Phytochemical Profile and Total Antioxidant Capacity of *Sempervivum ruthenicum* Koch Hydroethanolic Extract

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The scope of this work is a phytochemical analysis and antioxidant activity assay of a Sempervivum ruthenicum Koch hydroethanolic extract. The hydroethanolic extract was prepared from the dried leaves of the plant by maceration in a water and methanol mixture (50:50 v/v). The total phenolic content of the extract was calculated to be 3.0501±0.0272 mg/mL and the total flavonoid content was determined in a concentration of 3.113±0.0394 mg/mL. The HPLC-DAD analysis revealed phenolic acids and flavonoids, which were quantified. The most prevalent phenolic acids in the extracts were gallic and ellagic acids, with concentrations of 1.2443±0.0475 mg/mL, respectively 0.6339±0.0026 mg/mL. The heteroside astragalin was present in high concentration of 1.1934±0.0754 mg/mL. The DPPH free radical scavenging assay revealed the EC50 value of the extract to be 2.5788±0.003 mg/mL. These results suggest a good scavenging ability of the extract, which is due to the abundance of polyphenolic compounds. The antioxidant activity of the extract demonstrates a high scavenging ability at low doses. Sempervivum ruthenicum Koch shows a promising phytochemical profile that suggests it's use in pathological conditions that involve high oxidative stress.

Keywords: Sempervivum ruthenicum, HPLC-DAD, polyphenols, DPPH, antioxidant

During the last 20 years, phytotherapy has been gaining a special interest due to the vast abundance of natural compounds with pharmacological properties, that can be used either as substitutes for synthetic drugs or as complementary treatments in a wide variety of diseases. Natural compounds have the advantage of exhibiting fewer side effects than synthetic drugs and can also be used to obtain derivatives with enhanced pharmacological properties [1,2].

Sempevivum ruthenicum Koch (common Houseleek) is a variety of the genus *Sempervivum* (*Crassulaceae*) endemic to the Dobrogea region of Romania. The species is differentiated from other members of the genus by a characteristic red color at the base of its yellow petals [3]. The plant can only be found in dry, rocky terrains, having multiple adaptations in order to survive in an arid environment, such as succulent leaves and well developed roots.

Members of the *Sempervivum* family are well known in folk medicine, being used in a variety of disorders such as bacterial otitis, burn wounds and peptic ulcers [4-6]. Most sources mention the usage of either fresh leaf juice or decoctions, to obtain a pharmacological effect, however, no phytochemical analysis of this plant has been published apart from those made by Gomeyuk et al. [7,8] that highlights the presence of polyphenolic compounds and flavonoids [9].

The quantitative analysis and total antioxidant capacity of a *Sempervivum ruthenicum* Koch ethanolic extract was presented in a previous study [10], highlighting the presence of both polyphenols and flavonoids. The aim of this work is to characterize a hydroethanolic extract in order to determine its polyphenol and flavonoid content, and to evaluate its antioxidant properties, noting the differences between the different solvents used in order to obtain the extracts.

Experimental part

Materials

Plant material was harvested form the *Cheile Dobrogei* Park during flowering (20th August - 10th September). The plants were identified at the Pharmaceutical Botanic Department of the Faculty of Pharmacy, where a voucher specimen was preserved. All harvested plants had a mean diameter of 7.5 cm ± 2 cm. The leaves were removed from the stem and were air-dried at a constant temperature of 25 ± 2°C and relative humidity of 50% ±10% for 6 months. After obtaining the dry plant material, the water content was calculated to be 87.75%, according to the Romanian Pharmacopoeia 10th Edition [11].

All reagents and standards used were purchased from Sigma-Aldrich (Sigma-Aldrich GMBH, Munich, Germany). All reagents used were of analytical quality.

Preparation of the hydroethanolic extract

Ten grams of dry plant material were ground into a fine powder and passed through a no. 7 sieve and were macerated in a water-ethanol mixture (50-50 v/v) in order to obtain a concentration of 100 mg/mL [12]. Maceration took place for 14 days, with daily stirring for 1 minute every 8 hours. The macerate was filtered through a Whatman paper (42 porosity) three times, until a brown, clear extract was obtained. The extract was conserved in a borosilicate glass container at 2-8°C.

Total phenolic content

The method employed by Tekeli [13] with slight alterations was used to determine the total phenolic content of the hydroethanolic extract. Briefly, the standard curve of pyrogallol was draw by using 6 dilutions in absolute methyl alcohol (0.05, 0.1, 0.5, 1.0, 2.6 and 6 mg/mL). 100 µL of each dilution were vortexed with 500 µL double distilled water and 100 µL Folin-Ciocalteu Reagent, allowing the mixture to stand for 6 min. Afterwards, 1 mL

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sodium carbonate 7% and 500 µL double distilled water were added to the reaction mixture, which was left at room temperature for 90 min. The absorbance of the samples was read at 760 nm using a UV-Vis UV-6300PC (VWR) spectrophotometer. The plant extract was assayed according to the same procedure. The total phenolic content was calculated as pyrogallol equivalents (mgPIR/mL). The assay was done in triplicate, and all results are expressed as mean ± SD.

Total flavonoid content

Total flavonoid content was determined according to Chantiratikul et al. [14]. Quercetin was used as reference, and the total flavonoid content was determined as quercetin equivalents. A standard quercetin solution of 1% was prepared in absolute methanol, from which 6 dilutions were made: 0.05, 0.1, 0.5, 1.0, 2.5 and 5 mg/mL. 100 µL of each dilution were vortexed with 500µL double distilled water and 100 µL sodium nitrate 5%, allowing the mixture to react for 6 min. Afterwards, 150 µL aluminum chloride solution (10%) were added, allowing a reaction time of 5 minute and finally 200 µL sodium hydroxide solution (1M) were added. The absorbance was read at 510 nm using a UV-Vis UV-6300PC (VWR) spectrophotometer. The plant extract was subjected to the same assay conditions. All experiments were done in triplicate, and the results are expressed as mean ± SD.

HPLC analysis

To identify and quantify the bioactive compounds in the plant extract, a standardised HPLC method for determining phenolic compounds was used, described in the USP 30-NF25 Pharmacopoeia [15].

The equipment used included a Agilent 1200 chromatogram with quaternary pump, DAD, thermostat, degas system and autosampler. The chromatographic column employed was a C18 Zorbax XDB 250 mm X 4.6 mm; 5µm. The eluents consisted of phosphoric acid (A) 0.1% and acetonitrile (B), and employed a linear gradient as follows: 10% B for 13 min, 22% B for 1 min, 40% B for 3 min and 10% B for 1 min. The column temperature was 35 °C and the flow rate was 1.5 mL/min. The injection volume was 20 µL and the total elution time was 20 min. Detection was carried out using the DAD system at 310 nm, 335 nm and 360 nm, simultaneously. Standards used included: E-resveratrol, Z-resveratrol, caffeic acid, chlorogenic acid, cinnamic acid, ellagic acid, vanillin, gallic acid, ferulic acid, astragalin, isorhamnetin, kaempferol, scutellarin, rutoside and quercetin. The hydroethanolic extract vas injected 4 times (each injection noted by *I*). The results were expressed as mean ± SD.

Determination of Antioxidant Activity Using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Method

The method used to determine the total antioxidant capacity of the *Sempervivum ruthenicum* Koch was described in a previous study [10]. Briefly, a 4% 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution was prepared in absolute methanol and stored in the absence of light. Seven dilutions of plant extract were prepared, with concentrations ranging from 1 mg/mL to 100 mg/mL. 100 µL test sample were mixed with 3.9 mL DPPH stock solution and the mixture was left to react in the absence of light for 30 min. The absorbance was read at 517 nm using a UV-Vis UV-6300PC (VWR) spectrophotometer. A blank sample using a hydroethanolic mixture (50/50 v/v) was used as negative control. The total antioxidant capacity of the plant extract was calculated using the following equation (1):

$$\%SA \quad \frac{A_{control} \quad A_{sample}}{A_{control}} \times 100 \qquad (1)$$

Where A is the absorbance measured at 517 nm. The efficient concentration needed to inhibit 50% of the DPPH free radical (EC50) was calculated by linear interpolation [16]. All experiments were done in triplicate, and the results are expressed as mean ± SD.

Statistical analysis

Results were expressed as mean ± standard deviation. Statistical analysis was carried out using BM SPSS Statistics 17 (IBM IBM, Armonk, New York, NY, USA) by applying ANOVA. Significant differences among samples were evaluated by Tukey's test at a significance level of 0.05.

Results and discussions

Total phenolic and flavonoid content

The total phenolic content of the analysed plant extract showed a value of 3.0501±0.0272 mg/mL and the total flavonoid content was determined at a value of 3.113±0.0394 mg/mL. In a previous study conducted on the same plant species, we determined the total phenolic content of a pure ethanolic extract of *Sempervivum ruthenicum* Koch dried leaves [10] with a value of 1.0344±0.0237 mg/mL. The solvent mixture used for extraction suggests a more effective recovery of phenolic compounds in comparison with absolute ethanol.

HPLC analysis

The HPCL analysis of the hydroethanolic plant extract revealed the presence of a number of polyphenols and flavonoids. The polyphenolic acids identified included gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, cinnamic acid and ellagic acid. The compounds were also quantified, with the results presented in table 1.

A total concentration of 1.9892±0.0608 mg/mL polyphenolic acids was calculated from the results, accounting for 65.21% of the total phenolic compounds determined previously. The remainder of phenolic compounds have yet to be identified and quantified. Previous studies show the presence of phenolic acids both in *Sempervivum ruthenicum* Koch [10], as well as in other species of the genus [4-9]. These findings suggest that S*empervivum* species represent a good source of natural

Table 1

POLYPHENOLIC ACIDS QUANTIFIED BY HPLC-DAD FROM A *SEMPERVIVUM RUTHENICUM* KOCH HYDROETHANOLIC EXTRACT

	Polyphenolic acids (mg/mL)						
	Gallic acid	Chlorogenic acid	Caffeic acid	Ferulic acid	Cinnamic acid	p-coumaric acid	Ellagic acid
Mean value	1.2443	0.0073	0.0183	0.0450	0.0142	0.0258	0.6339
Standard deviation	0.0475	0.0007	0.0017	0.0019	0.0046	0.0015	0.0026

Table 2
FLAVONOIDS QUANTIFIED BY HPLC-DAD FROM A *SEMPERVIVUM RUTHENICUM* KOCH HYDROETHANOLIC EXTRACT

	Flavanols (mg/mL)			Heterosides (mg/mL)		Flavones (mg/mL)
	Kaempferol	Quercetin	Isorhamnetin	Astragalin	Rutoside	Scutellarein
Mean	0.2462	0.0499	0.0171	1.1934	0.1296	0.6264
Standard deviation	0.0006	0.0038	0.0031	0.0754	0.0118	0.1034

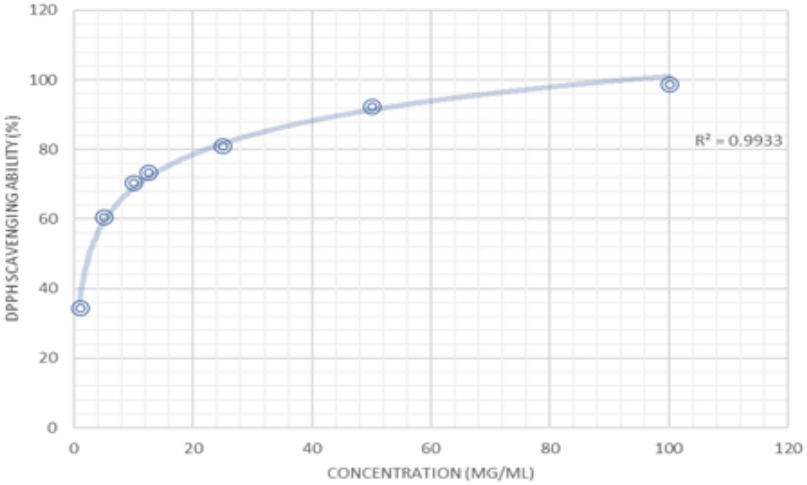

Fig. 1. Antioxidant activity of the *Sempervivum ruthenicum* Koch hydroethanolic extract

polyphenols. The main phenolic acid identified was gallic acid, with a concentration of 1.2443±0.0475 mg/mL, which was also found in other similar studies [17].

The flavonoids identified and quantified by HPLC-DAD analysis include isorhamnetin, astragalin, kaempferol, quercetin, scutellarin, isorhamnetin and rutoside. The flavonoids were divided into three groups according to their chemical structure: flavanols (kaempferol, quercetin and isorhamnetin), heterosides (astragalin and rutoside) and flavones (scutellarein). The quantification of the identified flavonoids can be seen in table 2.

The most prevalent flavonoid identified in the hydroethanolic extract was astragalin with a concentration of 1.1934±0.0754 mg/mL. The presence of all identified flavonoids was confirmed in other *Sempervivum* species [17], however the exact concentration of these compounds was not previously determined.

The biological activities of the compounds identified in *Sempervivum ruthenicum* Koch include anti-inflammatory [5,18,19], antioxidant [20,21], antimicrobial [5,22] and immunomodulatory effects [6,23]. Both traditional medicine and current research suggest the potential use of *Sempervivum* species in pathological states that involve high oxidative stress and infections.

DPPH Radical Scavenging Method

The DPPH method is being used for nearly 50 years to evaluate the ability of a compound to act as a free radical scavenger or hydrogen donor, and also to evaluate its antioxidant capacity. The EC50 (efficient concentration value) is used to interpret the results of this method and is defined as the concentration of compound that causes 50% loss of the DPPH activity, which changes colour. The EC50 value of the hydroethanolic plant extract was 2.5788±0.003 mg/mL. In comparison, our previous study [10] on the ethanolic extract of *Sempervivum ruthenicum* Koch revealed a EC50 value of 4.6112±0.08 mg/mL which was significantly higher. These values correlate with the total polyphenol content and total flavonoid content, suggesting that the mixture of solvents is more effective in recovering the antioxidant compounds from the dried plant material. Similar studies were carried out on different *Sempervivum species*, showing a good antioxidant potential [17]. Karabegovic et al. [24] have determined a EC50 value of 0.0246±0.45 mg/mL for a ultrasound assisted methanolic extract of *S. tectorum*, which highlights the high concentration of antioxidant compounds in the species, that can be recovered by more advanced extraction techniques. The authors showed that the extraction technique plays a key role in the recovery of antioxidant compounds, which is confirmed by the comparison between the antioxidant activity of the ethanolic and hydroethanolic extracts of *Sempervivum ruthenicum* Koch [10]. Figure 1 displays the DPPH radical scavenging activity of the *Sempervivum ruthenicum* Koch hydroethanolic extract.

Conclusions

The phytochemical characterization of *Sempervivum ruthenicum* Koch hydroethanolic extract shows the presence of polyphenolic acids and flavonoids with high concentrations of both types of compounds. The antioxidant activity of the extract demonstrates a high scavenging ability at low doses. *Sempervivum ruthenicum* Koch shows a promising phytochemical profile that suggests its use in pathological conditions that involve high oxidative stress.

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