

Comparison of Polyphenolic Content and Antioxidant Capacity of Five Romanian Traditional Medicinal Plants

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The aim of this study was the quantitative determination of total phenolic and flavonoid content for five ethanolic plant extracts (Urtica dioica, Artemisia absinthium, Arnica montana, Achillea millefolium and Helleborus purpurascens). Also, their antioxidant activity was evaluated by Oxygen Radical Absorbance Capacity (ORAC) and Trolox Equivalent Antioxidative Capacity (TEAC) assays. All the studied extracts showed a higher antioxidant activity than Trolox, used as standard. The values of antioxidant activity determined by both methods increased in the following order: H. purpurascens, U. dioica, A. montana, A. millefolium, A. absinthium. The relationship between the total phenolic and flavonoid content of plant extracts and their antioxidant activity was also studied. The obtained results showed that A. absinthium and A. millefolium extracts, which contained the largest amount of phenolic and flavonoid compounds exhibited the highest level of total antioxidant capacity. Our in vitro assays indicate that the studied plant extracts are a significant source of natural antioxidants, which might be helpful in preventing the diseases caused by oxidative stress.

Keywords: ethanolic plant extracts, phenolic content, flavonoids, antioxidant capacity, ORAC assay, TEAC assay

In living systems, free radicals are constantly generated and they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases and extensive lysis [1].

Free radicals are generated as a result either of normal metabolic activity or environmental factors. In the presence of exogenous factors such as radiation, smoking, pollutants, organic solvents, pesticides, etc [2] or endogenous metabolic processes (normal aerobic respiration, stimulated polymorphonuclear leukocytes and macrophages), highly reactive free radicals are formed, resulting in diseases including cancer, diabetes, arteriosclerosis and accelerated ageing [3, 4]. Active oxygen, in the form of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) or singlet oxygen (1O_2) is a product of normal metabolism, which attacks biological molecules resulting in cell and tissue injury.

Synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tert-butylhydroquinone present high toxicity and are suspected of being responsible for liver damage and carcinogenesis in laboratory animals [4]. Therefore, recent investigation turned researchers attention to the development of natural antioxidants with strong antioxidant properties but low cytotoxicity, instead of synthetic ones [1, 5, 6]. It was mentioned that medicinal plants contain various free radical scavenging molecules, such as vitamin C, D, E and other endogenous metabolites [7-9]. Among the plant metabolites, polyphenolic compounds (phenolic acids, flavonoids) are widely distributed in plants and exhibit antioxidant activities based on the free radicals scavenging capacity [10].

Evaluation of the total antioxidant capacity of fruits, vegetables, and plant products cannot be performed accurately by any single method due to the complex nature of phytochemicals [11].

In this paper, our objective was to evaluate the antioxidant capacity of five plant extracts (*Urtica dioica*,

Artemisia absinthium, *Arnica montana*, *Achillea millefolium* and *Helleborus purpurascens*) used in the folk medicine of Romania, as tea or decoction, in order to use them in obtaining different pharmaceutical products. Two methods were used for the evaluation of antioxidant capacity of these extracts, one based on the oxygen radical absorbance capacity (ORAC assay) and one based on the ability of plant extracts to scavenge the generated radical cation in comparison with a standard (TEAC assay).

Experimental part

Plant materials and extraction. *U. dioica*, *A. absinthium*, *A. montana*, *A. millefolium* and *H. purpurascens* were bought from local market of Bucharest, Romania and authenticated by Dr. Elvira Gille from Stejarul Piatra Neamt Biological Research Center. Aerial parts of the first four plants and rhizomes of *H. purpurascens* were dried in air, at room temperature and, then, grounded. 7.5 g of each plant were extracted in aqueous ethanol of 70% concentration (v/v), in a ratio of 1:10 (w/v), on a shaker, at 200 rpm, at room temperature, for 8 h. Then, the extracts were filtered over Whatman No. 1 paper. The filtrates were placed in plastic bottles and stored at -20 °C until use.

Determination of total phenolic content. The phenolic content of the five plant extracts was determined spectrophotometrically using a slightly modified Folin Ciocalteu reagent assay [12]. To 150 μL plant extract, 750 μL of Folin Ciocalteu reagent were added. The mixture was kept at room temperature, for 5 min. Then, 4 mL of 15 % Na_2CO_3 were added and the volume was brought to 15 mL with distilled water. The mixture was kept at room temperature, for 30 min and the absorbance of the developed colour was recorded at 765 nm, using an UV/VIS spectrophotometer (Jasco V 650). Caffeic acid was used as standard for calibration curve. The phenolic content was expressed as mg caffeic acid/g dry weight (d.w.).

Determination of flavonoid content. The aluminium chloride colorimetric method [13] was used to measure

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Table 1
TOTAL PHENOLIC AND FLAVONOID CONTENT OF *U. DIOICA*, *A. ABSINTHIUM*, *A. MONTANA*,
A. MILLEFOLIUM AND *H. PURPURASCENS*

Plant species	Total phenolic content* (mg caffeic acid/g d.w.)	Total flavonoid content* (mg quercetin/g d.w.)
<i>U. dioica</i>	90.09 ± 2.82	31.03 ± 1.93
<i>A. absinthium</i>	178.76 ± 1.58	52.43 ± 2.22
<i>A. montana</i>	97.16 ± 1.37	38.62 ± 1.50
<i>A. millefolium</i>	131.59 ± 2.96	46.59 ± 2.31
<i>H. purpurascens</i>	39.18 ± 2.60	19.32 ± 2.78

Each value in the table was obtained by calculating average of three experiments ± standard deviation.

the flavonoid content of the plant extracts. From each plant extract 0.5 mL were added to a mixture of 1.5 mL methanol, 0.1 mL 10 % aluminium chloride, 0.1 mL sodium acetate and 2.8 mL distilled water. The mixture was allowed to stand at room temperature, for 30 min. The absorbance was measured at 415 nm using an UV/VIS spectrophotometer (Jasco V 650). Quercetin was used as standard for the calibration curve. Flavonoid content was expressed as mg quercetin/g dry weight (d.w.).

Antioxidant activity determination. In order to determine the antioxidant activity of the plant extracts, two methods were applied:

- the Oxygen Radical Absorbance Capacity (ORAC) assay and
- the Trolox Equivalent Antioxidative Capacity (TEAC) assay.

We used the ORAC method described in [14] with some modifications. The reaction mixture was prepared by adding 50 µL of 0.42 µM fluorescein to 100 µL test sample and 1.8 mL phosphate buffer, pH 7.30. This mixture was incubated at 37 °C, for 15 min. Then, 50 µL of 640 mM 2,2'-azobis[2-methyl-propionamidin] dihydro-chloride (AAPH) was added, as a peroxy radical generator. The working solutions and a standard solution of Trolox were daily prepared. The intensity of relative fluorescence was monitored at every 0.004 min, for 80 min, on a Perkin Elmer LS 55 spectrometer with fluorescent filters (excitation 489 nm, emission 515 nm). The ORAC values, expressed as µM Trolox equivalents (µM TE) were calculated according to the following relation:

$$ORAC (\mu M TE) = \frac{C_{Trolox} \cdot (AUC_{sample} - AUC_{blank}) \cdot k}{(AUC_{Trolox} - AUC_{blank})} \quad (1)$$

where:

- C_{Trolox} = Trolox concentration,
- k = the sample dilution factor,
- AUC = the area below the fluorescence decay curve of the sample, blank, and Trolox, respectively.

The TEAC assay described in [15] was slightly modified by us. The ABTS radical cation was generated by mixing 7 mM 2,2'-azino-bis(3-ethyl-benzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) stock solution with 2.45 mM potassium persulfate (1:1, v/v) and incubation for 12–16 h in the dark, at room temperature, until the reaction was complete and the absorbance was stable. The absorbance of the ABTS radical solution was equilibrated to 0.700 (± 0.02) by diluting with bidistilled water. Then, 1 mL reagent was mixed with 100 µL test sample (0.05–1 mg/mL) and the absorbance was measured after 6 min, at 734 nm, using an UV/VIS spectrophotometer (Jasco V 650). The

annihilation activity of free radicals was calculated according to the following formula:

$$\%Inhibition = \frac{(A_{blank} - A_{sample})}{A_{blank}} \cdot 100 \quad (2)$$

where:

- A_{blank} = blank absorbance
- A_{sample} = sample absorbance.

At the same time, a Trolox calibration curve was prepared for a concentration range of 0–250 µM, and the inhibition percentage obtained for the sample was interpolated to calculate the concentration in Trolox equivalents (µM TE).

Statistical analysis. Data were expressed as means ± standard deviation (S.D.) of the means for three separate experiments (n=3). Statistical analysis was performed using Student's test. Differences were considered significant at $p < 0.05$.

Results and discussions

Total phenolic and flavonoid content

The total phenolic content of the ethanolic extracts, expressed as caffeic acid equivalents/g dry weight extract is shown in table 1. The results revealed significant differences between the values obtained for the five studied plants. *A. absinthium* had the highest phenolic content among the five plant extracts. The values of phenolic content decreased in the following order: *A. absinthium* > *A. millefolium* > *A. montana* > *U. dioica* > *H. purpurascens*.

We analyzed the flavonoid content of these ethanolic extracts, expressed as quercetin equivalents/g dry weight extract (table 1). The highest flavonoid content was found for the ethanolic extract of *A. absinthium*, followed by the next studied plants: *A. millefolium*, *A. montana*, *U. dioica* and *H. purpurascens*.

Antioxidant activity

Various methods for the evaluation of the antioxidant activity of different plant products are recommended because the composition of a plant extract is complex [16-18]. A true measure and a standard method of total antioxidant capacity are not yet available [19, 20].

The methods for measuring antioxidant capacity are basically classified into two groups, depending on the reaction mechanism: methods based on hydrogen atom transfer (HAT) and methods based on electron transfer (ET) [21]. The majority of HAT-based assays applies a competitive scheme, in which the antioxidant and the substrate compete for thermally-generated peroxy radicals through the decomposition of azo-compounds. ET-based assays measure the capacity of an antioxidant in reduction of an oxidant which changes its colour when

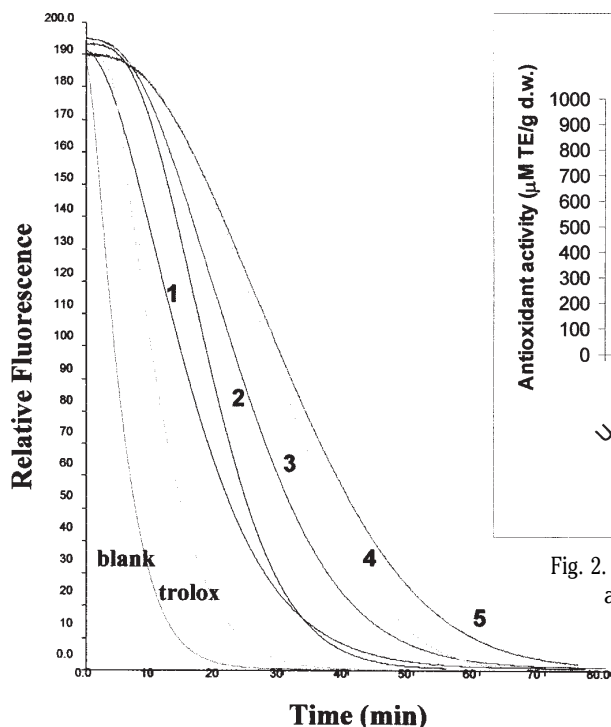


Fig. 1. Fluorescence decay curve during the ORAC assay in the presence of various samples (1. *H. purpurascens*, 2. *U. dioica*, 3. *A. montana*, 4. *A. millefolium*, 5. *A. absinthium*) and the synthetic antioxidant, Trolox

reduced. The degree of colour change is correlated with the sample antioxidant concentration.

In this paper, the antioxidant capacity of the studied ethanolic plant extracts was evaluated by measuring peroxyl radical scavenging activity (ORAC assay-HAT method) and by measuring the inhibition of the ABTS cationic radical (TEAC assay-ET method).

During the ORAC assay, free radicals are generated by the water soluble compound AAPH. The peroxyl radicals thus generated quench the fluorescence of fluorescein over time. The decrease in fluorescence intensity was registered for the five plant extracts and a synthetic antioxidant, Trolox (fig. 1).

The antioxidants from plant extracts block the peroxyl radical mediated oxidation of fluorescein. In the presence of Trolox, the fluorescein molecule was protected against AAPH radical-induced damage. The extracts provided a similar protection profile for the fluorescence molecule as observed with the Trolox antioxidant.

The values of the antioxidant capacity for the five plant extracts determined by ORAC and TEAC assays are presented in figure 2.

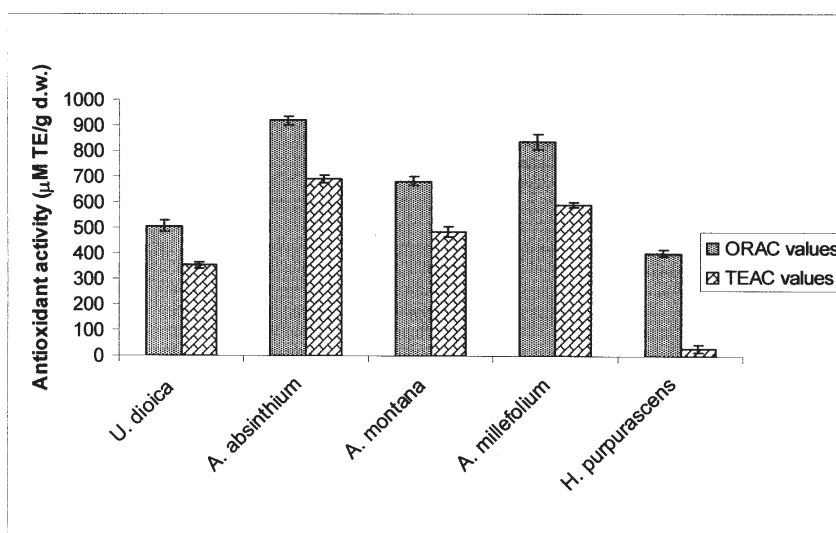


Fig. 2. Antioxidant activity of *U. dioica*, *A. absinthium*, *A. montana*, *A. millefolium* and *H. purpurascens* determined by the ORAC assay and TEAC assay

The highest antioxidant capacity, measured by ORAC assay was observed with *A. absinthium* and *A. millefolium* extracts (917.89 ± 15.83 and $835.24 \pm 30.78 \mu\text{M TE/g d.w.}$, respectively). Lower but important ORAC values were observed with *A. montana* and *U. dioica* (682.22 ± 17.32 and $505.21 \pm 22.38 \mu\text{M TE/g d.w.}$, respectively). The lowest ORAC value was exhibited by *H. purpurascens* ($401.97 \pm 12.30 \mu\text{M TE/g d.w.}$).

The antioxidant capacity measured by TEAC assay for the investigated extracts varied widely, ranging from $690.62 \pm 13.79 \mu\text{M TE/g d.w.}$ (for *A. absinthium* extract) to $31.01 \pm 15.40 \mu\text{M TE/g d.w.}$ (for *H. purpurascens* extract). The medium values were identified for *A. millefolium*, *A. montana* and *U. dioica* extracts (589.92 ± 11.71 , 486.06 ± 20.63 and $352.90 \pm 11.47 \mu\text{M TE/g d.w.}$, respectively).

Correlation between ORAC assay and TEAC assay

The TEAC values of the studied plants were slightly lower than the ORAC values. Comparing the antioxidant capacity values of the five plant extracts determined by the TEAC method and the ORAC method, we found a good correlation between them (the correlation coefficient, $R^2 = 0.9083$) (fig. 3).

The differences in the antioxidant capacities found between the two methods were partially due to the different nature of the two assays. The TEAC assay is rapid and requires simple conventional laboratory equipment. The ORAC assay is more sensitive, reproducible, but requires some very expensive equipment. Previously studies reported that ORAC method mimicks antioxidant

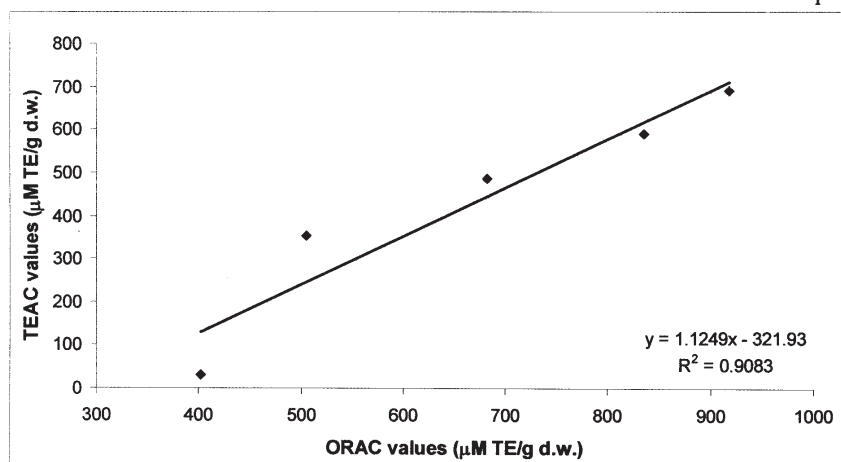


Fig. 3. Correlation between ORAC values and TEAC values for the studied plants

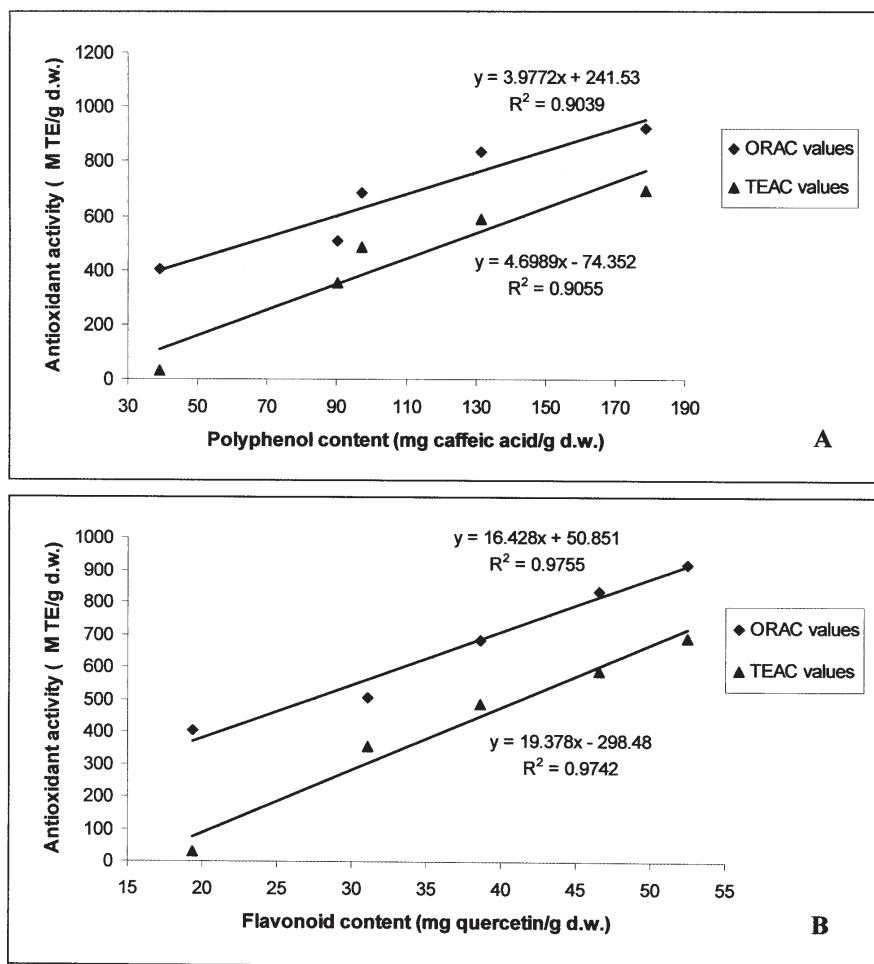


Fig. 4. Relationship between polyphenolic (A) and flavonoid (B) content and the antioxidant activities by ORAC and TEAC for the studied plants

capacity of polyphenols in biological systems better than other methods because it uses biologically relevant free radicals [20, 22 - 24].

Correlation between the total phenolic and flavonoid content and the antioxidant activity

Several studies have evaluated the relationship between the antioxidant activity of plant products and their phenolic content [7, 25]. In our case, the overall relationship between the antioxidant activities by ORAC and TEAC and total phenolic content for the five tested medicinal plants was a positive and highly significant linear correlation ($R^2=0.9039$, $R^2=0.9055$, respectively) (fig. 4A). The correlation coefficients for the antioxidant activities by ORAC and TEAC and flavonoid content of the medicinal plants were also calculated ($R^2=0.9755$, $R^2=0.9742$, respectively) (fig. 4B).

The obtained data showed that *A. absinthium* and *A. millefolium* extracts which contain large amounts of phenolic and flavonoid compounds exhibit a higher total antioxidant capacity. Our studies are in accordance with other papers which observed that polyphenolic compounds are very important plant constituents because their hydroxyl groups confer free radicals scavenging ability [1, 26, 27].

Conclusions

The comparative study on antioxidant capacity of five plant extracts (*U. dioica*, *A. absinthium*, *A. montana*, *A. millefolium* and *H. purpurascens*) used in Romanian folk medicine was achieved by two methods: ORAC (oxygen radical absorbance capacity) and TEAC (Trolox equivalent antioxidative capacity). The obtained results demonstrated that all studied plant extracts showed a high ability to scavenge the free radicals comparative with the standard (Trolox). The TEAC values of the studied plants were slightly

lower than the ORAC values, but we found good correlation between the antioxidant capacity results obtained with the TEAC method and the ORAC method ($R^2=0.9083$).

In this paper, we studied the relationship between the antioxidant activities of plant extracts and their total phenolic and flavonoid contents, quantitatively determined by spectrophotometric methods. The obtained data showed good correlation between the antioxidant activities by ORAC and TEAC assays and phenolic content ($R^2=0.9039$, $R^2=0.9055$, respectively) and, also, between the antioxidant activities by ORAC and TEAC assays and flavonoid content ($R^2=0.9755$, $R^2=0.9742$, respectively). Among assayed plant extracts, *A. absinthium* and *A. millefolium* which contained large amounts of phenolic and flavonoid compounds exhibited the highest total antioxidant capacity.

In conclusion, our results showed that the polyphenolic compounds significantly contributed to the antioxidant capacity of the medicinal plants and they may represent an alternative to allopathic medicine.

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