ANTIOXIDANT POTENTIAL OF SOME PLANTS USED IN FOLK MEDICINE IN ROMANIA

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Abstract

The aim of the study was to analyze the antioxidant activity of *Ajuga reptans*, *Anthyllis vulneraria*, *Impatiens noli-tangere*, *Lilium candidum*, *Ononis arvensis*, *Plantago media*, *Rhinanthus serotinus*, *Thymus serpyllum*, and *Veronica beccabunga* used in ethnomedicine in Romania. *In vitro* antioxidant assays were performed with 2,2-diphenyl-1-picrylhydrazyl (DPPH), modified chemiluminescence and oxygen radical absorbance capacity (ORAC) assays on the ethnomedicinally used parts of the species. The 50% ethanolic extracts of plants exerted a similar radical-scavenging effect measured by DPPH and chemiluminescence assays ($R^2 = 0.8811$). However, considerably higher values were measured by the ORAC method and these data did not correlate with the results of the chemiluminescence and the DPPH assays. The studied vegetal products from *L. candidum*, *P. media*, *Rh. serotinus*, *Th. serpyllum*, and *V. beccabunga* showed the highest total antioxidant capacity values by all used methods, while *I. noli-tangere* expressed the lowest antioxidant capacity. The study highlights the promising phytochemical values of ethnomedicinally used plants from Romania.

Rezumat

Scopul acestui studiu a fost analiza activității antioxidante asupra *Ajuga reptans*, *Anthyllis vulneraria*, *Impatiens noli-tangere*, *Lilium candidum*, *Ononis arvensis*, *Plantago media*, *Rhinanthus serotinus*, *Thymus serpyllum*, și *Veronica beccabunga*, utilizate în România, în medicina populară. Statusul antioxidant, *in vitro* a fost evaluat prin metoda 2,2-difenil-1-picirilhidrazil (DPPH), chemiluminiscență modificată și prin metoda ORAC (*oxygen radical absorbance capacity*) asupra produselor vegetale a acestor plante utilizate în etnomedicină. Extractele etanolicе 50% au exercitat o activitate antioxidantă asemănătoare, determinată atât cu DPPH cât și prin chemiluminiscență ($R^2 = 0.8536$). Prin metoda ORAC s-au obținut valori ridicate, care nu s-au corelat cu rezultatele obținute prin chemiluminiscență sau DPPH. Produsele vegetale studiate provenind de la *L. candidum*, *P. media*, *Rh. serotinus*, *Th. serpyllum*, și *V. beccabunga* au prezentat valori ridicate ale capacității antioxidante prin fiecare metodă, în timp ce *I. noli-tangere* a prezentat valori scăzute. Aceste studii subliniază valorile fitochimice promițătoare ale plantelor utilizate în medicina populară, în România.

Keywords: chemiluminescence, DPPH, ethnomedicine, ORAC

Introduction

The traditional ethnomedicinal knowledge on plant use relies mostly on the archaic folklore in Romania. The ethnobotanical fieldworks having been done in several regions since the 60s involve the vernacular names, home treatments, and special terminology of plants applied in the local human and veterinary medicine. Based on these earlier works, ethnobotanical field trips were carried out in various geographical regions from Romania, since 2008. Our collected data were compared with scientific databases to select species for antioxidant activity studies.

In the present work, nine plants were selected and evaluated based on their frequent use in the local

ethnomedicine in Romania, and on phytochemical and pharmacological data reported earlier. *Ajuga reptans* L. (*Lamiaceae*) is a perennial plant living in humid fields [48]. It is known as "carpenter's herb" due to its supposed ability of stem bleeding [23]. The plant contains e.g. anthocyanins [46], ecdysteroids [49], phenylpropanoid glycosides [15], and iridoids [36]. Traditionally, the leaves are widely used for wounds healing and for respiratory disorders in Austria [50], and also for their anti-inflammatory effect tested in colitis [15].

Anthyllis vulneraria L. or commonly named kidney vetch (*Fabaceae*) occurs in dry meadows, sunny pastures, and roadsides mostly on calcareous soil [48]. Flowers and leaves contain flavonoids,

saponins [34], polyphenols and tannins, while the leaf is rich in proanthocyanidins [18]. The herb is traditionally used for inflammatory disorders, acne, and wounds [34].

Impatiens noli-tangere L. or touch-me-not balsam (*Balsaminaceae*) is a 20-180 cm high annual plant living in damp and shady habitats [48]. The aerial part of the species contains e.g. flavonoids (kaempferol, quercetin, rutin), coumarins (scopoletin), dieicosyl ether, and α -spinasterol-3-O- β -D-glucopyranoside [9].

Lilium candidum L. or white lily (*Liliaceae*), also found as a popular cultivated plant, spontaneously grows in the fields. The white flowers contain flavonoids (kaempferol and its derivatives), linaline, jatrophine, and carotenoids. The fully open flowers are used as juice, ointments, and tinctures [7]. The bulb and the flowers can be used internally as astringent, demulcent, emmenagogue, emollient and expectorant, and externally as a poultice to tumours [21]. Its pollen grains have been used in the treatment of epilepsy [10].

Ononis arvensis L., syn.: *O. hircina* Jacq. (field restharrow, *Fabaceae*) is a 50-100 cm high perennial plant which occurs in humid grasslands and pastures [25, 48]. The aerial part contains essential oils [12], flavonoids [27, 43], hydroxyl-cinnamic acids [42], isoflavons, α -onocerin, scopoletin, and scopolin [40, 44], phenolic acids, flavonols, flavones, and flavanones [13]. The herb was described as a remedy for typhus and hernia [6], and as an aphrodisiac [6, 8].

Plantago media L. or hoary plantain (*Plantaginaceae*) is a perennial species living in dry grasslands [48]. It contains e.g. flavonoids [2] and iridoids [20]. The plant has an antitumor and antioxidant effect [20, 28].

Rhinanthus serotinus (Schönh.) Oborny (greater yellow-rattle, *Scrophulariaceae*) is a 40-60 cm high annual species. It can be found in grasslands and humid fields [25]. The plant contains aucubin and other iridoide glycosides [22], phenolic acids, flavons, flavonols, and flavanones, as well [13].

Thymus serpyllum L. (wild thyme, *Lamiaceae*) is a 2-10 cm high perennial species occurring in grasslands of sandy soil [25]. The aerial part contains essential oils (including mostly thymol and carvacrol), phenolic acids, flavonols, flavonones, and flavones [4, 5, 31]. The plant is officially described in the 8th Hungarian Pharmacopoeia [24] and in the 7th European Pharmacopoeia [17]. The aerial part has been used for its bronchospasmolytic, expectorant, and antimicrobial effect for a long time [16].

Veronica beccabunga L. or European speedwell (*Scrophulariaceae*) living at the edge of streams and rivers reaches 5-50 cm height [48]. The aerial part contains flavonoid aglycones such as apigenin [35]. The herb is traditionally used as a laxative and appetizing drug [45], for wounds, rashes [26], burns [39], and against pneumonia [38].

Several studies have been carried out on the phytochemical and pharmacological data, as well as on the antioxidant potential of plants used ethnomedicinally in various regions of the world up to the present [1, 41]. Considering the earlier reported ethnomedicinal and phytochemical data on the mentioned nine species, the aim of this study was to assess the antioxidant capacity of their different parts used in local ethnomedicine in Romania.

Materials and Methods

Materials

Plant samples

The selected nine species were collected in 2013 - 2014 with a special emphasis on their communicated collection time and parts used in ethnomedicine (Table I). Voucher specimens with unique codes were deposited at the Department of Pharmacognosy, University of Pécs, Hungary. The plant samples were dried at room temperature, ground and stored in the dark.

Table I
Collected parts, harvesting places and times of the selected plants

Plant taxa	Voucher code	Collected part	Harvesting place coordinates (Latitude/Longitude)	Harvesting time
<i>Ajuga reptans</i>	T02_AR	Aerial part	grassland 47°55'00"/23°05'00"	July 2013
<i>Anthyllis vulneraria</i>	L04_AV	Aerial part	grassland 46°16'27"/25°29'15"	August 2013
<i>Impatiens noli-tangere</i>	M02_INT	Aerial part	edge of beech forest 46°13'59"/25°27'21"	July 2014
<i>Lilium candidum</i>	T01_LC	Leaf, flower	garden 47°55'00"/23°05'00"	July 2013
<i>Ononis arvensis</i>	P05_OA	Aerial part	hayfield 46°10'37"/25°22'36"	July 2013
<i>Plantago media</i>	M03_PM	Leaf	grassland 46°13'59"/25°27'21"	July 2013

Plant taxa	Voucher code	Collected part	Harvesting place coordinates (Latitude/Longitude)	Harvesting time
<i>Rhinanthus serotinus</i>	M04_RS	Aerial part	grassland 46°13'59"/25°27'21"	July 2013
<i>Thymus serpyllum</i>	PS05_TS	Aerial part	grassland 46°10'37"/25°22'36", 46°11'29"/25°22'56", 46°14'00"/25°23'00"	July 2013 and 2014
<i>Veronica beccabunga</i>	M06_VB	Aerial part	edge of stream 46°13'59"/25°27'21"	July 2013

Data analysis

The data collected were compared with ethnomedicinal and phytochemical studies of major databases (PubMed, Science Direct, and Scopus). During the comparison, similarities and differences of the records were taken into consideration.

Extraction of plant samples

0.25 g of each plant sample was ground, then 5 mL of 50% ethanol was added. The solutions were shaken at room temperature for 30 minutes (200 rpm). The extracts were filtered through a 0.45 µm pore-size filter (Mini-Uniprep, Whatman), and were stored at -20°C until performing the analyses.

Chemicals and reagents

The antioxidant capacity of each studied plant extract was determined by three different antioxidant assays: a modified enhanced chemiluminescence (ECL) based antioxidant assay described by Muller *et al* [33], the 1,1-Diphenyl-2-picryl-hydrazyl (DPPH stable free radical) method [30, 32], and a modified oxygen radical absorbance capacity (ORAC) assay [11, 19]. All chemicals were of analytical grade and highly purified water (ion exchange + reverse osmosis, < 1µS) was used throughout the experiments.

Chemiluminescence-based antioxidant assay

The method is based on the antioxidant capacity-dependent inhibition of enhanced chemiluminescence (ECL) of luminol triggered by the action of peroxidase (POD) enzyme. The following chemicals were used in our experiments: POD from horseradish (Sigma-Aldrich), 50 mM phosphate buffer pH 7.4 containing 1 mg/mL bovine serum albumin (BSA, Serva), H₂O₂ (Molar Chemicals), citric acid (Ph.Hg. Eur), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich), luminol and para-iodophenol (Sigma-Aldrich). For the assay, POD and ECL working solutions were prepared separately and the reaction was triggered by the addition of H₂O₂ in the presence of Trolox standards and/or plant extracts.

Peroxidase and H₂O₂ working solutions: POD stock (in phosphate buffer saline (PBS)) was diluted with the BSA containing phosphate buffer and was kept on ice (15 µU/mL). 10 M H₂O₂ was

diluted with 0.1% citric acid to reach a concentration of 1360 µM and it was also kept on ice protected from light. The reagents were always prepared freshly prior to measurements.

ECL reagent: Luminol and p-iodophenol were prepared in 0.2 M boric acid/NaOH buffer, pH 9.6 and kept at 4°C (being stable for at least 1 month).

Trolox standard: 1 mM Trolox (dissolved in 50% ethanol) was prepared freshly every week and Trolox standards were diluted with the same solution which was used for the samples.

ECL antioxidant assay: The reaction was adapted to a plate reader using 96-well white optical plates (Perkin-Elmer). The enzyme working solution and the detection reagent were premixed in a ratio of 200 µL POD to 70 µL ECL reagent. Into each well, 20 µL Trolox/blank/sample and 270 µL of POD-ECL reagent were pipetted and the reaction was initiated by automated injection of 20 µL ice-cold H₂O₂ in citric acid. The final concentrations of the components in the wells were as follows: 0.97 µU/mL POD, 101.6 µM luminol, 406.4 µM p-iodophenol, 88 µM H₂O₂. Chemiluminescence signal was monitored for 20 min at 64 s intervals.

DPPH assay

DPPH, methanol and Trolox were obtained from Sigma-Aldrich. For the assay, 4 mg DPPH in 100 mL methanol (0.1 mmol/L) was prepared and kept in the fridge. Trolox standards were prepared in 50% ethanol. The assay was also adapted to a plate reader using standard 96-well plates (Sarstedt). Into each well, 20 µL Trolox/blank/sample and 180 µL DPPH solution were pipetted and the absorbance was read at 517 nm after 30 minutes of incubation in the dark at 25°C.

ORAC assay

The method of Gillespie *et al* [19] was adapted to our laboratory conditions: the concentration of Na-fluorescein was reduced and the concentration of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was enhanced. 4 µM Na-fluorescein (FL) stock was prepared in high purity water (stable for 1 week at 4°C). The working FL solution was made freshly by diluting the stock with 75 mM K-phosphate buffer (pH 7.5) at a 1:99 ratio (40 nmol/L FL working concentration). AAPH was also

prepared before the measurements in phosphate buffer (9.22 mM). Trolox standards were used as described above. Into each well of black optical plates (Perkin-Elmer), 25 μ L of blank/standard/sample and 150 μ L of diluted FL were pipetted and the plates were preheated to 37°C for 20 min. The outer wells of the plates were filled with 200 μ L phosphate buffer, and only the inner 6 x 10 matrix was used for the assay. The reaction was initiated by automated injection of 25 μ L AAPH solution into each well and fluorescence intensities were immediately monitored for 80 min (490/520 nm) at 150 s intervals. The final concentrations of the components in the wells were as follows: FL 30 nM, AAPH 1.15 mM [11, 19].

Equipments and interpretation of data

The chemiluminescence-based antioxidant assay was performed using a Biotek Synergy HT plate reader equipped with programmable injectors. At each luminescence reading the measuring time/well was 0.2 s and the light output was followed for 20 min at 64 s measuring intervals. For quantification of the data, a calibration range of 0 - 150 μ M Trolox was prepared in 50% ethanol and a 32-fold dilution of the plant extracts was made in 50% ethanol (n = 12 replicates for each sample). The antioxidant capacity of the plant extracts was calculated from the regression equation obtained for the standards, multiplied by the dilution factor and expressed as μ M Trolox equivalent (TE). TE was then referred to 1 g of initial dry material for each plant.

The DPPH assay was performed by a Perkin-Elmer EnSpire Multimode reader. Trolox standards were used in 50% ethanol in the range of 0 - 200 μ M. Antioxidant capacities were calculated in two ways: a) using the equation of the calibration line; b) expressing the antioxidant activity of the extracts in % by the formula: $(A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100$ [29]. TE values were also calculated for 1 g of dried plant.

For the ORAC assay fluorescence intensities were monitored for 80 min at 150 s sampling intervals using the Biotek Synergy HT plate reader. The area under the curve (AUC) was determined for the blank and this light output was subtracted from each standards/samples. In this way, net AUC (nAUC) was used for quantification of antioxidant capacity.

Results and Discussion

Antioxidant capacity of the studied plants

Antioxidant capacity tests

There are a lot of approaches in the literature to measure the antioxidant capacity of plant origin samples. The principles of different assays are adequately described, however, working with a

complex mixture of diverse nature of antioxidants can give only a partial view of the action of the components in the sample. In order to make narrower the wide spectrum of antioxidant molecules one can use different extraction methods (polar and apolar as well). The usage of 50% ethanol is a widely applied technique with the advantage of getting both lipid- and water soluble molecules on the one hand, but with the disadvantage of getting a very complex mixture of antioxidants on the other. The extracts are poor in proteins, which is favourable for having low background signals when plant peroxidases might be involved in the reaction.

In our experiments, 50% ethanolic extracts were studied by three assays: the widely accepted DPPH free radical assay, and a modified peroxidase-based enhanced chemiluminescence method and an ORAC assay. Each method was adapted to microplate readers to enhance the throughput and reliability of the tests and to make the application of sufficient number of replicates in the analyses easy. In the ECL assay, we stabilized the light emission of the enhanced chemiluminescence emission by protecting the POD enzyme from auto-oxidation with a considerably high (1 mg/mL) albumin concentration in the buffer during the light emission process. Our antioxidant CL assay reagent with p-iodophenol enhancer gave an almost constant light signal for 20 minutes with less than 10% decrease in the light output during the measuring period. The top light signals of the blanks were in the range of 1.3 to 1.4 x 10⁶ photons/s (cps), which made the measurements easy and precise. Trolox was used as a standard, and the antioxidant activity was calculated based on the total light output during the measurement by integration of the light emission curve (area under curve, AUC). We also evaluated the data by subtracting the AUC levels of the samples from that of the blank that was suggested by Muller et al. [33], but it did not provide any advantages. Using a calibration range of 0 to 150 μ M Trolox, a good linearity was observed (typically $R^2 = 0.999$) with a limit of detection (LOD) of 8.0 μ M Trolox.

ECL vs. DPPH and ORAC assay of the studied plant extracts

Neither the solvents for extraction, nor the plant samples gave significant background signals in the applied tests (<< 1%). On the other hand, in the ECL assay all plants exerted antioxidant activity while in the DPPH assay *Impatiens noli-tangere* showed a much less antioxidant capacity (around the detection limit). All the other studied plants were active in both test systems with a close correlation between the ECL and DPPH groups.

In the analysis of total antioxidant capacity (TAC) of 9 plant species by ECL and DPPH assays (n = 12

replicates), a comparison of the two methods by linear regression approach gave the following equation: $y = 0.5997x + 68.452$, $R^2 = 0.8811$ where y is the ECL, while x is the DPPH method. Students' t -test gave a p value of 0.195 which means that there are no significant differences between the ECL and the DPPH groups.

In the literature, DPPH data are usually interpreted as the decolouration activity of the samples/standards expressed in percentage of the blank (I/IC_{50}) [14].

However, besides the percentage TAC evaluation of our samples, for a better comparison of the two methods, we also calculated the Trolox equivalents (TE) in the DPPH assay represented in Table II. In our experimental conditions, the IC_{50} value for Trolox was $16.77 \mu\text{M}$. The DPPH scavenging activity of the examined plant extracts is shown in Figure 1.

Figure 1.

Antioxidant capacity of 9 tested plants from Romania using the DPPH assay ($n = 12$ replicates, mean $\pm$ SD) expressed as scavenging activity (%)

Table II shows the analysis of the total antioxidant capacity (TAC) of the 9 studied plant species by DPPH, ECL and ORAC assays ($n = 12$ each). The comparison of the ECL and DPPH methods by

linear regression approach gave a fair correlation as described above. However, the ORAC assay did not show any correlation with the other two assays (mean $\pm$ SD) and gave much higher TAC values.

Table II

TAC analysis of the 9 studied plant species by 3 different antioxidant assays

Investigated plant species	DPPH	ECL	ORAC
	TE/g dried plant ($\mu\text{mol/g}$)	TE/g dried plant ($\mu\text{mol/g}$)	TE/g dried plant ($\mu\text{mol/g}$)
	mean $\pm$ SD	mean $\pm$ SD	mean $\pm$ SD
<i>Ajuga reptans</i>	101.00 ± 8.45	137.30 ± 2.19	907.90 ± 72.36
<i>Anthyllis vulneraria</i>	26.41 ± 2.61	73.46 ± 6.49	348.90 ± 25.17
<i>Impatiens noli-tangere</i>	detection limit	52.78 ± 4.26	116.30 ± 11.31
<i>Lilium candidum/tepals</i>	39.18 ± 4.43	86.97 ± 4.47	1800.00 ± 125.30
<i>Lilium candidum/leaf</i>	54.73 ± 3.92	117.50 ± 3.76	1142.00 ± 93.85
<i>Ononis arvensis</i>	79.06 ± 7.00	145.10 ± 1.40	961.80 ± 64.94
<i>Plantago media</i>	155.00 ± 0.99	161.40 ± 0.45	1274.00 ± 18.44
<i>Rhinanthus serotinus</i>	156.50 ± 4.51	155.30 ± 0.72	1230.00 ± 79.53
<i>Thymus serpyllum</i>	165.50 ± 1.73	161.40 ± 0.08	958.60 ± 77.61
<i>Veronica beccabunga</i>	167.10 ± 14.95	160.30 ± 0.18	473.60 ± 43.48

When dealing with antioxidant assays there is a great choice among different tests but basically two types of mechanisms are involved and measured: hydrogen atom transfer (HAT) assay and singlet electron transfer assay (SET). These tests are called non-enzymatic total antioxidant activity

measurements because they are based on the formation of oxygen radicals and their scavenging by non-proteinaceous substances. The widely used oxygen radical absorbance capacity (ORAC) method as a HAT type procedure is simple, however, having the disadvantage that it is strongly

temperature dependent, which makes microtiter adaptations uncertain [37]. Our modified ECL method also belongs to the HAT group. The test is criticized for giving a low level of luminescence and a potential ability of improper restoration of light emission after exhaustion of antioxidants present in the sample. In our case, the light output was in the range of 10^6 cps and there was a complete restoration of the light signal after the lag period due to the antioxidant activity. The DPPH method belongs to the SET group but in certain conditions hydrogen atom transfer mechanisms are also involved. The test is simple but there are many factors influencing the results and making the interpretation difficult. Small molecules might exert a higher antioxidant action in this assay because of an easier access to the DPPH moiety. The sensitivity of the method is less than those of other assays (e.g. our ECL assay). In this study, we calculated the linearity of the DPPH assay by Trolox standardization receiving a linear response between 10 - 200 μ M Trolox concentrations ($R^2 = 0.9971$). These data can be converted to an inhibitory capacity of 1 - 79%. Therefore, we suggest the usage of TE values for the DPPH assay as well, besides the IC_{50} values.

In our case, all of the studied plant species showed antioxidant capacity by ECL, DPPH and ORAC assays. The applied assays gave comparable data (except those for the ORAC method) however, all the used techniques gave information on the antioxidant activity of the compounds in the extracts. Phenolic compounds have been identified in the selected species [2, 4, 5, 7, 9, 13, 15, 28, 31, 34, 35, 43], therefore, it seems to be highly probable that these molecules are at least partially responsible for the measured antioxidant activity. In our future studies the plant samples will be investigated by chromatographic methods to identify the active metabolites responsible for the antioxidant capacity of the species.

Conclusions

Medicinal herbs are very complex in composition, therefore, an initial analysis of their total antioxidant activities might be the first step in deciding their potential use in complementary medicine. In the present work, a modified ECL, ORAC and the classic DPPH assays were applied for TAC analyses of the investigated plant extracts. ECL and DPPH methods gave similar results for the studied plants except in the case of *Anthyllis vulneraria*, *Impatiens noli-tangere*, *Lilium candidum*, and *Ononis arvensis*. On the other hand, both studied parts of *Lilium candidum*, and the herb of *Ajuga reptans*, *Ononis arvensis*, *Plantago media*, *Rhinanthus serotinus*, *Thymus serpyllum* and

Veronica beccabunga showed the highest TAC values in the ECL assay, while the ORAC method gave about 5-10 times higher TAC levels, except for *Anthyllis vulneraria*, *Impatiens noli-tangere*, and *Veronica beccabunga*. It should be noted that the Trolox equivalent data calculated from the calibration line were basically in good agreement with the inhibitory capacity (as %) based on the DPPH method. Therefore, it is suggested that the evaluation of the DPPH technique is complete when both the % inhibitory capacity and the Trolox equivalents are evaluated simultaneously. The correlation between the antioxidant activity and the concentration of distribution of phenolic compounds should be further investigated to determine the responsible ingredients in the studied species. The systematic documentation and evaluation of our data can serve as a new source for finding antioxidant plants and for isolation of bioactive compounds which can be used in further phytochemical investigations.

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