ACTA SCIENTIARUM POLONORUM *Acta Sci. Pol., Hortorum Cultus 12(3) 2013, 3-11*

ACTIVITY AND TOTAL PHENOLIC CONTENT OF *Alnus glutinosa* AND *Alnus incana* LEAVES

Olga V. Mushkina[1], Natalia S. Gurina[1], Mira M. Konopleva[2], Wieslawa Bylka[3], Irena Matlawska[3]

[1]Belarusian State Medical University, Minsk, Republic of Belarus

[2]Vitebsk State Medical University, Vitebsk, Republic of Belarus

[3]Poznań University of Medical Sciences, Poznań, Poland

Abstract. *Alnus glutinosa* and *A. incana* (Betulaceae), are a small to medium size tree, native to the Northern Hemisphere. The leaves of this species are used in the Republic of Belarus as a source of antioxidants. The aim of this work was investigation of antioxidant activities and total phenolics content in various extracts obtained using water, mixture water with ethanol (from 9:1 to 2:8), and ethanol from *A. glutinosa* and *A. incana* leaves. Phenolics content was determined by method with Folin-Ciocalteu reagent and calculated on ellagic acid. The antioxidant activities were measured utilising 1,1-diphenyl-2 picryl-hydrazyl (DPPH) radical scavenging test. The highest phenolics concentration was observed in the extracts prepared by extraction with the mixture of water:ethanol from 7:3 to 3:7 and ranged from 17.82% to 18.96% for *A. glutinosa* and from 10.82% to 12.55% for *A. incana*. This extracts exhibited the highest free radical scavenging activity ranging from 49.21% to 49.42% and from 41.28% to 41.67% for *A. glutinosa* and *A. incana* respectively, comparable to the activity of quercetin. Therefore the mixture of water:ethanol from 7:3 to 3:7 should be used for preparing extracts from this species for medicinal purposes. Results also indicated the existence of a high correlation between antioxidant activity and total phenolics content.

Key words: leaf extracts, DPPH, hiperoside, phenilic acids

INTRODUCTION

A. glutinosa L. (Gaertn.) (Black Alder European, Alder or Common Alder) and *A. incana* (L.) Moench. (Grey, Speckled Alder) belong to genus Alnus L., family Betulaceae, subfamily Betuloideae. *A. glutinosa* is a moderately-sized tree or large shrub native to Europe, including Britain, Fennoscandia and extends into North Africa, Asia

Corresponding author: Wiesława Bylka, Department of Pharmacognosy, Poznan University of Medical Sciences, 60-781 Poznań, Święcickiego 4, Poland, e-mail: wieslawabylka@tlen.pl

Minor and Western Siberia, usually growing in moist woods or pastures or by streams. The Latin name *glutinosa* implies that the buds and young leaves are slightly sticky with a resinous gum. *A. incana* is a species of alder with a wide range across the cooler parts of the Northern Hemisphere. The wood of the gray alder is somewhat paler then the wood of the black alder.

Alders are a pioneer species particularly well adopted to wet sites, important for river ecosystems and river bank stabilization.

Alders are classified as an actinorhizal tree species and belong to a few non-leguminous plants (Fabaceae), with a capacity for symbiosis with Frankia bacteria forming nitrogen-fixing root nodules. The accumulation of nitrogen increased together with the rise in atmospheric CO_2 and, at the same time, the plant biomass grew, too. As a result of the increase in the nitrogen accumulation and easy decomposition of their leaves, Alders fertilize the soil and can be used as a pre-crop during recultivation of postindustrial areas [Mingeot et al. 2010].

Alders are highly resistant to *soil salinity* and environmental pollution with heavy metals. The leaves of both alder species, in comparison with other tree leaves, preserve green foliage longer in the fall because of nitrogen remobilization.

In the leaves of black alder, the presence of hiperoside, together with phenolic acids: ellagic, *cis*-, *trans*-ferulic, sinapic, caffeic, *cis*-, *trans*-p-coumaric, p-hydroxybenzoic and isovanilinic, and in the leaves of graya the presence of hiperoside were confirmed chromatographically (TLC).

The total content of phenolic compounds (with Folin-Ciocalteu reagent, expressed as ellagic acid), and flavonoids (expressed as hiperoside) as well as ellagic acid and hiperoside (with the use of HPLC method) was measured in various leaf samples of both species. In black alder leaf the content of phenolic compounds was 8.91% to 19.33%, ellagic acid, 0.89% to 1.91%, and hiperoside from 0.41% to 1.03%, whereas in gray alder leaf content of flavonoids was 1.22% to 2.65% and hiperoside 1.08% to 2.12%. Additionally, content of hiperoside, gallic, ellagic, caffeic, chlorogenic acids from the water and ethanolic extracts of the leaves of both species were determined by HPLC method (Tab. 1) [Mushkina and Gurina 2007, Mushkina 2008].

Table 1. Content of hiperoside and phenolic acids in the extracts of *A. glutinosa* and **A. incana* leaf, according to Mushkina and Gurina 2007, Mushkina 2008

Solvent	Flavonoid (%)		Phenolic acids (%)					
	Hiperoside		Gallic		Ellagic		Chlorogenic	Caffeic
Ethanol	0.65	1.86*	0.08	0.04*	1.01	0.26*	1.93*	
Water	0.44	1.18*	0.33	0.11*	0.37	0.46*	0.03*	0.60*

The monographs of the leaves of both species in the Republic of Belarus Pharmakopoeia recommends HPLC methods for hiperoside and ellagic acid determination, whereas the method with Folin-Ciocalteu reagent is advised to determine the phenolics

content. *Alni glutinosae folia* (black alder leaf) does not contain less than 5.0% of phenolic compounds expressed as ellagic acid, not less than 0.5% of ellagic acid and not less than 0.3% of hiperoside. The standardisation of *Alni incanae folia* (gray alder leaf) concerns the content of hiperoside (not less than 1.0%) [Republic of Belarus Pharmakopoeia 2008].

The bark of *Alnus glutinosa* contains tannic acid (16–20%), triterpenes (lupenon, glutinon, taraxerol), and sterols (*β*-sitosterol) [Pasich and Kowalewski 1966, Pasich et al. 1966].

The different plant parts of both species (leaves, bark, cones) are used in folk medicine.

Due to their astringent and hemostatic properties, the decoctions from the leaves are used locally for gargling in the treatment of sore throat and pharyngitis and, internally of intestinal bleeding [PDR for Herbal Medicines 2004]. The infusions of the bark of both species, especially of *A. incana,* are used in the treatment of the gastrointestinal tract and skin diseases, as well as for gargling in mouth and throat infections [Hoppe 1958, Stević et al. 2010]. The bark of *A. glutinosa* possess anti-inflammatory activity [Cahen and Poisson 1971] and has been used to treat swellings, and rheumatism [Middleton et al. 2005].

So far, the investigation of the biological activity of both species has been limited. Only anti-inflammatory, antioxidant and hepatoprotective activity resulting from membrane stabilization and improvement of liver function by ellagotannins from the cones of black alder have been found [Buniatian et al. 1998].

Due to its emetic properties, the fresh bark of *A. glutinosa* can be used to induce vomiting in the case of ingestion of a poisonous substance. In cosmetics, the decoction of the bark has been used for coloring hair and in foot baths for tired and aching feet. The dry cones or water extracts of the cones of *A. glutinosa* are used for disinfection of aquariums.

The aim of this work was investigation of antioxidant activities (DPPH), and determination of total phenolic content (Folin-Ciocalteu reagent) in various extracts obtained using water, mixture water with ethanol, and ethanol in *A. glutinosa* and *A. incana* leaves.

MATERIAL AND METHODS

Plant collection and extraction. The samples of the leaves of *A. glutinosa* and *A. incana* were collected from the botanical garden of the Medical University in Vitebsk in May, 2009 and the voucher specimens are deposited in the herbarium of the Pharmacognosy and Botany Department in Vitebsk Medical University, the Republic of Belarus.

Chemicals. The 1,1-diphenyl-2 picrylhydrazyl radical (DPPH), Folin-Ciocalteu (FC) reagent and ellagic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), quercetin from Merck (Darmstadt, Germany), whereas the other chemicals used in this experiments were analytical quality and solvents were purchased from POCh (Gliwice, Poland).

Preparation of plant extracts. The leaves were dried at room temperature and crushed. Each 0.5 g of the samples of the leaves was extracted in a boiling water bath for 40 min with 30 ml of H_2O or a mixture of H_2O and ethanol (from 9:1 to 2:8) or 96% of ethanol. The obtained extracts were centrifugated at 3000 rpm for 5 min. One milliliter of the proper solvent was added to one milliliter of each supernatant to obtain the following samples: AG: 1–10 and AI : 1–10.

Determination of total phenolic compounds. The total phenolic contents was determined with the use of the Folin-Ciocalteu reagent [Chandra and Gonzalez de Mejia 2004].

Calibration curve. The calibration curve was constructed using freshly made ethanolic solutions of ellagic acid at concentration of 0.5 mg/ml. 0.1, 0.2, 04, 0.5, 0.6 ml of these solutions (containg 0.05, 0.1, 0.2, 0,25 and 0.30 mg/ml, respectively) were put into 25 ml volumetric flasks, then 0.5 ml of the FC reagent and 10 ml of water were added to each solution. After 5 min of incubation (in darkness at room temperature), they were diluted to 25 ml with 15% sodium carbonate solution and incubated in darkness at room temperature for the next 40 min. The absorbance of the test solutions was measured at 760 nm by the comparison with the compensation liquid (the mixture consisting of 10 ml of distilled water, 0.5 ml of FC reagent, and diluted to 25 ml with 15% aqueous solution of sodium carbonate).

The equation obtained for the calibration curve (2–12 μg/ml) was: $y = 79.268x + 0.0239$; $r^2 = 0.9964$.

Determination of phenolics. 0.1 ml of AG 1–10 or AI 1–10 extracts (see Tab. 2) were put into the 25 ml volumetric flasks, then 0.5 ml of the Folin-Ciocalteu reagent and 10 ml of water were added and, next, they were treated according to the procedure described in the case of the calibration curve.

The total phenolics content (x) of the extracts expressed as the ellagic acid equivalent per gram of the dry plant material was determined according to the formula:

$$x = \frac{c \cdot 15 \cdot 10^5}{m}$$

where:

c – amount of ellagic acid (from calibration curve), mg/ml;

$15 \cdot 10^5$ – dilution factor;

m – mass of the plant sample, mg.

DPPH radical-scavenging test. The reaction mixture contained 0.6 ml of the plant extracts (AG 1–10 and AI 1–10) or 0.6 ml of quercetin (standard substance) at concentration of 10 mg/ml and 4.2 ml of a 0.01% m/v solution of DPPH in ethanol was shaken and left standing at room temperature for 30 min in the dark. The absorbance of the reaction mixture was measured at 517 nm against the blank (UV/VIS Spectrophotometer Lambda 35 Perkin Elmer). The inhibition of the DPPH radical by the sample was calculated according to the following formula:

$$\text{DPPH scavening activity}\,(\%) = \frac{(A_0 - A_1)\cdot 100\%}{A_0}$$

where:

A_0 – the absorbance of control;

A_1 – the absorbance of the samples

Statistical analysis. The experiments were done in sixplicate. The results are given as a mean ± standard deviation.

RESULTS AND DISCUSSION

A. glutinosa L. (Gaertn.) and *A. incana* (L.) Moench belong to Alder genus (Alnus L.), family Betulaceae, and are classified as an actinorhizal tree species, with a capacity for symbiosis with Frankia bacteria forming nitrogen-fixing root nodules. Alders fertilize the soil and can be used as a pre-crop during recultivation of postindustrial areas.

The different plant parts of both species (leaves, bark, cones) are used in folk medicine.

The leaves of *A. glutinosa* and *A. incana* are used in the Republic of Belarus as a source of antioxidants [Republic of Belarus Pharmakopoeia 2008], whereas ellagitannins from the cones of *A. glutinosa* are constituents of the herbal drug ALTAN, recommended as a hepatoprotective agent [Buniatian et al. 1998].

In this work, the antioxidant activity and the total content of phenolics in *A. glutinosa* and *A. incana* leaves extracts, prepared by extraction with water, water and ethanol mixtures and ethanol as solvents were investigated. The antioxidant activities were measured by DPPH scavenging assay. DPPH is a stable free radical**,** which loses its absorption band at 517 nm, on accepting an electron or a free radical species which results in discoloration from violet to yellow [Chandra and Gonzalez de Mejia 2004].

The total phenolics content was determined using a spectrophotometric technique, by the method of Folin-Ciocalteu reagent and calculated by comparison with a standard curve generated by analyzing ellagic acid. On the basis of the results obtained from our data, it can be stated that the highest phenolics concentration was observed in the extracts prepared by extraction with the mixture of water:ethanol from 7:3 to 3:7 and ranged from 17.82% to 18.96% for *A. glutinosa* and from 10.82% to 12.55% for *A. incana* (Tab. 2).

The extracts from the leaves of both species, prepared by extraction with the mixture of water:ethanol from 7:3 to 3:7, exhibited the highest free radical scavenging activity ranges from 49.21% to 49.42% and from 41.28% to 41.67% for *A. glutinosa* and *A. incana* respectively (Tab. 3), comparable to the activity of 0.6 ml of quercetin at concentration of 1 mg/ml (52.47%). As illustrated in Fig. 1, a high correlation was found between DPPH values and total phenol content ($r^2 = 0.9385$).

Table 2. Phenolic contents in the *A. glutinosa* (AG) and *A. incana* (AI) leaf extracts

	Solvent		Phenolics x ± Δx (%)	
			AG	AI
1	H_2O		15.72 ± 0,46	7.29 ± 0.13
2	H_2O:EtOH	9:1	15.98 ± 0,40	8.85 ± 0.31
3		8:2	16.42 ± 0,64	9.76 ± 0.35
4		7:3	17.82 ± 0,61	10.82 ± 0.28
5		6:4	18.56 ± 0,64	11.54 ± 0.35
6		5:5	18.72 ± 0,57	11.84 ± 0.41
7		4:6	19.66 ± 0,62	12.24 ± 0.41
8		3:7	18.96 ± 0.74	12.55 ± 0.40
9		2:8	17.72 ± 0,50	11.33 ± 0.41
10	EtOH 96°		9.13 ± 0,32	10.19 ± 0.32

Table 3. Antioxidant activity of the *A. glutinosa* (AG) and *A. incana* (AI) leaf extracts

	Solvent		Antioxidant activity x ± Δx (%)	
			AG	AI
1	H_2O		47.30 ± 1.15	40.09 ± 0.65
2	H_2O:EtOH	9:1	47.13 ± 1.17	39.77 ± 0.83
3		8:2	47.37 ± 1.61	40.89 ± 1.07
4		7:3	49.42 ± 1.46	41.28 ± 0.49
5		6:4	49.50 ± 1.61	41.37 ± 0.73
6		5:5	49.80 ± 1.26	40.84 ± 1.34
7		4:6	49.86 ± 1.19	41.21 ± 1.24
8		3:7	49.21 ± 1.56	41,67 ± 0.80
9		2:8	46.97 ± 1.69	39.36 ± 0.98
10	EtOH 96°		40.70 ± 1.77	38.55 ± 0.99

According to literature, the presence of hiperoside together with various phenolic acids in the leaves of both species, were confirmed [Mushkina and Gurina 2007, Mushkina 2008]. Phenolic compounds such as phenolic acids and flavonoids as well as other soluble compounds present in the extracts, including proteins, peptides, polysaccharides and pigments could be responsible for the antioxidant activity [Zhu 2011, Prior et al. 2005].

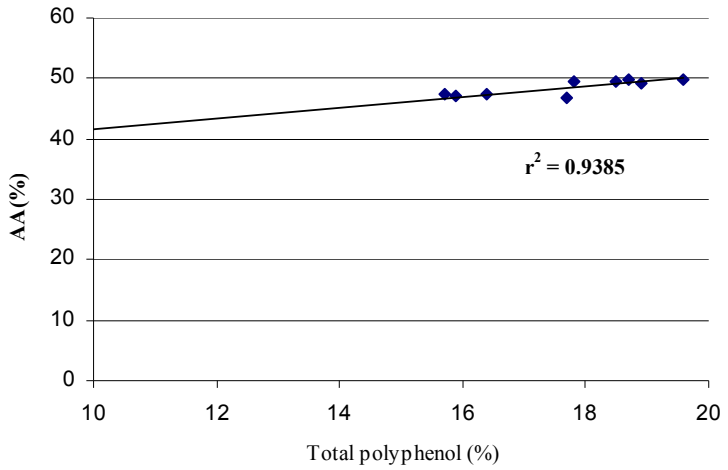

Fig. 1. Correlation of DPPH values and total phenols content

Antioxidant activity of *A. glutinosa* and *A. incana* leaves extracts is probably determined by polyphenols eg. ellagic acid and flavonoids. Ellagic acid is present in some fruit, such as pomegranates, raspberries or strawberries. It exhibits antioxidant, anti-inflammatory, antiproliferative, apoptosis-inducing activities, inhibit growth of cancer cells [Landet 2011]. Flavonoids, ubiguitous in the plants are scavengers of oxygen-derived free radicals and possess anti-inflammatory, antiallergic, anticarcenogenic properties [Masuoka et al. 2012].

This activity can mainly be credited to the absorbing, neutralizing and quenching free radicals or/and the metal chelating potential. The plant antioxidants retard or prevent the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions and have been effective in the prophylaxis against aging and against different human diseases such as cancer and arteriosclerosis.

CONCLUSION

In the present work, the antioxidant activity of extracts from *A. glutinosa* and *A. incana* leaves was proved for the first time. According to our investigations the antioxidant activity of extracts of both species, and the phenolics content depends on the solvent used for the extractions and were the highest in the extracts prepared by extraction with the mixture of water:ethanol from 7:3 to 3:7. We observed that the content of phenolic compounds in the extracts correlated with antioxidant activity.

Summing up, the leaves of *A. glutinosa* and *A. incana* as a high source of antioxidants can be recommended as an ingredients of medicines for free radical dependent diseases.

REFERENCES

Buniatian N.D., Chikitkina V.V., Jakovleva L.V., 1998. The hepatoprotective action of ellagitannins. Eksperimental'naia Klinicheskaia Farmakologiia 61, 53–55.

Cahen R., Poisson J., 1971. Pharmacological study of *Alnus glutinosa* Gaertn. Bark. C. R. Seances Soc. Biol. Fil. 165, 820–822.

Chandra S., Gonzalez de Mejia E., 2004.Polyphenolic Compounds, Antioxidant Capacity, and Quinone Reductase Activity of an Aqueous Extract of *Ardisia compressa* in Comparison to Mate (*Ilex paraguariensis*) and Green (*Camellia sinensis*) Teas. J. Agric. Food Chem. 52, 3583–3589.

Hoppe H.A., 1958. Europäische Drogen. Cram, de Gruyter & CO, Hamburg.

Masuoka N., Matsuda M., Kubo I., 2012. Characterization of the antioxidant activity of flavonoids. Food Chem. 131, 541–545.

Middleton P., Steward F., Al-Quahtani S., Egan P., Abdulrahman A., Byres M., Middleton M., Shoeb M., Nahar L., Delazar A., Sarker S., 2005. Antioxidant, Antibacterial activities and General Toxicity of *Alnus glutinosa*, *Fraxinus excelsior* and *Papaver rhoedas*. Iran. J. Pharm. Res. 2, 81–86.

Mingeot D., Baleux R., Watillon B., 2010. Characterization of microsatellite markers for black alder (*Alnus glutinosa* [L.] Gaertn.). Conservation Genetetic Resources 2, 269.

Mushkina O.V., 2008.Standardisation of the *Alnus glutinosa* and *Alnus incana* leaves. Vest. VGMU 7, 144 (www.vinti.ru).

Mushkina O.V., Gurina N.S., 2007.Quantitative determination of the phenolic compounds in the leaves of *Alnus glutinosa.* Vest. Pharm. 38, 3 (www.vinti.ru).

Landet J.M., 2011. Ellagitannins, ellagic acid and their derived metabolites: A review about source, metabolism, functions and health. Food Research International. 44, 1150–1160.

Pasich B., Kowalewski Z., 1966.Triterpenoid compounds in plant material. Part X Isolation of lupenon from the bark of *Alnus glutinosa* (L.) Gaertn. Dissert. Pharm. Pharmacol.18, 275–280.

Pasich B., Kowalewski Z., Grzybek P., 1966.Triterpenoid and sterol compounds in plant material. Part XII. Isolation of glutinon, taraxerol and β-sitosterol from the bark of *Alnus glutinosa* (L.) Gaertn. Dissert. Pharm. Pharmacol. 18, 385–390.

PDR for Herbal Medicines. 2004. 3rd edn. Medical Economics Company Montvale, New Yersey.

Prior R.L., Wu X.L., Schaich K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J. Agric. Food Chem. 53, 4290–4302.

Republic of Belarus Pharmakopoeia., 2008. Molodechno, Tipographia, Pobieda 389–391, 392–394.

Stević T., Savikin K., Zdunić G., Stanojković T., Juranić Z., Janković T., Menković N., 2010. Antioxidant, cytotoxic, and antimicrobial activity of *Alnus incana* (L.) ssp. *incana* Moench and *A. viridis* (Chaix) DC ssp. *viridis* extracts. J. Med. Food 13, 700–704.

Zhu K.X., Lian C.X., Guo X.N., Peng W., Zhou H.M., 2011. Antioxidant activities and total phenolic contents of various extracts from defatted wheat germ. Food Chem. 126, 1122–1126.

DZIAŁANIE ANTYOKSYDACYJNE I ZAWARTOŚĆ FENOLI W LIŚCIACH *Alnus glutinosa* I *Alnus incana*

Streszczenie. *Alnus glutinosa* i *A. incana* (Betulaceae), są niewielkimi drzewami, występującymi w stanie naturalnym na obszarach półkuli północnej. Liście obu gatunków są stosowane na terenie Białorusi jako źródło antyoksydantów. Celem naszej pracy było oznaczenie aktywności antyoksydacyjnej oraz zawartości sumy polifenoli w różnych wyciągach otrzymanych z liści *Alnus glutinosa* i *A. incana* przez ekstrakcję wodą, mieszaniną wody z etanolem (9:1 do 2:8) i etanolem. Zawartość polifenoli oznaczano metodą z odczynnikiem Folin-Ciocalteu, w przeliczeniu na kwas elagowy. Aktywność antyoksydacyjną mierzono, stosując 1,1–diphenyl–2 picrylhydrazyl radical (DPPH) scavenging test. Zaobserwowano, że najwyższa zawartość polifenoli występowała w wyciągach otrzymanych przez ekstrakcję mieszaninami woda:etanol od 7:3 do 3:7 i wynosiła od 17,82% do 18,96% dla *A. glutinosa* i od 10,82% do 12,55% dla *A. incana*. Te ekstrakty wykazywały też najwyższą zdolność zmiatania rodnika w granicach od 49,21% do 49,42% dla *A. glutinosa* i od 41,28% do 41,67% dla *A. incana*, porównywalną z aktywnością kwercetyny. Stąd mieszanina woda:etanol od 7:3 do 3:7 powinna być stosowana w celu otrzymywania wyciągów stosowanych w celach leczniczych. Wyniki wskazują też na korelację między aktywnością antyoksydacyjną a zawartością związków fenolowych.

Słowa kluczowe: ekstrakty z liści, DPPH, hiperozyd, kwasy fenolowe

Accepted for print – Zaakceptowano do druku: 10.09.2012