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POLYPHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF AN EXTRACTIVE FRACTION FROM *ABIES ALBA* BARK

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POLYPHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF AN EXTRACTIVE FRACTION FROM *ABIES ALBA* BARK (Abstract): The wood of mature *Abiesalba*Mill. (silver fir) trees is used in construction and furniture industries, the bark being the main waste product. **Aim:** This study aimed to investigate possible therapeutic applications of *Abiesalba* bark waste. **Material and methods:** A raw extract and four extractive fractions were obtained from silver fir bark. They were also studied regarding their polyphenolic content (Folin-Ciocalteu assay). The ethyl acetate extractive fraction was further studied regarding its ability to scavenge free radicals (DPPH, superoxide anion and hydroxyl radicals) and to chelate ferrous ions. **Results and discussion:** The ethyl acetate fraction contained the highest amount of polyphenols (47.72 ± 0.38 g gallic acid equivalents/100 g). Its DPPH scavenging and ferrous ions chelating abilities (EC_{50} = 7.9± 0.1 and 1.56 ± 0.05μg/ml) were comparable to those of the positive controls, catechin (EC_{50} = 7.10± 0.05 μg/ml) and EDTA-Na_2 (EC_{50} = 1.27± 0.01μg/ml), respectively. It also scavenged superoxide anion and hydroxyl radicals with EC_{50} values of 53.30± 5.91 and 63.12 ± 1.78μg/ml, respectively. **Conclusions:** The ability of the ethyl acetate fraction to scavenge free radicals and chelate ferrous ions justifies further studies on the possible therapeutic applications of its antioxidant potential. **Keywords:***ABIES ALBA*MILL., POLYPHENOLS, DPPH RADICAL, SUPEROXIDE ANION RADICAL, HYDROXYL RADICAL, FERROUS IONS.

In the last years, there was a special interest for the study of polyphenols, especially proanthocyanidins – condensed flavan-3,4-diol structures (1, 2). One of the most studied extracts is Pycnogenol® (Horphag Research Ltd, Geneva, Switzerland). This is an extract isolated from the maritime pine (*Pinusmaritima* Mill.) bark containing proanthocyanidins, phenolic acids and flavonoids. Pycnogenol® acts as a scavenger for reactive oxygen species. Therefore, it reduces the incidence of mutagenic DNA damage and protects normal cells from the cytotoxic effects of oxidative stress. Pycnogenol® stimulates the activity of certain intracellular enzyme systems with antioxidant effects (superoxide-dismutase, catalase, glutathione-peroxi-

dase, glutathione-reductase) (3). The promising results of these investigations on Pycnogenol® justify the present study aiming to evaluate the antioxidant potential of polyphenols from another conifer bark.

Abiesalba Mill. (*Pinaceae*, silver fir) is naturally spread in the highlands of Romania. It is also planted as forest species, its wood being used as raw material in furniture industry or as building material. The aim of this research was to explore possible medicinal applications for the bark - the main waste from the industrial processing of the wood of mature silver fir trees. For this purpose, a raw extract and four fractions were isolated from silver fir bark using a fast and inexpensive method. The ethylacetate fraction, having the highest polyphenolic content, was subjected to several *in vitro* antioxidant assays.

MATERIAL AND METHODS

Plant material. Abiesalba Mill. bark was collected in February 2008 in Bicaz area, Neamț district. The bark samples were dried at room temperature in the dark for two weeks.

*Extraction and fractionation.*Silver fir bark (150 g), dried and powdered, was extracted with 1500 ml of 80% methanol for 1 h using a magnetic stirrer (FALC F-30ST magnetic stirrer). The extraction was repeated two times. The combined extracts were concentrated under reduced pressure (Büchi R-210 rotavapor, Büchi V-850 pressure controller, Büchi V-700 vacuum pump) and then lyophilized (Unicryo TFD 5505 freeze-dryer). 17.44 g of dry raw extract were obtained. 16 g of the raw extract were suspended in 160 ml of double distilled water and successively extracted with 5×160 ml of diethyl ether, 9×160 ml of ethyl acetate and 10×160 ml of n-butanol. The extractive fractions were concentrated under reduced pressure while the remaining aqueous phase was lyophilized.

*Quantification of total phenolic content.*Total phenolic content was determined by the Folin-Ciocalteu method (4). Extracts (1 mg/ml in DMSO; 40 µL) were mixed with water (3.16 ml) and Folin-Ciocalteu's phenol reagent (0.2 ml). After 5 min., 20% sodium carbonate solution (0.6 ml) was added. The solutions were mixed and left standing at room temperature. After 2 h the absorbance was measured at 765 nm. The results were expressed as g of gallic acid equivalents (GAE)/100 g extract.

*DPPH radical scavenging assay.*Free radical scavenging activity was evaluated by testing the ability to scavenge diphenylpicrylhydrazyl (DPPH) radical (5). Different concentrations of the sample to be tested (0.31-5 mg/ml in DMSO; 50 µL) were added to a solution of DPPH in methanol (A_{517nm}=1.00 ± 0.05; 2.95 ml). After 5 min., the absorbance was measured spectrophotometrically at 517 nm. (+) Catechin hydrate was used as positive control. Percent DPPH radical-scavenging activity was calculated using the formula: $100 \times (A_{start}-A_{end})/(A_{start})$ where A_{start} is the absorbance before adding the sample and A_{end} is the absorbance measured 5 min. after adding the sample.

*Superoxide anion radical scavenging assay.*Superoxide anion radical scavenging activity was evaluated according to a described procedure with minor changes (6, 7). All reagents were prepared in 100 mM phosphate buffer (pH 7.4). The reaction mixture contained different concentrations of sample (20.16-322.56 µg/ml), nitroblue-tetrazolium (25 µM), beta-nicotinamide adenine dinucleotide reduced (NADH, 78 µM) and phenazine methosulphate (10

μM). After 2 min. the absorbance was read at 560 nm. (+) Catechin hydrate was used as positive control. Percent superoxide anion radical scavenging activity was calculated using the formula: 100 × ($A_{control}$– A_{sample})/($A_{control}$).

*Hydroxyl radical scavenging assay.*Hydroxyl radical scavenging activity was assayed by the deoxyribose method (8, 9). Briefly, the sample (0.62-10 mg/ml in ethanol; 95 μL) was mixed with 5 mM 2-deoxy-D-ribose (0.4 ml) and phosphate-saline buffer (0.1 M; pH 7.4) up to a final volume of 1.6 ml. After addition of 2 mM $FeSO_4 \times 7H_2O$ (0.2 ml), the mixture was incubated at 37ºC for 1 h. Then 1% thiobarbituric acid (1 ml) and 2.8% trichloroacetic acid (1 ml) were added followed by heating for 15 min. at 100ºC. The mixture was cooled and the absorbance was measured at 532 nm. (+) Catechin hydrate was used as positive control. Percent hydroxyl radical scavenging activity was calculated using the formula: 100 × ($A_{control}$– A_{sample})/($A_{control}$).

*Ferrous ion chelating assay.*Ferrous ion chelating activity was estimated according to a literature procedure (10, 11) with minor changes. The assay was carried out in 20 mM potassium phosphate buffer (pH 7.2). The sample was mixed with a solution of 2 mM $FeCl_2 \times 4H_2O$ (0.05 ml). After 1 min., 5 mMferrozine (0.2 ml) was added followed by vigorous shaking. The absorbance of the ferrozine-ferrous ions complex was measured at 562 nm. Ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2) served as positive control. The percentage of ferrous ion chelating activity was calculated using the formula: 100× ($A_{control}$– A_{sample})/($A_{control}$).

RESULTS AND DISCUSSION

*Extraction and fractionation.*In order to obtain a polyphenol-rich extract, *Abiesalba* Mill. bark was extracted with 80% methanol - a common solvent used for polyphenols extraction from vegetal sources. The hydromethanolic raw extract (AA) was fractioned with solvents of different polarities; four extractive fractions were obtained containing phytochemicals soluble in diethyl ether (AA-1), ethyl acetate (AA-2), n-butanol (AA-3) and water (AA-4). The three organic fractions were concentrated under reduced pressure leading to 1.30 g of AA-1, 2.04 g of AA-2 and 5.67 g of AA-3. The remaining aqueous phase was lyophilized and 6.84 g of AA-4 were obtained. Extracts were stored at -20°C until use.

*Quantification of total phenolic content.*Fraction AA-2 contained the largest amount of polyphenols (47.72 ± 0.38 g gallic acid equivalents/100 g). The lowest polyphenolic content was determined in fraction AA-1 (15.02 ± 0.31 g gallic acid equivalents/100 g) (tab. I).

TABLE I

Total phenolic content of raw extract and extractive fractions from*Abies alba* Mill. bark

Extract / Extractive fraction	**Total phenolic content (g gallic acid equivalents/100 g extract or extractive fraction)**
AA	29.86 ± 1.07
AA-1	15.02 ± 0.31
AA-2	47.72 ± 0.38
AA-3	26.92 ± 0.14
AA-4	27.20 ± 0.30

values are means of three replicates ± SD

Both normal-phase and reversed-phase HPLC-DAD analysis of silver fir bark extracts revealed the presence of low molecular phenolics in fractions AA-1 and AA-2 while fractions AA-3 and AA-4 contained proanthocyanidin-type polymers (data not shown). Highly polymerized proantho cyanidins are poorly absorbed in the gut and poorly metabolized by the intestinal microflora. In addition, polymeric proantho cyanidins bind to proteins, starch and enzymes forming complexes which cannot be absorbed. Low molecular polyphenols have a high bioavailability being absorbed in the gut (12). Fraction AA-2 contained the largest amount of polyphenols, mainly low molecular compounds and therefore it was chosen for further studies.

DPPH radical scavenging assay. Scavenging of free radicals is one of the main mechanisms by which polyphenols act as antioxidants. Polyphenols scavenge DPPH radical by donating hydrogen atoms of phenolic hydroxyls and transfering an electron from phenolic hydroxyls or phenoxide anions. Both the solvent and the redox potentials of polyphenols affect the mechanism of radical scavenging. Polar solvents (methanol, ethanol) form strong hydrogen bonds with phenolic hydroxyl groups and therefore, in polar solvents, electron transfer is the major mechanism by which polyphenols scavenge DPPH radical; in apolar solvents, hydrogen donation prevails (13). Fraction AA-2 and catechin scavenged DPPH radical in a concentration dependent manner. According to the EC_{50} values, the scavenging ability of fraction AA-2 (EC_{50} = 7.9 ± 0.1 μg/ml) was comparable to that of catechin (EC_{50} = 7.10 ± 0.05 μg/ml) (tab. II).

Superoxide anion radical scavenging assay. Despite a low reactivity and a low capacity of crossing cell membranes, superoxide anion radical is toxic due to its capacity to promote DNA damage. Superoxide anion radical does not react with DNA directly. Its DNA damaging potential is due to the conversion into highly reactive hydroxyl radical through iron-induced redox reactions (14). The effect of fraction AA-2 on superoxide anion radical was evaluated by measuring the inhibition of NADH-dependent nitrobluetetrazolium reduction. In this assay, superoxide anion radical is generated in a NADH-phenazinemethosulphate system. Once generated, superoxide radical reduces nitro bluetetrazolium to blue formazan which is measured spectrophotometrically at 560 nm. In the presence of antioxidants, the formazan production is diminished; the decrease in absorbance at 560 nm is proportional with the superoxide scavenging activity (6, 7). Both fraction AA-2 and catechin scavenging effects were concentration dependent. Fraction AA-2 scavenging activity increased from 24.13±0.69% at 20.16 μg/ml to 82.81± 0.79% at 322.56 μg/ml. At the same concentrations, catechin scavenged superoxide radical by 32.57±0.06% and 92.53±0.12%, respectively. According to the EC_{50} values, fraction AA-2 (53.30 ± 5.91 μg/ml) had a lower scavenging activity in comparison to catechin (39.53 ± 0.95 μg/ml) (tab. II).

Hydroxyl radical scavenging assay. Hydroxyl radical is considered to be the most deleterious reactive oxygen species. As it has an extremely short half-life and a low diffusion capacity, hydroxyl radical will damage the closest biomole-

cules immediately after its generation. In case hydroxyl radical is produced in immediate vicinity of nucleic acids, it may oxidize DNA directly. The effect of fraction AA-2 on hydroxyl radical was estimated using 2-deoxy-D-ribose degradation assay (8, 9). Hydroxyl radicals generated by iron (II) sulfate heptahydrate in phosphate buffer damage 2-deoxy-D-ribose with the release of thiobarbituric-acid reactive material which is spectrophotometrically quantified at 532 nm. The decrease of absorbance at 532 nm indicates hydroxyl radical scavenging activity. Hydroxyl radical scavenging activity of fraction AA-2 increased from 17.06±0.18% at 15.62 µg/ml to 70.41±0.76% at 250 µg/ml. The scavenging effects of the positive control, catechin, were 27.14±0.97% at 15.62 µg/ml and 76.36±0.34% at 250 µg/ml. As compared by the EC_{50} values, fraction AA-2 (63.12 ± 1.78 µg/ml) showed less hydroxyl radical scavenging activity than the positive control, catechin (46.16 ± 0.72 µg/ml) (tab. II).

*Ferrous ion chelating assay.*Ferrous ions are involved in the generation of reactive oxygen species, mainly hydroxyl radical *via* autoxidation and Fenton/Haber-Weiss reactions. Iron-induced reactive oxygen species inhibit tumor suppressor genes increasing the risk of cancer development (14). Ferrous ions chelation is another mechanism by which polyphenols act as antioxidants. The ferrous ions chelating activity was evaluated by the ferrozine assay. Ferrozine binds ferrous ions forming red coloured complexes with a strong absorbance at 562 nm. Chelating agents reduce ferrozine-ferrous ions complex formation with a decrease in absorbance at 562 nm (10, 11). Fraction AA-2 and EDTA-Na_2 chelated ferrous ions in a concentration-dependent manner. The positive control, EDTA-Na_2 and fraction AA-2 showed comparable chelating effects (EC_{50} = 1.27 ± 0.01 µg/ml and 1.56 ± 0.05 µg/ml, respectively) (tab. II).

TABLE II

EC_{50} values (µg/ml) of fraction AA-2 in different antioxidant assays

Sample	**DPPH radical scavenging activity**	**Superoxide anion scavenging activity**	**Hydroxyl radical scavenging activity**	**Ferrous ion chelating activity**
AA-2	7.9 ± 0.1	53.30 ± 5.91	63.12 ± 1.78	1.56 ± 0.05
catechin	7.10 ± 0.05	39.53 ± 0.95	46.16 ± 0.72	n.d.
EDTA-Na_2	n.d.	n.d.	n.d.	1.27 ± 0.01

values are means of three replicates ± SD; n.d. – not determined

CONCLUSIONS

This study demonstrated that an ethyl acetate extractive fraction (AA-2) isolated from the bark of mature *Abiesalba* Mill. trees effectively scavenged free radicals (DPPH, superoxide anion and hydroxyl radicals) and chelated ferrous ions. Our findings justify further studies on the identification of the active constituents and *in vivo* antioxidant activity of fraction AA-2.

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