# PHENOLIC PROFILE AND ANTIOXIDANT ACTIVITY OF A *SEMPERVIVUM RUTHENICUM* KOCH ETHANOLIC EXTRACT

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## ABSTRACT

The current goal of this study is a phytochemical analysis of a *Sempervivum ruthenicum* Koch ethanolic extract, in order to find potential medical applications. The ethanolic extract was prepared from the dried leaves of the plant by maceration in absolute ethanol. Total phenolic content was assessed with a slightly modified Folin-Ciocalteu method. A HPLC analysis was conducted and the total antioxidant activity of the extract was assessed with a DPPH method. The total phenolic content of the extract was calculated at 1.0344 ± 0.0237 mg/mL. After conducting the HPLC analysis on the ethanolic extract, phenolic acids, flavonoids and glycosides were identified and quantified. The most prevalent phenolic acids in the extracts were gallic and ellagic acids, with concentrations of 0.5137 ± 0.0110 mg/mL, respectively 0.3139 ±0.0046 mg/mL. The glycoside astragalin is also present in relatively high concentrations, of 0.0929 ± 0.0049 mg/mL. The EC value for the ethanolic extract was 4.6112 ± 0.08 mg/mL. These results suggest a good scavenging ability of the extract, which is due to the abundance of phenolic compounds found within. The ethanolic extract of *Sempervivum ruthenicum* Koch harvested from the Dobrogea Region of Romania has a complex phenolic profile with high levels of gallic and ellagic acids, and a strong anti-oxidant activity which can be exploited both in traditional medicine and in phytotherapeutic preparations.

***Keywords: anti-oxidant, chromatography, DPPH, phenolics, Sempervivum***

## INTRODUCTION

Since the beginning of medicine, natural remedies were used for therapeutic purposes for a large spectrum of ailments. These remedies were used empirically, with uncertainty regarding the exact composition of the plant material used or the extracts obtained by processing. However, with the progress of analytical techniques and technologies, laboratories all over the world began screening known medicinal plants for bioactive molecules that can be used for treating diseases [1]. Antibiotics and antioxidants are currently being used in combating pathological agents such as bacteria and fungi. Such compounds have been

derived from natural sources and are the only line of defense against infections [2], [3]. Screening for natural bioactive molecules derived from plants is one of the leading trends in pharmaceutical research, due to the diversity and complexity of natural products.

*Sempervivum ruthenicum* Koch e.g. 'houseleek' is a member of the Crassulaceae family, is a rare plant, growing on harsh rocky terrain. It has a radial disposition, having a short stem, numerous leaves placed in helicoidal position around the stem. Flowering occurs after a average of 3-4 years. The main stem extends upwards forming a leafy thin stem which holds the flowers. The rosettes produce offsets on stolons that can vary in length and are firmly attached to the parent plant [4]. Due to its harsh living environment it's metabolism is adapted for survival and it can be theorized that the bioactive molecules it produces may be of use in pharmaceutical research. However, the literature on this plant is limited to mostly traditional remedies and old phytochemical studies [5], [6], [7], [8].

The current goal of this study is a phytochemical analysis of a *Sempervivum ruthenicum* Koch ethanolic extract, to find potential medical applications.

## MATERIALS AND METHODS

### Biological material

The plant species selected for this study was *Sempervivum ruthenicum* Koch, a rare plant found in the rocky terrain of south-eastern region of Romania. The plant material was harvested exclusively from the region called 'Cheile Dobrogei' during its flowering period (30th of June – 20th of July). The plant was positively identified at the Faculty of Pharmacy of Constanta, The Botanical Department, where a voucher specimen of the plant is stored. The harvested plant material was washed with double distilled water, dried and weight. The plant was dried for 6 months at a constant temperature of 20 ºC ± 2 ºC and a constant relative humidity of 50% (±10%). After drying the plant material was weight and the total moisture content was calculated [9]. The leaves were separated from the remaining plant material (roots, stems, flowers) and were ground to a fine powder using a mortar and pestle. The powder was then passed through a no. 7 sieve and was stored at room temperature for further use.

### Chemicals and reagents

All chemicals, reagents and standards used for this study were purchased from Sigma-Aldrich (Munich, Germany), Chromadex (Wesel, Germany), and Extrasynthese (Lyon, France). All chemicals, reagents and standards were of analytical quality.

### Preparation of the extract

The ethanolic extract was prepared from the dried leaves of the plant by maceration in absolute ethanol, as proposed by Sukhdev et al. [10]. Ten grams (10 g) of powdered dry leaves were macerated in 100 g absolute ethyl alcohol for 14

days, shaking the maceration vial manually at 8-hour intervals for 1 minute. The crude extract was then filtered through Whatman no. 42 papers until the final product was a clear, dark green liquid. The final extract was then placed in a sterilized glass vial and stored at -8° C for later use.

**Total phenolic content**

To determine the total phenolic content of the extract, the method employed by Shirazi et al. [11] was used, with minor modifications consisting of using a pyrogallol standard. The standard curve of the pyrogallol solution was drawn by preparing 6 dilutions of this compound in methyl alcohol (0.05, 0.1, 0.5, 1.0, 2.5, and 5 mg/mL). 100 µL of these solutions were mixed with 500 µL of double distilled water and 100 µL Folin-Ciocalteu reagent (Sigma-Aldrich GMBH, Munich, Germany), allowing the mixture to react for 6 minutes. After the reaction time expired, 1 mL sodium carbonate solution 7% and 500 µL double distilled water were added to the reaction mixture. The absorbance of the samples was recorded after 90 minutes, at 760 nm, using a UV-Vis UV-6300PC (VWR) spectrophotometer. The same procedure was followed for the plant extract that had a concentration of 10% (m/m). The total phenolic content was calculated as pyrogallol equivalents (mgPIR/mL). All experiments were run in triplicate, and the results are expressed as mean ± SD.

**Analysis by HPLC**

To identify and quantify the bioactive compounds in the plant extract, a standardized HPLC method for determining phenolic compounds was used, described in the USP 30-NF25 Pharmacopoeia [9].

The equipment used included am Agilient 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; 5 µm. The eluents consisted of phosphoric acid (A) 0.1% and acetonitrile (B), and employed a linear gradient as follows: 10% B for 13 minutes, 22% B for 1 minute, 40% B for 3 minutes and 10% B for 1 minute. 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 minutes. 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, rutin and quercetin. The ethanolic extract vas injected 4 times (each injection noted by 'I'). The results were expressed as mean ± SD.

**Anti-oxidant activity**

The ability of scavenging the free radical 2,2-diphenyl-1- picrylhydrazyl (DPPH) was assayed according to the method proposed by Ravichandran et al. [12] with minor modifications. The DPPH 4% stock solution was prepared using absolute methanol and was stored in the dark. Seven dilutions of the plant extract were prepared in concentrations of 100, 50, 25, 12.5, 10, 5, and respectively 1 mg/mL.100 µL of the prepared solutions were vortexed for 30s with 3.9 mL of

DPPH stock solution. The mixture was left to stand in the dark for 30 minutes, and the absorbance was measured at 517 nm using a UV-Vis UV-6300PC (VWR) spectrophotometer. A blank sample with 100 μL absolute ethanol was used as negative control. The scavenging ability of the extract dilutions were calculated using the following equation:

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

where A is the absorbance measured at 517 nm. The EC50 (efficient concentration) is the concentration necessary to inhibit 50% of the DPPH free radical and was calculated by linear interpolation from the obtained results [13], [14]. All experiments were run in triplicate and the results are expressed as mean ± SD.

**Statistical analysis**

The data has been subjected to analysis of variance (ANOVA) using IBM SPSS Statistics 17 (IBM IBM, Armonk, New York, NY, USA)

## RESULTS AND DISCUSSION

**Total phenolic content and analysis by HPLC**

The total phenolic content of the extract was calculated at 1.0344 ± 0.0237 mg/mL.

After conducting the HPLC analysis on the ethanolic extract, phenolic acids, flavonoids and glycosides were identified and quantified. The phenolic acids quantified included gallic acid, chlorogenic acid, caffeic acid, ferulic acid, ellagic acid and cinnamic acid. All these compounds were identified according to their retention times and quantified using standard curves.

Other bioactive compounds were also identified, such as kaempferol, astragalin, quercetin and isorhamnetin, however, only the former and second compounds could be quantified. The results of the HPLC analysis can be observed in Table 1.

*Table 1. HPLC quantification of bioactive compounds*

| Injection no. | Polyphenolic acids (mg/mL) | | | | | | Flavonols (mg/mL) | Glycosides (mg/mL) |
|---|---|---|---|---|---|---|---|---|
| | Gallic Acid | Chlorogenic Acid | Caffeic Acid | Ferulic Acid | Cinnamic Acid | Ellagic Acid | Kaempferol | Astragalin |
| I1 | 0.5009 | 0.0028 | 0.0071 | 0.0048 | 0.0037 | 0.31176 | 0.0177 | 0.0929 |
| I2 | 0.5251 | 0.0026 | 0.0058 | 0.0045 | 0.0038 | 0.31934 | 0.0240 | 0.0883 |
| I3 | 0.5083 | 0.0030 | 0.0064 | 0.0048 | 0.0025 | 0.30882 | 0.0160 | 0.0896 |
| I4 | 0.5204 | 0.0032 | 0.0050 | 0.0046 | 0.0030 | 0.31594 | 0.0142 | 0.1010 |
| Average | 0.5137 | 0.0029 | 0.0061 | 0.0047 | 0.0033 | 0.31396 | 0.0180 | 0.0929 |
| SD | 0.0110 | 0.0002 | 0.0008 | 0.0001 | 0.0006 | 0.00462 | 0.0042 | 0.0049 |

The compounds quantified by the HPLC analysis account for 92.37% of the total phenolic content of the extract. Given this result, it can be assumed that de difference can be attributed to phenolic bioactive compounds with different molecular structures that have not yet been identified.

The most prevalent phenolic acids in the extracts were gallic and ellagic acids, with concentrations of 0.5137 ± 0.0110 mg/mL, respectively 0.3139 ±0.0046 mg/mL. The glycoside astragalin is also present in relatively high concentrations, of 0.0929 ± 0.0049 mg/mL. Gallic acid accounts for 49.66% of the total phenolic content of the extract, and ellagic acid for 30.34%, making both acids the main bioactive constituents of the ethanolic extract of *Sempervivum ruthenicum* Koch.

A similar study was conducted by Stojkovic et al. [15] on the plant species *Sempervivum tectorum* Koch, used in Southern Serbia traditional medicine. The authors used a crude leaf extract and identified several glycosides such as astragalin and rutin, with astragalin presenting a concentration of 195 ± 5 μg/g. Also, the authors identified several free organic acids such as maleic acid, ascorbic acid, citric acid, fumaric acid, succinic acid and oxalic acid, all of which were not identified in the present study. In contrast, the concentration of astragalin in the current study corresponds to 929 ± 49 μg/g, which is significantly higher.

**Anti-oxidant activity**

The total scavenging ability of the dilutions was calculated, and the results can be viewed in Table 2. The EC value for the ethanolic extract was 4.6112 ± 0.08 mg/mL. These results suggest a good scavenging ability of the extract, which is due to the abundance of phenolic compounds found within.

*Table 2. Antioxidant activity after the DPPH assay*

| Concentration of extract (mg/mL) | Total DPPH scavenging ability (%) | EC50 (mg/mL) |
|---|---|---|
| 100 | 95.6531±0.1 | 4.6112±0.08 |
| 50 | 90.4098±0.42 | |
| 25 | 78.8070±0.21 | |
| 12.5 | 68.5775±0.2 | |
| 10 | 62.9418±0.35 | |
| 5 | 50.7876±0.62 | |
| 1 | 23.5733±0.86 | |

## CONCLUSION

*Sempervivum ruthenicum* Koch is considered a rare plant species in Romania, and it possesses a unique biochemical composition which cand be exploited. The ethanolic extract of *Sempervivum ruthenicum* Koch harvested from the Dobrogea Region of Romania has a complex phenolic profile with high levels of gallic and ellagic acids, and a strong anti-oxidant activity which can be exploited both in traditional medicine and in phytotherapeutic preparations.

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