Seeds of *Arnica montana* and *Arnica chamissonis* as a potential source of natural antioxidants

URSZULA GAWLIK-DZIKI[1*], MICHAŁ ŚWIECA[1], DANUTA SUGIER[2], JOANNA CICHOCKA[1]

[1]Department of Biochemistry and Food Chemistry
University of Life Sciences
Skromna 8
20-704 Lublin, Poland

[2] Department of Industrial and Medicinal Plants
University of Life Sciences
Akademicka 15
20-950 Lublin, Poland

[*]corresponding author: e-mail; urszula.gawlik@up.lublin.pl

S u m m a r y

Arnica sp. infusions and tinctures play a great role in the traditional and contemporary medicine. Seeds of plantation-grown *Arnica montana* L. (AM) and *Arnica chamissonis* var. *foliosa* Less. (AC) are a good source of bioactive compounds such as phenolic acids and flavonoids. In both studied genera higher levels of phenolic acids and flavonoids were detected in water extracts. Regardless of plant genus, predominant fractions of phenolics were determined in seeds, comprising chlorogenic, caffeic acid, quercetin and kaempferol. All samples show high antioxidant activity, however, their levels depend on the extraction procedure and used material. The free radicals were scavenged most effectively by extracts of AC seeds (30.3% – SASA, 31.3% – DPPH and 39.8% – ABTS, respectively). It should be noted that lipid peroxidation was strongly inhibited by both studied tinctures. It is noteworthy that studied samples show positive correlations between inhibition of lipid peroxidation ability and total flavonoids and phenolic acids content ($r=0.89$ and $r=0.83$, respectively). Results obtained from this study show that *Arnica* seeds extract, either alone or in combination with other active principles, can be used in cosmetic, nutraceutical and pharmaceutical applications.

Key words: *antioxidant activity, Arnica, free radicals, phenolic compounds*

INTRODUCTION

Oxidative damage caused by reactive oxygen species (ROS) has been frequently suggested to be associated with the pathogenesis of various conditions such as aging, arthritis, cancer, inflammation and heart diseases in human body. ROS can readily react with and oxidize most biomolecules including carbohydrates, proteins, lipids and DNA. There is increasing evidence that the accumulation of ROS in biological system causes oxidative damage to the tissue, which affects cellular integrity and functions.

A great number of aromatic, spicy, medicinal and other plants contain chemical compounds of antioxidant properties. Recently, food companies are increasingly promoting the benefits of natural antioxidants which are secondary plant metabolites. The trend has been to investigate a variety of plants as new potential sources of antioxidants [1]. This sector promotes products named functional food, food supplements, nutraceuticals or cosmeceuticals [2].

A. montana (AM) is the most widely employed medicinal plant in the clinics either by itself or in combination with other herbs, although, the antioxidant properties of this plant are much less documented. Preparations of AM flowers have been used both in traditional and homeopathic medicine for topical treatment of post-trauma effects and inflammatory diseases. Alcoholic, as well as oily extracts, are prepared and used in gels, creams, ointments or as arnica oil [3].

Within the antioxidant literature the number of studied residual sources have been augmented considerably, which is caused by a value adding recycling interest of agro- and food industry, but also increasing information on the specific location of active compounds [2]. It can be concluded that the antioxidant activity of *Arnica* sp. was poorly investigated. Therefore, testing of their antiradical properties is of interest, primarily in order to find new promising sources of natural antioxidants for functional foods and nutraceuticals. Wild-growing AM plants are under natural protection and do not give stable yields in commercial plantations. For this reason, many trials were undertaken to find other *Arnica* sp. with comparable properties. In our field experiments the highest yields of fresh mass were obtained for *Arnica* plants propagated from seedlings and by fragmentation of the pattern plant. In this reason we compared the phenolic profiles and antioxidant abilities of remaining seeds, as novel sources of natural antioxidants.

MATERIALS AND METHODS

Plant material

The *Arnica* genus belongs to the *Asteraceae* family. In the study different parts of cultivated AM and AC were tested. A field experiment was carried out in 2006 on three-year-old plantation of AM grown on grey-brown podsolic soil with granulometrical composition of heavy loamy sand. The soil was characterised by mean con-

tent of humus, very low phosphorus, potassium and magnesium content, and was acid in reaction. In the autumn of 2005, all the plots were subjected to phosphorus-potassium fertilisation in following doses: 24.0 kg P and 66.4 kg K per ha, whereas nitrogen in the dose of 40.0 kg was applied in two equal doses: in spring, before the beginning of vegetation, and after the heads harvest. During vegetation plants were weeded by hand three times, and inter-rows were cultivated. Immediately after harvest seeds of AM and AC were dried in the temperature of 35°C.

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH); Tween 40; 2,2'-Azinobis (3 ethylbenzthiazo-linesulfonic acid) diammonium salt (ABTS); linoleic acid; haemoglobin; 3-(2-pirydyl)-5,5-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS); nicotinamide adenine dinucleotide (NADH) were purchased from Sigma (St. Louis, USA). All solvents and pure chemicals were of HPLC grade and were obtained from Merck (Darmstadt, Germany) and Sigma (St. Louis, USA).

Extracts preparation

Ethanolic (tincture) and water (infusion) extracts were prepared following Polish Pharmacopoeia V [4]. In the water extracts, 2 g of seeds were extracted with hot distilled water, cold off and centrifuged. In the next step, infusion was dribbled and top up with distilled water to 100 ml. In ethanolic extracts 2 g of seeds was filled with 100 ml of 70% ethanol and left in darkness for 2 weeks.

Determination of total phenolic compounds

The amount of total phenolics was determined using Folin-Ciocalteau reagent [5]. The amount of total phenolics was expressed as a gallic acid equivalents (GAE) in mg per ml of sample.

Determination of phenolic acids content

The amount of total phenolic acids was determined following the Szaufer-Hajdrych [6], phenolic acids content was expressed as a caffeic acid equivalent (CAE) in μg per ml of sample, using the calibration curve.

Determination of flavonoids content

Total flavonoids content was determined according to the method described by Bahorun [7]. Total flavonoids were calculated as a quercetin equivalent (QE) in mg per ml of sample using the calibration curve.

HPLC analysis

Samples were analysed with a Varian ProStar HPLC System separation module (Varian, Palo Alto, USA) equipped with Varian ChromSpher C18 reverse phase column (25 mm x 4.6 mm) column and ProStar 325 UV-Vis detector according to Gawlik-Dziki and Świeca [8]. The column thermostat was set at 35°C. The mobile phase consisted of 1% acetic acid (solvent A) and methanol (solvent B) and flow rate of 0.8 ml\min. At the end of gradient, the column was washed with 100% methanol and equilibrated to initial condition for 10 min. Chromatographic peaks were defined by comparing retention times with those of standards. Quantitative determinations were carried out with external standard calculation using calibration curves of standards. The gradient elution for phenolic acids determination was used as follow: 0 min, 14% B; 15 min, 14% B; 50 min, 50% B; 60 min, 40% B; 76 min, 55% B; 78 min, 100% B. Phenolic acids were detected at λ=270 nm. Better resolution of flavonoids was performed on the gradient: 0 min, 15% B; 17 min, 55% B; 30 min, 20% B; 37 min, 100% B. Flavonoids were detected at λ=370 nm.

Superoxide anion scavenging activity (SASA)

The superoxide anion scavenging activity was determined according to the method described by Liu et al. [9]. The affinity of test material to quench superoxide anion was evaluated according to equation:

$$\mathrm{SASA\%} = \frac{[(A_{(0)} - A_A)]}{A_{(0)}} \times 100,$$

where: $A_{(0)}$ – absorbance of control, A_A – absorbance of sample.

Determination of free DPPH radicals scavenging activity

The free radical scavenging activity was measured according to Brand-Williams et al.[10]. The affinity of test material to quench DPPH free radical was evaluated according to equation:

$$\mathrm{scavening\%} = \frac{[(A_{C(0)} - A_{A(t)}]}{A_{C(0)}} \times 100,$$

where: $A_{C(0)}$ – absorbance of control at 0 min., $A_{A(t)}$ – absorbance of sample after 10 min.

Determination of ABTS radicals scavenging activity

The experiments were carried out using an improved ABTS decolorization assay [12]. $ABTS^{+\bullet}$ was generated by oxidation of ABTS with potassium persulfate. The affinity of test material to quench ABTS free radical was evaluated according to equation:

$$\text{scavening}\% = \frac{[(A_C - A_A)]}{A_C} \times 100 ,$$

where: A_C – absorbance of control, A_A –absorbance of sample.

Reducing power

Reducing power was determined by the method of Oyaizu [12].

Metal chelating activity

Chelating power was determined by the method of Guo et al. [13]. The percentage of inhibition of ferrozine – Fe^{2+} complex formation was given below in formula:

$$\%\text{inhibition} = [1 - \frac{A_P}{A_C}] \times 100 ,$$

where: A_C – absorbance of the control, A_P – absorbance in the presence of sample.

Inhibition of linoleic acid peroxidation

The antioxidant activity was determined as the degree of inhibition on the haemoglobin-catalyzed peroxidation of linoleic acid according to Kuo et al. [14]. The antioxidative activity of the sample was calculated as

$$AA[\%] = \frac{1 - (A_s - A_0)}{A_{100} - A_0} \times 100 ,$$

where: Ao – absorbance of the control (without haemoglobin), As – absorbance in the presence of sample, A_{100} – absorbance without sample.

Statistical analysis

All experimental results were mean ±SD of three parallel measurements and data were evaluated by using one-way analysis of variance (Tukey test). P values <0.05 were regarded as significant.

RESULTS AND DISCUSSION

Quantitative analyses of phenolic compounds

It is well-known that active constituents of *Arnica* are sesquiterpene lactones (e.g. helenalin, dihydrohelenalin) and flavonoids (e.g. quercetin, patuletin) as well as phenolic acids (e.g. caffeic, chlorogenic) [15]. The anti-inflammatory effects are explainable mainly by the inhibition of transcription factors NF-λB and NF-AT caused by sesquiterpene lactones. On the other hand, flavonoids and phenolic acids are mainly bound with antioxidant and antibacterial activity [16]. Irrespective of plant genus, much more phenolic acids and flavonoids were extracted by 70% ethanol (tab. 1). Predominant fraction of phenolic acids determined in seeds regardless of plant genus comprised chlorogenic and caffeic acids. Potential therapeutic and nutraceutical abilities of plant material are identified with flavonoids presence. Our studies clearly show that AM extracts contain significantly greater amounts of rutin, quercetin and kaempferol than AC samples. It should be also noted that, in comparison to AM extracts, AC seeds contain far more luteolin and apigenin (tab. 2). Until now, there is a little about chemical composition of *Arnica* seeds. It should be also emphasized that in *Arnica* sp. differences in secondary metabolite profiles are closely bound with place (altitude) and cultivation conditions [17]. It is noteworthy that our studies were performed on non-hydrolysed samples that can be used in pharmaceutical, nutraceutical and food industry.

Table 1.

The contents of total phenolics, phenolic acids and flavonoids in tested *Arnica* seeds samples

	total phenolics content [GAE mg/ml]		total phenolic acids content [CAE μg/ml]		total flavonoids content [QE mg/ml]	
	tincture	infusion	tincture	infusion	tincture	infusion
AM	1.66±0.12	5.13±0. 29	1.44±0.02	0.55±0.08	2.49±0.03	0.47±0.11
AC	1.97±0.16	2.91±0.09	1.39±0.09	0.30±0.03	2.57±0.02	0.24±0.06

Table 2.

Phenolic compounds detected by HPLC analysis in tested *Arnica* seeds tinctures

compound [μg/ml]	chlorogenic acid	caffeic acid	ferulic acid	synapinic acid	*O*-coumaric acid	luteolin	quercetin	kaempferol	apigenin
AM	28.39 ±2.52	2.90 ±0.12	0.21 ±0.02	0.07 ±0,01	3.23 ±0.05	1.27 ±0.06	7.33 ±0.09	38.77 ±1.23	0.15 ±.02
AC	15.25 ±0.88	2.19 ±0.15	0.17 ±0.03	0.93 ±0.07	0.50 ±0.08	35.75 ±2.09	4.11 ±0.11	3.06 ±0.14	0.4 ±0.02

Free radical scavenging activity

Most of antioxidant potential in herbs and spices is due to the properties of phenolic compounds which can act as reducing agents, free radical scavengers and hydrogen donators [18]. Recent reports have described antioxidants and compounds with radical scavenging activity present in many herbs and spices. Therefore, radical scavenging of AM and AC seeds extracts was determined.

Superoxide anions (O_2^-), the single-electron reduced form of molecular oxygen, is a precursor to active free radicals that have the potential of reacting with biological macromolecules, thereby inducing tissue damage [19]. Although O_2^- itself is quite unreactive compared to other radicals, biological systems convert it into more reactive species, e.g. OH radicals [20].

Taking superoxide anion scavenging properties into account it was observed that AC extracts were about six times more effective. It should be noted that both studied extracts exhibited relatively high ability for hydrogen donating (DPPH). Studies on ABTS+ scavenging ability clearly showed that infusion of AC was significantly more effective than analogical samples of AM (39.8 and 20.7%, respectively). In conclusion, free radicals were more effective scavenged by the extracts prepared from seeds of AC. Taking antioxidant activity into account, it is clear that the active compounds of AC seeds were better extracted using hot water (fig. 1). These results suggest that the antiradical capacity of AC seeds extracts is mainly bound with hydrophilic compounds.

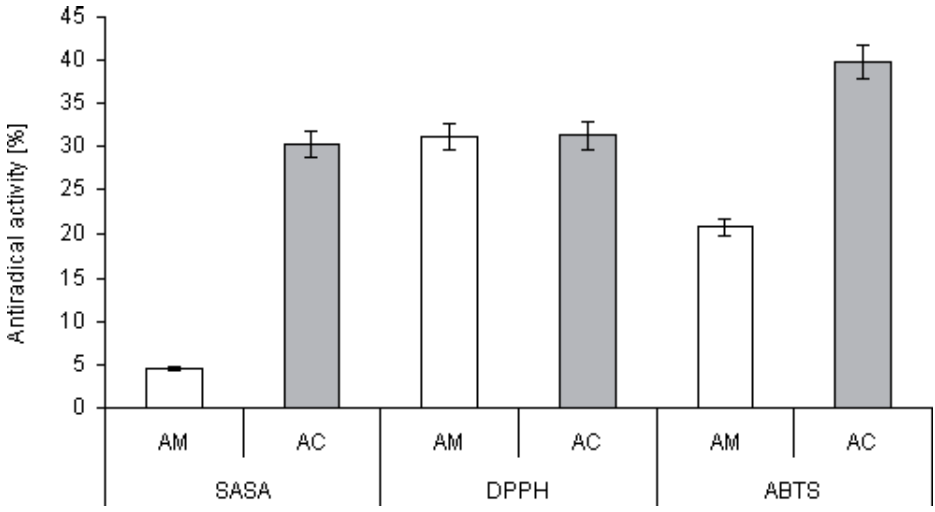

Figure 1. Free radical scavenging activity of the Arnica seeds extracts. AM – A. montana, AC – A. chamissonis, SASA – superoxide anion scavenging activity, DPPH – DPPH radicals scavenging activity, ABTS – ABTS radicals scavenging activity

Reducing power

Reductive ability can also determine the antioxidant capacity of investigated samples, however, the mechanism of activity comes from the structure of active compounds. There are various abilities among these mechanisms: (1) the ability to protect the initiation of chain reaction, (2) to chelate intermediate metal ion, (3) to destruct peroxides, (4) to neutralize free radicals and finally comes (5) their reducing ability [20]. It is noteworthy that both *Arnica* species possessed high reducing power. The influence of extraction medium was significant only in AM. The highest reducing power possessed ethanolic extracts of AM, however, also AC extracts exhibited comparable activities (Fig. 2A).

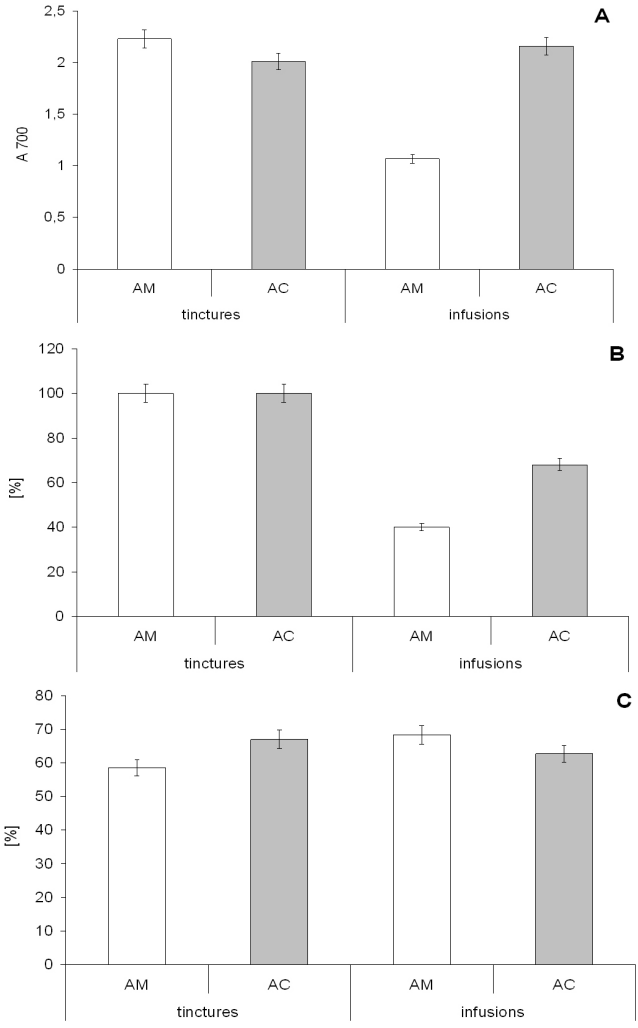

Figure 2. Antioxidant activity of *Arnica* samples: in (A) reducing activity, (B) lipid peroxidation, and (C) iron chelating; AM – *A. montana*, AC – *A. chamissonis*

Inhibition of lipid peroxidation

Cell homeostasis is closely bound with correct functionality of cell membrane. Membrane lipids peroxidation can negatively influence signaling cascades and finally cause pathological process. Oxidative damage of membranes can influence many things: destroy ion channels, mechanisms of active transports or inactivation of membrane enzymes. In chronic diseases, such as Alzheimer's or Parkinson's disease, significantly increased LPO products were observed [19]. Judging by this observation, lipid protection seems to be very important. All studied tinctures inhibited lipids peroxidation at a very high level. The activities of hydrophilic extracts were significantly lower (40 and 68% for AM and AC infusions, respectively). It is clearly visible that compounds responsible for inhibition of lipids peroxidation were extractable with 70% ethanol and probably possess lipophilic character. Results obtained in these studies can suggest using seeds extracts as an alternative source of lipid peroxidation inhibitors for cosmetics and food industry (Fig. 2B).

Metal chelating activity

Several plant extracts have had their antioxidant activity detected when chelating catalytic metals. Intermediate metal ions play an important role in the Fenton reaction. This process is closely bound with the formation of free radicals, especially hydroxy- and peroxyradicals. Inhibition of this reaction can be performed by inactivation or chelating of iron ions [21]. Studies of metal chelating activities showed that the kind of solvent used in extraction procedure was not significant. Irrespective of plant genus, all studied seeds extract exhibited relatively high reducing power (about 60%). The highest activities were found in the AC tinctures and AM infusions (67 and 68.3%, respectively, Fig. 2C).

Many studies have shown that the phenolic contents of plants can be correlated with their antioxidant activities [22]. Małolepsza and Urbanek [23] suggest that flavonoid complexes included in plant samples provide better results than single compounds. The analysis of the correlation between the total phenolic compounds, flavonoids and phenolic acids content and antioxidant activities showed significant dependence in the case of free radical neutralizing properties, chelating power, reducing power and lipid peroxidation inhibition and the total phenolic compounds ($r=-0.77$, $r=0.65$, $r=-0.92$ and $r=-0.97$, respectively). It should be also noted that positive correlations were between inhibition of lipids peroxidation and content of flavonoids ($r=0.89$) and total phenolics acids ($r=0.83$) (Tab. 3).

Table 3.

Relationships between total phenolics, flavonoids and phenolic acids content and antioxidant activity of *Arnica* seeds extracts (means show correlation coefficient)

antioxidant activity	total phenolics	total flavonoids	total phenolic acids
reducing activity	**–0.92**	0.47	0.39
chelating activity	**0.65**	–0.29	–0.28
inhibition of LPO	**–0.97**	**0.89**	**0.83**
radical scavenging activity	**–0.77**	0.23	0.13

These results indicate that biological active compounds of *Arnica* seeds associate themes together and show synergistic or antagonistic effects. It was also clear that antioxidant potential of biological sample is not restricted to the concentration of active compounds but is also closely bound to mutual relationships between them. In earlier studies, Sherwin [24] defined the benefits that come from using the mixture of antioxidant compounds. He claims that it is possible to complement active mechanisms of biological compounds and reduce the methodological problems such as varying solubility and colour of a simple compound solution.

CONCLUSION

In conclusion, the biologically active compounds of *Arnica* sp. seeds possessed a wide spectrum of antioxidant activities. All studied samples were very rich in lipophilic and hydrophilic compounds with a comparable activities. AM may be an alternative for AM, naturally protected and difficult to cultivate. This study shows also that *Arnica* seeds extract, either alone or in combination with other active principles, can be used in cosmetic, nutraceutical and pharmaceutical applications.

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NASIONA *ARNICA MONTANA* I *ARNICA CHAMISSONIS* JAKO POTENCJALNE ŹRÓDŁO NATURALNYCH ANTYOKSYDANTÓW

URSZULA GAWLIK-DZIKI[1*], MICHAŁ ŚWIECA[1], DANUTA SUGIER[2], JOANNA CICHOCKA[1]

[1]Katedra Biochemii i Chemii Żywności
Uniwersytet Przyrodniczy
ul. Skromna 8
20-704 Lublin
[2]Katedra Roślin Przemysłowych i Leczniczych
Uniwersytet Przyrodniczy
ul. Akademicka 15
20-950 Lublin

[*]autor, do którego należy kierować korespondencję: e-mail: urszula.gawlik@up.lublin.pl

S t r e s z c z e n i e

Nalewki i napary z arniki odgrywają wielką rolę w medycynie. Jak wykazano w badaniach, nasiona pozyskane z uprawy *Arnica montana* L. (AM) i *Arnica chamissonis* var. *foliosa* Less (AC) stanowią dobre źródło związków bioaktywnych takich jak kwasy fenolowe i flawonoidy. W przypadku obydwu badanych gatunków wyższą zawartość kwasów fenolowych i flawonoidów oznaczono w ekstraktach wodnych. Niezależnie od gatunku rośliny dominującymi frakcjami związków fenolowych były kwas chlorogenowy i kawowy oraz kwercetyna i kempferol. Wszystkie badane próby wykazywały wysoką aktywność przeciwutleniającą, jakkolwiek jej poziom zależał od metody ekstrakcji użytego materiału. Wolne rodniki były najwydajniej zmiatane przez ekstrakty otrzymane z nasion AC (30,3% – SASA, 31,3% – DPPH i 39,8% – ABTS). Należy podkreślić, że peroksydacja lipidów była silnie hamowana przez nalewki. Należy odnotować, że stwierdzono dodatnią korelację pomiędzy zdolnością do hamowania peroksydacji lipidów a zawartością flawonoidów i kwasów fenolowych (odpowiednio r=0,89 i r=0,83). Otrzymane wyniki potwierdzają, że nasiona AM i AC mogą być użyte jako łatwo dostępne źródło naturalnych antyoksydantów dla przemysłu farmaceutycznego i medycznego, m.in. jako suplementy diety.

***Słowa kluczowe**: właściwości przeciwutleniające, Arnica, wolne rodniki, związki fenolowe*