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Secondary metabolites in *in vitro* cultures of Siberian medicinal plants: Content, antioxidant properties, and antimicrobial characteristics

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Abstract:
Introduction. Wild-crafting leads to the local extinction of many medicinal plants that are rich in phenolic substances. *In vitro* cultivation of cells and organs of higher plants can be the optimal solution to this problem. The research objective was to study the biosynthetic activity of *in vitro* extracts of wild Siberian plants.
Study objects and methods. The study featured callus, cell suspension, and hairy root extracts of such Siberian medicinal plants as *Eleutherococcus senticosus*, *Codonopsis pilosula*, *Platanthera bifolia*, and *Saposhnikovia divaricata*. They were obtained by *in vitro* cultivation using modified nutrient media of Murashige and Skoog and Gamborg. The content of secondary metabolites was studied using the methods of thin-layer and high-performance liquid chromatography. A set of *in vitro* experiments tested the antioxidant and antimicrobial activity of the extracts.
Results and discussion. All the samples demonstrated a high content of secondary metabolites of phenolic nature. Flavonoglycosides, apigenin, and rutin were found to be the predominant biologically active substances in the callus extracts. Flavonoglycosides dominated in the suspension extracts. The root extracts contained more caffeic acid, rutin, ecdysteroids, quercetin, apigenin, cardiofolin, and coleofolide than the callus and suspension cultures. The list of prevailing secondary metabolites in the root extracts included rutin, apigenin, coleofolide, and quercetin. All the extracts showed antimicrobial and antioxidant activity.
Conclusion. All the extracts demonstrated good antioxidant and antimicrobial properties. Therefore, they can be used for the production of pharmaceuticals and biologically active food supplements as they can be helpful against infectious diseases, as well as oncological, cardiovascular, and neurodegenerative diseases linked to oxidative stress.

Keywords: Callus culture, cell suspension culture, hairy roots, medicinal plant, secondary metabolite, phenolic substances, antioxidant, oxidative stress, antimicrobial properties, extraction

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INTRODUCTION

According to World Health Organization (WHO), medicinal herbs receive a lot of attention in medicine worldwide. Currently, more than 50 000 plant species are used in herbal and allopathic medicine [1]. About 60% of medicinal plants are harvested from their natural habitat, the proportion of cultivated pharmaceutical plants being negligible [2–4]. Many medicinal plant species become extinct as a result of environmental degradation [5]. From 4000 to 10 000 species of medicinal plants have become endangered in the recent decades [3, 6].

In vitro cultivation of cells (callus, suspension cultures) and organs (hairy roots) of higher plants can be a good alternative to wild-crafting [7, 8]. *In vitro* methods have a lot of advantages in terms of secondary metabolites production. First, climatic chambers with their controlled environment do not depend on the weather conditions. Second, these methods allow for a

greater control over production of biologically active substances (BAS) in sterile conditions [9].

Polyphenols are the best known and most numerous metabolites with more than 8000 identified compounds, including phenolic acids, flavonoids, anthocyanins, and stilbenes. Plant polyphenols have excellent biotechnological prospects as they possess anticarcinogenic, antioxidant, antimicrobial, and anti-inflammatory properties [10].

Phenolic BAS with antimicrobial and antioxidant properties can be obtained from many plants [11]. The present research featured secondary metabolites obtained from *in vitro* cultures of wild medicinal plants growing in the Siberian Federal District, namely spiny eleuterococcus (*Eleutherococcus senticosus*), Asian bell (*Codonopsis pilosula*), butterfly orchid (*Platanthera bifolia*), and siler (*Saposhnikovia divaricate).*

The rhizomes and roots of *E. senticosus* owe their pharmacological properties to eleutherosides, which are special glycosides, conventionally marked as A, B, B_1, C, E, F, and G. In addition, *E. senticosus* contains polysaccharides, lipids, essential oils, tannins, and flavonoids, which make it a popular immunomodulatory agent. This plant is described in the Russian Pharmacopoeia [12].

C. pilosula has a general tonic and immunomodulatory effect [13]. *C. pilosula* proved to be a source of several neutral and acidic polysaccharides with immunomodulatory and antitumor properties [14].

The chemical composition of *P. bifolia* remains understudied. Its young tubers are known to contain mucus (up to 50%), which consists mainly of proteins (≤ 15%), sugar (≤ 1%), starch (≈ 27%), coumarin, mineral salts, traces of essential oil and alkaloids, and a small amount of calcium oxalate [15]. Salep possesses anti-inflammatory, antiseptic, tonic, tonic, and anticonvulsant properties [16].

S. divaricata (Turcz.) Schischk. owes its antipyretic, analgesic, hypotensive, antimicrobial, and antitumor properties to various useful substances in their roots. The list includes chromones, triterpenoids of cimifugine and *β*-glycosylcymiosyl sitosterol, steroids *β*-D-glycoside and *β*-sitosterol, coumarins, e.g. emperorin, scopoletin, psoralen, deltoin, bergapten, felloperin, and xanthotoxin [17].

The research objective was to study the biosynthetic activity of callus, cell suspension, and hairy root *in vitro* cultures of *E. senticosus*, *C. pilosula*, *P. bifolia*, and *S. divaricate.* The study also featured the antimicrobial and antioxidant properties of the biologically active substances produced by their cell cultures.

STUDY OBJECTS AND METHODS

The callus, cell suspension, and hairy root cultures of spiny eleuterococcus (*Eleutherococcus senticosus* L.), Asian bell (*Codonopsis pilosula* L.), butterfly orchid (*Platanthera bifolia* L.), and siler (*Saposhnikovia divaricate* L.) were obtained from their seeds. According to aseptic regulations, the seeds were washed in a surfactant solution and sterilized for 1 min in a 0.1% $HgCl_2$ solution. After being rinsed three times in distilled sterile water, the seeds were planted on agar nutrient media in 60 mm Petri dishes in order to obtain sterile seedlings.

The callus cultures of *E. senticosus* were grown on a nutrient medium which consisted of 50.00 mL of MS (Murashige and Skoog) macrosalts (20×), 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.07 g of jasmonic acid, and 20.00 g of agar (per 1 liter of distilled water) [18]. The callus cultures of *E. senticosus* were grown on a nutrient medium which consisted of 50.00 mL MS (Murashige and Skoog) macrosalts (20×), 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 0.07 g of jasmonic acid, and 20.00 g of agar (per 1 liter of distilled water) [18].

The callus cultures of *C. pilosula* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts (20×), 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 2.00 g of Tween 80, and 20.00 g of agar (per 1 liter of distilled water).

Callus cultures of *P. bifolia* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts (20×), 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 1.00 g of chitosan, and 20.00 g of agar (per 1 liter of distilled water).

The callus cultures of *S. divaricata* were grown on a nutrient medium of the following composition which consisted of 50.00 mL of MS macrosalts, 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), and 20.00 g of agar (per 1 liter of distilled water).

The first seedlings appeared after 6–8 weeks. The callus cultures were induced to eight-week-old sterile seedlings with 2–4 leaves: the leaves were cut into pieces and planted on agar medium in 60 mm Petri dishes. The first calli formed on days 7–14. The callus cultures were allowed to grow for 28 days.

The cell suspension cultures were grown in 250 mL flasks (30–40 mL of suspension per flask) in a shaker (100 rpm): 300–400 mg of callus cultures were placed in 25–30 mL of liquid nutrient media.

The cell suspensions of *E. senticosus* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts (20×), 1.00 mL of MS microsalts, 30.00 g

of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.07 g of jasmonic acid (per 1 liter of distilled water).

The cell suspensions of *C. pilosula* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts, 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 2.00 g of Tween 80 (per 1 liter of distilled water).

The cell suspensions of *P. bifolia* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts, 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D), 1.00 g of chitosan (per 1 liter of distilled water).

The cell suspensions of *S. divaricata* were grown on a nutrient medium which consisted of 50.00 mL of MS macrosalts, 1.00 mL of MS microsalts, 30.00 g of sucrose, 0.10 g of meso-inositol, 0.50 mg of vitamin B_6, 0.50 mg of nicotinic acid, 0.10 mg of vitamin B_1, 1.00 mg of kinetin, and 0.50 mg of 2,4-dichlorophenoxyacetic acid (2,4-D) (per 1 liter of distilled water).

The cell suspension cultures were maintained under 16 h of light and 8 h of dark for 14–21 days.

The root cultures (hairy roots) were obtained from the leaves of 14–28-day-old seedlings. The seedling explants were inoculated with a wild strain of *Agrobacterium rhizogenes* A4 grown on a YEB nutrient medium for 48 h in the dark at 26°C in a shaker that performed circular motions with an amplitude of 5–10 cm and rotation speed of 90 rpm. The medium consisted of 5 g/L of peptone, 1 g/L of yeast extract, 5 g/L of sucrose, and 0.5 g/L of $MgCl_2$ [19].

The transformation was conducted according to the following pattern. After a pair of leaves appeared, the aerial part of the seedlings was separated from the roots, and the leaves, the caulicle, and the hypocotyl were cut into 1.0–1.5 cm segments. After that, the leaf rib was carefully pricked with an insulin syringe needle along the epicotyl and hypocotyls, attempting to reach the vascular system in the center and of the plant. The explants were subsequently transferred onto the YEB medium and kept in a magnetic bath for 10–100 for a more efficient transformation. The incubation time was 48 h. After the incubation of the explants with agrobacterium, the plant material was rinsed in sterile water and transferred onto solid Gamborg B5 medium. To eliminate *A. rhizogenes*, the medium contained 500 mg/L of claforan [20]. The Petri dishes with the explants were placed in a light chamber, where they stayed until they developed transformed roots.

After the roots reached a certain size, they were transplanted onto a fresh hormone-free B-5 nutrient medium to eliminate *A. rhizogenes* completely. The roots were cultivated in the dark at 23°C for 35 days using a shaker (100 rpm). They were subsequently transplanted into a fresh medium as the contamination with the agrobacterium increased.

Secondary metabolites were extracted from the biomass of callus, cell suspension, and hairy root cultures with 70% ethanol by placing 3.0 g of dried biomass of callus, cell suspension, and hairy root cultures in a 50 mL plastic test tube. Together with an appropriate amount of ethyl alcohol, the portion was placed in a shaker and stirred for 60 min. Table 1 demonstrates the extraction parameters.

The resulting extracts were filtered, and the filtrates were centrifuged at 3900 rpm. The solvent was then removed from the extracts by evaporation under reduced pressure in a rotary evaporator. The flask was weighed to measure the extract yield. The dry extract was dissolved in a suitable solvent, which underwent thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) to study the composition of biologically active substances.

The TLC was conducted according to the standard specified in the Russian Pharmacopoeia, Chapter 1.2.1.2.0003.15.

The HPLC was performed using a Shimadzu LC-20 Prominence chromatograph (Shimadzu, Japan) with a Shimadzu SPD20MA diode array detector and a Zorbax C-18 column (150×4.6 mm, phase particle size = 5 μm). The mobile phase included acetonitrile (solvent A) and 0.1% trifluoroacetic acid (B). The HPLC involved gradient and isocratic separation; the wavelength during detection was 276 nm.

The biologically active substances were identified in two ways. First, the UV spectra and retention times of the peaks in the chromatograms were compared with the corresponding parameters in the chromatographically

Table 1 Extraction of secondary metabolites from the biomass of callus, cell suspension, and hairy root cultures with 70% ethyl alcohol

Plant	Water duty	Time, min	Temperature, °C
Callus cultures			
Eleutherococcus senticosus	1:10	60	60
Codonopsis pilosula	1:10	60	40
Platanthera bifolia	1:10	60	40
Saposhnikovia divaricata	1:10	60	40
Suspension cultures			
Eleutherococcus senticosus	1:10	60	60
Codonopsis pilosula	1:10	30	40
Platanthera bifolia	1:5	30	40
Saposhnikovia divaricata	1:10	60	40
Root cultures			
Eleutherococcus senticosus	1:10	60	60
Codonopsis pilosula	1:10	60	40
Platanthera bifolia	1:10	60	40
Saposhnikovia divaricata	1:10	60	40

pure samples. The chromatograms were processed in the LabSolutions. Second, the biologically active substances were identified using high performance liquid chromatography combined with tandem mass spectrometry (HPLC-MS).

The DPPH method made it possible to assess the antioxidant activity of the extracts as stated in [21]. First, the optical absorption of a 2,2-diphenyl-1-picryl-hydrazyl (DPPH) in methanol solution was measured at 515 nm. The DPPH solution and the antioxidant solution were mixed, and the optical density was measured again after 10 min. The antioxidant activity was calculated by the formula:

$$AA = \left(E_{DPHH} - \frac{E_{ex}}{E_{DPPH}}\right) \cdot 100\% \quad (1)$$

where E_{DPPH} and E_{ex} – the optical density of the DPPH solution and the antioxidant solution, respectively.

The antimicrobial properties of the extracts were determined in relation to the opportunistic and pathogenic test strains on a solid nutrient medium (diffusion method) and in a liquid nutrient medium. The test strains involved *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Proteus vulgaris* ATCC 63, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* EMTC (Russian Collection of Extremophilic Microorganisms and Type Cultures) 34, *Leuconostoc mesenteroides* EMTC 1865, *Shigella flexneri* ATCC 12022, and *Shigella sonnei* ATCC 25931.

The diffusion method determined the antimicrobial activity of the extracts according to the following pattern. The test strain was inoculated on beef-extract agar using the spread plate technique. A paper disc with the nutrient medium served as control, and a disc with an antibiotic ciprofloxacin served as reference. The Petri dishes were incubated at 35–37°C for 24 h. The results depended on the presence and size (mm) of the microorganism-free transparent zone around the disc. The diameter of the inhibition zones was measured with an accuracy of 1 mm with a vernier caliper.

The second method involved incubating the test strains in 96-well culture plates. Overnight broth cultures were re-suspended in a Mueller Hinton plate (*C. albicans* – in Sabouraud's medium) until the number of microorganisms reached the seed dose of ~10^5 CFU/mL. The cell suspension and the extracts were simultaneously introduced into the wells in an amount of 1/10 of the total volume. MRS medium served as control, ciprofloxacin (10 μg/mL) – as reference; the total suspension volume in each well was 200 μL, the test was performed in duplicate. The wells were incubated at 35°C in a shaker (580 rpm). After 24 h, the optical density was measured using a multi-reader at 595 nm. Bactericidal activity was assessed by the change in the optical density in comparison with the control. In the wells where cell growth stopped or slowed down, the optical density was lower than in the wells with normal microbial growth. Ciprofloxacin served as reference because it is known as a standard for this group of antibacterial medications. It is also effective against Gram-negative microorganisms and staphylococci, including some strains that are resistant to other antibiotics.

Statistical data were processed using Microsoft Office Excel 2007 and the paired Student's t-test. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

The resulting callus, cell suspension, and hairy root cultures of spiny eleuterococcus (*Eleutherococcus senticosus* L.), Asian bell (*Codonopsis pilosula* L.), butterfly orchid (*Platanthera bifolia* L.), and siler (*Saposhnikovia divaricate* L.) were dried *in vitro* and extracted with ethyl alcohol (see Table 1 for extraction parameters).

Tables 2–4 demonstrate the content of secondary metabolites in the callus, suspension, and root extracts.

The chromatographic tests showed that the biomass of callus, suspension, and root cultures of *E. senticosus*, *C. pilosula*, *P. bifolia*, and *S. divaricata* accumulated such secondary metabolites as phenolic acids, flavistonoids, ecdysanthonoids, and ecdysanthonoids.

Table 2 shows that the callus extracts proved rich in flavonoglycosides, apigenin, and rutin. Codonopsin, cardiofolin, and coleofolide were less abundant. The

Table 2 Content of secondary metabolites in callus extracts

Secondary metabolite	Content of secondary metabolites, mg/kg			
	E. senticosus	*C. pilosula*	*P. bifolia*	*S. divaricata*
Caffeic acid	2.430 ± 0.120	0.400 ± 0.020	0.800 ± 0.040	4.200 ± 0.210
Rutin	3.080 ± 0.150	3.440 ± 0.170	3.550 ± 0.180	0.650 ± 0.030
Total ecdysteroids	0.030 ± 0.002	0.100 ± 0.010	0.070 ± 0.010	0.470 ± 0.020
Mangiferin	0.180 ± 0.010	0.360 ± 0.020	7.370 ± 0.370	0.140 ± 0.010
Quercetin	1.170 ± 0.060	0.650 ± 0.030	0.120 ± 0.010	0.780 ± 0.040
Total flavonoglycosides	8.070 ± 0.400	7.560 ± 0.380	6.680 ± 0.330	4.310 ± 0.220
Apigenin	2.740 ± 0.140	4.710 ± 0.240	5.230 ± 0.260	4.210 ± 0.210
Codonopsin	0.760 ± 0.040	0.390 ± 0.020	0.440 ± 0.020	0.510 ± 0.030
Cardiofolin	0.950 ± 0.050	0.810 ± 0.040	0.350 ± 0.020	0.540 ± 0.030
Coleofolide	0.810 ± 0.040	0.470 ± 0.020	0.210 ± 0.010	3.910 ± 0.200

Table 3 Content of secondary metabolites in suspension extracts

Secondary metabolite	Content of secondary metabolites, mg/kg			
	E. senticosus	*C. pilosula*	*P. bifolia*	*S. divaricata*
Caffeic acid	2.43 ± 0.12	0.41 ± 0.02	0.82 ± 0.04	4.29 ± 0.21
Rutin	3.24 ± 0.16	3.21 ± 0.16	3.31 ± 0.17	0.34 ± 0.02
Total ecdysteroids	0.13 ± 0.01	0.13 ± 0.01	0.77 ± 0.04	0.67 ± 0.03
Mangiferin	0.38 ± 0.02	0.96 ± 0.05	8.71 ± 0.44	0.44 ± 0.02
Quercetin	1.18 ± 0.06	0.41 ± 0.02	0.27 ± 0.01	0.17 ± 0.01
Total flavonoglycosides	8.27 ± 0.41	6.21 ± 0.31	1.48 ± 0.07	2.37 ± 0.12
Apigenin	1.74 ± 0.09	1.71 ± 0.09	1.23 ± 0.06	1.12 ± 0.06
Codonopsin	0.76 ± 0.04	4.39 ± 0.22	2.44 ± 0.12	5.51 ± 0.28
Cardiofolin	0.35 ± 0.02	0.31 ± 0.02	0.95 ± 0.05	0.21 ± 0.01
Coleofolide	4.81 ± 0.24	5.47 ± 0.27	6.21 ± 0.31	3.91 ± 0.20

Table 4 Content of secondary metabolites in root extracts

Secondary metabolite	Content of secondary metabolites, mg/kg			
	E. senticosus	*C. pilosula*	*P. bifolia*	*S. divaricata*
Caffeic acid	9.43 ± 0.47	8.40 ± 0.42	8.80 ± 0.44	7.20 ± 0.36
Rutin	63.08 ± 3.15	13.44 ± 0.67	53.55 ± 2.68	30.65 ± 1.53
Total ecdysteroids	6.03 ± 0.30	7.10 ± 0.36	6.07 ± 0.30	5.47 ± 0.27
Mangiferin	7.18 ± 0.36	8.36 ± 0.42	3.37 ± 0.17	5.14 ± 0.26
Quercetin	12.17 ± 0.61	14.65 ± 0.73	17.12 ± 0.86	12.78 ± 0.64
Total flavonoglycosides	2.07 ± 0.10	5.56 ± 0.28	6.68 ± 0.33	6.31 ± 0.32
Apigenin	32.74 ± 1.64	14.71 ± 0.74	75.23 ± 3.76	12.12 ± 0.61
Codonopsin	1.76 ± 0.09	0.59 ± 0.03	0.54 ± 0.03	0.31 ± 0.02
Cardiofolin	3.95 ± 0.20	11.81 ± 0.59	10.35 ± 0.52	7.61 ± 0.38
Coleofolide	17.81 ± 0.89	20.47 ± 1.02	40.21 ± 2.01	63.91 ± 3.20

callus extract of *P. bifolia* had the maximal amount of mangiferin. The extracts of *S. divaricata* and *E. senticosus* demonstrated the biggest amount of caffeic acid, while *E. senticosus* had the highest content of quercetin.

According to Table 3, flavonoglycosides appeared to be the predominant secondary metabolites in the suspension extracts. However, their content was much lower in the suspension extracts of *C. pilosula*, *P. bifolia*, and *S. divaricata* by 17.8, 77.8, and 45.0%, respectively, in comparison with callus extracts. The suspension extract of *P. bifolia* had the highest content of mangiferin: its content increased by 18.2% in comparison with the callus extract. The contents of caffeic acid, rutin, total ecdysteroids, and quercetin followed the same pattern as in the callus extracts. As for apigenin, its content in the suspension extracts of *E. senticosus*, *C. pilosula*, *P. bifolia*, and *S. divaricata* decreased by 36.5, 63.7, 76.5, and 73.4%, respectively. The content of codonopsin in the suspension extracts of *C. pilosula*, *P. bifolia*, and *S. divaricata* increased by 10.2, 4.5, and 9.8 times, respectively. The suspension extracts of *E. senticosus*, *C. pilosula*, and *P. bifolia* demonstrated a higher biosynthesis of coleofolide in comparison with callus extracts.

Table 4 shows that the content of caffeic acid, rutin, ecdysteroids, quercetin, apigenin, cardiofolin, and coleofolide in the root extracts was higher than in callus and suspension extracts. Rutin, apigenin, coleofolide, and quercetin were found to be the dominant biologically active substances in the root cultures.

Secondary metabolites of medicinal plants often demonstrate various types of biological activity, e.g. antimicrobial or antioxidant. Experiments *in vitro* proved that caffeic acid possesses antimicrobial, antimycotic, and immunomodulatory properties, as well as the ability to absorb free radicals [22]. Other studies [23, 24] also revealed its antibacterial properties.

Rutin is known for its antioxidant properties, which were found superior to those of vitamins C and E [25–27]. The antioxidant action of this flavanoid can be explained by its ability to activate antioxidant enzymes [25]. Quercetin is one of the most powerful antioxidative polyphenols [28, 29]. It also possesses anti-inflammatory, antimicrobial, anticarcinogenic, and antiviral properties [30]. Mangiferin is also known worldwide for its experimentally confirmed antioxidant, radioprotective, and immunomodulatory properties [31].

The obtained results made it possible to study the antioxidant activity of the callus, suspension, and root extracts (Fig. 1).

Figure 1 shows that all the samples exhibited antioxidant properties. The root extracts demonstrated the maximal antioxidant activity. The antioxidant activity of the extracts obtained from the biomass of hairy roots was 4.2–10.1 times (depending on the

species) higher than in callus extracts and 4.0–4.9 times higher than in suspension extracts. The root extract of *E. senticosus* had the best antioxidant properties. The revealed pattern is consistent with that for phenolic biologically active substances, where the root extracts also demonstrated the greatest accumulation (Tables 2–4).

Table 5 shows the antimicrobial activity by the diffusion method, while Figs. 2–4 show the results of the optical density method.

According to Table 5, all the extracts possessed antimicrobial activity against the tested strains. The best antimicrobial properties belonged to root extracts. The diameter of the lysis zone was 18.0–23.0 mm: in the callus and suspension extracts, this value did not exceed 17.5 mm. These results correlate with the results obtained for the antimicrobial properties of extracts in a liquid nutrient medium (Figs. 2–4).

CONCLUSION

The present research featured callus, cell suspension, and hairy root cultures of spiny eleuterococcus (*Eleutherococcus senticosus* L.), Asian bell (*Codonopsis pilosula* L.), butterfly orchid (*Platanthera bifolia* L.), and siler (*Saposhnikovia divaricate* L.). The TLC and HPLH tests showed a high content of secondary metabolites belonging to phenolic acids, flavonoids, ecdysteroids, and xanthones.

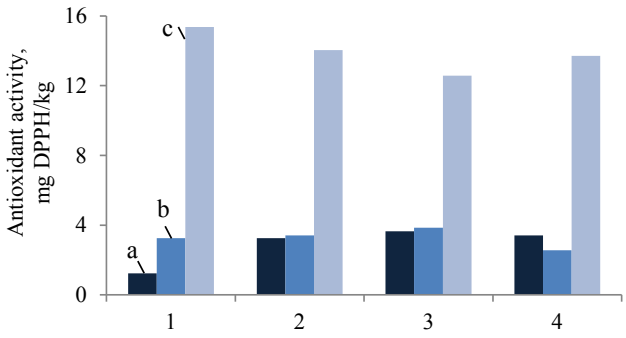

Figure 1 Antioxidant activity of callus (a), suspension (b), and root (c) extracts obtained from *Eleutherococcus senticosus* (1), *Codonopsis pilosula* (2), *Platanthera bifolia* (3), and *Saposhnikovia divaricate* (4)

For the callus extracts, the list of prevailing biologically active substances included flavonoglycosides apigenin, and rutin. Their total content depended on the plant species and varied from 4.31 to 8.07 mg/g for flavonoglycosides, from 2.74 to 5.23 mg/kg for apigenin, and from 3.08 to 3.55 mg/kg for rutin. The callus extract of *P. bifolia* appeared to have the highest content of mangiferin (7.37 mg/kg).

In case of all suspension extracts, flavonoglycosides dominated. The suspension extract of *P. bifolia* had the highest content of mangiferin: the concentration

Table 5 Antimicrobial activity of callus, suspension, and root extracts of *Eleutherococcus senticosus*, *Codonopsis pilosula*, *Platanthera bifolia*, and *Saposhnikovia divaricate* (diffusion method)

Test strain	Diameter of inhibition zone of test strains, mm, for callus, suspension, and root extracts					
	E. senticosus	*C. pilosula*	*P. bifolia*	*S. divaricata*	Control	Ciprofloxacin
E. coli ATCC 25922	15.0 ± 0.8	13.0 ± 0.7	14.0 ± 0.7	12.0 ± 0.6	0	23.0 ± 1.2
	13.0 ± 0.7	15.0 ± 0.8	17.0 ± 0.9	14.0 ± 0.7		
	22.0 ± 1.1*	23.0 ± 1.2	22.0 ± 1.1	20.0 ± 1.0		
S. aureus ATCC 25923	13.0 ± 0.7	12.0 ± 0.6	10.0 ± 1.0	13.0 ± 0.7	0	21.0 ± 1.1
	15.0 ± 0.8	13.0 ± 0.7	13.0 ± 0.7	12.0 ± 0.6		
	19.0 ± 1.0	19.0 ± 1.0	18.0 ± 0.9	20.0 ± 1.0		
P. vulgaris ATCC 63	12.0 ± 0.6	12.0 ± 0.6	16.0 ± 0.8	12.0 ± 0.6	0	22.0 ± 1.1
	11.0 ± 0.6	12.0 ± 0.6	13.0 ± 0.7	14.0 ± 0.7		
	20.0 ± 1.0	21.0 ± 1.1	21.0 ± 1.1	19.0 ± 1.0		
P. aeruginosa ATCC 9027	17.0 ± 0.9	11.0 ± 0.6	15.0 ± 0.8	12.0 ± 0.6	0	24.0 ± 1.2
	17.0 ± 0.9	15.0 ± 0.8	14.0 ± 0.7	14.0 ± 0.7		
	21.0 ± 1.1	23.0 ± 1.2	22.0 ± 1.1	19.0 ± 1.0		
C. albicans EMTC 34	15.0 ± 0.8	11.0 ± 0.6	12.0 ± 0.6	14.0 ± 0.7	0	23.0 ± 1.2
	12.0 ± 0.6	14.0 ± 0.7	11.0 ± 0.6	14.0 ± 0.7		
	21.0 ± 1.1	21.0 ± 1.1	20.0 ± 1.0	21.0 ± 1.1		
L. mesenteroides EMTC 1865	15.0 ± 0.8	14.0 ± 0.7	13.0 ± 0.7	12.0 ± 0.6	0	23.0 ± 1.2
	14.0 ± 0.7	16.0 ± 0.8	15.0 ± 0.8	16.0 ± 0.8		
	22.0 ± 1.1	20.0 ± 1.0	21.0 ± 1.1	22.0 ± 1.1		
Sh. flexneri ATCC12022	16.0 ± 0.8	15.0 ± 0.8	15.0 ± 0.8	12.0 ± 0.6	0	22.0 ± 1.1
	16.0 ± 0.8	17.5 ± 0.9	16.0 ± 0.8	16.0 ± 0.8		
	20.0 ± 1.0	21.5 ± 1.1	21.0 ± 1.1	22.0 ± 1.1		
Sh. sonnei ATCC 25931	15.5 ± 0.8	15.0 ± 0.8	12.0 ± 0.6	14.0 ± 0.7	0	20.0 ± 1.0
	17.5 ± 0.9	17.0 ± 0.9	14.5 ± 0.8	16.5 ± 0.9		
	20.0 ± 1.0	18.0 ± 0.9	19.5 ± 1.0	19.0 ± 1.0		

*line 1 – callus cultures, line 2 – suspension cultures, line 3 – root cultures

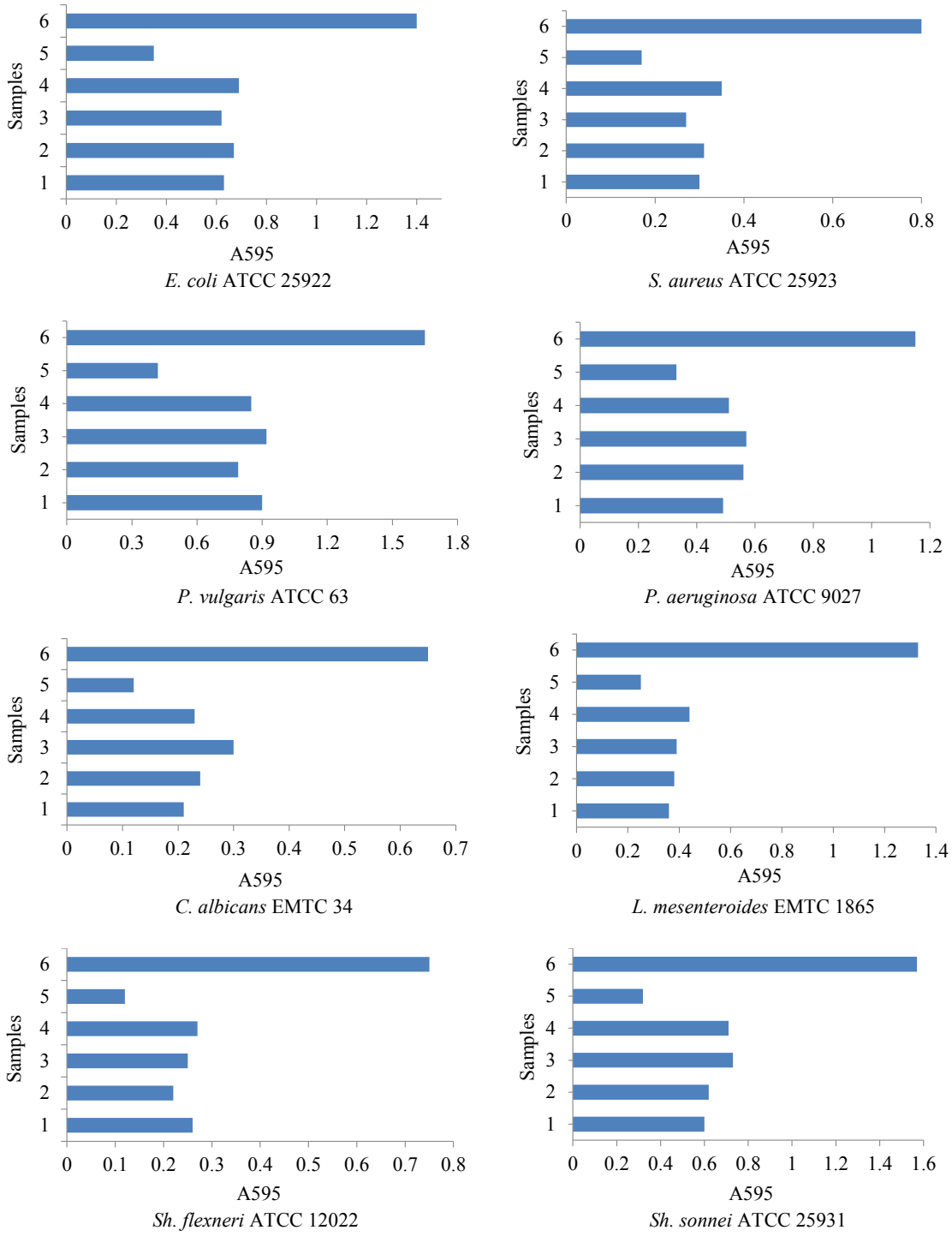

Figure 2 Antimicrobial activity of callus extracts (optical density method): 1 – *Eleutherococcus senticosus*; 2 – *Codonopsis pilosula*; 3 – *Platanthera bifolia*; 4 – *Saposhnikovia divaricata*; 5 – ciprofloxacin; 6 – control

of this xanthone glycoside increased by 18.2% in comparison with the callus extract. All the suspension extracts demonstrated the same pattern as the callus ones in relation to caffeic acid, rutin, ecdysteroids, and quercetine. The content of apigenin was lower than in the callus extracts, while that of codonopsin increased. The suspension extracts of *E. senticosus*, *C. pilosula*, and *P. bifolia* also demonstrated a higher biosynthesis of coleofolide than the callus extracts.

All the root extracts had an even higher content of caffeic acid, rutin, ecdysteroids, quercetine, apigenin, cardiofolin, and coleofolide. The list of prevailing biologically active substances included rutin (13.44–63.08 mg/kg), apigenin (12.12–75.23 mg/kg), coleofolide (17.81–63.91 mg/kg), and quercetin (12.17–17.12 mg/kg).

The experiments *in vitro* revealed antioxidant activity in all the samples. The maximal antioxidant activity belonged to the hairy root extracts.

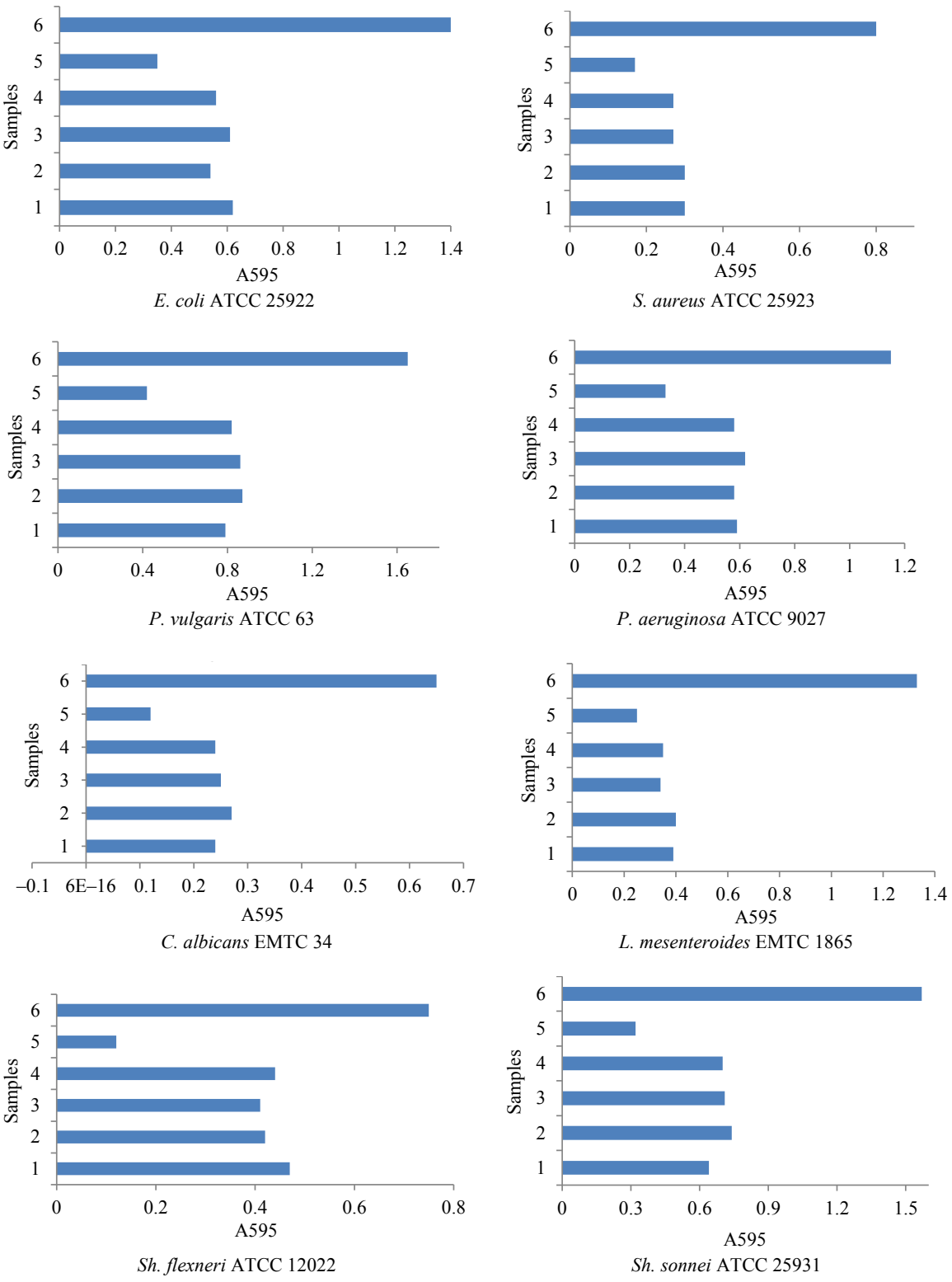

Figure 3 Antimicrobial activity of suspension extracts (optical density method): 1 – *Eleutherococcus senticosus*; 2 – *Codonopsis pilosula*; 3 – *Platanthera bifolia*; 4 – *Saposhnikovia divaricata*; 5 – ciprofloxacin; 6 – control

All the samples demonstrated antimicrobial activity against test strains of *Escherichia coli*, *Staphylococcus aureus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Candida albicans*, *Leuconostoc mesenteroides*, *Shigella flexneri*, and *Shigella sonnei*. The root extracts demonstrated the maximal antimicrobial properties.

Further research could cover such issues as isolation of individual phenolic substances from extracts of medicinal plants *in vitro*. This raw material can serve

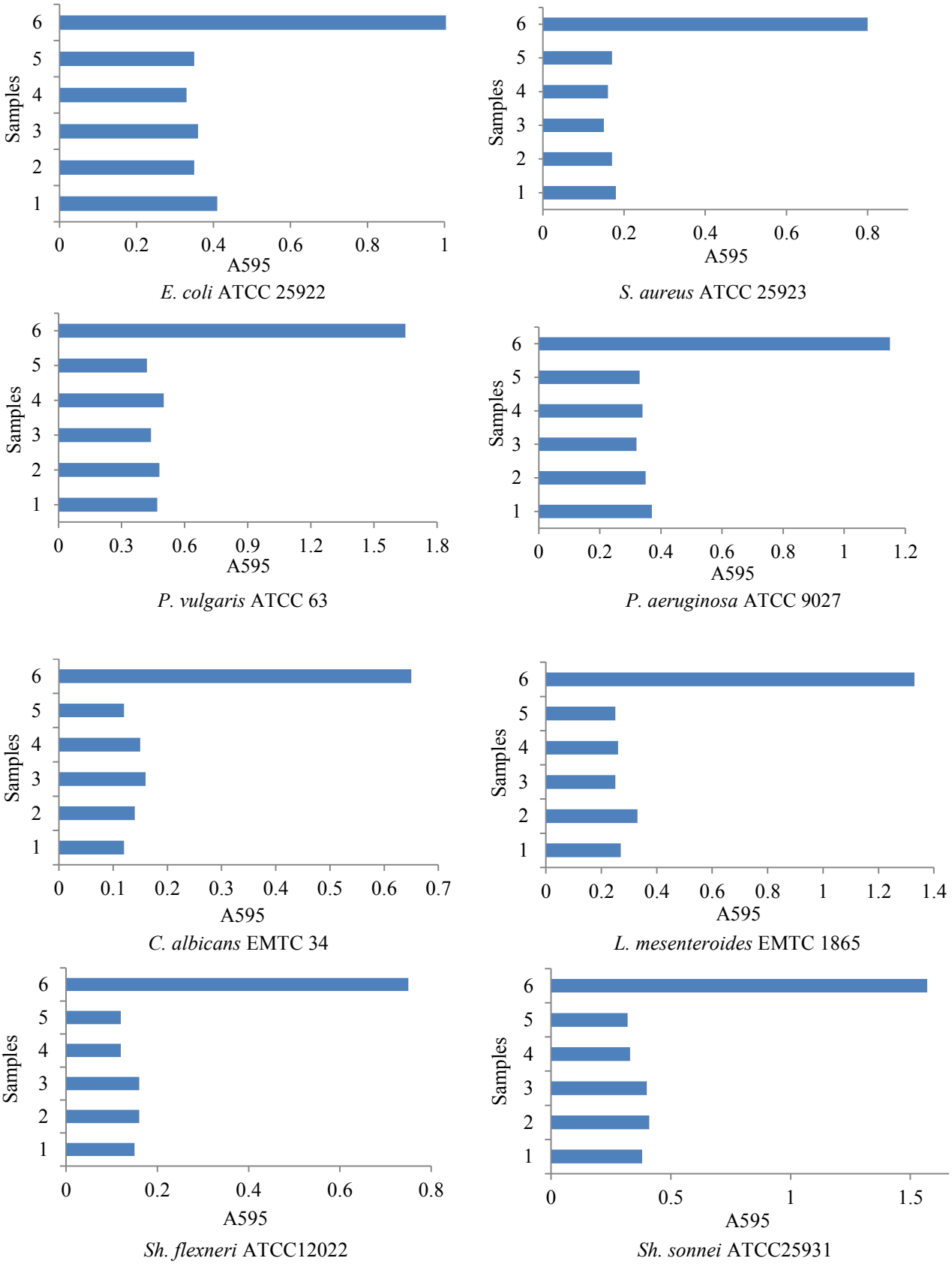

Figure 4 Antimicrobial activity of root extracts (optical density method): 1 – *Eleutherococcus senticosus*; 2 – *Codonopsis pilosula*; 3 – *Platanthera bifolia*; 4 – *Saposhnikovia divaricata*; 5 – ciprofloxacin; 6 – control

as basis for medications and biologically active food supplements for the prevention and treatment of infectious diseases or conditions linked to oxidative stress.

CONTRIBUTION

I.S. Milentyeva prepared the test samples and described the content, antioxidant activity, and antimicrobial properties of the secondary metabolites. V.M. Le studied the content of secondary metabolites in the callus extracts. O.V. Kozlova wrote the introduction. N.S. Velichkovich studied the antioxidant properties of callus, suspension, and root extracts. A.M. Fedorova researched the antimicrobial properties of the root extracts in liquid growth medium. A.I. Loseva described the research results.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of the present article.

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