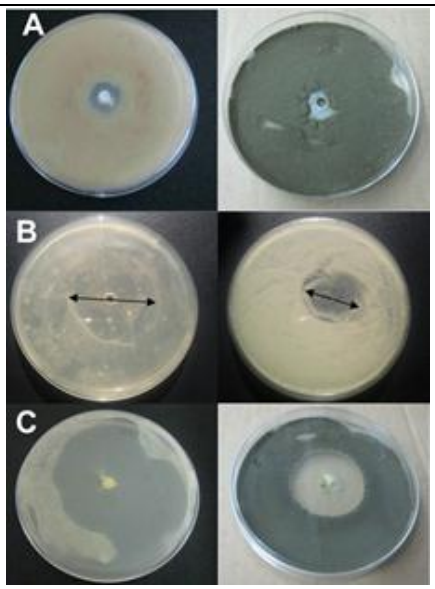

Figure 3.
Antifungal activity of negative control (A), *H. sphondylium* extract (B) and positive control (C) against *Penicillium hirsutum* (left) and *Aspergillus niger* (right)

As the plants of the *Apiaceae* family contain substances that inhibit the germination process (organic aromatic acids, terpenoids, phenolic compounds, coumarins, furocoumarins etc.) [16], the germination inhibitory effect was studied for the hydroalcoholic extract of *H. sphondylium* [5]. The results presented represent the mean of 10 replicates. The *H. sphondylium* extract strongly influences the increase in the length of embryonary roots, both after 3 and after 6 days from the start of the experiment (Figures 4 and 5).

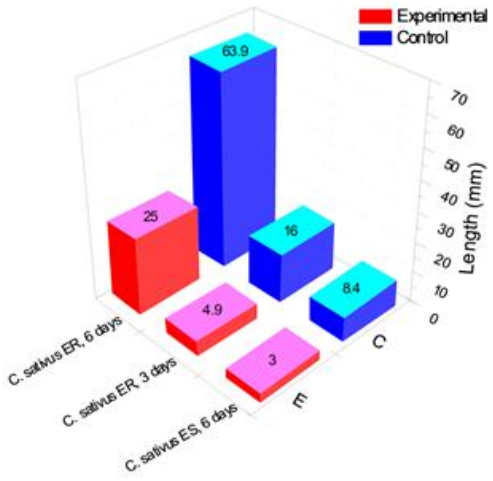

Figure 4.
The increase in length of the *C. sativus* embryonary stems (ES) after 6 days and embryonary roots (ER) after 3 and 6 days; R – reference, E - experimental

Figure 5.
Cucumis sativus plantlets after 3 days (left) and 6 days (right); up – reference, down - experimental

Thus, compared to the control (C), the experiment using diluted hydroalcoholic extract (E) presents a decrease of 69.5% of the average root length increase. A reduction in the average increase in length of embryonary roots of 60.86% compared to the control experiment was also recorded at 6 days after initiation of the experiment. Regarding lateral roots formation, 3 days after initiation of the experiment, in the control experiment, 60% of plantlets had lateral roots, while the hydroalcoholic treated plantlets did not show any lateral roots. The *H. sphondylium* extract also influenced the increase in the length of the embryonary stem of the cucumber plantlets. Thus, compared to the reference, it was observed a decrease of 64.29% of the average increase in length of the embryonary stems.

Conclusions

In the present work we report the analytical characterization of the Romanian native *Heracleum sphondylium* L. subsp. *sphondylium* extract, in terms of chemical composition (which revealed 38 main components). The hydroalcoholic extract revealed a good antioxidant potential, as well as a very important *in vitro* antifungal activity on the studied fungal lines. It can therefore be used as a natural antifungal agent for the treatment of fruits and vegetables in the postharvest period. The extract also influences the growth in length of embryonary roots; lateral roots formation and the embryonary stem growth for *Cucumis sativus*. These findings could have applications in the use of the proposed extract as an environmentally-friendly and safe bio-herbicide. Also, this would be a very important factor for the commercial cultivation of the plant.

Acknowledgements

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