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EVALUATION OF POLYPHENOLIC PROFILE AND ANTIOXIDANT ACTIVITY OF SOME SPECIES CULTIVATED IN THE REPUBLIC OF MOLDOVA

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Abstract: *The aim of this study was to assess the polyphenolic composition and antioxidant capacity of the medicinal plants from the collection of the Scientific Center of Medicinal Plants Cultivation of Nicolae Testemitanu State University of Medicine and Pharmacy: agrimony - Agrimonia eupatoria L. (Rosaceae), chicory - Cichorium intybus L. (Asteraceae.), artichoke - Cynara scolymus L. (Asteraceae) and St John's-wort - Hypericum perforatum L. (Hypericaceae). A qualitative and quantitative characterization of the main phenolic compounds from ethanolic extracts was carried out by HPLC method. The total polyphenolic and flavonoid contents were measured by spectrophotometric methods. The in vitro antioxidant activity of these extracts was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity (TEAC), 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical cation scavenging and metal chelating activity. Considering the obtained results, the following order in antioxidant activities was established: A. eupatoria> C. inthybus> H. perforatum> C. scolymus. All species contain significant amounts of polyphenolic compounds and they may be considered an important source of natural antioxidants for pharmaceutical uses.*

Keywords : *plant extracts, polyphenols, flavonoids, antioxidant*

INTRODUCTION

Interest in natural antioxidants, especially those derived from medicinal plants, has increased in recent years. Phenols represent a class of mainly natural compounds and are characterized by the presence of multiples of phenol structural units. The scientific studies revealed that use of polyphenols-rich vegetable products is correlated with the increased longevity and decreased incidence of cardiovascular diseases. In addition to their antioxidant properties, polyphenols have been reported to exhibit other multiple biological effects, such as, antiviral, antibacterial, anti-inflammatory, vasodilatory, anticancer and antiischemic [3]. Nowadays, interest in the possible health benefits of polyphenol compounds has increased owing most of all to their potent antioxidant and free radical scavenging activities observed in vitro. Synergistic effects of the polyphenolic compounds as mixtures in preventing human LDL oxidation may reflect that advantage are found in the uses of a polyphenols-rich vegetable products in preventing LDL oxidation and perhaps a host of cardiovascular diseases. Interest in natural antioxidants, especially those derived from medicinal plants, has increased in recent years, including through multiple studies and publications [5].

Scientific Center of Medicinal Plants Cultivation (SCMPC) of Nicolae Testemitanu State University of Medicine and Pharmacy (SUMPh) from the Republic of Moldova show a great interest in research of medicinal plants rich in polyphenolic compounds. The ongoing project of research include the study of plants: agrimony - *Agrimonia eupatoria* L. (Rosaceae), chicory - *Cichorium intybus* L. (Asteraceae),

artichoke - *Cynara scolymus* L. (Asteraceae) and St John's-wort - *Hypericum perforatum* L. (Hypericaceae).

Agrimonia eupatoria L. has a long tradition of folk use for a variety of conditions: liver, gastrointestinal, bile duct, and pulmonary diseases, edemas, obesity and diabetes [15]. Agrimony is considered a good regenerator when given as an infusion or decoct, and it can be used in liver pathologies, alone or (more often) in combination with other medicinal plants. A number of non-clinical studies reported various activities for its extracts: antibacterial and antiviral, hepatoprotective, neuroprotective, and anti-inflammatory through a variety of cytokine and other signaling molecule regulation [6]. Tannins, phenolic acids, triterpenoids, flavonoids (rutin, quercetin, kaempferol, luteolin, and apigenin), essential oils, vitamin K were identified in the herbal product from this species [23].

Cichorium intybus L. has been used in food as salads, its roots have been employed as a coffee substitute, and all its parts have been attributed a variety of potential health benefits: anti-inflammatory, hypolipidemic, gastroprotective, analgesic, antidiabetic. It is claimed that its active components stimulate digestion, detoxify the body, decrease the level of cholesterol and glucose in blood, and because they are anorexic and laxative, chicory is indicated in obesity [2, 16]. The aerial parts of chicory (*Cichorii herba)* contain cichoriin, arginine, choline, chicoric acid (dicaffeoyl tartaric acid), bitter principles, and microelements: Fe, P, Ca. The plant also serves as a source of vitamins: A, C, E, K, PP and flavonoids. The roots are rich in bitter triterpenic substances, fructose, tannins and volatile oil [9, 22].

Cynara scolymus L. is a perennial thistle plant which cultivation dates back to ancient Greece and Rome [25]. Artichoke has been used in traditional medicine for centuries all over Europe as a specific liver and gallbladder remedy as well for high cholesterol and digestive disorders [7]. Artichoke leaves (*Cynarae folia)* has shown cholesterol-lowering and lipid-lowering activity in rats and humans [19]. Chemical components, mainly of polyphenolic nature (caffeic acid, chlorogenic acid and cynarin), contained in artichokes, are very important substances for human nutrition since they are involved in the prevention of cancer, cardiovascular diseases; osteoporosis, diabetes mellitus and neurodegenerative diseases [4, 17].

Hypericum perforatum L. contains condensed anthracene derivatives (hypericin), accompanied by isomers. Also contains polyphenols, tannins, flavonoids, amino acids, carotenoids, vitamin C, choline, resins, mineral substances, prenilfloroglucinols (hyperphorine). Volatile oil consisted of 1% (α- pinen, cineol, cadinen, mircen, cariophenylene). *Hyperici herba* exhibits psychotropic, euphoric, sedative, anxiolytic action, can be administered in depressive disorders by hypericin. Flavanoids increase capillary resistance and permeability, it is collagogue and hepatoprotective through polyphenols. By tannins it is widely used as anti-inflammatory and astringent, hyperforin determine bacteriostatic and bactericid action. Hypericin and its isomers exhibit a photodynamic effect (hypericism) [1, 24].

One of the current directions of development of the pharmaceutical industry is the obtaining and uses of extracts from medicinal plants containing polyphenols, therefore the SCMPC of Nicolae Testemitanu SUMPh researches plants as a potential source of local raw material for natural medicinal formulations. For these reasons, the aim of the present study was to determine the antioxidant activities of plants cultivated in the pedo-climatic condition of Republic of Moldova by using different antioxidant tests including total phenolic compound, $DPPH^{\cdot}$ free radical scavenging, $ABTS^{\cdot+}$ radical cation scavenging and metal chelating activity.

MATERIALS AND METHODS

A. Chemicals and Reagents

The chemicals used in the present work were purchased from various companies: Folin-Ciocalteu reagent, gallic acid, hyperoside, chlorogenic, caffeic and ferulic acids, apigenin, biapigenin, quercetin, quercitrin, DPPH (2,2-diphenyl-1- picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Trolox, HPLC solvents were purchased from Sigma-Aldrich (Germany); rutin and luteolin were purchased from Merck (Germany); EDTA, TPTZ (2,4,6-tris(2-pyridyl)-striazine) from HiMedia Laboratories (India); ethanol used were from Luxfarmol Ltd. (Moldova). All other chemicals were of analytical reagent grade.

B. Plant material

The aerial parts of the *A. eupatoria*, *H. perforatum*, *C. inthybus* and leaves of *C. scolymus* were collected from the SCMPC of Nicolae Testemitanu SUMPh, according to the pharmaceutical monograph recommendations. In order to be used for extraction, the dry plant materials were grinded with a laboratory mill to a fine powder. A Soxhlet apparatus was used for obtaining hydro-alcoholic extracts.

The solvent used was ethanol 70%, and the ratio between the vegetal material and the solvent was 1:10, the resulting solutions were kept at 4°C, in dark sealed bottles until further use in the antioxidant studies.

C. HPLC Analysis

For the identification and qualitative determination of phenolic compounds, Agilent Technologies 1200 series liquid chromatograph, thermostat-TCC G 13116A series DE 63063824, detector-DAD G 1315B series DE 43603042, manual injector-P / N 7725i, degasser G 1322A series YP 62359783 were used. Working conditions: analytical column Zorbax Eclipse XBD-C 8 250 mm x 4.6 mm, 5 µm, type collar precolumn; mobile phase: acetonitrile: water (30:70), with the addition of glacial acetic acid 1%; flow rate 1 ml/min; temperature: 40 °C; injection volume 20 µl, detection: 360 nm ultraviolet. There were used as reference standards the following phenolic compounds:chlorogenic acid, caffeic acid, luteolin, hyperoside, ferulic acid, apigenin, biapigenin, rutin, quercetin, quercitrin. Calibration curves in the 0.5-50 µg/mL range with good linearity (R2 > 0.999) for a five point plot were used. The chromatographic data were processed using Data Analysis software from Agilent.

D. Determination of Total Phenolic Content (TPC)

The total phenolic content of the *A. eupatoria*, *C. inthybus, C. scolymus, H. perforatum* extracts was determined spectrophotometrically according to the Folin-Ciocalteu method [10, 21]. using galic acid as a standard. In brief, the reaction mixture was prepared by mixing extracts with Folin-Ciocalteu reagent and solution of 7% sodium carbonate. After the 90 minutes incubation at room temperature, in the dark, the absorbance was determined spectrophotometrically at 760 nm. The TPC was determined from extrapolation of calibration curve which was made by preparing gallic acid solution. The estimation of the phenolic compounds was carried out in triplicate. The TPC was expressed as milligrams of gallic acid equivalents (GAE) per ml extract.

E. Determination of Total Flavonoid Content (TFC)

The total flavonoid content was determined spectrophotometrically according to aluminium chloride colorimetric method

[13,14]. Each plant extracts were dissolved in methanol and mixed with 0.1 mL of 10% aluminium chloride hexahydrate, 0.1 mL of 1 M potassium acetate and 2.8 mL of deionized water. After the 40 minutes incubation at the room temperature, the absorbance of the reaction mixture was determined spectrophotometrically at 415 nm. Rutin was chosen as a standard (the concentration range: 0.05 to 0.1 mg/mL) and the total flavonoid content was expressed as milligram RE per g of dry extracts by extrapolation on calibration curve (R2 = 0.999).

F. DPPH radical scavenging activity assay

The free radical scavenging activity of the fractions was measured *in vitro* by 2,2′- diphenyl-1-picrylhydrazyl (DPPH) assay [11, 12]. The stock solution was prepared by dissolving 24 mg DPPH with 100 ml methanol and stored at 20°C until required. The working solution was obtained by diluting DPPH solution with methanol to obttain an absorbance of about 0.98±0.02 at 517 nm using the spectrophotometer. A 3 ml aliquot of this solution was mixed with 100 µl of the sample at various concentrations (10 - 500 µg/ml). The reaction mixture was shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The control was prepared as above without any sample. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the following equation (1):

$$AA\ DPPH\ (\%) = Acontrol - A\ Sample\ /\ A\ Control * 100 \qquad (1)$$

where, $A_{control}$ is the optical density of the control (containing all reagents except for the extract) and A_{sample} is the optical density in the presence of the extract. The extract concentration providing 50% of free radical scavenging activity (IC_{50}) was calculated from the graph of the radical scavenging activity percentage against extract concentration.

G. ABTS radical scavenging activity

The 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid), commonly called ABTS cation scavenging activity was performed [18]. ABTS solution (7 mM) was reacted with potassium persulfate (2.45 mM) solution and kept for overnight in the dark to yield a dark coloured solution containing ABTS radical cations. The ABTS radical cation was diluted with 50% methanol for an initial absorbance of about 0.70±0.02 at 745 nm, with temperature control set at 30°C. Free radical scavenging activity was assessed

by mixing 300 µl of test sample with 3.0 ml of ABTS working standard in a microcuvette. The antioxidant capacity of test samples was expressed as IC_{50} (anti-radical activity), the concentration necessary for 50% reduction of ABTS.

H. Ferric Reducing Antioxidant Potential Assay (FRAP Assay)

Ferric reducing antioxidant potential (FRAP) of the extracts was evaluated according to the method proposed by Benzie and Strain [8]. Briefly, FRAP reagent was prepared by mixing in 25 mL acetate buffer (30 mM; pH 3.6), 2.5 mL TPTZ solution (10 mM) and 2.5 mL ferric chloride solution (20 mM). The mixture was incubated for 15 min at 37 °C before use. EDTA was employed as a standard in this assay, and its calibration curve was obtained by using its concentrations ranging from 50 mg/L to 500 mg/L in water. The results were reported as µg of EDTA equivalents (EDTAE) per g dry weight.

RESULTS AND DISCUSSION

A. Polyphenolic compounds analysis

The HPLC profile of the polyphenolic compounds (table I) revealed the presence of 3 phenolic compounds for *A. eupatoria* and for *C. inthybus* (quercetin, rutin and apigenin), 5 phenolic compounds for *C. scolymus* (luteolin, apigenin, caffeic, chlorogenic and ferulic acids) and 3 flavonoid glycosides (rutin, quercitrin, hyperoside), a flavonoidic aglycone (quercetin), one caffeoylquinic acid (chlorogenic acid) and a bioflavonoid (biapigenin) for *H. perforatum,* respectively. Rutin has been detected in highest amounts in *Agrimoniae herba* (19.3 mg/ml). Quercetin has been detected only in *A. eupatoria* (2.05 mg/ml), C. *inthybus* (1.84mg/ml) and in *H. perforatum* (5.35 mg/ml). Ferrulic acid have been identified in low concentration only in *C. scolymus* (0.1 mg/ml). The species with high amount of apigenin are *A. eupatoria* (2.05 mg/ml) and *C. inthybus* (1.84mg/ml), less content is revealed in *C. scolymus* (0.13 mg/ml).

TABLE 1: HPLC PHENOLIC PROFILES OF *A. EUPATORIA*, *C. INTHYBUS*, *C. SCOLYMUS* AND *H. PERFORATUM* EXTRACTS

Compound	Retention time (t_R)	Samples			
		Agrimoniae herba (mg/ml)	***Cicorii herba*** (mg/ml)	***Cynarae folia*** (mg/ml)	***Hyperici herba*** (mg/ml)
Rutin	2.4±0.002	19.3±0.001	3.05±0.004	—	17.63±0.023
Hyperozide	3.3±0.054	—	—	—	7.59±0.008
Quercitrin	3.5±0.002	—	—	—	2.54±0.0009
Quercetin	6.9±0.002	2.05±0.016	1.84±0.030	—	5.35±0.030
Biapigenin	13.7±0.009	—	—	—	2.77±0.018
Luteolin	29.1±0.19			2.1 ±0.005	—
Apigenin	33.1±0.17	4.62±0.012	5.02±0.003	0.13±0.001	—
Caffeicacid	5.6±0.07	—	—	1.35 ±0.16	—
Ferulic acid	12.8±0,1	—	—	0.14 ±0.001	—
Chlorogenic acid	5.60±05			5.02±0.006	0.3±0.030

*- not found, below limit of detection; results are presented as the mean of triplicate determinations ± standard deviation

The results of quantitative analysis regarding the content of total polyphenols and flavonoids are shown in table 2. The highest content of flavonoids is recorded in *Hyperici herba* (49.1±0.02 mg RE/g) and the lowest in *Cicorii herba (*16.9±0.042 mg RE/g). Regarding polyphenols the highest values were obtained in the case of *Agrimoniae herba* (124.5±0.032 GAE/g) and in decreasing content - *Cicorii herba* (109.0±0.240 GAE/g), *Hyperici herba* (62.4±0.018 GAE/g) *Cynarae folia* (52.6 ±0.103 GAE/g, respectively).

TABLE 2: TPC AND TFC IN A. EUPATORIA, C. INTHYBUS, C. SCOLYMUS, AND H. PERFORATUM EXTRACTS

Samples	TFC (mg RE/g dw)	TPC (mg GAE/g dw)
A. eupatoria	37.2±0.024	124.5±0.032
C. inthybus	16.9 ± 0.042	109.0±0.240
C. scolymus	28.1 ±0.1	52.6 ±0.103
H. perforatum	49.1 ± 0.02	62.4 ±0.018

*Results are presented as the mean of triplicate determinations ± standard deviation

B. Antioxidant activity assay

Antioxidant properties of medicinal plants *A. eupatoria*, *C. inthybus, C. scolymus* and *H. perforatum* were determined by three methods, DPPH, ABTS and FRAP. The results are shown in table 3.

The scavenging effect of plant extracts, as determined by DPPH method, is measured as percentage of inhibition (%) of DPPH radical, and the antioxidant capacity of extracts from ABTS measured as Trolox equivalents (TE) and FRAP method measured as EDTA equivalents. All 4 plant extracts were able to reduce the DPPH radical with different degrees of scavenging activity. The most pronounced antioxidant capacity was found for *Hyperici herba* (IC_{50} 19.08 µg/ml)**,** then followed by *Agrimoniae herba* (IC_{50} 45.55 µg/ml)*, Cicorii herba* (IC_{50} 90.79±0.04 µg/ml*)* and the lowest antioxidant capacity have shown *Cynarae folia* (IC_{50} 92.27 µg/ml). ABTS radical cation scavenging assay is another method widely used to evaluate the free radical scavenging potential of antioxidants. *A. eupatoria* and *C. scolymus* extracts proved to be efficient scavengers of ABTS radical cation, according to the IC_{50} values (59.18±0.30 mmol/L TEAC and 57.15±0.05 mmol/L TEAC, respectively), *H. perforatum* had the lowest activity among the other extracts (22.74±0.01 mmol/L TEAC).

TABLE 3: ANTIOXIDANT ACTIVITY OF A. EUPATORIA, C. INTHYBUS, C. SCOLYMUS AND H. PERFORATUM EXTRACTS

Samples	DPPH, IC_{50} µg/ml	ABTS, µM TE/g	FRAP, µM EDTAE/g
Agrimoniae herba	45.55±0.01	59.18±0.30	98.07 ±0.003
Cicorii herba	90.79 ±0.04	31.29±0.25	97.25 ±0.012
Cynarae folia	92.27 ±0.1	57.15±0.05	68.5±0.6
Hyperici herba	19.08±0.12	22.74±0.01	77.36 ±0.05
Trolox	5.02 ±0,008	-	-
EDTA	-	-	99.58

*- not determined, results are presented as the mean of triplicate determinations ± standard deviation

Phenolic compounds are secondary metabolites of plants and these compounds are generally playing an important role in chelating redox-active metal ions and scavenging free radicals. Therefore, in this study, the iron (II) chelating activity of ethanol extracts from plants was screened. *Agrimoniae herba* showed the highest iron (II) chelating activity (98.07**±**0.003 mMol EDTAE/g extract), whereas the *C. scolymus* leaves had the lowest activity among the other extracts (68.5±0.6 mMol EDTAE/g extract). Statistical evaluation has revealed a significant difference between methods of antioxidant determination, and using more methods in comporation represent a need for a proper evaluation. Accumulated evidences suggest that antioxidant activity of herbal products are correlated with the content chemical constituents mainly represented by polyphenols More aspects of this plants, as well as the activity and mechanisms of its action are to be revealed by future studies.

CONCLUSIONS

As a conclusion, in the present study there were evaluated the antioxidant activities and phenolic contents of 4 plant materials from the collection of SCMPC: *Agrimoniae herba, Cichorii herba, Cynarae folia* and *Hyperici herba.* All species contain significant amounts of polyphenols and may be considered an important local source of natural antioxidants for pharmaceutical uses.

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