INVESTIGATION OF ANTIOXIDANT ACTIVITY OF DIFFERENT EXTRACTS OF *HELICHRYSUM ITALICUM* FROM BULGARIA

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Abstract

Helichrysum italicum from the daisy family Asteraceae have been used in the folk medicine for many years for preventive purposes and for restoring the body after a heart attack. The plant material from H. italicum was provided by the Institute of Roses and Essential Oils, Kazanlak, Bulgaria. The antioxidant activity of different H. italicum extracts was evaluated by various methods, such as ABTS, CUPRAC, FRAP and DPPH.

Keywords: *Helichrysum italicum, TPC, antioxidant activity, DPPH, ABTS, CUPRAC, FRAP*

1. INTRODUCTION

Medicinal plants or medicinal herbs have been discovered and used in folk medicine since ancient times. They are important regarding the discovery and isolation of new drugs and APIs (active product ingredients) as reserpine, quinine, digitoxin etc. [1, 2].

Helichrysum italicum is plant of the *Asteraceae family*. More than 500 species are widely distributed between the Mediterranean area, Africa, Asia and Australia. The origin of the name *Helichrysum* comes from the Greek words for Sun and gold (Ήλιος and χρυσός) as describing the bright yellow color of the flower heads.



Helichrysum italicum

Helichrysum Italicum is commonly known as the curry plant or everlasting plant as it grows on dry, rocky, or sandy ground around the Mediterranean area [3].

For first time the genus *Helichrysum* was mentioned for medicinal uses in 3rd – 2nd century B.C. in the work of Greek Theophrastus of Eresos "Historia Plantarum" [4].

Some of the most examined species of this genus for the last years are *Helichrysum arenarium* (L.) Moench [5], *Helichrysum stoechas* (L.) Moench [6], *Helichrysum graveolens* (M. Bieb.) Sweet [7] and *Helichrysum italicum* (Roth) G. Don [8]. Of course the interest of all these species comes from the traditional medicine. Have been reported that *Helichrysum italicum* has helped in inflammatory and

allergy conditions such as the ones related with the respiratory tract, and skin conditions [9, 10]. The essential oil of *Helichrysum italicum* has been used in the aromatherapy [11].

The activity of the Helichrysum italicum extracts of consist of different biological activity compound classes as the most common are terpenes and phenolic compounds. As the variety of specialized metabolites Helichrysum italicum possess a wide range of biological activities [12].

Sacchetti and co-workers [13] have reported the antioxidant activity of supercritical CO_2 extract of *Helichrysum italicum* dried flower heads from plants grown in north-east Italy. The authors used DPPH and *β*-carotene bleaching test methods. Also the samples were tested for their ability to scavenger superoxide radicals. They have compared wild *Helichrysum italicum* with cultivated in fields, commercial one and from nursery cultures. The obtained results of Sacchetti for the first three samples were pretty similar. The difference they found is in plants from the nursery cultures they have linked not only to the chemo type but also to a different qualitative and quantitative characterization of the polyphenol fraction responsible for the scavenger activity.

Very interesting is research described from Pereira and co-workers [14]. As the tea is the most common drink in the World they have reported the antioxidant activity results for *Helichrysum italicum* subsp. *picardii* (everlasting) compared to Green tea (*Camellia sinensis*)and rooibos tisane (*Aspalathus linearis*). In nowadays a lot of scientific community has begun focusing on halophytes as sources of functional drinks. The authors has explored for the first time *H. italicum* subsp. *picardii* as a potential source of innovative herbal beverages with potential health promoting properties. They have made infusions and decoctions from all of the drugs parts (roots, vegetative aerial organs – stems and leaves, flowers) and have evaluated both *in vitro* antioxidant and anti-diabetic activities. The obtained results they expressed relating to 'a cup-of-tea' and compared with the green tea (*Camellia sinensis*) and rooibos tisane (*Aspalathus linearis*) ones. Tisanes from the everlasting's above-ground organs, particularly flowers, have high polyphenolic content. Several phenolics have been identified of which the main are chlorogenic and quinic acids, dicaffeoylquinic acid isomers and gnaphaliin A. The antioxidant activity of beverages from the everlasting's above-ground organs matched or surpassed that of green tea and rooibos. Its anti-diabetic activity was moderate and toxicity low. From their results can be concluded that the everlastings are potential source of innovative and functional herbal beverages.

Helichrysum italicum is commonly used in Bulgaria for their various biological properties, including anti-inflammatory [15], antioxidant [16, 17], and antimicrobial activities [18-20]. This study attempts to contribute to the lack of knowledge about the antioxidant activity for *Helichrysum italicum* grown in Bulgaria.

2. MATERIALS AND METHODS

2.1. Plant Materials

The used herb *H. italicum* was grown in the area of Kazanlak (Bulgaria), harvested in phase mass flowering, year 2018. The plant material was kindly provided by Silviya Mollova, Institute for roses and aromatic plants – Kazanlak, Bulgaria.

2.2. Chemicals and reagents

Chromatographic grade methanol (MeOH) and ethanol (EtOH) were used for HPLC analyses (VWR, Austria). Water for HPLC was prepared with Millipore purifier (Millipore, USA). Ammonium acetate (NH_4Ac), cupper(II) chloride, glacial acetic acid, sodium acetate trihydrate, ferric chloride hexahydrate, gallic acid, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox,) and reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), neocuproine (2,9-dimethyl-1,10-phenanthroline), 2,4,6- tripyridyl-s-triazine (TPTZ), Folin–Ciocalteu reagent were purchased from Sigma-Aldrich.

2.3. Preparation of extracts

The milled plant material was weighed with 0.0001 g precision and 5 g have been used for analysis. The initial milled material was extracted with different solvents (methanol, ethanol, 70 % methanol, 60 % ethanol and ethyl acetate) via ultrasonic bath for 30 minutes with 100 ml extract solution at room temperature. The obtained extracts were filtered under vacuum. The extractions have been carried out three times. The supernatants were combined and evaporated under vacuum with at 40 °C. The volume of all samples was adjusted to 60 ml and filtered through syringe filter with pore size of 0.45 µm prior for analysis.

2.4. Determine of total phenolic content (TPC)

The total phenolic content in different extracts was determined with colorimetric method using Folin-Ciocalteau's reagent [21] with slight modifications [22]. Calibration curve was achieved using as standard ethanolic solution of gallic acid at concentrations between 50 and 1000 µg/ml. Briefly, 50 µl of extract or gallic acid standard was mixed with 3 ml distilled water, 250 µl of 0.2 M Folin-Ciocalteu's reagent and 1.7 ml of 7.5 % sodium carbonate (Na_2CO_3) solution. The tested samples were incubated for 2h in dark at room temperature. The absorbance of the samples was measured at 765nm with a spectrophotometer (Camspec M508, England), using a blank sample. The total phenolic content was expressed as mg gallic acid equivalent per grams of dry weight (mg GAE/g DW) based on the calibration curve.

2.5. Trolox Equivalent Antioxidant Capacity (TEAC)

2.5.1. ABTS assay

The Trolox Equivalent Antioxidant Capacity (TEAC) was determined by using the colorimetric method reported from Re et al. [23] with slight modifications. For this assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$) solution was prepared by dissolving 7 mM of ABTS in 2.45 mM $K_2S_2O_8$. This mixture was shaken for 12 – 16 h at ambient temperature in the dark until obtaining a stable oxidative state. For the study of the extracts, the $ABTS^{\bullet+}$ stock solution was diluted with methanol until absorbance became 0.70 ± 0.02 at 734 nm. Sample analysis was performed as follows: 3 ml of ABTS solution and 30µl of sample or standard were mixed. Absorbance of sample was measured at 734 nm with a spectrophotometer Camspec M508, England after samples incubation at 25 °C for 5 min. The calibration curve was plotted by using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) as a standard. The results were expressed as mmol Trolox equivalents per g of dry weight (mM TE/g DW).

2.5.2. Cupric ion reducing antioxidant capacity (CUPRAC) assay

The different *H. Italicum* extracts were investigated by cupric ion reducing antioxidant capacity (CUPRAC) method described by Apak et al [24]. In test tubes were mixed 1 ml $CuCl_2$ solution (10 mM), 1 ml neocuproine alcoholic solution (7.5 mM) and 1M NH_4Ac buffer solution (pH = 7), 0.2 ml tested extract or trolox and 0.9 ml water (final volume, 4.1 ml). Absorbance against a blank sample was measured at 450 nm with a spectrophotometer Camspec M508, England after 30 min in dark at room temperature. Calibration curve was achieved using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) as a standard ethanolic solution at concentration ranges between 0.045 and 1.5 mM. The trolox equivalent antioxidant capacity was plotted as mmol Trolox equivalents per g of dry weight (mM TE/g DW).

2.5.3. Ferric reducing antioxidant power (FRAP) assay

All extracts were investigated by ferric reducing antioxidant power (FRAP) method described by Benzie and Strain [25]. The FRAP reagent was freshly prepared before analyzes by mixing 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6- tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in dd H_2O in a ratio 10:1:1. The In test tubes were mixed 0.15 ml tested extract or standard Trolox and 2.85 ml FRAP reagent. Absorbance against a blank sample was measured at 593 nm with a spectrophotometer Camspec M508, England after 15 min in dark at room temperature. Calibration curve was achieved using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid

(Trolox) as a standard ethanolic solution at concentration ranges between 0.045 and 1.5 mM. The trolox equivalent antioxidant capacity was plotted as mmol Trolox equivalents per g of dry weight (mM TE/g DW).

2.5.4. DPPH assay

Antioxidant activity was measured according to Brand-Williams [26] procedure. In the test tubes were mixed 100 µl of extract or Trolox and 2.9 mL of 0.12 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent, which was prepared with 4.8 mg DPPH dissolved in 100 mL CH_3OH). The mixtures were shaken and then incubated for 30 min at room temperature. The absorbance was recorded at 517 nm with a spectrophotometer Camspec M508, England. To quantify the antioxidant activity a standard Trolox curve (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is used with concentration from 0.045 to 1.5 mmol Trolox. The results were expressed as mM TE/g dry weight.

2.6. Statistical Analysis

The analyses were made in triplicate. Statistical differences between samples were tested using ANOVA. The data were expressed as mean ± SD. The differences between different extracts were analyzed by one-way analysis of variance (ANOVA) followed by a Post-Hoc Test. The comparison between groups was performed by using Student's test. The significance level was set at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Yield and Analysis of total phenolic content (TPC)

The phenolic compounds are widely spread in the plants and contribute significantly to the overall antioxidant activity that potentially has beneficial effects on the human health. Therefore, it is very important to evaluate the total phenol content of the different extracts. As a first step in this study, we have estimate the total phenol content (TPC) of the different *Helichrysum italicum* extracts.

The yield of the polyphenols depends on the type of solvent. On Figure 1 is shown the percentage of yields of different extracts from *H. italicum*. The results show that 60% EtOH and 70% MeOH are suitable for deriving the polyphenol compounds, with high yields of 18.78% and 17.92%, respectively. The ethyl acetate extract owns the lowest yield (4.78%). The amount of total phenols and antioxidant activity depends on the yield of phenolic compounds.

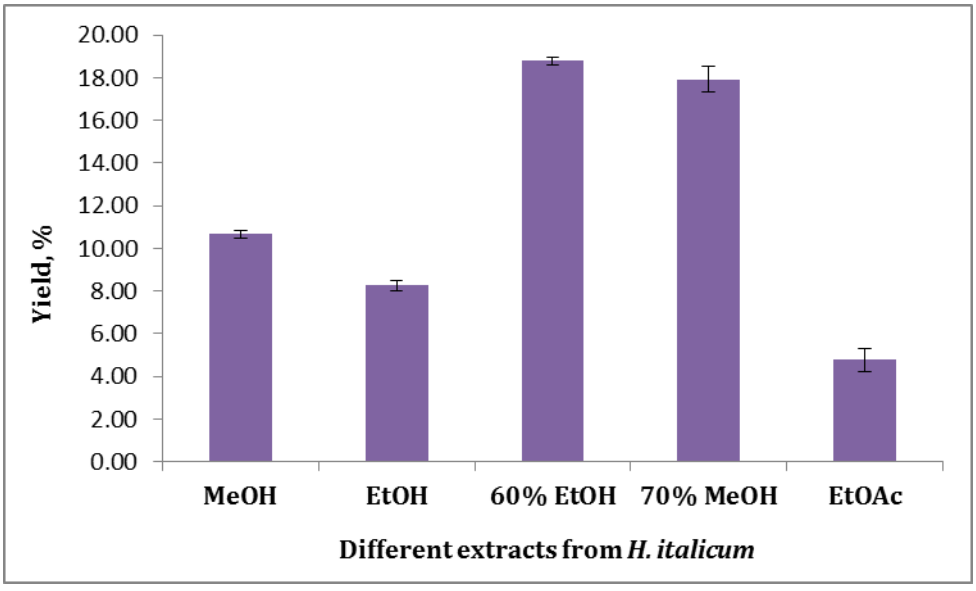


Figure 1. Percentage of yields of different extracts from *H. italicum*.

The total phenolic content (TPC) of the examined extracts is shown in Table 1 and Figure 2. The values of TPC are in the range from 10.06 – 65.18 mg GAE/g DW. It's obvious that 60 % EtOH and 70 % MeOH extracts are characterized by a high content of total phenols. The results were statistically processed at $p > 0.05$.

Extracts	mg GAE/g DW	CUPRAC	FRAP	ABTS	DPPH
MeOH	31.79 ± 0.17	152.48 ± 1.99	163.36 ± 6.14	76.02 ± 4.92	73.18 ± 3.51
EtOH	21.81 ± 0.60	106.86 ± 4.02	106.92 ± 3.53	107.51 ± 4.20	58.35 ± 5.25
60% EtOH	65.18 ± 1.29	307.58 ± 1.51	257.37 ± 14.01	100.47 ± 9.85	132.38 ± 1.15
70% MeOH	61.92 ± 3.09	303.23 ± 12.88	289.34 ± 7.45	100.47 ± 9.85	144.36 ± 7.01
EtOAc	10.06 ± 0.81	53.84 ± 0.99	54.89 ± 3.43	73.25 ± 5.36	24.58 ± 2.00

Table 1. TPC (mg GAE/g DW) and antioxidant activity of different *H. italicum* extracts. The results of antioxidant activity were expressed as mM TE/g DW.

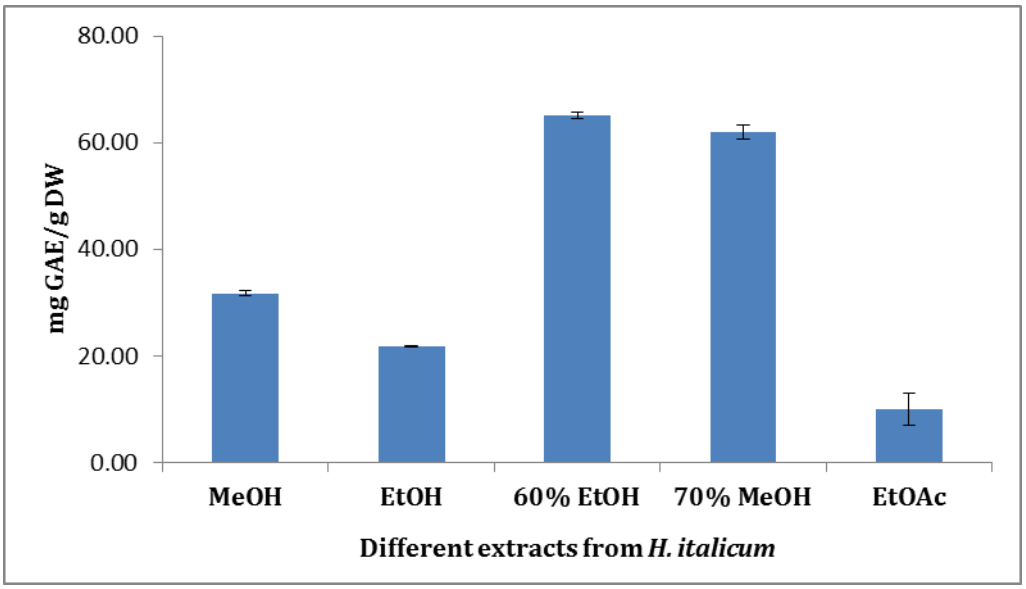


Figure 2. Total phenolic content (TPC) of different extracts from *H. italicum* was evaluated using Folin-Ciocalteau's reagent.

The results show that 60% EtOH is the most suitable solvent for the extraction of polyphenols. Additionally, we can assess the effect of the solvent on the extracting of the polyphenol components. In this case, the ethyl acetate as a solvent extracts about 15% of the total phenolic compounds, ethanol about 22% and methanol about 32%.

3.2. Antioxidant activity

Reactive Oxygen Species (ROS) cause damage to biomolecules such as proteins, nucleic acids, lipids and carbohydrates which are the main structures of the human cells [27]. They are generated by oxidative stress and are responsible for the aging process. Antioxidants have a beneficial effect on the human body. In fact, antioxidants do not allow the generation of ROS and also play an important role in their inactivation. Although human cells are protected from oxidative damage by antioxidants, they are sometimes not sufficient to prevent ROS induced cellular damage [28].

Depending on the mechanism of antioxidant reaction, the methods were divided in hydrogen atom transfer (HAT) and single electron transfer (SET) techniques. The HAT method is based on the antioxidant's ability to quench free radicals through a hydrogen donor. SET methods are characterized on the one electron transfer reductive ability of the tested antioxidant versus a radical species. FRAP and CUPRAC are single electron transfer methods. ABTS and DPPH methods were regarded as methods using both hydrogen and single electron transfer [29, 30].

The antioxidant activity of different *H. italicum* extracts varies significantly. The antioxidant activity of the different extracts was evaluated by various methods, such as the ability to deactivate free radicals as well the ability to reduce copper (Cu^{2+}) and ferric (Fe^{3+}) ions using ABTS, DPPH, CUPRAC and FRAP analysis respectively. The results of the antioxidant activity are presented in Table 1 and Figure 3.

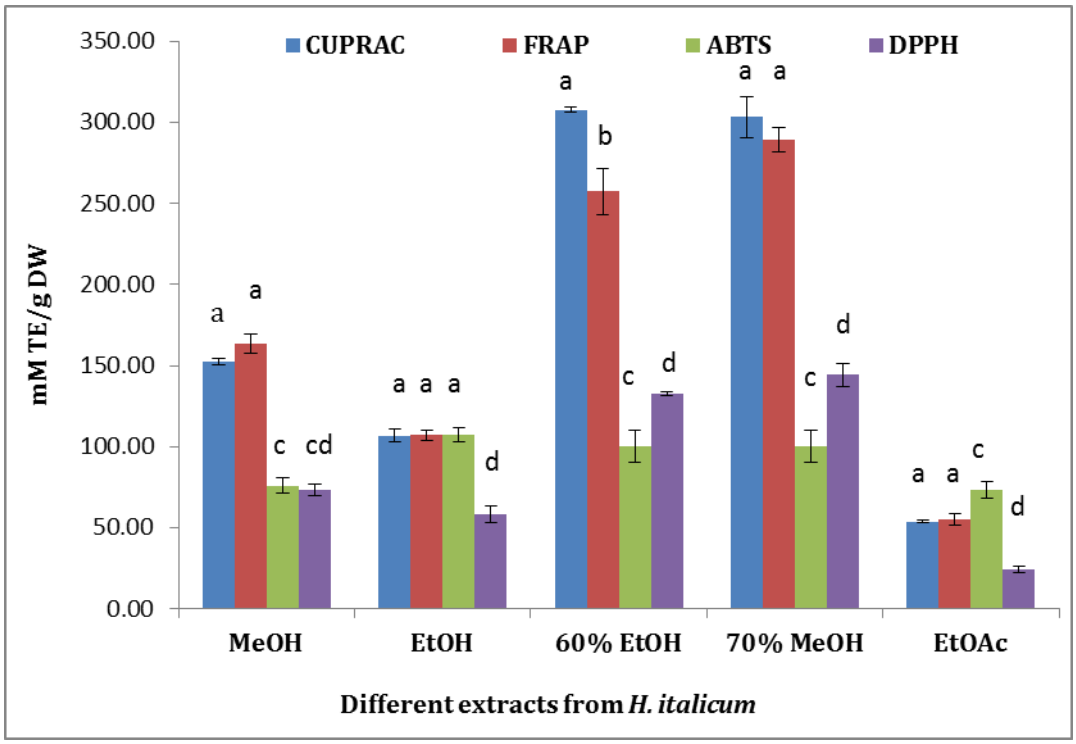


Figure 3. Antioxidant activity of different extracts from *H. italicum* was evaluated using CUPRAC, FRAP, ABTS and DPPH methods. The differences between different extracts were analyzed by one-way analysis of variance (ANOVA) followed by a Post-Hoc Test. The comparison between groups was performed by using Student's test.

The TEAC results are determined by ABTS analysis are presented in Table 1 and Figure 3. The analysis is based on the ability of the antioxidants to decolorize the stable blue/green form of $ABTS^{\bullet+}$. The TEAC grade of the different extracts from *H. italicum* varies 73.25 – 107.51 mM TE/g DW (Table 1, Figure 3). The results for antioxidant activity show that the EtOH, 60 % EtOH and 70 % MeOH extracts are characterized the highest TEAC.

The DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples. DPPH is a stable nitrogen free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation [31]. The antioxidant activity of the different extracts was investigated by DPPH analysis. Research has shown that aqueous alcoholic extracts have high antioxidant properties. The TEAC values for 60 % EtOH and 70 % MeOH 132.38 ± 1.15 and 144.36 ± 7.01 mM TE/g DW respectively (Table 1, Figure 3). The ethyl acetate extract show low antioxidant activity 24.58 ± 2.00 mM TE/g DW.

The CUPRAC methods are based on single electron transfer and it is characterized to the reduction of copper ions. The obtained results of the antioxidant study of the different extracts using the reduction of copper (Cu^{2+}) ions vary widely (53.84 - 307.58). The highest antioxidant properties were observed in 60 % EtOH and 70 % MeOH 307.58 ± 1.51and 303.23 ± 12.88 mM TE/g DW respectively (Table 1, Figure 3). The resulting high values for aqueous alcoholic extracts obtained by the CUPRAC method correlate very well with the high content of total phenols.

The FRAP assay is a typical ET-based method that measures the reduction of ferric ion (Fe^{3+}) –ligand complex to the intensely blue colored ferrous (Fe^{2+}) complex by antioxidants in acidic media. Antioxidant activity is determined as increase of absorbance at 593 nm [32]. The reducing capacity of a compound may serve as an important indicator of its potential antioxidant activity. The highest antioxidant activity were observed in 60 % EtOH and 70 % MeOH 257.37 ± 14.01 and 289.34 ± 7.45 mM TE/g DW respectively (Table 1, Figure 3).

Despite the various mechanisms of scavenging radicals, the analysis of antioxidant activity shows that water-alcoholic extracts exhibit the highest antioxidant capacity. The high antioxidant activity of 60% ethanol and 70% methanol correlates with the total phenolic content. The ethyl acetate extraction shows the lowest antioxidant activity (Table 1). This can explain the fact that phenolic compounds show high solubility in aqueous alcoholic solvents like 60 % EtOH and 70 % MeOH. The aqueous alcoholic extracts demonstrate the highest grade to deriving of TPC than organics like methanol, ethanol and ethyl acetate. Therefore, aqueous ethanolic extracts are preferred in medical practice and pharmacy.

	mg GAE/g DW	***CUPRAC***	***FRAP***	***ABTS***	***DPPH***
mg GAE/g DW	1				
CUPRAC	0.9993	1			
FRAP	0.9834	0.9871	1		
ABTS	0.5233	0.5238	0.4902	1	
DPPH	0.9891	0.9924	0.9945	0.5701	1

Table 2. Correlations between TPC/TEAC and TEAC/TEAC of the different *H. italicum* extracts.

The results of all analyzed extracts allowed us to establish a correlation between the total phenolic content (mg GAE/g) and antioxidant capacity (mM TE/g), expressed as R. Table 2 shows the correlation between the total phenolic content (TPC) and the different TEAC methods. The obtained values ($p > 0.05$) indicate that R between the total phenolic content (TPC) (mg GAE/g) and trolox equivalent antioxidant capacity (TEAC) (mM TE/g) are in the range of 0.5233 to 0.9993. The obtained values for R are 0.9993 for CUPRAC versus to Folin, 0.9891 for DPPH versus Folin and 0.9834 for FRAP versus Folin. From these results it can be concluded that the best correlation is established between TPC and CUPRAC. The high correlation between the content of CUPRAC and TPC may be due to the fact that both analyzes are based on the same reaction mechanism. It has been found that the antioxidant activity of phenols is mainly due to their oxidation-reducing properties, which allows them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [33].

4. CONCLUSIONS

In conclusion, present study revealed the *in-vitro* antioxidant activity of different extracts from *Helichrysum italicum* using four methods – CUPRAC, FRAP, ABTS and DPPH. The presence of phenolic compounds is responsible for the activity. Further investigations are required to find out the active component of the extracts and to confirm the mechanism of action.

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