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METABOLIC PROFILING OF *ARTEMISIA SANTHONICUM* AND *ARTEMISIA LERCHIANA* BY GC/MS

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In the flora of Bulgaria, the genus *Artemisia* L. is represented by 13 species. This study is devoted to the study of two morphologically similar species - *Artemisia lerchiana* Stechm and *Artemisia santonicum* L. **Materials and methods.** The objects of study were methanol and acetone extracts from the dried aerial part and the fractions obtained from them. We used TLC and GC-MS methods. The antioxidant activity of the extracts was assessed by the method with DPPH reagent. **Results and discussion.** In the lipophilic fractions of the methanol extract of *A. lerchiana* and *A. santonicum* fatty acids, higher alcohols and sterols was found, and *A. santonicum* is distinguished by a high content of C18:3, C18:1, C18:2 fatty acids. Organic acids, phenolic acids, mono- and disaharas were found in the polar fractions. In the acetone extract triterpenes, organic and phenolic acids, and sugars were determined. Using TLC profile differences for the studied species were established. When comparing the antioxidant properties, it was found that the methanol extracts of the studied species have the greatest activity compared to the acetone extracts, while the extracts from *A. santhonicum* exhibited stronger activity than *A. lerchaina*. **Findings.** A comparative study of the profile of the metabolites of *A. santhonicum* and *A. lerchaina* was carried out. It has been shown that the methanol extract of *A. santhonicum* has a richer metabolite composition and a higher antioxidant activity. The data obtained are new to the studied representatives of the genus Artemisia L.

***Key words**: exudate flavonoids, fatty acids, phenolic acids, DPPH.*

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Genus *Artemisia* L. (Asteraceae) includes more than 500 species with distribution in the temperate zones of Europe, Asia and North America [1]. The predominant biological types are perennial herbaceous plants and shrubs.

In the Bulgarian flora, the genus is represented by 13 species [2]. The objects of the present study are two morphologically similar species – *Artemisia lerchiana* Stechm and *Artemisia santonicum* L. with limited distribution in Bulgaria. They belong to *Artemisia maritima* group with typical occurrence on saline and dry habitats mainly in the eastern part of the country, often along the Black Sea coast. The two species exist as semi shrubs or perennial herbs. They form numerous flowering stems and sterile shoots, with massive partly lignified roots. The main difference between the species is that involucrum scales of *A. lerchiana* are densely covered by white and gray hairs, while these of *A. santonicum* are glabrous. With regard to chemical composition, the both species have been studied mainly for content of essential oil and sesquiterpene lactones [3–7]. The most studies about *A. santonicum* refer to its biological activity. Antidiabetic, antibacterial, antioxidant activities have been reported of the species [8–10].

Principal aim of the study was to make a comparative analysis of the metabolic profiles of the two

closely related *Artemisia* species – *A. santonicum* and *A. lerchiana* distributed in Bulgaria.

MATERIALS AND METHODS

Plant material: Aerial parts of the studied species were collected from the Bulgarian Black Sea in the summer of 2018 year in the phenological stage beginning of flowering. *A. lerchiana* was collected from Byalata laguna locality, close to Balchik (43°24'24.92"N/ 28°14'31.21"E, 31 m a.s.l.). *A. santonicum* was collected from locality, close to Primorsko (42°14'42.49"N/ 27°45'22.82"E, 13 m a.s.l.).

Preparation of extracts: *Methanol extract* and their fractions were obtained according to Berkov et al. [11]. Sample of 100 mg of dried plant material as well as internal standards of 50 μg of nonadecanoic acid, 50 μg of ribitol and 50 μg of 3,4 dichloro-4-hidroxy benzoic acid were placed in 2 mL Eppendorf tubes and extracted with 1 mL of MeOH for 24 h at room temperature. Aliquot of 800 μL was transferred into another Ependorf tube, 500 μL H_2O and 500 μL of $CHCl_3$ were added, and after vortexing for 2 min, the mixture was centrifuged. The chloroform fraction was separated, evaporated and transmethylated with 2% of H_2SO_4 in MeOH at 60 ºC for 18 h, than lipids were extracted with *n*-hexane (2×500 μL), which was dried with anhydrous Na_2SO_4 and evaporated to obtain lipid fraction. An aliquot of 100 μL from the aqueous fraction was placed in glass vial and evaporated to obtain polar fraction. The rest of aqueous fraction was hydrolyzed with 0.5 mL of 1N NaOH for 18 h at 60 ºC. After acidification to pH 1–2 with conc. HCl, the phenolic compounds were extracted with EtOAc (2×500 μL) which was dried with anhydrous Na_2SO_4 and evaporated to obtain phenolic fraction. The obtained extractions and fractions were silylated with 50 μL of N,O-bis-(trimethylsilyl)trifluoro-acetamide (BSTFA) in 50 μL of pyridine for 2 h at 50 °C.

Acetone exudates: Air dried plant material, not powdered, was briefly (2–3 min) rinsed with acetone at room temperature to dissolve the lipophilic components accumulated on the surface. After evaporation of the acetone, the residues were subjected to further analysis.

GC–MS analysis: The GC–MS spectra were recorded on a Termo Scientific Focus GC coupled with Termo Scientific DSQ mass detector operating in EI mode at 70 eV. ADB-5MS column (30 m × 0.25 mm × 0.25 μm) was used. The temperature program was: 100…180 ºC at 15 ºC × min^{-1}, 180…300 ºC at 5 ºC × min^{-1} and 10 min hold at 300 ºC. The injector temperature was 250 ºC. The flow rate of carrier gas (Helium) was 0.8 mL × min^{-1}. The split ratio 1:10 1 μL of the solution was injected. The metabolites were identified as TMSi derivatives comparing their mass spectra and Kovats Indexes (RI) with those of an on-line available plant specific database. The measured mass-spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. RI of the compounds were recorded with standard n-hydrocarbon calibration mixture (C9-C36) (Restek, Cat no. 31614, supplied by Teknokroma, Spain) using AMDIS 3.6 software.

Thin layer chromatographic analysis: The acetone exudates were examined for flavonoid aglycones by co-*TLC* with authentic compounds. Toluene:dioxane:acetic acid (95:25:4, v/v/v) was used for the development of the exudates on HPTLC Silica gel 60 F_{254} (Merck 5548) and (Merck 5744) plates. Toluene:methylethylketone:methanol (60:25:15, v/v/v) and (30:10:10, v/v/v) were used on Polyamid 11 F_{254} (Merck 5557) and (Merck 5555) plates. Water: acetic acid (60/40, v/v) was used on Cellulose F_{254} (Merck 5574) and (Merck 5725) plates. Chromatograms were viewed under UV light at 336 nm before and after spraying with ‘‘Naturstoffreagenz A’’, 1% solution of diphenylboric acid-ethanolamine complex in methanol.

Free radical scavenging activity: The effect of methanol extracts on DPPH radicals was estimated according to Karabegović et al. (2011). Results are presented as IC_{50} values (μg mL^{-1}) – extract concentration providing 50% inhibition of the DPPH solution. The IC_{50} values were calculated by Software Prizm 3.00. All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Lipid, polar and phenolic fractions of methanol extracts of *A. santhonicum* and *A. lerchiana* were analyzed for chemical composition by GC/MS. The results are presented in Table 1. Fatty acids and alcohols were determined as the main components of lipid fractions of the both species. The high content of unsaturated fatty acids (C18:3, C18:1, C18:2) was noticeable. This result is consistent with previous studies relevant to the species *A. lerchiana* [12]. In the lipid fraction of *A. santhonicum*, doubly higher content of unsaturated fatty acids and sterols than of *A. lerchiana* was found. The high content of unsaturated fatty acids implies good antibacterial and antifungal properties of the plant [12].

Table 1. ***Metabolites identified of methanole extracts of studied Artemisia species***

Compounds	RI	*A. santhonicum**	*A. lerchiana**
Fatty alcohols			
Dodecanol	1562	–	12±4,5
Tetradecanol	1760	23,5±1,9	5,4±0,3
Hexadecanol	1957	35,4±0,9	15,2±7,7
Octadecanol	2154	22,4±3,7	15,8±2,7
Tetracosanol	2741	2,8±1,2	23,1±4,1
Fatty acids			
Octanoic acid (Caprylic acid, C8:0)	1575	43,2±2,2	216±8,6
Tetradecanoic acid (C14:0)	1722	38,8±1,3	8,4±0,1
Hexadecanoic acid (Palmitic acid, C16:0)	1929	812,8±12,1	271,1±3,2
Octadecadienoic acid (Linoleic acid, C18:2)	2089	691,1±5,3	267,5±3,5
Octadecatrienoic acid (Linolenic acid, C18:3)	2097	1042±21	125,4±13
Octadecenoic acid (Oleic acid, C18:1) (Z)	2098	1011±64	126,5±4,2
Octadecenoic acid (Oleic acid, C18:1) (E)	2102	104,5±4,9	22±0,4
Octadecanoic acid (Stearic acid, C18:0)	2132	420,8±12	78,4±1,6
Sterols			
Silane	2297	18,8±0,1	24,6±0,1
Stigmasterol	2412	–	24±0,5
β-Sitosterol	2614	118,3±1,2	0,2±0,06
Organic acids			
Succinic acid	1310	52,4±2,2	179,2±10,8
Glyceric acid	1340	48,8±6,9	–
Malic acid	1488	133,4±8,1	288,9±4,5
Pyroglutamic acid	1512	358,4±12,9	417,7±3,1
Saccharides			
Fructose 1	1793	6,1±0,8	1,3±0,5
Fructose 2	1803	20,3±1,8	13,1±1,1
Glucose	1889	–	42±2,8
Disaccharide	2579	16,1±1,2	5,4±0,9
Sucrose	2628	493,9±12	425,2±21
Disaccharide	2770	33,9±2,2	82,9±3,7
Free phenolic acids			
Salicylic acid	1516	2,8±0,7	16,3±2,2
4(p)-hydroxybenzoic acid	1640	10,4±2,3	0,4±0,05
Gentisic acid	1770	4±1,7	4,9±0,7
Vanilic acid	1776	3±0,8	5,6±0,8
Protocatechuic acid	1813	12,3±2,1	15,5±2,1
Quinic acid	1840	697,8±15	362,9±19
Syringic acid	1884	0,9±0,1	1,8±0,2
Hydroxycinnamic acid-p-*trans*	1948	–	4±0,5
Ferulic acid *trans*	2103	1,8±0,4	–
Caffeic acid	2142	43,6±3,5	15,8±2,1
Phenolic acid after alkaline hydrolysis			
Salicylic acid	1516	4,5±0,6	13,1±1,8
4(p)-hydroxybenzoic acid	1640	4,3±0,5	4,9±1,6
Vanilic acid	1776	8,7±0,3	8,5±0,4
Gentisic acid	1770	–	1,8±1,1
Protocatechuic acid	1813	35,1±8,2	1,3±0,01
Hydroxycinnamic acid-p-	1948	1,5±0,3	7,7±0,9
Ferulic acid	2103	3,3±1,1	8,7±1,6
Caffeic acid	2142	9,4±2,2	1,5±0,5

Legend: *Values are expressed as percentages of peak area of standard [μg/mL].

In the polar fraction, organic acids mono- and disaccharides were determined. Malic acid and pyroglutamic acids were detected as the most abundant organic acids. Free phenolic acids also identified in this fraction as well as in the crude methanol extracts. Quinic acid was the main in the both species. In the phenolic fractions, alkaline hydrolysable phenolic acids were determined. In the acetone exudates of the studied species, mainly nonpolar compounds were identified. The results are presented in Table 2.

Table 2. ***Metabolites identified of acetone exudates of studied Artemisia species***

Compounds	RI	*A. santhonicum**	*A. lerchiana**
Lipids			
Hexadecanoic acid (Palmitic acid, C16:0)	1929	1,25	1,28
Hexadecanol	1957	0,16	–
Octadecanol	2154	0,04	0,63
Silane	2297	0,55	–
Triterpene 1	2481	2,33	–
Triterpene 2	2519	–	1,17
Phenolic acids			
4(p)-hydroxybenzoic acid	1435	0,22	0,81
Vanilic acid	1776	0,56	0,13
Gentisic acid	1770	0,1	–
Protocatechuic acid	1813	0,34	2,81
Quinic acid	1843	0,21	1,7
Hydroxycinnamic acid-p-	1948	–	0,16
Ferulic acid	2104	0,02	–
Caffeic acid	2142	0,1	–
Chlorgenic acid	3099	1,17	0,5
Organic acids			
Succinic acid	1310	0,77	–
Malic acid	1488	0,37	–
Saccharides			
Monosacharide	–	0,89	–
Fructose 1	1793	0,53	–
Fructose 2	1803	3,06	2,45
Fructose 3	1830	2,38	1,92
Glucose	1889	–	1,68
Sucrose	2628	9,44	10,7

Legend: *Values are expressed as percentages of peak area of detected peaks.

Table 3. ***Exudate flavonoids identified of aerial parts of studied Artemisia species***

Flavonoid aglycones	Studied species	
	Artemisia santonicum	*Artemisia lerchiana*
Apigenin	Trace	Trace
-4'-Me	●	–
6-Hydroxyapigenin (*scutellarein*)		
-6-Me	●	●
-6,4-Me	●	●
Luteolin	●	●●
6- Hydroxyluteolin		
-6-Me	●	●●
-6,3'-Me	●	●
Quercetin	Trace	–
6-Hydroxyquercetin (*quercetagetin*)		
-6-Me	Trace	–
-3,6,4'-Me	–	●

Legend: Me – methyl ether.

It is important to note the presence of triterpenes in the exudates of both species which are the substances with important biological activity – anti-inflammatory, cytotoxic, anti-microbial, antitumor etc. [13, 14]. The established triterpenes in the studied species have different spectral data, indicating that the species differ in this class of compounds. Also, phenolic and organic acids were detected as well as mono- and disaccharide.

Additionally, acetone exudates of the studied species were checked by TLC for presence of flavonoid aglycones. Multi-component flavonoid profiles of both species were established (Fig. 1). Due to the complex composition, not all of the observed flavonoids were identified. By a comparison of the position and color of the flavonoid spots with those of authentic compounds on three different sorbents - silica gel, polyamide, and cellulose ten flavonoids were identified. Luteolin (**1**), apigenin (**2**), 6-hydroxyluteolin-6 methyl ether (**3**), 6-hydroxyluteolin-6,3'-dimethyl ether (**4**) scutelarein 6-methyl ether (**5**), scutelarein 6,4'-dimethyl ether (**6**) and were determined as common flavonoid aglycones for the both species.

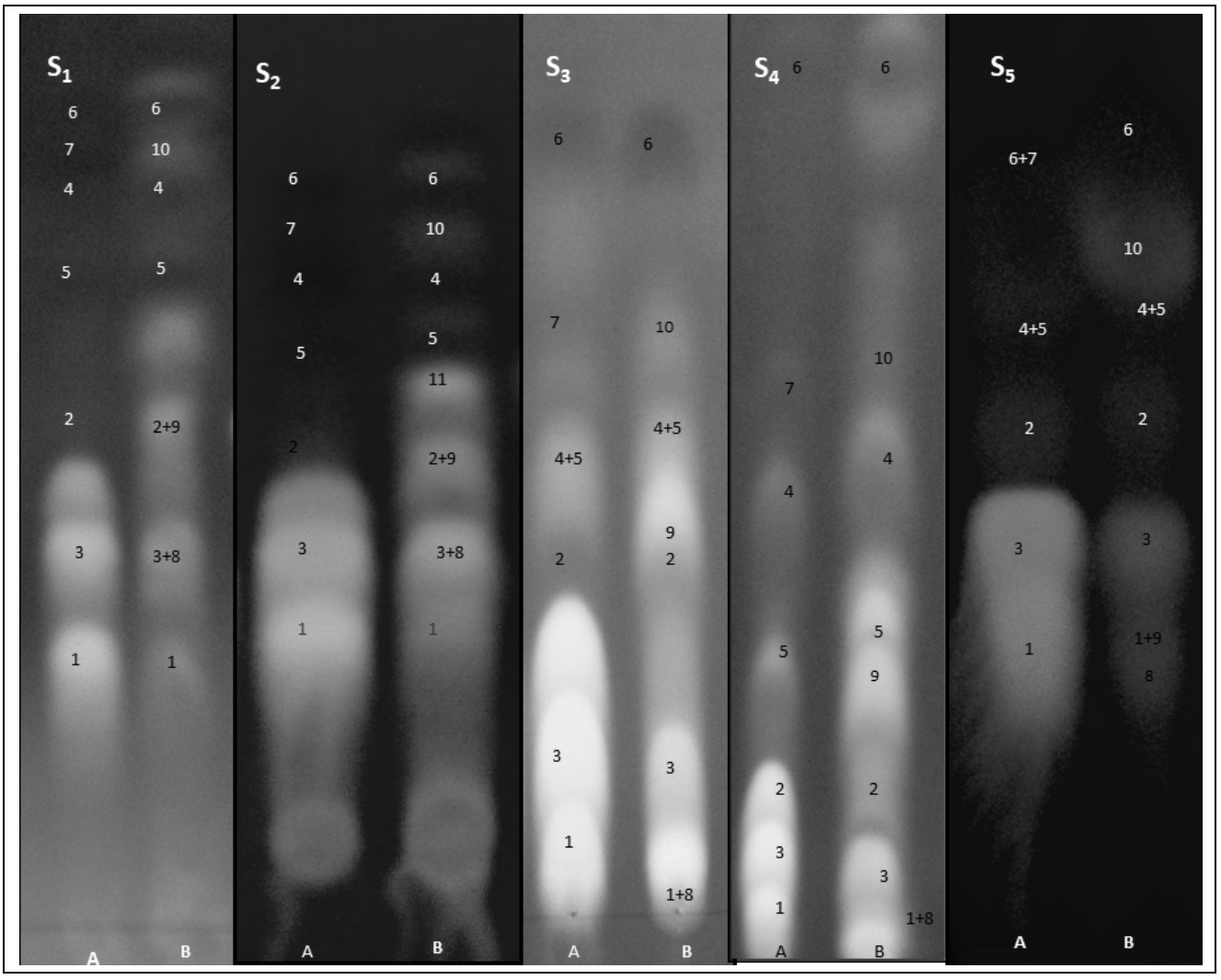

Fig. 1. TLC chromatograms of studied acetone exudates: **A** – *Artemisia lerchiana* acetone exudate; **B** – *Artemisia santhonicum* acetone exudates; S_1 – toluene:dioxane:acetic acid (95:25:4) on (Merck 5548) and on (Merck 5744) S_2 – silica gel plates; S_3 – toluene:methylethylketone:methanol (60:25:15) and (30:10:10) S_4 – on (Merck 5555) polyamide plates; S_5 – water: acetic acid (60/40) on (Merck 5574) cellulose plates; **1** (luteolin), **2** (apigenin), **3** (6-hydroxyluteolin 6-methyl ether), **4** (6-hydroxyluteolin 6,3'-dimethyl ether), **5** (scutelarein 6-methyl ether), **6** (scutelarein 6,4'-dimethyl ether), **7** (quercetagetin 3,6,4'-trimethyl ether) **8** (quercetin), **9** (quercetagetin 6-methyl ether), **10** (apigenin 4-methyl ether)

Quercetagetin 3,6,4'-trimethyl ether (**7**) was found for *A. lerchiana* exudate. Quercetin (**8**) and quercetagetin-6 methyl ether (**9**) as well as apigenin 4-methyl ether (**10**) were determined in *A. santhonicum* exudate. The reported data are new for the studied species. Both species can be distinguished well by their profiles of flavonoid aglycones by TLC analysis. The received results are in agreement with the previously published data on the flavonoid composition of *Artemisia* species of subgenus Seriphidium (Bess)Rouy. Exudate flavonoids (**1**, **2**, **3**, **4**, **7**, **8**, **9**) identified in the present study for *A. lerchiana* and *A. santhonicum*, have been reported for *Artemisia barrelieri* Besser from Spain of subgenus Seriphidium [15].

The results of free radical scavenging evaluation are presented as IC_{50} values (μg mL^{-1}) – extract concentration providing 50% inhibition of the DPPH solution. All studied extracts showed significant activity. Difference in free radical scavenging activity between methanol extracts and acetone exudates was found. The last showed lower activity probably due to the non-polar compounds contained in them and their lower activity. Methanol extracts and acetone exudate of *A. santhonicum* displayed stronger activity that that of *A. lerchaina* (Fig. 2). The results could be explain with the higher content of fatty and phenolic acids of *A. santhonicum* extract and exudate.

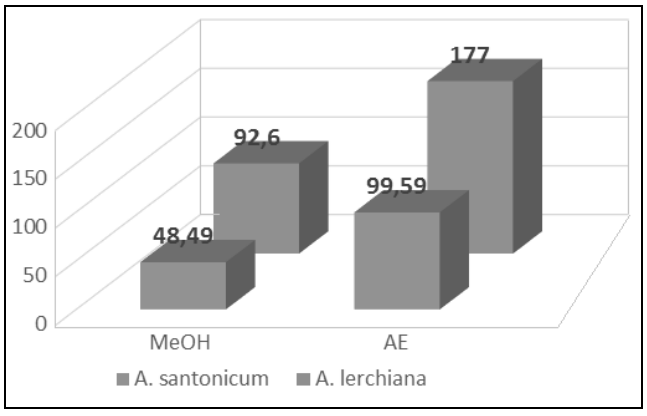

Fig. 2. Free radical scavenging activity of studied samples of *Artemisia lerchiana* and *Artemisia santonicum*

CONCLUSIONS

GC/MS and TLC analysis of methanol extracts and acetone exudates as well as their antiradical activities of *Artemisa santhonicum* and *A. lerchaina* were examined in the present study. The results revealed that the both species accumulated similar metabolites. The observed differences in qualitative and especially quantitative metabolite composition of the studied species find expression in antradical properties of their extracts. Methanol extract and acetone exudate of *Artemisa santhonicum* exhibited more complex metabolite profile and higher free radical scavenging activity than *A. lerchaina.* The reported data are new for the studied species.

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ИЗУЧЕНИЕ МЕТАБОЛИЧЕСКОГО ПРОФИЛЯ *ARTEMISIA SANTHONICUM* L. И *ARTEMISIA LERCHIANA* L. МЕТОДОМ ГХ-МС

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Artemisia L. является одним из самых значительных родов семейства Asteraceae, представители которого широко распространены в Евразии, в Северной и Южной Африке, Северной Америке. Во флоре Болгарии род *Artemisia* L. представлен 13 видами. Данное исследование посвящено изучению двух морфологически сходных видов – *Artemisia lerchiana* Stechm и *Artemisia santonicum* L. **Целью** работы являлась характеристика качественного состава вторичных метаболитов данных видов и оценка их антиоксидантных свойств.

Материалы и методы. Объектами изучения служили метанольный и ацетоновый экстракты из высушенной надземной части и полученные из них фракции. В работе использовали методы ТСХ и ГХ-МС. Антиоксидантную активность экстрактов оценивали по методу с реактивом DPPH.

Результаты и обсуждение. В результате проведенных исследований в липофильных фракциях метанольного экстракта *A. lerchiana* и *A. santonicum* было установлено наличие ненасыщенных жирных кислот, высших спиртов и стеролов, причем *A. santonicum* отличается большим содержанием жирных кислот C18:3, C18:1, C18:2. В полярных фракциях были обнаружены органические кислоты, фенолокислоты, моно- и дисахара. В составе ацетонового экстракта обоих видов определены тритерпены, органические и фенолокислоты, сахара. Методом ТСХ с использованием стандартных образцов идентифицировано десять флавоноидов, установлены различия профиля для изучаемых видов. При сравнении антиоксидантных свойств установлено, что наибольшей активностью обладают метанольные экстракты изучаемых видов по сравнению с ацетоновыми, при этом извлечения из *A. santhonicum* проявляли более сильную активность, чем *A. lerchaina*.

Выводы. Проведено сравнительное изучение профиля метаболитов *A. santhonicum* и *A. lerchaina*, установлены различия в качественном составе веществ. Показано, что метанольный экстракт *A. santhonicum* имеет более насыщенный состав метаболитов и более высокую антиоксидантную активность. Полученные данные являются новыми для изучаемых представителей рода *Artemsia* L.

Ключевые слова: *Artemisia santhonicum L., Artemisia lerchiana L., флавоноиды, фенолокислоты, жирные кислоты, DPPH.*

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