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ARCTIUM TOMENTOSUM MILL. CULTIVATED IN SIBERIA: CHEMICAL COMPOSITION OF LEAVES, ANTIOXIDANT ACTIVITY OF RAW MATERIALS, EXTRACTS AND INDIVIDUAL SUBSTANCES

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Researchers from various countries are engaged in the search for biologically active substances with antioxidant properties. Medicinal plants are among the most accessible sources of such substances. One such source is the species of the genus *Arctium L.* The most widespread species of this genus in Russia is *Arctium tomentosum* Mill., which has also been introduced into industrial cultivation. Research on wild and cultivated varieties of *Arctium tomentosum* is scarce or entirely absent.

The dominant groups of biologically active substances in the leaves of cultivated *Arctium tomentosum* include polysaccharides, hydroxycinnamic acids, tannins, and triterpenoid saponins, with peak accumulation (excluding polysaccharides) occurring in mid-summer. The dominant groups of compounds in liquid and dry extracts are the same as in the raw plant material. Chlorogenic acid is predominant in all extracts, while 4-caffeoylquinic acid is detected only in the 70% liquid extract.

The 70 and 40% extracts from the leaves exhibit high antioxidant and antiradical activity compared to the aqueous extract, containing significantly higher concentrations of hydroxycinnamic acids, coumarins, carotenoids, and chlorophyll.

The obtained results highlight the potential for further research into ethanol-based extraction complexes from *A. tomentosum* leaves as a source for creating agents with complex, including antioxidant, effects.

Keywords: *Arctium tomentosum*, antioxidant activity, antiradical activity, chemical composition.

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Introduction

Maintaining redox homeostasis plays a central role in health and disease prevention. Oxidative stress arises due to an imbalance between reactive oxygen species (ROS) and antioxidants. Excessive ROS leads to the degradation of lipids, proteins, and nucleic acids, potentially causing oxidative damage to cells, overexpression of oncogenes, formation of mutagens, induction of atherogenic activity, or inflammation [1, 2]. Apart from ROS, another significant source of cellular damage is xenobiotics, which generate reactive metabolites-products of enzymatic reactions that are electrophilic in nature and capable of damaging intracellular macromolecules. Targets for such reactive metabolites include enzymes, cellular structures, and nucleic acids. By alkylating or arylating these molecules, reactive metabolites can induce irreversible modifications [3–5].

The researchers worldwide have been actively searching for biologically active substances with antioxidant properties. Medicinal plants are the most accessible sources of such compounds. The genus *Arctium L.* is one such source. For example, *Arctium tomentosum* Mill. Despite being a common wild species, *Arctium tomentosum* has been introduced into industrial cultivation due to its high demand in the food, nutraceutical, and pharmaceutical industries. Experimental studies on the antioxidant activity of *Arctium tomentosum* herb [6], *Arctium lappa* leaves [7–9], fractions from *A. lappa* leaves [10], and the lactone onopordopicrin from *A. lappa* leaves [11] are available in the literature. However, a review of the available publications reveals that research on *Arctium tomentosum* is scarce. These gaps formed the basis and objective of this study.

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Materials and Methods

The collection of raw material from the *Arctium tomentosum* and the production of a dry extract were carried out in 2023 in Altayskoye village (Altai Krai) at the production facility of Visterra LLC. The leaves were harvested throughout the vegetative period, from June to October 7, with 10-day intervals (except in October). The leaves were dried using air-shadow drying techniques.

The total tannin content was determined titrimetrically according to the guidelines of General Pharmacopoeia Monograph (GPM) 1.5.3.0008.15, «Determination of Tannin Content in Medicinal Plant Raw Materials and Herbal Preparations» [12]. Ascorbic acid content was measured titrimetrically following the Russian Pharmacopoeia (RF) XV edition, «Rosehip Fruit» monograph [12]. The total coumarin content was determined according to the State Pharmacopoeial Monograph (SPM) for «Melilotus Herb» [13]. Chlorophyll content was measured spectrophotometrically as described in [14], and carotenoid content was determined using the spectrophotometric method from the «Rosehip Fruit» monograph of RF XV [12]. Flavonoid content was assessed spectrophotometrically following the monograph of RF «Peppermint Leave» [12], while hydroxycinnamic acid content was determined according to the RF «Nettle Leaves» [12]. Polysaccharide content was measured spectrophotometrically using the «Burdock Roots» RF XV edition [12], and the total content of sesquiterpene lactones was determined using the method described by K.S. Rybalko [15]. Anthocyanin content was evaluated based on the «Dried Aronia Berries» RF XV edition [12]. Saponin content was measured spectrophotometrically and recalculated into oleanolic acid equivalents [16].

The results are presented as the mean ± standard deviation of fivefold experiments. $P<0.05$ was considered to indicate a statistically significant difference. All physical and chemical methods used were validated for linearity, precision, and accuracy. Validation was conducted following the requirements of RF XV, and the analysis met all relevant standards [12].

Hydro-alcoholic extracts (40 and 70%) were prepared using ethanol as the extraction solvent. The raw material was extracted three times (material-to-solvent ratio 1 : 15) under reflux on a water bath for 45 minutes each time. The obtained filtrates were combined and concentrated under vacuum at a temperature of 50–60 °C. The dry aqueous extract, industrially produced by Visterra LLC, was obtained using proprietary technology currently undergoing patent application.

Individual compounds were isolated from raw materials using column chromatography with an ethyl acetate fraction in glass columns (2.5 cm in diameter) filled with silica gel with particle sizes of 100–250 μm. Elution was carried out using a mixture of ethyl acetate and ethanol. Purification of individual compounds was achieved via rechromatography in a thin layer of sorbent on Kieselgel$_{60}$ F^{254} plates.

Compound identification and quantification were performed using high-performance liquid chromatography coupled with diode-array detection and mass spectrometry (HPLC-DAD-TOF-MS) by comparison with standard compounds (chlorogenic acid and caffeic acid, all manufactured by Phytolab, Germany). HPLC-DAD-TOF-MS analysis was performed using an Agilent 1260 Infinity LC chromatograph (USA), liquid chromatograph-mass spectrometer 6550 QTOF LC/MS, equipped with diode-array and mass spectrometric detectors. Chromatography was conducted on a Zorbax Extend-C18 column (100×4.6 mm; 3.5 μm) under gradient elution. Mobile phase A consisted of 0.1% formic acid solution, and mobile phase B consisted of acetonitrile for gradient chromatography. The gradient program was as follows: 0.0–2.0 min: 5% B; 2.0–10.0 min: 80% B; 10.0–13.0 min: 80% B; 13.0–13.1 min: 5% B; 13.1–15.0 min: 5% B. Flow rate: 0.8 mL/min; wavelength: 320 nm; injection volume: 0.2 μL; thermostat temperature: 30°C. Conditions of mass spectrometric analysis: electrospray ionization (ESI), the mode of registration of negative ions, and the four most intense fragmentary ions; fragmentation energy – 10 eV; temperature of the ionization source (gas): 200 °C; drying gas current: 14 l/min; spray gas pressure: 35 psig; curtain gas temperature: 350 °C; curtain gas current: 11 l/min; capillary voltage (VCap): − 3500 V; Nozzle voltage: − 1000 B; scanning mode, scanning range: 100–1200 m/z; detection time: 0.0 to 16.0 min. Chromatographic purity was determined by the internal normalization method.

The pharmacological study was conducted *in vitro*. Antioxidant activity was assessed using a titrimetric method based on the reaction of reducing substances present in the extracts with potassium permanganate (the titrant).

The extracts were prepared in a 1 : 10 ratio, heated in a water bath for 15 minutes, and then cooled for 45 minutes. In a 50 mL beaker, 8 mL of freshly boiled and cooled water, 1 mL of 20% H_2SO_4, and 0.05 N $KMnO_4$

aqueous solution were added to the extract and mixed. Titration was performed with the extracts until the pink color disappeared [17]. The concentration of reducing substances, expressed as rutin equivalents (mg/mL), corresponds to the value of antioxidant activity and was calculated using the was calculated using the following equation given below:

$$B = \frac{C_k \times V_k \times V_0}{V_x \times m}$$

Where B is the concentration of reducing substances (mg/mL) required to titrate 1 mL of 0.05 N $KMnO_4$ aqueous solution; C_K is the concentration of rutin in the solution used for titration of 1 mL of 0.05 N $KMnO_4$ aqueous solution (mg/mL); V_k is the volume of rutin solution used for titration (mL); V_0 is the volume of the analyzed solution (mL); V_x is the volume of the sample solution used for titration (mL); m is the weight of the sample (g).

Antiradical activity (ARA) and inhibition degree were studied using a spectrophotometric method [18, 19], which evaluated the extracts' ability to inactivate the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich, USA). Preparation of the DPPH Solution: 0.04 g of DPPH was dissolved in a 100 mL volumetric flask with slowly added 96% ethanol, with the final volume adjusted after dissolution. From this solution, an aliquot of 10 mL was taken, transferred to a 100 mL volumetric flask, and diluted with 96% ethanol. The solution was stored in a dark place. On the next stage three solutions were prepared:

– 1 mL of the corresponding extract (hydro-alcoholic or dry aqueous) + 2 mL of the corresponding solvent (40%, 70% ethanol, aqua) + 2 mL of DPPH reagent (A_1);

– 3 mL of the corresponding solvent + 2 mL of DPPH reagent (A_2), control for reagent;

– 1 mL of the corresponding extract + 4 mL of the corresponding solvent (A_3), control for extract.

The solutions were left in a dark place for 30 minutes. Further the optical density of the samples was measured at 517 nm, using 96% ethanol as the reference solution.

When assessing a potential ARA, two indicators were considered: antioxidant activity and the degree of inhibition (ID). Antiradical activity was calculated using the following equation given below:

$$ARA = \frac{(A_2 - A_1)}{A_2 \times 100\%}$$

Where A_1 is the optical density of the tested solution with DPPH; A_2 is the optical density of the DPPH solution without the tested sample.

ID was calculated using the following equation given below:

$$ID = \frac{(A_2 - (A_1 - A_3)}{A_2 \times 100\%}$$

Where A_1 is the optical density of the test sample with DPPH; A_2 is the optical density of the DPPH solution without the tested sample; A_3 is the optical density of the test sample without DPPH.

Standard substances, including rutin and ascorbic acid (both from Phytolab, Germany), were used.

To prepare the rutin solution, a sample weighing 0.05 g was placed into a 50 mL volumetric flask. Then, 40 mL of 60% ethanol was added, and the solution was heated in a water bath at 60 °C until the rutin completely dissolved. The solution was cooled to room temperature, and the volume was adjusted to the mark with 60% ethanol. To prepare the ascorbic acid solution, a sample weighing 0.05 g was placed into a 50 mL volumetric flask. Freshly boiled and cooled water was gradually added, with stirring until the ascorbic acid fully dissolved. The volume was then adjusted to the mark with water.

The solutions of chlorogenic and caffeic acids were prepared as follows: 10 mg of chlorogenic or caffeic acid was placed into a 50 mL volumetric flask, and 10 mL of 96% ethanol was added, followed by thorough mixing. After the substances dissolved, the volume was adjusted to the mark. The solutions have a shelf life of 3 months when stored in well-sealed containers in a cool, light-protected place.

Results

The data presented in Table 1 summarize the average monthly values (excluding October) for each group of biologically active substances (BAS) based on five replicates. The dominant BAS groups in the leaves of *Arctium tomentosum* include polysaccharides (PSCs), hydroxycinnamic acids (HCAs), tannins, and triterpenoid saponins. All BAS groups exhibit a similar dynamic. Thus, at the beginning of vegetation, the leaves primarily contain PSCs, HCAs, tannins, and triterpenoid saponins. The peak accumulation of most BAS groups, except PSCs, occurs in mid-summer (July). By the end of the vegetation period (October), the content of all BAS groups, except PSCs, decreases. The decrease in content compared to July's peak values is as follows: 16% for tannins, 24% for flavonoids, 40% for HCAs, 2.7% for saponins, 11% for sesquiterpene lactones, 30% for ascorbic acid, 150% for carotenoids, and 21% for chlorophyll. In contrast, the total content of PSCs increases by 79% from the July values at the end of the vegetation period.

Based on these data on biologically active substances (BAS) content across phenological phases, further studies (extract preparation and pharmacological activity evaluation) focused on leaf samples collected on July 22, 2023, and October 7, 2023. The dry aqueous extract was obtained from raw materials collected on October 7, 2023. Table 2 presents the content of BAS in liquid and dry extracts obtained from *Arctium tomentosum* leaves. The dominant BAS in the extracts are the same as in the raw plant material – tannins, hydroxycinnamic acids (HCAs), polysaccharides (PSCs), and saponins.

Using HPLC-DAD-TOF-MS, the main HCAs and their concentrations in the extracts were determined (Table 3). According to the data in the table, the identified HCAs include caffeic acid, chlorogenic acid, and 4-caffeoylquinic acid. Chlorogenic acid was the predominant compound in all extracts, while 4-caffeoylquinic acid was found only in the 70% hydro-alcoholic extract.

The antioxidant activity (AOA) and antiradical activity (ARA) of 40% and 70% hydro-alcoholic extracts, as well as the dry aqueous extract, were determined on the next stages of the study. The results are presented in Tables 4 and 5.

Table 1. Content of Biologically Active Substances in *Arctium tomentosum* leaves (n=5, P=95%, t=2.57)

BAS Group	(Content as % of absolutely dry raw material, total monthly values)					
	May	June	July	August	September	October
Total tannins	1.64±0.03	3.18±0.09	4.34±0.08	3.86±0.08	3.92±0.09	3.63±0.09
Total flavonoids in terms of luteolin	0.15±0.003	0.49±0.001	0.82±0.001	0.79±0.01	0.65±0.01	0.62±0.01
Total HCAs in terms of chlorogenic acid	2.98±0.05	3.62±0.001	5.52±0.01	4.89±0.08	5.36±0.009	3.26±0.09
Total PSCs in terms of fructose	4.03±0.08	4.30±0.07	4.39±0.07	4.58±0.07	4.87±0.07	7.86±0.10
Total saponins in terms of oleanolic acid	1.95±0.03	2.84±0.07	2.89±0.07	2.85±0.07	2.84±0.07	2.81±0.07
Total coumarins	0.27±0.005	0.31±0.005	0.84±0.02	0.98±0.02	0.82±0.02	0.74±0.01
Total sesquiterpene lactones	0.16±0.003	0.18±0.003	0.18±0.003	0.18±0.003	0.17±0.003	0.16±0.003
Ascorbic acid	0.40±0.007	0.52±0.008	0.59±0.008	0.51±0.008	0.43±0.007	0.41±0.008
Total carotenoids in terms of β-carotene	0.89±0.02	0.96±0.02	0.98±0.02	0.95±0.02	0.86±0.02	0.83±0.02
Chlorophyll	0.23±0.005	0.28±0.005	0.37±0.007	0.38±0.007	0.32±0.006	0.29±0.005

Table 2. Content of Biologically Active Substances in Extracts from *Arctium tomentosum* leaves (n=5, P=95%, t=2.57)

BAS Group	Content as % of absolutely dry raw material				
	40% hydro-alcoholic extract		70% hydro-alcoholic extract		Dry aqueous extract
	July	October	July	October	
Total tannins	3.48±0.1	2.92±0.1	2.35±0.1	1.95±0.08	3.81±0.2
Total flavonoids in terms of luteolin	0.90±0.05	0.74±0.04	0.07±0.002	0.05±0.001	0.06±0.002
Total HCAs in terms of chlorogenic acid	2.44±0.1	1.53±0.06	5.83±0.2	3.43±0.1	1.27±0.05
Total PSCs in terms of fructose	1.26±0.06	2.17±0.1	0.49±0.01	0.78±0.02	8.06±0.4
Total saponins in terms of oleanolic acid	1.75±0.05	1.64±0.06	1.95±0.03	1.81±0.03	0.53±0.04
Total coumarins	0.28±0.01	0.24±0.01	0.65±0.01	0.57±0.01	0.11±0.05
Total sesquiterpene lactones	0.02±0.001	0.015±0.001	0.06±0.002	0.05±0.002	0.005±0.0001
Ascorbic acid	0.37±0.01	0.24±0.009	0.09±0.001	0.06±0.003	0.35±0.01
Total carotenoids in terms of β-carotene	0.31±0.01	0.18±0.01	0.89±0.02	0.74±0.02	0.09±0.001
Chlorophyll	0.08±0.003	0.03±0.001	0.41±0.01	0.33±0.001	0.01±0.001

Table 3. Content of Main Hydroxycinnamic Acids in Extracts

Retention time tR, min	M_w	Molecular Formula	m/z			λmax, nm	Content, mg/kg				
			[M-COOH]$^-$	[M-H]$^-$	[M-CH_2COOH]$^-$		40% hydro-alcoholic extract		70% hydro-alcoholic extract		Dry aqueous extract
							July	October	July	October	
17.4–17.7	180.1	Caffeic acid $C_9H_8O_4$	135.04	179	107.05	240, 298, 322	19.3±1.1	12.5±0.4	24.7±1.4	14.3±0.3	9.4±0.4
22.01–22.28	354.1	4-Caffeoylquinic acid $C_{16}H_{18}O_9$	707.17	353.09	191.06	246, 325	–	–	2.9±0.1	1.6±0.4	–
22.9–23.3	354.1	Chlorogenic acid (3-Caffeoylquinic acid) $C_{16}H_{18}O_9$	707.18	353.09	191.06	246, 326	50.3±2.4	28.7±1.2	68.4±3.1	39.6±1.6	28.1±1.4

Table 4. Antioxidant Activity of Extracts , mg/mL ($p<0.05$, n=9)

Object of study	Antioxidant Activity (mg/mL)			
	In terms of rutin		In terms of ascorbic acid	
	July	October	July	October
70% hydro-alcoholic extract	125±0.46	118.4±0.65	405±3.1	383.68±3.42
40% hydro-alcoholic extract	107.14±0.54	102.27±.0.75	347.14±2.38	331.36±2.76
Dry aqueous extract	–	66.17±0.51	–	214.41±1.45
Chlorogenic Acid	129±1.31		407±3.03	
Caffeic Acid	115±0.93		388±1.67	

Table 5. Antiradical Activity and Degree of Inhibition of Extracts, % ($p<0.05$, n=9)

Extragent	ARA, %		ID, %	
	July	October	July	October
70% hydro-alcoholic extract	76.25±0.66	81.15±0.35	88.06±0.55	98.41±0.77
40% hydro-alcoholic extract	76.44±0.39	79.71±0.54	87.91±0.61	84.42±0.43
Dry aqueous extract	–	42.65±3.82	–	46.48±0.18
Chlorogenic Acid	101±1.14		112±0.96	
Caffeic Acid	98±0.87		109±0.75	
Ascorbic Acid	106±1.03		119±1.22	

Higher antioxidant activity was exhibited by extracts obtained with 70% and 40% hydro-alcoholic extract from leaves harvested in July (125 and 107 mg/mL, respectively, in terms of rutin equivalents, and 405 and 347 mg/mL in terms of ascorbic acid equivalents). Compared to the dry aqueous extract, the 70 and 40% hydro-alcoholic extracts contained significantly higher concentrations of hydroxycinnamic acids, coumarins, carotenoids, and chlorophyll (Table 2). These groups of biologically active substances, as reported in the literature, have antioxidant properties [20, 21]. The peak accumulation of these BAS in the leaves occurred in July, as shown in Table 1. The AOA of the 70 and 40% hydro-alcoholic extracts from leaves collected in October was slightly lower, with a difference of no more than 5% (118.4 and 102 mg/mL in terms of rutin equivalents, and 383.68 and 331 mg/mL in terms of ascorbic acid equivalents). This reduction may be attributed to the decline in BAS content affecting antioxidant activity by October. The industrially produced dry aqueous extract demonstrated lower AOA due to its significantly reduced content of phenolic compounds (HCAs, coumarins, flavonoids) and chlorophyll, partially compensated by higher concentrations of ascorbic acid and tannins.

The 70 and 40% hydro-alcoholic extracts from leaves, collected in both July and October, exhibited high Antiradical Activity (ARA) and inhibition degree (ID), significantly outperforming the aqueous extract. The similarity in ARA and DI values between summer and autumn hydro-alcoholic extracts may be influenced not only by the content of phenolic compounds, chlorophyll, and vitamins but also by specific individual compounds. Moreover, some authors suggest that high ARA and ID in extraction complexes could be linked to the high polysaccharide content [22, 23], which increases in *Arctium tomentosum* leaves towards the end of the growing season and transfers to the respective extracts.

In the study by Lou et al., it was shown that hydro-alcoholic fractions have the highest antioxidant activity compared to aqueous, butanol, ethyl acetate and ether fractions from *A. lappa* leaves [10]. This correlates with the results we obtained (hydro-alcoholic extracts are significantly more active than the aqueous extract). Possible

mechanisms of the antioxidant effect of extracts include: blocking the production of free radicals; preventing peroxidation of not only lipids, but also proteins and fats; blocking the action of the enzyme xanthine oxidase; protective action on enzymes – superoxide dismutase and peroxidase.

Conclusion

The dominant of biologically active substances in the leaves of *Arctium tomentosum* include polysaccharides, hydroxycinnamic acids, tannins, and triterpenoid saponins. The peak accumulation of these substances in raw materials (excluding polysaccharides) occurs in July. The dominant groups of substances in both hydro-alcoholic and dry aqueous extracts correspond to those in the original raw materials. Chlorogenic acid is the predominant compound in all extracts, while 4-caffeoylquinic acid is found only in the 70% hydroalcoholic extract. High antioxidant and antiradical activities were observed in the 70 and 40% hydroalcoholic extracts from the leaves. Compared to the aqueous extract, these ethanol extracts contained significantly higher concentrations of hydroxycinnamic acids, coumarins, carotenoids, and chlorophyll. The results demonstrate the potential for further research of *A. tomentosum* leaves as sources for developing products with antioxidant effects. Further research is required to clarify the individual antioxidant components and the underlying pharmacological mechanisms of these effects.

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Conflict of Interest
The authors of this work declare that they have no conflicts of interest.

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