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Free Radicals Scavenging and Antioxidant Activity of European Mistletoe (*Viscum album*) and European Birthwort (*Aristolochia clematitis*)

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In this study, ethanolic extracts obtained from European mistletoe (Viscum album) and European birthwort (Aristolochia clematitis) were investigated for total polyphenols and flavonoid contents, Fe^{2+} chelating ability and free radicals scavenging activity. Scavenging activity was studied for 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH·), superoxide anion, hydroxyl radical, hydrogen peroxide and nitric oxide. The obtained results indicate a higher content in polyphenols and flavonoids in case of birthwort extract comparatively to mistletoe extract. The polyphenols contained by the two alcoholic extracts have the ability to act as hydrogen donors and can chelate Fe^{2+}; hydrogen donating ability using DPPH· assay and Fe^{2+} chelating ability were found to be higher for birthwort extract. Also, the two vegetal extracts had a good scavenging activity against superoxide anion, hydroxyl radical and nitric oxide, while for hydrogen peroxide the activity was low. Generally, free radicals scavenging activity was superior in case of birthwort extract comparatively to mistletoe extract.

Keywords: free radicals, scavenging activity, antioxidant activity, European Mistletoe, European Birthwort

Oxidative stress, defined as an imbalance between oxidants and antioxidants in favor of oxidants, leads to multiple biochemical changes in animal and human organism, which are causative factors of several chronic diseases, such as cardiovascular diseases, mutagenesis and cancer, several neurodegenerative disorders and aging process [1]. Medicinal plants have been a readily available source of drugs since ancient times and even today almost 50% of the new drugs have been patterned after phytochemicals [2]. Recognizing the medicinal significance of indigenous plants, World Health Organization (WHO), in its 1997 guideline, states that "effective locally available plants can be used as substitutes for drugs" [1].

Plants have almost limitless ability to synthesize aromatic substances, most of which are phenols or their substituted derivatives [3-5]. Polyphenols exhibit several biological effects such as anti-inflammatory, anti-microbial, anti-carcinogenic, anti-HIV, cardio-protective and neuro-protective. Various classes of dietary polyphenols are under investigations for their anticancerous properties in order to design novel strategies for chemoprevention and chemotherapy [6]. For instance, studies have found that genistein, an isoflavone from soy, can inhibit the growth of various cancer cell lines including leukemia, lymphoma, prostate, breast, lung and neck cancer cells [7]. In addition to their potential as anticancerous agents, an important role of plant polyphenols as natural modulators of cancer multidrug resistance (MDR) has been realized recently [8, 9].

Viscum album is a species of mistletoes (*Santalaceae* or *Viscaceae* family), the species originally so-named and also known as European mistletoe or Common mistletoe to distinguish it from other related species. It is native to Europe and south-western Asia and it growths as semi-parasite on leafy and coniferous trees. Mistletoe provides medicinal material with a very wide spectrum of pharmacological activities; the most important in therapy is the anti-neoplastic activity [10-12].

Aristolochia clematitis, known as European birthwort, is an herbaceous plant in the *Aristolochiaceae* family, which is native to Europe. It was formerly used as a medicinal plant (though poisonous), but nowadays it is not used any more internally (*per os*). A recent study suggests that birthwort is the cause for endemic nephropathies in Romania, Bulgaria, Serbia and Croatia where the plant is unintentionally consumed through flour [13].

The purpose of this study was to investigate total polyphenols and flavonoids content, reducing power, Fe^{2+} chelating ability and free radicals scavenging activity of two ethanolic extracts obtained from mistletoe (*Viscum album*) and birthwort (*Aristolochia clematitis*).

Experimental part

Obtaining vegetal extracts

Aerial parts (*herba*) of the dried plants were minced and then extracted with 60% ethanol in a ratio of 1:10 (w:v) for 3 h at 60°C. The obtained homogenate was filtered using no. 1 Whatman filter paper and the filtrate was then centrifuged. The obtained extracts were diluted with 60% ethanol in 1:50 ratio (v:v) for all determinations, excepting for the reducing power assay, in which the used dilution was 1:10 (v:v).

Total polyphenols content assay

Total polyphenols content was determined by mixing 500 µL extract (diluted 1:50, v:v, with 60% ethanol), with 4.5 mL distillated water, 0.2 mL Folin Cicalteu reagent and 0.5 mL 20% Na_2CO_3. Water was added to total volume 10 mL. Caffeic acid was utilized for calibration curve [14].

Flavonoid content assay

Flavonoid content was estimated by a colorimetric method described in [15]. Briefly, diluted extract was incubated for 10 min with 2.5 mL $AlCl_3$ reactive. Absorbance at 430 nm was measured using a Jasco V-670

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spectrophotometer. Quercitin was utilized for calibration curve.

Reducing power assay

The reducing properties of plant extracts were determined by assessing the ability of the sample extract to reduce $FeCl_3$ solution, as described in [16]. 200 µL extract in the appropriate dilution (1:10, v:v) were mixed with sodium phosphate buffer (*p*H 6,6), 1 mM $FeSO_4$ and 1% potassium ferricyanide. After incubation of the mixture for 20 min at 50°C, thricloroacetic acid was added and then the mixtures were centrifuged; 2.5 mL of resulting supernatant was mixed with an equal volume of water and 0.5 mL 0.1% $FeCl_3$. The absorbance was measured at 700 nm.

Ferrous metal ions chelating assay

The ability of plant alcoholic extracts to chelate Fe^{2+} was determined using a modified method [17]. Briefly, 150 µL of fresh prepared 500 µM $FeSO_4$ was added to a reaction mixture containing 1.5 mL 0.1 M Tris-HCl (*p*H 7.4) and 850 µL diluted alcoholic extract (1:50, v:v). The mixture was incubated for 5 min, before the addition of 150 µL 0,25% 1,10 *o*-phenantroline (w:v). The absorbance was measured at 510 nm using a Jasco V-670 spectrophotometer. The chelating activity was calculated using the following formula:

$$\%\ Fe(II)\ chelation = \frac{A_c - A_s}{A_c} \times 100 \quad (1)$$

where:
Ac = absorbance of control;
As = absorbance of the sample.

Free radicals scavenging activity assays
DPPH radical

Scavenging activity of 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH·) was investigated according to the method described in [18]. Briefly, to the alcoholic solution of DPPH· (100 µM), 100 µL diluted alcoholic extract were added. Equal amount of ethanol was added to the control. Absorbance was measured at 517 nm. The scavenging of DPPH radical was calculated used the following formula:

$$\%\ Inhibition = \frac{A_c - A_s}{A_c} \times 100 \quad (2)$$

where:
Ac = absorbance of control;
As = absorbance of the sample.

Superoxide anion

The evaluation of superoxide anion scavenging activity of mistletoe and birthwort alcoholic extracts was based on the method described in [19], with slight modifications. Superoxide radicals were generated in PMS-NADPH reaction system. To the mixture containing 16 mM Tris-HCl buffer (*p*H 8.0), 50 µM NBT, 78 µM NADPH and 100 µL plant alcoholic extract, 1 mL of 10 µM PMS solution was added. After 5 min, absorbance was measured at 560 nm using a Jasco spectrophotometer. The inhibition percentage of superoxide anion was calculated using the following formula:

$$\%\ Inhibition = \frac{A_c - A_s}{A_c} \times 100 \quad (3)$$

where:
Ac = absorbance of control;
As = absorbance of the sample.

Hydroxyl radical

In the reaction mixture containing 120 µL 20 mM deoxyribose, 400 µL 0.1 M phosphate buffer (*p*H 7.4), 40 µL 20 mM hydrogen peroxide and 40 µL 500 µM $FeSO_4$, 100 µL diluted alcoholic extract (1:50, v:v) and 800 µL distilled water were added. The reaction mixture was incubated at 37°C for 30 min, and the reaction was then stoped by addition of 500 µL of 2.8% TCA (trichloroacetic acid); this was followed by the addition of 400 µL of 0.6% TBA (thiobarbituric acid) solution. The tubes were incubated in boiling water for 20 min. Absorbance of the resulting solution was measured at 532 nm [20]. The inhibition percentage of hydroxide anion was calculated using the following formula:

$$\%\ Inhibition = \frac{A_c - A_s}{A_c} \times 100 \quad (4)$$

where:
Ac = absorbance of control;
As = absorbance of the sample

Hydrogen peroxide

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer (*p*H 7.4). The decrease in absorbance of hydrogen peroxide solution in the presence of plants extracts at 230 nm was determined after 10 min. A control solution containing phosphate buffer and hydrogen peroxide, but without vegetal extracts, was used. The percentage inhibition of hydrogen peroxide was calculated using the following formula:

$$\%\ Inhibition = \frac{A_c - A_s}{A_c} \times 100 \quad (5)$$

where:
Ac = absorbance of control;
As = absorbance of the sample.

Nitric oxide

At physiological *p*H, sodium nitroprusiate in aqueos solution generates nitric oxide which interacts with oxygen to produce nitrite ions that can be evaluated using Greiss reagent. For this experiment, sodium nitroprusiate (10 mM) in saline phosphate buffer was mixed with diluted alcoholic extract (1:50, v:v) and incubated at room temperature for 30 min. After incubation, 1.5 ml Greiss reagent was added. The absorbance was read at 540 nm. The same reaction mixture without the plant alcoholic extract, but with equivalent amount of alcohol served as control [21].

The ability of extract to scavenge NO was calculated in % inhibition using the following equation:

$$\%\ Inhibition = \frac{A_c - A_s}{A_c} \times 100 \quad (6)$$

where:
Ac = absorbance of control;
As = absorbance of the sample.

Data analysis

The results were expressed as mean values (±SD) of 3 determinations. The mean values and standard deviation were calculated with the EXCEL program from Microsoft Office package.

Results and discussions

Total polyphenols and flavonoids content

Table 1 illustrates total polyphenols content and flavonoids content of mistletoe and birthwort. The results revealed that *Viscum album* contain 6.33 mg total

Table 1
TOTAL POLYPHENOLS AND FLAVONOID CONTENT OF MISTLETOE *(VISCUM ALBUM)* AND BIRTHWORT (*ARISTOLOCHIA CLEMATITIS*)

Compound	Mistletoe (*Viscum album*)	Birthwort (*Aristolochia clematitis*)
Total polyphenols	6.33 ± 1.21mg/g dry plant	11.04 ±2.52 mg/dry plant
Flavonoids	9.72 ± 2.81 μg/g dry plant	23.87 ± 3.33μg/g dry plant

Table 2
FREE RADICALS SCAVENGING ACTIVITY OF MISTLETOE *(VISCUM ALBUM)* AND BIRTHWORT (*ARISTOLOCHIA CLEMATITIS*) ETHANOLIC EXTRACTS

Free radical	% Inhibition	
	Mistletoe	Birthwort
DPPH radical	7.20 ± 2.71	24.77 ± 3.28
Superoxide anion	16.66 ± 3.62	35.55 ± 2.76
Hydroxyl radical	34.44 ± 1.91	31.13 ± 2.91
Hydrogen peroxide	0.37 ± 0.12	0.55 ± 0.19
Nitric oxide	28.43 ± 4.43	46.56 ± 3.44

phenolics/g dry plant and flavonoids in quantity of 9.72 μg equivalent quercitin/g dray plant. In case of birthwort, total polyphenols content was 11.04 mg/dry plant, while the content in flavonoids was 23.87 μg equivalent quercitin/g dray plant. The obtained results indicate a higher content in polyphenols and flavonoids in case of birthwort extract comparatively to mistletoe extract.

Reducing power

The reducing capacity of a compound may be a good indicator of its potential antioxidant activity. The reducing power of mistletoe crude alcoholic extract was estimated at 0.10 ± 0.02 equivalent 1mM $FeSO_4$ and for birthwort at 0.14 ± 0.02 equivalent 1mM $FeSO_4$. Reducing power obtained for *Viscum album* alcoholic extract, for this dilution, is similar with the results obtained in [22] for *Viscum album* methanolic extract harvested from cashew tree.

Ferrous metal ions chelating activity

Because intracellular iron is potentially dangerous to the cell, it is tightly bound to either proteins or small-molecular ligands and sequestered in a specific pool. Circulating iron is bound to transferrin and taken up into cells via the transferrin receptor. Inside the cell, Fe^{3+} is reduced to Fe^{2+}, which is bound to small-molecular weight compounds and constitutes labile iron pool. To keep this pool as small as possible, the excess iron is stored and sequestered in ferritin. Chemicals and their metabolites are able to reduce the ferritin–bound Fe^{3+} and this cause the release of Fe^{2+} into cytoplasm. Also, Fe^{2+} ions react with hydrogen peroxide to produce hydroxyl radicals ($HO^{\cdot}$), via Fenton reaction, and that can be a strong initiator for lipid peroxidation. Fe^{2+} can also stimulate lipid peroxidation by decomposing hydroperoxides into peroxyl and alkoxyl radicals, that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation [23]. The ability of *Viscum album* and *Aristolochia clematitis* ethanolic extracts to chelate Fe^{2+} was 54.68 ± 2.77% and 54.71 ± 3.05%, respectively. The obtained values for the two extracts indicate the ability of polyphenols to bound Fe^{2+} as chelates and their involvement in antioxidant mechanisms.

Free radicals scavenging activity

The free radicals scavenging activities for *Viscum album* and *Aristolochia clematitis* ethanolic extracts are showed in table 2.

$DPPH^{\cdot}$ scavenging ability of the plant extracts may be attributed to their hydrogen donating ability; $DPPH^{\cdot}$ is a stabile free radical and it accepts a hydrogen radical to become a stable diamagnetic molecule. Birthwort alcoholic extract decreased the concentration of DPPH radical much more than the mistletoe extract, probably due to the higher content in polyphenols and flavonoids. The scavenging activity calculated for *Viscum album* in % inhibition was 7.2%. This value is comparable with the one obtained in case of polyphenols extracted in methanol from *Viscum album* harvested from cocoa and cashew trees, at a concentration similar with the concentration of our extract [22].

Oxygen free radicals are part of a larger group of molecules called reactive oxygen species (ROS) that are all more strongly oxidizing than molecular oxygen itself. This group includes superoxide anion, hydroxyl radical and hydrogen peroxide [24]. Oxygen free radicals and other free radicals are constantly formed in human and animal body by normal metabolic processes, as the reduction of oxygen to water by the mitochondrial electron transport chain. Xenobiotics can dramatically enhance ROS production if they are able to penetrate into mitochondria and interact with one or several of the electron transport chain complexes in the inner mitochomdrial membrane, thus blocking the normal electron flow.

Superoxide anion ($O_2^{\cdot -}$) is generated by a number of enzyme systems, by autooxidation reactions and by nonenzymatic electron transfers that univalently reduce

molecular oxygen. For example, a small percentage of electrons leaks away for the main stream of mitochondrial respiratory chain, leading to univalent reduction of molecular oxygen, which generates superoxide anions. The both alcoholic extracts investigated in this study showed superoxide anion scavenging activity; the percentage for inhibition was found as 16.66% for *Viscum album* and 35.55% for *Aristolochia clematitis* (table 2).

Hydroxyl radical ($HO^{\cdot}$) has a very short life-time and it is therefore present in extremely low concentrations. This radical, very reactive, has the ability to attack all the molecules in the body, inclusive the benzene rings of aromatic compounds, producing hydroxilated compound [25]. Hydroxyl radical, the most reactive free radical, was scavenged by mistletoe ethanolic extract in a percentage of 34.44%, while birthwort extract scavenged hydroxyl radical in a percentage of 31.13%. For total phenolics concentration in extracts, the scavenging activity against hydroxyl radical is appreciable.

Hydrogen peroxide (H_2O_2) is highly important because of its ability to penetrate biological membranes. Hydrogen peroxide itself is not very reactive, but may be toxic to cell because in Fenton reaction it may give rise to hydroxyl radicals [23]. The ability of *Viscum album* and *Aristolochia clematitis* ethanolic extracts to scavenge hydrogen peroxide was found as 0.37%, respectively 0.55% inhibition percentage. These results indicate a low ability of the two extracts to scavenge hydrogen peroxide.

Nitric oxide (NO) can cause myocyte relaxation, and plays an important role in the regulation of coronary circulation and myocard contractility. It has also been recognized that nitric oxide, by inhibition of platelet aggregation and leukocyte adhesion, may defend endothelial cell against damage [26]. Overproduction of NO is associated with vasodilatation, resistance to constrictor stimuli, endothelial cell damages and increased vascular permeability [27]. The endothelial cell damages and dysfunction in overproduction of NO are explained by its free radical nature and its high reactivity. NO diffuse out and can reach adjacent cells where it reacts with the iron-sulfur centers of several important enzymes from the mitochondrial electron transport chain and/or ribonucleotide reductase, the enzyme necessary for DNA synthesis [26]. Birthwort ethanolic extract showed a nitric oxide scavenging percentage of 46.56%, higher than the one obtained for mistletoe (28.43%). Scavinging activity of nitric oxide expressed by birthwort alcoholic extract is superior to the one determined in case of *Desmodium gangeticum* [1].

Free radicals have been shown to play an important role in tumor formation and tumor progression. Several classes of chemotherapy substances act by producing reactive oxygen species. For this reason, antioxidants are used by some patients during cancer treatment in the hopes of reducing the side effects of chemotherapy. Reactive oxygen species scavenging activity of polyphenols and Fe^{2+} chelating ability of *Viscum album* and *Aristolochia clematitis* can be an explanation for the use of these two plants in the complementary and alternative therapy of cancer.

Conclusions

The results obtained in this study indicate that mistletoe and birthwort alcoholic extracts contain polyphenols with antioxidant activity.

The polyphenols contained by mistletoe and birthwort extracts have the ability to reduce Fe^{3+} ions and to chelate ferrous metal ions. Also, they presented hydrogen donating abilities to DPPH free radical to become a stable diamagnetic molecule.

Oxygen free radicals (superoxide anion, hydroxyl radical, hydrogen peroxide and nitric oxide) were annihilated by mistletoe and birthwort alcoholic extracts.

Free radicals scavenging activity and antioxidant activity of mistletoe and birthwort explain the use of these plants during cancer treatments for reducing the side effects of chemotherapy.

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