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EVALUATION OF BIOACTIVE COMPOUNDS WITH ANTIOXIDANT ACTIVITY OF *HELICHRYSUM ARENARIUM* (L.) MOENCH. INFLORESCENCES EXTRACTS

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Abstract

The aim of this study was to evaluate the bioactive compounds with antioxidant activity in aqueous, 70% ethanolic and 80% methanolic extracts of Helichrysum arenarium inflorescences. Phytochemical screening was performed on the three extracts to detect the presence of secondary metabolites such as alkaloids, flavonoids, phenolic compounds, glycosides, phytosterols and tannins. The total flavonoid content was determined by aluminium chloride colorimetric assay at 420 nm. Total phenol content was determined with Folin-Ciocalteu reagent at 765 nm. Antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method and the total antioxidant capacity (TAC) assay. The results showed that among the three samples studied, the 70% ethanol extracts had the highest polyphenol and flavonoid values. Also, the antioxidant activity of the 70% ethanol extract was higher than that of the aqueous and 80% methanol extracts. The high content of phenols and flavonoids indicated that these compounds contribute to the antioxidant activity of Helichrysum arenarium. Phytochemical examination revealed the presence of alkaloids, flavonoids, phenols, glycosides, phytosterols and tannins in all extracts.

Key words*: Helichrysum arenarium, phenols, flavonoid, phytochemical screening, antioxidant activity.*

INTRODUCTION

Helichrysum arenarium (L.) Moench. (also known as "Sandy everlasting", "Dwarf everlasting", "Immortelle", and "Siminoc" in popular Romanian folklore) belongs to the *Compositae* family and is an herbaceous perennial plant naturally distributed in Central, Eastern and South-Eastern Europe, North of Balkans, West Siberia, Central Asia, Mongolia, and China (Pljevljakušić et al., 2018). In Romania it grows in the spontaneous flora of the countryside, in sandy or calcareous places.

The plant is rich in bitter substances, tannins, flavonoids, saponins, apigenin, astragalin, colorants, glycosides, volatile oil, mineral salts. The pharmacological profile of *Helichrysum arenarium* has recently been improved by new research (Dănăilă-Guidea et al., 2022).

It revealed additional effects by identifying primary phytochemical ingredients that stimulate bile secretion production and circulation. (Les et al., 2017). The antioxidant, antidiabetic, and neuroprotective properties of *Helichrysum* species were investigated in order to obtain novel therapeutic products. Various studies regarding the composition of *Helichrysum arenarium* have shown that the presence of phenolic and flavonoid compounds provides antibacterial and antioxidant properties against pathogens (Czinner et al., 2000).

The inflorescences of *H. arenarium*, being rich in many bioactive compounds, have been used in European herbal medicine for its various beneficial properties for human health (Grădinaru et al., 2014). The *H. arenarium* essential oil demonstrated antimicrobial activity (Moghadam et al., 2014) against various microorganisms, including pathogenic strains. Other authors (Tepe et al., 2005) reported the antioxidant activity of the methanol extracts of various *Helichrysum* species.

H. arenarium plant was extensively investigated in different parts of the world. However, the plant has been less investigated in Romania in terms of bioactive compounds with antioxidant activity of inflorescence aqueous and alcoholic extracts. Therefore, the

aim of this study was to evaluate the bioactive compounds with antioxidant activity of inflorescences *Helichrysum arenarium* various extracts.

MATERIALS AND METHODS

Plant material. Dried inflorescences of *Helichrysum arenarium* (producer: Hypericum Impex Srl, Cluj-Napoca Romania) were purchased from Bucharest city commercial herb centres (Figure 1).

Figure 1. *H. arenarium* inflorescences material sources (producer: Hypericum Impex Srl, Cluj-Napoca, from Romania)

Samples preparation

Microwave-assisted extraction (MAE) method. The plant material consisting of dried flowers was weighed (1 g) and mixed with three different solvents (30 ml): 70% ethyl alcohol, 80% methanol and distilled water. Each sample (1 g/30 ml solvent) was placed in a microwave oven at 700 W, temperature = 85-90°C, extraction time 4 min (Pan et al., 2003). After microwave-assisted extraction, samples were cooled and filtered. The extracts were then analyzed for total phenol content, flavonoids and antioxidant activity.

Determination of total phenolic content (TPC). The concentration of phenolic compounds in the extracts was determined by the Folin-Ciocalteau method (Singleton, 1999). Samples were reacted with Folin- Ciocalteau reagent. After the formation of blue colored compounds, a Na_2CO_3 solution was added for alkalinization and the samples were then incubated for 2 h in the dark. After incubation, samples are measured spectrophotometrically (Eppendorf UV-Vis) at 750 nm against a similarly prepared control, but in which the sample extract is replaced by distilled water.

For the determination of polyphenol concentration, a calibration curve was performed, starting from standard gallic acid solutions (50-500 mg/l). The concentration of phenols (mg GAE/l) in the samples was determined from the equation of the standard gallic acid curve (y = 0.0009x + 0.0032; R = 0.9955) obtained for different gallic acid concentrations (Figure 2).

The total phenolic content (TPC) in all samples was calculated using formula:

$$TPC = c \times V/M,$$

where:

TPC - Total phenol content (mg/g dry matter);
c = Concentration determined from the calibration curve (mg GAE/ml);
V = Extraction volume (ml);
M = Mass of plant material in the extract (g).

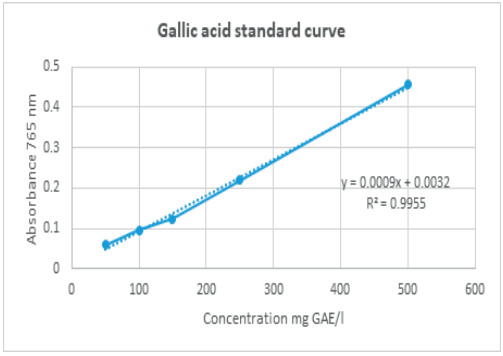

Figure 2. Gallic acid standard curve and the regression equation

Determination of total flavonoids content (TFC). The total content of flavonoids in the samples was determined by the colorimetric method with aluminum chloride (Chang et al., 2002). The reaction mixture consisted of: 1 ml sample/standard, 3 ml methanol, 200 µl $AlCl_3$, 200 µl 1 M potassium acetate and 5.6 ml distilled water.

The absorbance of this reaction mixture was recorded at 420 nm using a UV spectrophotometer (Eppendorf UV-VIS). The concentration of flavonoids (mg quercetin equivalent/ml) in the samples was determined based on the standard calibration curve (y = 0.009x + 0.0538; R^2 = 0.9913) obtained for different concentrations of quercetin (25, 50, 100, 150 and 200 µg/ml) (Figure 3).

The total flavonoid components in the extracts in quercetin equivalents (QE) were calculated by the following formula:

$$TFC = C \times V/M$$

where:

TFC = total flavonoid contents, milligram per gram of sample extract in QE;

C = the concentration of quercetin established from the calibration curve, mg/mL;

V = the volume of extract, milliliter;

M = the weight of sample extract (g).

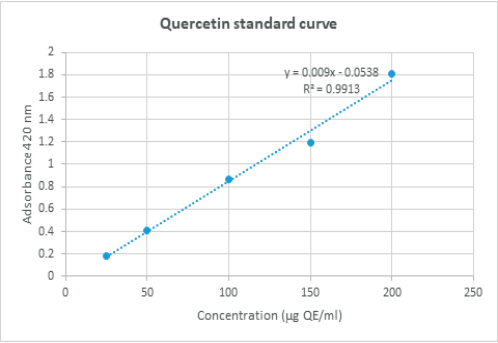

Figure 3. Quercetin standard curve and the regression equation

Phytochemical screening of extracts. Phytochemical screening was carried out on the three extracts to detect the presence of secondary metabolites such as alkaloids, flavonoids, phenolic compounds, phytosterols, glycosides and tannins as described in literatures (Kokate et al., 2006).

Between 0.5 and 1.0 ml of each extract was used for each phytochemical test. The results were expressed based on the intensity of the colour developed by the reaction and were noted with "+++" highly present, "++" moderately present, "+" low and "-" absent.

Antioxidant activity

To evaluate the antioxidant properties of the samples, two methods were applied to determine the antioxidant activity: the DPPH method and the phosphomolybdate method to determine the total antioxidant capacity.

DPPH (2,2-difenil-1-picrilhidrazil) method. The spectrophotometric method for assessing the total antioxidant capacity of the samples is based on the absorbance decrease of the DPPH radical in the presence of antioxidants.

The free radical scavenging activity of each sample was determined with 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Braca et al., 2001). A volume of 200 µl of each sample of different concentrations (10-100 µg/ml) with 2 ml of 0.004% methanol solution of DPPH (0.1mM). After 30 minutes of incubation in the dark at room temperature, the color change from dark purple to light yellow was determined at 517 nm against 1 ml methanol (as blank) using a UV spectrophotometer (Eppendorf UV-VIS).

Different concentrations of ascorbic acid (10-200 µg/ml) were used as a standard agent.

The antioxidant capacity of the samples was expressed as inhibitory concentration, IC_{50} (µg/ml). The IC_{50} is the concentration of an antioxidant at which 50% inhibition of free radical activity is observed. The lower IC_{50} value indicates the greater overall effectiveness of the antioxidant. The IC_{50} of the samples was measured by spectrophotometric method at the λ_{max} of DPPH, 517 nm.

The percent inhibition was calculated by the following equation:

$$\text{Inhibition}(\%) : [(A_{control} - A_{sample})/(A_{control})] \times 100$$

where: $A_{control}$ is the absorbance of the control reaction.

All tests were performed in triplicate. Concentration of samples resulting in 50% inhibition on DPPH (IC_{50} value) were calculated.

Total antioxidant capacity (TAC). The total antioxidant capacity of the samples was evaluated by phosphomolybdate method (Prieto et al., 1999) using ascorbic acid as a standard. The reaction mixture consisted of: 0.3 mL extract combined with 3 mL reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. After the samples cooled to room temperature, the absorbance of the solution was measured at 695 nm against the blank using a spectrophotometer. Methanol (0.3 ml) was used as control.

The results were expressed in ascorbic acid equivalent in µg/ml extract based on the standard calibration curve (y = 0.0067x + 0.0029; R = 0.9982) obtained for different concentrations of ascorbic acid (10-200 µg/ml) (Figure 4). The higher absorbance value indicated higher antioxidant activity.

Statistical Analysis. Results were expressed as standard error of the mean (SEM) for triplicate measurements. The graphics were plotted by using Microsoft Office Excel 2010.

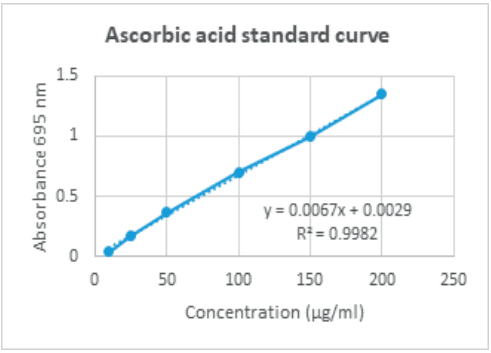

Figure 4. Ascorbic acid standard curve and the regression equation

RESULTS AND DISCUSSIONS

Microwave-assisted extraction (MAE). Dried flowers of *H. arenarium* were mixed with three different solvents: ethyl alcohol 70%, methanol 80% and distilled water. For extracting constituents from plant material, we used Microwave-assisted extraction (MAE) method.

In recent years, the use of the microwave oven for extracting constituents from plant material has shown tremendous research interest and potential.

Conventional techniques for extracting active constituents are time and solvent consuming, thermally unreliable, and the analysis of the many constituents in plant material is limited by the extraction step (Mandal et al., 2007). Traditional solvent extraction techniques for plant materials rely mainly on the correct choice of solvents and the use of heat and/or agitation to increase the solubility of the desired compounds and improve mass transfer. Usually, the traditional technique requires a longer extraction time, which leads to a severe risk of thermal degradation for most phytoconstituents. The fact that a single plant can contain up to several thousand secondary metabolites makes the need to develop fast and high-performance extraction methods an absolute necessity (Nyiredy, 2004).

The use and development of new techniques with shorter extraction times, reduced solvent consumption, increased concern for pollution prevention and special attention to thermolabile constituents.

New extraction methods including microwave-assisted extraction (MAE), supercritical fluid extraction (SCFE), pressurized solvent extraction (PSE) have attracted significant attention over the last decade.

Total phenolic and flavonoids content. The results for total phenolic and flavonoids content of *H. arenarium* extracts are shown in Figure 5. The total phenolic content of the 70% ethanol (19.26 ± 0.193 mg GAE/g dm) and 80% methanolic (11.48 ± 0.095 mg GAE/g dm) extracts was higher than the aqueous extract sample. Similarly, the content of flavonoids for both alcoholic extracts was greater (59.58 ± 0.125 mg QE/g dm in 70% ethanol and 50.48 ± 0.168 mg QE/g dm in 80% methanolic extracts) than that of aqueous extract (30.99 ± 0.199 mg QE/g dm) (Figure 5; Table 2).

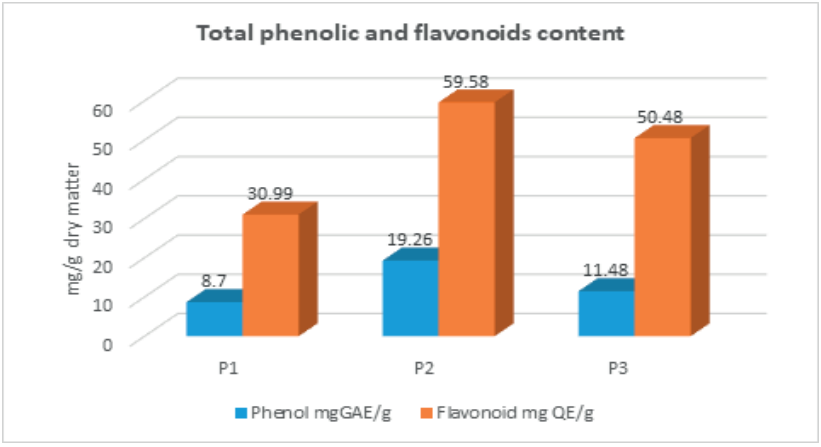

Figure 5. Total phenolics and flavonoids content in *H. arenarium* extracts
P1 - aqueous extract; P2 - 70% ethanol extract; 80% methanol extract

Czinner et al. (1999) suggested that the choleretic and hepatoprotective activities of *Helichrysum arenarium* inflorescence could be attributed to the antioxidant properties of its phenolic and flavonoid compounds. Various studies have revealed the presence of numerous bioactive compounds in *Helichrysum arenarium* inflorescences that have medicinal properties used in European phytotherapy (Grădinaru et al., 2014).

Phytochemical screening

Phytochemicals are currently enjoying increased attention due to exciting new findings on their biological activities (Cho et al., 2003). Alkaloids, flavonoids, phenols, glycosides, phytosterols and tannins detected in these extracts could implicate these phytochemicals as important bioactive agents in therapeutic action (Aliyu et al., 2013). The high phenolics content found in alcoholic extracts indicates high antioxidant potentials because the phenolics constituents can react with active oxygen radicals such as hydroxyl radical (Hussain et al., 1987), superoxide anion radical (Afanasev et al., 1989) and lipid peroxyl radical. The alcoholic extracts were found to have phenolic contents of 19.26 mg GAE/g dm for ethanol and 11.48 mg GAE/g dm and for methanol (Figure 5).

Phytochemical examination revealed the presence of alkaloids, flavonoids, phenols, glycosides, phytosterols and tannins in all extracts (Table 1, Figure 6).

Table 1. Phytochemical profile of *H. arenarium* extracts

Phytoconstituent	Reagent/test	Color change	Presence		
			Aqueous extract	Ethanolic extract	Methanolic extract
Alkaloids	Wagner	Reddish-brown	++	+++	+
Flavonoids	Alkaline test	Intense yellow	+++	+++	++
Phenols	Folin-Ciocalteu	Dark blue	++	+++	+++
Glycosides	Keller – Killani	Brown/green ring	++	++	++
Phytosterols	Salkowsky	Reddish-brown/Yellow	+++	++	+
Tannins	Ferric chloride test	Brown	++	+++	+++

Note: +++: highly present, ++: moderately present, +: low, -: absent.

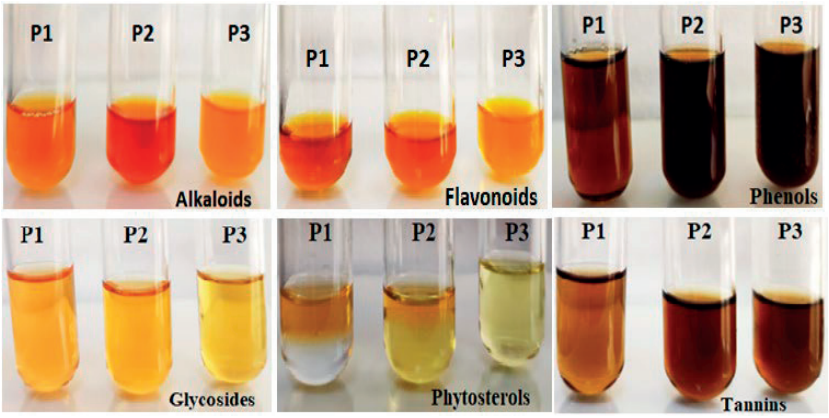

Figure 6. Presence of phytochemical compounds in *H. arenarium* extracts
P1 - aqueous extract; P2 - 70% ethanol extract; 80% methanol extract

Antioxidant activity

The antioxidant activity of *H. arenarium* extracts was determined via two methods (DPPH method and Total antioxidant capacity (TAC) by phosphomolybdate method). Assay based upon the use of DPPH radicals is among

the most popular spectrophotometric methods for determination of the antioxidant capacity of plant extracts because the radical compounds can directly react with antioxidants. Additionally, DPPH scavenging method has been used to evaluate the antioxidant activity of compounds due to the simple, rapid, sensitive, and reproducible procedures (Gonçalves et al., 2005).

In the present study, the $IC_{50\%}$ value of ascorbic acid, a well-known potent antioxidant, was 71.36±0.614 µg /ml.

The total antioxidant capacity (TAC) was based on the reduction of Mo (VI) to Mo(V) by the extract and subsequent formation of green phosphate/Mo(V) complex at acid pH.

The anti-radical activity of the samples with 70% ethanol displayed higher values (DPPH $IC_{50\%}$ = 109.5±0.341 µg /ml and TAC 202±11.78 µg /ml) than the samples with 80% methanol (DPPH $IC_{50\%}$ = 125.7±0.344 µg /ml and TAC 39.13±9.011 µg /ml) and distillated water (DPPH $IC_{50\%}$ = 151.2±0.263 µg/ml and TAC 9.41±0.129 µg /ml) by both methods. These results were presented in Figure 7 and Table 2.

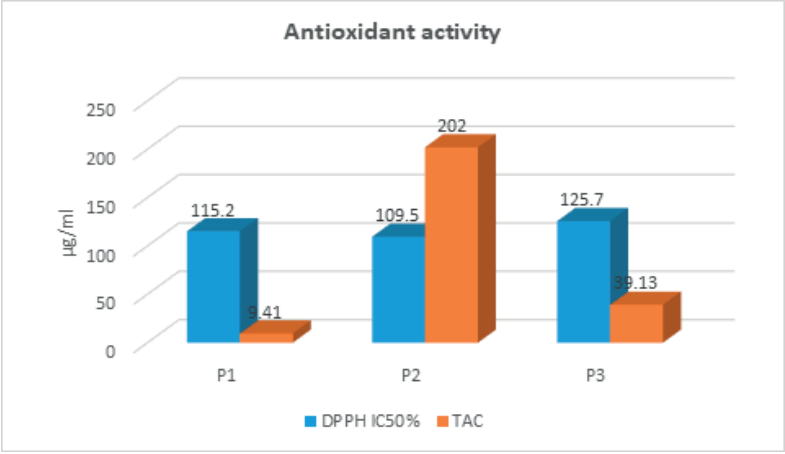

Figure 7. Antioxidant activity of *H. arenarium* extracts

The results for ethanol extracts and their antioxidant activity were in accordance with the total phenolic content. (TPC) and the concentration of flavonoids (TFC) (Table 2).

Table 2. Quantitative analyses of *H. arenarium* extracts

Sample	TPC mg GAE/g dm	TFC mg QE/g	DPPH IC50% µg /ml	TAC µg /ml
P1	8.70±0.087	30.99±0.199	151.2±0.263	9.41±0.129
P2	**19.26±0.193**	**59.58±0.125**	**109.5±0.341**	**202±11.78**
P3	11.48±0.095	50.48±0.168	125.7±0.344	39.13±9.011

Data are mean ± SEM for triplicate measurements
P1 - aqueous; P2 - 70% ethanol; 80% methanol

Various extracts (methanolic, ethanolic and 70% v/v ethanolic extracts) of Romanian sandy everlasting (harvested from Botoșani county), containing high amounts of polyphenols, have been tested for antioxidant properties (Babotă et al., 2018).

CONCLUSIONS

The 70% ethanol extract of *H. arenarium* showed the highest total flavonoid content and total phenolic content compared to other samples investigated. Moreover, ethanolic extract has highest DPPH free radical scavenging activity and TAC which could be related to its higher phenolic content. Phytochemical examination revealed the presence of alkaloids, flavonoids, phenols, glycosides, phytosterols and tannins in all extracts. The presence of various bioactive compounds in *Helichrysum arenarium* inflorescences may have beneficial properties for human health.

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