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# Cytotoxic, Antioxidant, and Antibacterial Properties of Eastern Baltic Plant Extracts

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**Abstract.**
Plant extracts are a source of new drugs and alternative therapies. This article describes the antioxidant and antibacterial properties of aqueous methanol extracts of Eastern Baltic *Glycyrrhiza glabra* L., *Salix alba* L., and *Echium vulgare* L., as well as their effects on the viability of human blood cells.

The aerial parts of the plants were harvested in the Kaliningrad Region, Russia. The phytochemical composition of their plant extracts was studied by the method of high-performance liquid chromatography (HPLC). The bioactive profile and antibacterial action were described using the methods of spectrophotometry and disk diffusion, respectively. Cytotoxicity of extracts was studied by WST-1 colorimetric analysis.

The extracts proved to contain phenolic compounds. The antioxidant activity of the *S. alba* extracts was four times higher than that of *G. glabra* and more than seven times higher than that of *E. vulgare*. The *G. glabra* extracts were active against both Gram-positive and Gram-negative bacteria while the *E. vulgare* extract samples inhibited Gram-negative bacteria only. As for cytotoxicity, *S. alba* and *E. vulgare* were able to reduce the viability of human T-lymphoblastic leukemia (Jurkat) cells and human blood mononuclear cells.

The extracts of *G. glabra*, *S. alba*, and *E. vulgare* demonstrated good prospects for biomedicine. Further detailed research may result in their eventual introduction into official medicine as potent therapeutic and preventive agents.

**Keywords.** Plants, *Glycyrrhiza glabra*, *Salix alba*, *Echium vulgare*, aqueous methanol extract, free radicals

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# Цитотоксические, антиоксидантные и антибактериальные свойства экстрактов растений Восточной Прибалтики

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**Аннотация.**
Растительные экстракты являются перспективным источником новых лекарственных средств и альтернативных методов лечения. Цель данного исследования – изучить антиоксидантные и антибактериальные свойства водно-спиртовых экстрактов восточно-балтийских растений, а также их влияние на клетки крови человека.
Наземные части солодки голой (*Glycyrrhiza glabra* L.), ивы белой (*Salix alba* L.), и синяка обыкновенного (*Echium vulgare* L.) собраны в Калининградской области (Россия). Фитохимический состав растительных экстрактов, полученных из данного растительного материала, изучался методом высокоэффективной жидкостной хроматографии (ВЭЖХ). Биологическая активность и антибактериальное действие описывались методами спектрофотометрии и диско-диффузии соответственно. Цитотоксичность экстрактов изучена с помощью колориметрического анализа WST-1.
Доказано наличие в экстрактах фенольных соединений. Антиоксидантная активность экстрактов *S. alba* оказалась в четыре раза выше, чем у *G. glabra*, и более чем в семь раз превышала антиоксидантную активность экстрактов *E. vulgare*. Экстракты *G. glabra* подавляли рост как грамположительных, так и грамотрицательных бактерий, а образцы экстракта *E. vulgare* оказались эффективны только в отношении грамотрицательных бактерий. *S. alba* и *E. vulgare* оказались способны снижать жизнеспособность клеток Т-лимфобластного лейкоза человека (Jurkat) и мононуклеарных клеток крови.
Экстракты *G. glabra*, *S. Alba*, и *E. vulgare* могут быть рекомендованы к использованию в области биомедицины. Требуется более подробное изучение для внедрения их в официальную медицину в качестве терапевтических и профилактических средств.

**Ключевые слова.** Растения, *Glycyrrhiza glabra*, *Salix alba*, *Echium vulgare*, водно-спиртовых экстрактов, свободные радикалы

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## Introduction

Medicinal plants that contain phenolic compounds, flavonoids, and phenol glycosides demonstrate great prospects as anticancer pharmaceuticals [1]. Studies that involve medicinal plant extracts cast light upon the effect of these beneficial substances on human cells [1].

Some medicinal plant extracts are known to contain chemical compounds able to permeate cell membranes and cause their apoptosis or necrosis. Insoluble nano- and microparticles may damage cell membranes or individual cellular compartments. High dilution factors may protect concentrated solutions from the possible effects of pH changes in the nutrient medium. However, some highly reactive solution components interact with organic compounds, even at low concentrations [2].

Human blood mononuclear cells are a population of immune cells. They are used to study cytotoxicity and biocompatibility of nanomaterials and soluble substances. Together with immortalized human T-lymphocyte cell line called Jurkat cells, mononuclear cells can be used to compare the cytotoxic effects of different agents on healthy and tumor blood cells [3]. Chemical exposure

tests make it possible to reveal the biocompatibility of blood mononuclear cells at different concentrations before introducing them into the blood stream [4].

Blood mononuclear cells are easy to isolate from peripheral blood, which makes them convenient for laboratory experiments. Due to their high sensitivity to chemicals, they can be used to detect such adverse effects as toxicity and inflammation [5].

*In-vitro* studies allow scientists to control the experimental parameters and focus on specific effects of different agents. The results can be extrapolated to other areas of medical research [6]. In addition, studies on the immunomodulatory effects of plant materials and extracts provide an insight into the interactome, yielding novel, and safe therapeutic approaches.

Screening plant extracts on mononuclear cells is important for several reasons. Many plants contain bioactive compounds, such as flavonoids, polyphenols, and alkaloids. These compounds have antioxidant, anti-inflammatory, and immunostimulant properties [7]. Understanding how these compounds interact with blood cells could lead to the development of new preventive and therapeutic natural drugs [8].

Plant extracts establish new biological therapies of immune diseases and cancer by affecting cell signaling pathways and modulating the immune response [9]. These interactions elucidate the potential benefits of herbal components and help to predict side effects of herbal medicines, thus adjusting them to modern safety standards.

Phytochemical screening reveals the effect of qualitative and quantitative profile of plant extracts on the survival of blood mononuclear cells. It casts light upon the mechanisms behind the cytotoxic effects of some medicinal plants [8].

*Glycyrrhiza glabra* L., commonly known as licorice, belongs to the legume family and is ubiquitous in Asia and Europe. It grows in abundance in the Kaliningrad Region, one of Russia's Baltic regions. *G. glabra* is used to treat throat infections, cardiovascular diseases, tuberculosis, and respiratory diseases. It has reliable antibacterial, anti-inflammatory, immunomodulatory, anticancer, hepatoprotective, anti-asthmatic, and antidiabetic activities [10]. These effects are due to such flavonoids as isoliquiritigenin, liquiritigenin, lichalocone, and glabridin, as well as to coumarins, chalcones, isoflavones, phenolic compounds, and triterpenoid saponins [11].

*Salix alba* L., or white willow, is a tree that grows 5–8 meters tall. Its thin, hanging branches form a characteristic dome top. It is native to Europe and is quite widespread in the Kaliningrad Region. *S. alba* contains such flavonoids as luteolin, apigenin, and quercetin. Other biologically active substances include phenoglycosides, tannins, phenolic acids, ascorbic acid, amino acids, saponins, essential oils, and polysaccharides. These substances may contribute to anti-ulcer, antioxidant, and anti-inflammatory effects [12].

*Echium vulgare* L., also known as viper's bugloss or blueweed, is native to Europe and the Kaliningrad Region. Some pharmacological studies confirmed its early ethnomedicinal properties *in vitro* and *in vivo*: *E. vulgare* extracts have antioxidant, analgesic, anxiolytic, anti-inflammatory, antibacterial, and antiviral effects. It contains naphthoquinones, flavonoids, terpenoids, and polyphenols [13, 14].

The bioactive profile of plants directly depends on the geobotanical environment [1]. In this respect, *G. glabra*, *S. alba,* and *E. vulgare* harvested in the Kaliningrad Region and the Eastern Baltic may have a bioactive profile different from that of the same plants harvested elsewhere. Herbal medicine is effective, low-toxic, and safe. Plant preparations are highly reliable as part of preventive treatment or against chronic and slowly developing diseases. Healthy people can also use them to improve their quality of life.

This study tested the antioxidant and antibacterial effects of extracts of Eastern Baltic *G. glabra, S. alba,* and *E. vulgare* on the viability of healthy and tumor human blood cell lines.

Eventually, official medicine may introduce these plants into clinical practice. As a source of new drug substances, they expand the possibilities of pharmacology and offer alternative therapeutic approaches. Ultimately, studies on the effects of plant extracts on mononuclear cells contribute to the integration of plant-based medicines into modern medical practices [15, 16].

## Study objects and methods

**Extract preparation.** The study featured extracts of *Glycyrrhiza glabra* L., *Salix alba* L., and *Echium vulgare* L. The plants were harvested in the Kaliningrad Region, Eastern Baltic, Northwestern Federal District of Russia, in July and August 2023. *G. glabra*, *S. alba*, and *E. vulgare* are not on any national or local list of rare wild plants, which means they are permitted for harvesting in the Russia. The species identification was obtained from the Herbarium of the Immanuel Kant Baltic Federal University, Kaliningrad, Russia (protocol No. 16/2023).

The extracts included aerial parts, i.e., leaves and stems. The aqueous methanol extracts provided a complete coverage of the secondary metabolome. Methanol extracts polyphenols and saponins, which have reliable therapeutic effects [17–20]. Methanol was added to a suspension of dry plant material in a weight-to-volume ratio of 1:20. After a one-minute vortexing (Pushchinskie Laboratorii, Russia), the extract was ultrasonicated in a UG-2060 ultrasonic bath (PiterLab, Russia) at room temperature for 15 min. After separating the precipitate on a centrifuge (Pushchinskie Laboratorii, Russia), the extracts were poured into separate flasks. The extraction involved a water-methanol mix at a methanol-to-water ratio of 80:20, v/v. As for the extractant, the ratio was 1:20 weight-to-volume. Then, we mixed the extract samples obtained in the first and second steps to remove

the solvent under reduced pressure in a Rotavapor R-300 vacuum rotary evaporator (Buchi Labortechnik AG, Switzerland) and a freeze-drying system (Labconco, USA) [21, 22].

**Total yield of *G. glabra*, *S. alba*, and *E. vulgare* extracts.** In line with the gravimetric method, we concentrated the extract samples using a Rotavapor R-300 vacuum rotary evaporator (Buchi Labortechnik AG, Switzerland) [22] and dried them in a Labconco Triad freeze dryer (Labconco, USA).

**Phytochemical profiling of *G. glabra*, *S. alba*, and *E. vulgare* extracts.** The method of high-performance liquid chromatography (HPLC) made it possible to study the content of secondary metabolites of phenolic nature in the obtained extracts. The experiment involved an LC-20AB Shimadzu Prominence chromatograph (Shimadzu, Japan) equipped with a binary pump, an SPD-M20A diode matrix detector, and a Zorbax 300SB-C18 4.6×250 mm 5 μm column (Agilent, USA). The separation had the following parameters: gradient elution mode, 40°C; mobile phase: eluent A – 0.1% tetrahydrofuran in double-distilled water; eluent B – acetonitrile; flow rate – 1 mL/min, analytical wavelengths – 254, 280, and 325 nm [22].

The components were identified by the retention times and the spectra of individual standard substances: 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, hyperoside, gallic acid, apigenin-7-O-glucoside (cosmosiin), catechin, astragalin, ferulic acid, caffeic acid, chlorogenic acid, neochlorogenic acid, acacetin, quercetin-3D-glucoside, ellagic acid, rutin, luteolin-7-glucoside (cynaroside), p-coumaric acid, caftaric acid, rosmarinic acid, chicoric acid, oenothein B, genistein, daidzein, formononetin, and morin (Massmedika, Russia). The concentrations of these compounds were calculated using calibration equations (3–5% error).

The results were summarized to specific values and calculated per dry weight of plant raw material.

**Cell cultivation.** All cell-related experiments were conducted under sterile conditions using a BMB-II-Laminar-S-1.5 system (Neoteric, Russia) in line with standard research methods [23]. A New Brunswick Galaxy 170 S incubator (Eppendorf, Germany) provided the following stable conditions: 37°C, 5% $CO_2$. To monitor the cell morphology, we used an inverted CKX53 light microscope (Olympus, Japan). A Countess II FL automated cell counter provided reliable cell count and viability data (Thermo Fisher Scientific, Singapore). To assess the cell viability, we stained the cells with 0.4% trypan blue stain (Invitrogen, USA). For this purpose, we mixed 10 μL cell suspension with 10 μL trypan blue solution before transferring the resulting mix to its well on the disposable cell counter slide (Invitrogen, USA). The measurement procedure followed the manufacturer's protocols [24, 25].

To culture human T-lymphoblastic leukemia (Jurkat) cells and human blood mononuclear cells, we used an RPMI-1640 nutrient medium (Gibco, UK), supplemented with 10% fetal bovine serum (Gibco, USA) and 1% antibiotic solution (penicillin and streptomycin, Gibco, USA). The Jurkat cells underwent three passages. The blood mononuclear cells were isolated immediately before the experiment according to the protocol described below.

**Human blood mononuclear cell isolation.** To obtain cellular material, blood was collected using vacuum systems for blood collection (Grener Bio-One, Austria) from the elbow vein of healthy donors (permission No. 5, May 16, 2016, Ethics Committee, Fabrika Innovation Park, Immanuel Kant Baltic Federal University). The heparinized blood samples were mixed with a cold solution (4°C) of phosphate-buffered saline (Sigma-Aldrich, USA) at a 1:1 ratio. This mix was then layered on a 3:1 Ficoll solution (Ficoll Paque Premium, $p$ = 1.077, Sigma-Aldrich, USA) in a 50 mL tube. The centrifugation was conducted in an 5702 centrifuge (Eppendorf, Germany) at 1,500 rpm at room temperature for 40 min. The resulting gradient ring was pipetted and mixed with nutrient medium. After that, we were able to assess the cell viability and total cellularity.

**Cell viability analysis by the WST-1 method.** To investigate the cytotoxic effect of the extracts, we used the WST-1 colorimetric assay (Roche Diagnostics GmbH, Germany). The method relies on the ability of viable cells to cleave cell dye molecules in water-soluble tetrazolium salt 1 to form formazan salts. This process presupposes staining with subsequent spectrophotometry. This procedure allowed us to count live cells. For this purpose, we seeded freshly isolated blood mononuclear cells and Jurkat cells at 50,000 cells per well (Fig. 1). The nutrient medium contained cells and extracts at concentrations of 10, 50, and 100 μg/mL in the experimental wells (E10, E50, E100, Fig. 1). The control wells contained no extracts. Calibration wells: C0, C10, C50, C100 (Fig. 1) contained extracts in the same concentrations as in the experimental wells but no actual cells. The plates spent 24 h in an incubator.

After 24 h of cultivation, we added 10 μL of warm (37°C) WST-1 dye to each well and mixed thoroughly. The plate spent 45 min in the incubator. Then, we measured the optical density of the cell suspensions in the wells with a Multiskan FC microplate photometer (Thermofisher, Germany) and a 450 nm optical filter. Each experimental group underwent a minimum of 12 repetitions. The obtained values were standardized to the average optical density in the control wells. The optical density values of the calibration wells were subtracted from the optical density values of the experimental wells with the same concentrations. The statistical processing and graphing relied on the GraphPad Prism 7.04 software (Graph Pad Software Inc., USA).

**Aliquoting the extract solutions.** The initial mass of the extract diluted in 50 mL 5% dimethyl sulfoxide water solution was 3.07 g, and the initial concentration

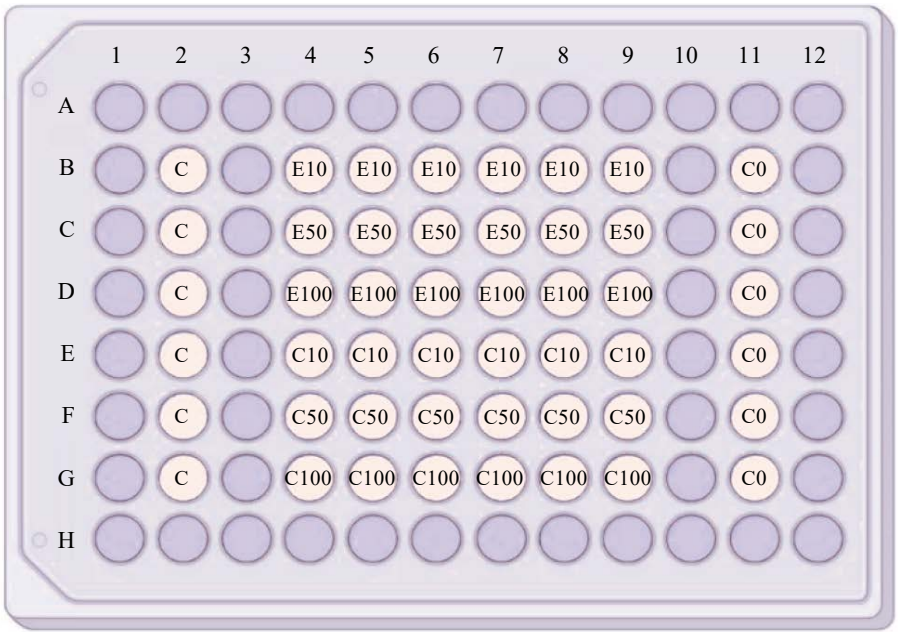



Figure 1. Cytotoxic effect of plant extracts on human blood mononuclear cells and Jurkat cells: C – control wells; E10, E50, E100 – experimental wells with solutions of extracts at concentrations of 10, 50, and 100 μg/mL, respectively; C0 – calibration wells without extract; C10, C50, C100 – calibration wells with extract solutions at concentrations of 10, 50, and 100 μg/mL, respectively

Рисунок 1. Цитотоксическое воздействие растительных экстрактов на мононуклеарные клетки крови человека и клетки линии Jurkat: C – контрольные лунки; E10, E50, E100 – экспериментальные лунки с растворами экстрактов в концентрациях 10, 50 и 100 мкг/мл соответственно; C0 – калибровочные лунки без экстракта; C10, C50, C100 – калибровочные лунки с растворами экстрактов в концентрациях 10, 50 и 100 мкг/мл соответственно

was 61.46 mg/mL. The concentrations of the stock solutions were obtained as follows:

$$C = \frac{1.000 \times m}{50} \quad (1)$$

where $C$ is the concentration of the initial extract solution, mg/mL, and $m$ is the mass of the initial extract solution, mg.

The dilution of the stock solutions into 1 mg/mL working solutions consisted of several steps. The plant extract solution was diluted with sterile distilled water at a ratio of 1:5, respectively, until the solution reached a concentration of 10.24 mg/mL. After that, the resulting solution was diluted with RPMI-1640 nutrient medium (Sigma-Aldrich, USA) at a ratio of 1:9 until 1.02 mg/mL.

The resulting working solutions were further diluted with RPMI-1640 nutrient medium (Sigma-Aldrich, USA) to working concentrations of 200 and 100 μg/mL as 1:4 and 1:9, respectively. The working solution with a concentration of 20 μg/mL was prepared by diluting the solution with a concentration of 200 μg/mL with RPMI-1640 nutrient medium (Sigma-Aldrich, USA) at a ratio of 1:9, respectively.

**Antioxidant properties of *G. glabra*, *S. alba*, and *E. vulgare* extracts.** To study the antioxidant activity of the extract samples, we appealed to spectrophotometric methods, i.e., ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), FRAP (ferric reducing antioxidant power), and DPPH (2,2-diphenyl-1-picrylhydrazyl). A series of standard solutions of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) served as standard. The results were expressed as milligrams of Trolox equivalent per gram of dry weight (mg/g).

The FRAP reagent consisted of 0.3 M acetate buffer solution at pH 3.6, 10 mM 2,4,6-tripyridyl-s-triazine solution in 40 mM HCl, and 20 mM $FeCl_3 \times 6H_2O$ solution at a ratio of 10:1:1. To implement the FRAP method, we mixed 20 μL extract and 300 μL reagent in a 96-well plate (Eppendorf, Germany). The resulting mix was incubated in a TS 1/20 SPU dry-air thermostat (Smolenskoye SKTB SPU, Russia) at 37°C for 10 min. The optical density test involved a CLARIOstar microplate reader (BMG Labtech, Germany) at 593 nm.

To implement the ABTS method, we mixed 20 μL extract and 300 μL $ABTS^{-+}$ cation radical solution in a 96-well plate (Eppendorf, Germany). Then, we studied the reaction progress by measuring the changes in optical density with a CLARIOstar microplate reader (BMG Labtech, Germany) at 734 nm.

The $ABTS^{-+}$ cation radical solution consisted of the ABTS reagent (Sigma-Aldrich, USA) mixed with a 2.45 mM potassium persulfate solution (Sigma-Aldrich, USA). It was incubated in the dark at room temperature for 16 h.

As part of the DPPH method, we added 20 μL extract and 300 μL of a fresh 0.1 mM solution of 2,2-diphenyl-1-picrylhydrazyl to a 96-well plate (Eppendorf, Germany). To study the reaction progress, we measured the change in the optical density with a CLARIOstar microplate reader (BMG Labtech, Germany) at 515 nm.

**Antibacterial properties of *G. glabra*, *S. alba*, and *E. vulgare* extracts.** The antibacterial profile was obtained by the disk diffusion method, which involved strains of *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, and *Candida albicans* (Kurchatov Institute, Russia).

The test strains of *P. aeruginosa*, *E. coli*, and *B. subtilis* grew on a dense Lysogeny broth nutrient medium (Biocomma, China) in an LSI-3016A Shaking Incubator thermostat (Daihan Labtech, South Korea). The cultivation involved stationary conditions at 37°C [26]. The test strain *C. albicans* grew on Sabouraud agar (Sigma-Aldrich, USA) under stationary conditions at 25°C [26].

As part of the disk diffusion method, the test strains were sown as a lawn on an agar nutrient medium at a bacterial suspension concentration of $1.5\times10^8$ CFU/mL. After that, we placed 5-mm-diameter paper disks soaked in 10 μL extracts on the lawn. A 5-mm-diameter disk impregnated with 10 μL of appropriate solvent served as negative control. The disks impregnated with kanamycin (for the bacteria) or fluconazole (for *C. albicans*) served as positive controls. The concentrations of kanamycin and fluconazole were 100 μg/disc.

The concentrations of plant extracts applied to the discs were 500 and 100 μg. They were selected experimentally. The Petri dishes were incubated in triplicates for 24 ± 0.5 h at optimal temperatures for each bacterial strain. The results were recorded as the presence and size, mm, of bacteria-free areas around the disc and taken as a mean value [26–27].

**Statistical analysis.** All experiments were triplicated, their results expressed as the mean ± standard deviation. The one-way ANOVA (*F*-test) made it possible to determine statistically significant differences between the mean values. It involved the GraphPad Prism 7.04 software (GraphPad Software Inc., USA) [27]. The tests that featured cell lines were normalized to the mean cell viability values, their results presented as mean values with error margins. The confidence intervals for the statistical differences between the control and experimental groups were presented as ranks (**** for $p < 0.0001$, ** for $p < 0.01$, and * for $p < 0.05$). The ranks were absent in cases of insignificance.

## Results and discussion

According to the gravimetry conducted, the total yields of the *Glycyrrhiza glabra* L., *Salix alba* L., and *Echium vulgare* L. extracts were 32.18, 13.82, and 20.96%, respectively. Figures 2–4 and Table 1 illustrate the results of the high-performance liquid chromatography (HPLC) analysis.

The *G. glabra* extract (Fig. 2) contained derivatives of daidzein (279.1 mg/kg), apigenin (567.1 mg/kg), formononetin (21.7 mg/kg), daidzein (579.6 mg/kg), genistein (160.2 mg/kg), and catechin (2014.7 mg/kg). The *S. alba* extract (Fig. 3) contained catechin and ferulic acid while the *E. vulgare* extract (Fig. 4) contained rosmarinic acid (3,479.0 mg/kg), astragalin (26.6 mg/kg), quercetin derivatives (315.0 mg/kg), apigenin (264.2 mg/kg), quercetin-3D-glucoside (239.8 mg/kg), rutin (861.6 mg/kg), hyperoside (257.3 mg/kg), and other polyphenolic compounds that accounted for its therapeutic properties [12].

The therapeutic value of medicinal plants depends on the bioactive substances they contain [12, 28]. To define the trends in the content of our bioactive substances, we compared our results with those published elsewhere.

The bioactive pharmacological profile of *S. alba* extracts was reported to include flavonoids, phenolic acids, and saponins [29–32]. In our case, we detected ferulic acid and quercetin-3D-glucoside, which was confirmed by other scientists [30, 33–34]. Quercetin-3D-glucoside was found in extracts of *Salix triandra, Salix acutifolia, S. alba,* and other plants; *S. triandra*, *S. acutifolia,* and *S. alba* extracts were also reported to contain rutin [30, 33, 34]. Rutin and quercetin are flavonoids that are exogenous low molecular weight antioxidants able to neutralize the action of active oxygen molecules [35]. Phenolic compounds are major contributors to the antioxidant activity of plant extracts [36, 37]; the magnitude depends on the *Salix* species and extractant [38–40].

The antioxidant effect of *S. alba* is due to its ability to inhibit reactive oxygen species. The anti-inflammatory effect of its extracts is traced to the inhibition of prostaglandins, the inflammatory markers, and the nitric oxide in macrophages [41].

Gligorić *et al*. [42] obtained 30.21% aqueous methanol *S. alba* extract, including 1.92 mg/g flavonoids and 6.25 ± 0.75 mg/g phenolic compounds. The phenolics included 1.60 mg/g rutin and 0.39 mg/g quercetin. The dry extracts also contained salicin (4.50 mg/g), gallic acid (0.59 mg/g), chlorogenic acid (1.89 mg/g), *n*-hydroxybenzoic acid (0.48 ± 0 mg/g), syringic acid (0.19 mg/g), *n*-coumaric acid (0.16 mg/g), and trans-cinnamic acid (0.03 mg/g). The composition of the plant extract depended on the extraction mode and the extractant. In our case, the extractant allowed us to obtain extracts with quercetin and ferulic acid, which are known for their reliable and diverse therapeutic effects [17–20, 33, 34].

In our research, the aqueous methanol extracts of *E. vulgare* demonstrated antioxidant activity, as well as the ability to inhibit Gram-negative bacteria: the lysis zone against *Pseudomonas aeruginosa* ranged from 6.5 to 10.0 mm. Other authors trace these properties to the presence of rosmarinic acid, astragalin, quercetin-derivatives, apigenin derivatives, and other polyphenolic compounds [12, 28]. Our research was consistent with some previous empirical findings [43–46]. Kapusterynska *et al*. [43] reported aqueous methanol

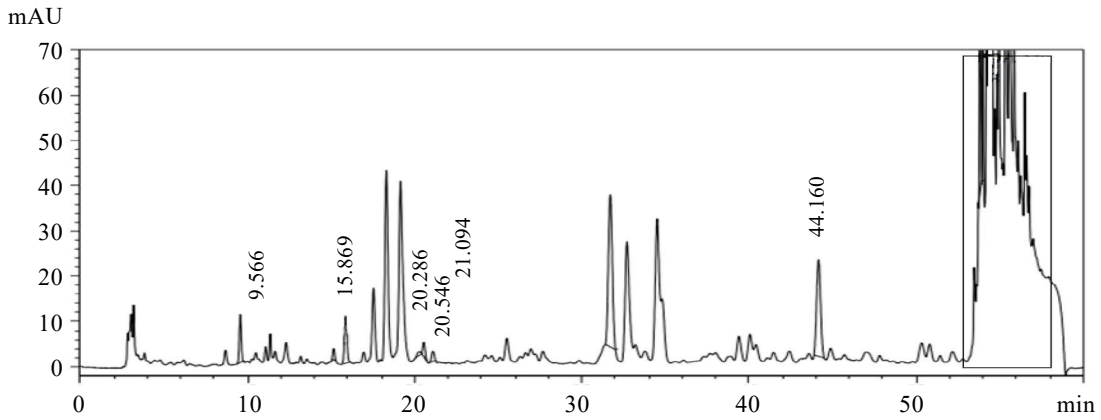



Figure 2. HPLC results for aqueous methanol extract of *Glycyrrhiza glabra* L.: 9.566 min – catechin; 15.869 min, 20.286 min, 20.546 min – apigenin derivatives; 21.094 min – genistein; 31.703 min – daidzein; 44.160 min – daidzein derivatives; and 53.803 min – formononetin

Рисунок 2. Результаты ВЭЖХ водно-спиртового экстракта *Glycyrrhiza glabra* L.: катехин – 9,566 мин; производные апигенина – 15,869 мин, 20,286 мин, 20,546 мин; генистеин – 21,094 мин; даидзеин – 31,703 мин; производные даидзеина – 44,160 мин; формононетин – 53,803 мин

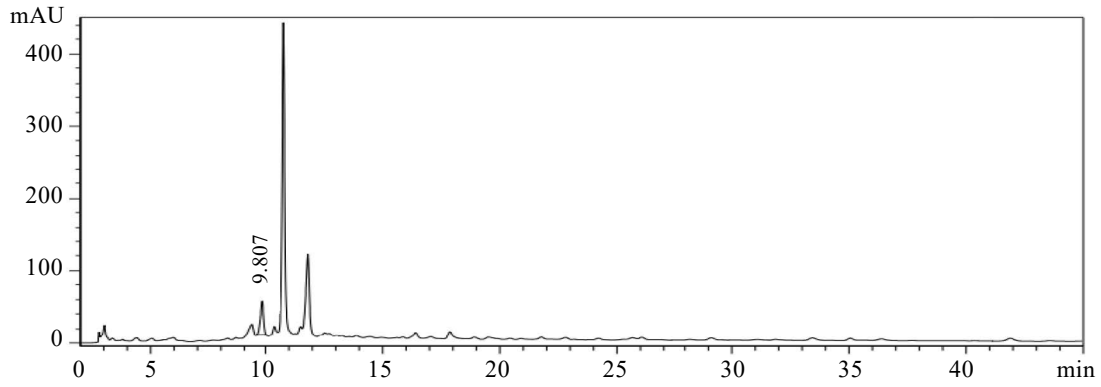



Figure 3. HPLC results for aqueous methanol extract of *Salix alba* L.: 9.824 min – catechin; 16.301 min – ferulic acid

Рисунок 3. Результаты ВЭЖХ для водно-спиртового экстракта *Salix alba* L.: катехин – 9,824 мин; феруловая кислота – 16,301 мин

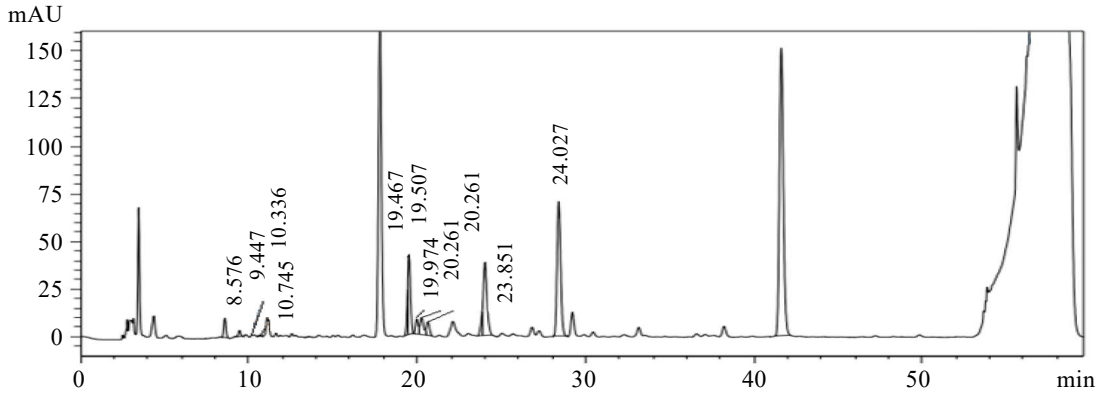



Figure 4. HPLC results for aqueous methanol extract of *Echium vulgare* L.: 8.576 min – neochlorogenic acid; 9.447 min – 2,5-dihydroxybenzoic acid; 10.336 min – chlorogenic acid; 10.745 min – caffeic acid; 19.467 min – hyperoside; 19.507 min – rutin; 19.974 min, 23.851 min – quercetin derivatives; 20.261 min – quercetin-3D-glucoside; 20.626 min – apigenin derivative; 24.027 min – astragalin; and 28.397 min – rosmarinic acid

Рисунок 4. Результаты ВЭЖХ водно-спиртового экстракта *Echium vulgare* L.: неохлорогеновая кислота – 8,576 мин; 2,5-дигидроксибензойная кислота – 9,447 мин; хлорогеновая кислота – 10,336 мин; кофейная кислота –10,745 мин; гиперозид – 19,467 мин; рутин – 19,507 мин; производные кверцетина – 19,974 мин, 23,851 мин; кверцетин-3D-глюкозид – 20,261 мин; производное апигенина – 20,626 мин; астрагалин – 24,027 мин; розмариновая кислота – 28,397 мин

Table 1. Secondary metabolites in *Glycyrrhiza glabra* L., *Salix alba* L., and *Echium vulgare* L. extracts

Таблица 1. Вторичные метаболиты в экстрактах *Glycyrrhiza glabra* L., *Salix alba* L., и *Echium vulgare* L.

| Bioactive substances | Retention time, min | Content, mg/kg | | |
|---|---|---|---|---|
| | | *Glycyrrhiza glabra* | *Salix alba* | *Echium vulgare* |
| Neochlorogenic acid | 8.6 ± 0.5 | –* | – | 56.1 ± 2.2 |
| 2,5-dihydroxybenzoic acid | 9.4 ± 0.5 | – | – | 284.5 ± 11.4 |
| Catechin | 9.6 ± 0.5 | 2,014.7 ± 72.5 | 9,590.0 ± 431.5 | – |
| Chlorogenic acid | 10.3 ± 0.5 | – | – | 203.2 ± 6.1 |
| Caffeic acid | 10.7 ± 0.5 | – | – | 40.6 ± 1.6 |
| Apigenin derivative | 15.8 ± 0.5 | 218.8 ± 74.3 | – | – |
| Ferulic acid | 16.3 ± 0.5 | – | 3.1 ± 0.1 | – |
| Hyperoside | 19.5 ± 0.5 | – | – | 257.3 ± 10.2 |
| Rutin | 19.6 ± 0.5 | – | – | 861.6 ± 31.0 |
| Quercetin derivative | 19.9 ± 0.5 | – | – | 14.5 ± 0.6 |
| Quercetin-3D-glucoside | 20.2 ± 0.5 | – | – | 239.8 ± 11.9 |
| Apigenin derivative | 20.3 ± 0.5 | 348.3 ± 8.7 | – | – |
| Apigenin derivative | 20.6 ± 0.5 | – | – | 264.2 ± 10.5 |
| Genistein | 21.1 ± 0.5 | 160.2 ± 5.2 | – | – |
| Quercetin derivative | 23.9 ± 0.5 | – | – | 43.2 ± 2.1 |
| Astragalin | 24.0 ± 0.5 | – | – | 26.6 ± 0.8 |
| Rosmarinic acid | 28.4 ± 0.5 | – | – | 3,479.0 ± 139.1 |
| Daidzein | 31.7 ± 0.5 | 579.6 ± 16.2 | – | – |
| Daidzein derivative | 44.2 ± 0.5 | 279.1 ± 12.5 | – | – |
| Formononetin | 53.8 ± 0.5 | 21.70 ± 0.97 | – | – |

Note: * – means below detection limit. Data presented as a mean ± SD ($n$ = 3).

Примечание: * – значение ниже предела обнаружения. Данные представлены в виде среднего значения ± SD ($n$ = 3).

extracts of *E. vulgare* to contain, catechol, and such catechin phenolic acids as gallic, benzoic, isoferulic, chlorogenic, vanillic, salicylic, ferulic, *n*-hydroxybenzoic, protocatecholic, alpha-coumaric, and *n*-coumaric acids [43]. Kuruüzüm-Uz *et al*. [44] described kaempferol 3-O-neohesperidoside, uridine, and rosmarinic acid as the main phytochemicals of *E. vulgare* extract.

Cytotoxic properties may significantly limit the potential use of plant extracts. We used spectrophotometry to assess the viability of healthy (blood mononuclear) cells and T-lymphoblastic leukemia (Jurkat) cells using the WST-1 staining.

We revealed a concentration-dependent decrease in cell viability after 24-hour exposure to each extract (Figs. 5–7). Jurkat cells were less sensitive to the inhibitory effects. The *S. alba* extract showed the highest cytotoxic effect against blood mononuclear cells, reducing their viability to 6.79% at a concentration of 100 μg/mL (Fig. 5). The Jurkat cell viability was reduced to 17%.

In contrast to the *S. alba* extract samples, those of *G. glabra* were able to reduce the viability of blood mononuclear cells only at the maximal concentration of 100 μg/mL (Fig. 6). Jurkat cells demonstrated no decrease in viability.

The *E. vulgare* extract samples (Fig. 7) exhibited less cytotoxic effect than *S. alba* (Fig. 5) but more than *G. glabra* (Fig. 6).

The extract samples of *S. alba*, *G. glabra*, and *E. vulgare* had different magnitudes of cytotoxicity towards human blood cells while Jurkat cells showed greater resistance to the inhibitory effects of all three extracts.

As for the cytotoxic effect on human cells, all three extracts reduced the cell viability of both types of cells. The decrease in the count of viable cells was concentration-dependent (Figs. 5–7). The extract samples of *G. glabra* had a cytotoxic effect on blood mononuclear cells only at the highest concentration value – 100 μg/mL (Fig. 6). The inhibitory effect of *S. alba* and *E. vulgare* extract samples was observed at a concentration of 10 μg/mL (Figs. 5, 7). Jurkat cells showed greater resistance to the cytotoxic action than blood mononuclear cells. Although the mechanisms of antitumor action of plant extracts are well-studied [11], their potential cytotoxicity and immunotoxicity require careful consideration [47, 48].

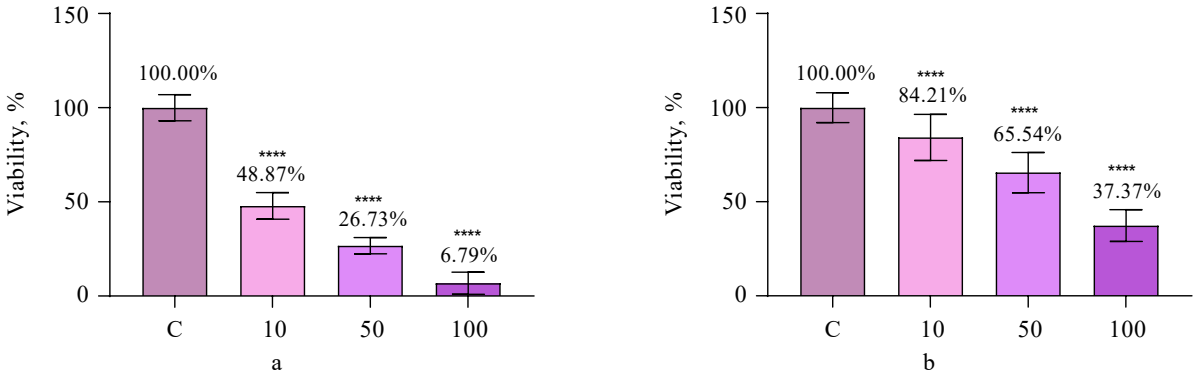
The difference in the cytotoxicity may be due to the differences in the content of bioactive substances that combine toxic and permeabilizing effects with potential therapeutic action. The *G. glabra* extracts demonstrated lower cytotoxicity, which renders it good prospects as a component of antitumor drugs [11].

The antioxidant activity results (Table 2) showed that the *S. alba* extract had significantly higher antioxidant activity than *G. glabra* and *E. vulgare*. The antioxidant activity of the *S. alba* extract was 634.9 μmol TE/g

(ABTS method), which was 4.0 times higher than the antioxidant activity of *G. glabra* extract and 7.2 times higher than that of the of *E. vulgare* extract. Similar results were obtained by DPPH and FRAP.

The antioxidant activity of the *E. vulgare* extract was 87.82 μmol TE/g (ABTS) and 74.58 μmol TE/g (DPPH). In our studies, the phytochemical composition of the extracts proved to be unique. They possessed reducing potential and exhibited antioxidant properties (55.80 μmol TE/g, FRAP). The empirical evidence was supported by the results of other researchers. In [45, 46, 49], the extracts of *E. vulgare* were rich in such natural antioxidants as phenolic acids, flavonoids, and tannins, which exhibited potent antioxidant activity. Alsanie *et al*. [50] obtained *E. vulgare* extract with 70%, v/v, water-methanol extractant, and it contained a lot of various phenolic compounds, especially gallic acid and quercetin. The content of flavonoids reached 35.98 mg quercetin/g, the content of phenolic compounds was 16.82 mg GA/g, and the total content of flavonoids was 35.98 mg quercetin/g. The antioxidant properties of the extract depended on the content of these compounds.

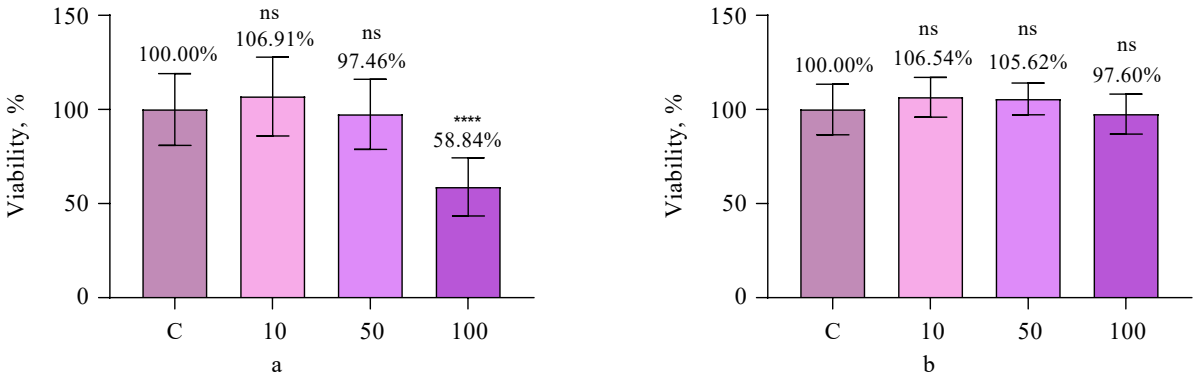
The mechanism by which flavonoids manifest their antioxidant potential in plants is also important. Flavonoids have a wide range of antioxidant mechanisms. As a rule, they suppress the activity of the enzyme peroxidase by




**** – marks significant difference from the control ($p < 0.0001$). Statistical analysis involved one-way ANOVA and *F*-test followed by Tukey's test in GraphPad Prism 7.04

Figure 5. Viability of blood mononuclear cells (a) and Jurkat cells (b) after culturing with *Salix alba* L. extract samples at different concentrations: C – control; 10, 50, 100 – extract concentration of 10, 50, 100 μg/mL, respectively; cultivation time – 24 h

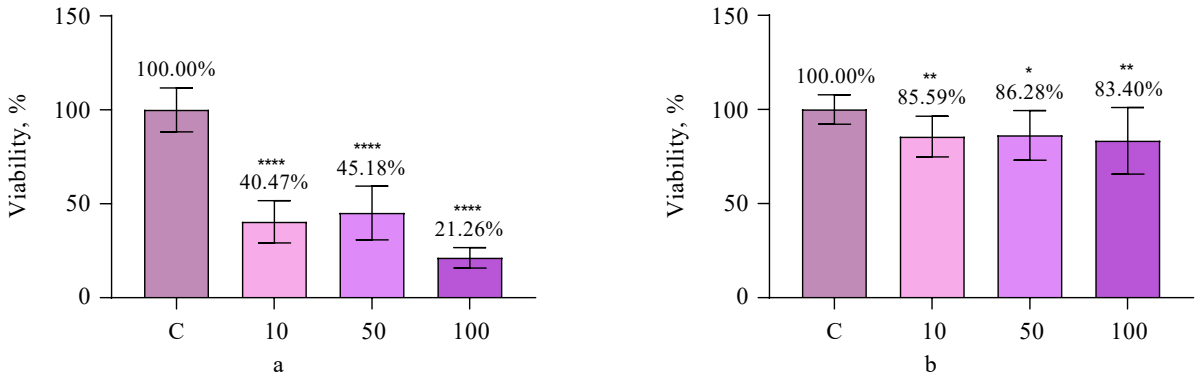
Рисунок 5. Жизнеспособность мононуклеарных клеток крови (a) и клеток линии Jurkat (b) после культивирования с образцами экстракта *Salix alba* L. в разных концентрациях: C – контроль; 10, 50, 100 – концентрация экстракта 10, 50, 100 мкг/мл соответственно; время культивирования – 24 ч




**** – marks significant difference from the control ($p < 0.0001$). Statistical analysis involved one-way ANOVA and *F*-test followed by Tukey's test in GraphPad Prism 7.04

Figure 6. Viability of blood mononuclear cells (a) and Jurkat cells (b) after culturing with *Glycyrrhiza glabra* L. extract samples at different concentrations: C – control; 10, 50, 100 – extract concentration of 10, 50, 100 μg/mL, respectively; cultivation time – 24 h; ns – non-significant differences

Рисунок 6. Жизнеспособность мононуклеарных клеток крови (a) и клеток линии Jurkat (b) после культивирования с образцами экстракта *Glycyrrhiza glabra* L. в различных концентрациях: C – контроль; 10, 50, 100 – концентрация экстракта 10, 50, 100 мкг/мл соответственно; время культивирования – 24 ч; ns – несущественные отличия




* – marks significant difference from the control ($p < 0.005$); ** – marks significant difference from the control ($p < 0.001$); **** – marks significant difference from the control ($p < 0.0001$). Statistical analysis involved one-way ANOVA and *F*-test followed by Tukey's test in GraphPad Prism 7.04

Figure 7. Viability of blood mononuclear cells (a) and Jurkat cells (b) after culturing with *Echium vulgare* L. extract samples at different concentrations: C – control; 10, 50, 100 – extract concentration of 10, 50, 100 μg/mL, respectively; cultivation time – 24 h

Рисунок 7. Жизнеспособность мононуклеарных клеток крови (a) и клеток линии Jurkat (b) после культивирования с образцами экстракта *Echium vulgare* L. в различных концентрациях: C – контроль; 10, 50, 100 – концентрация экстракта 10, 50, 100 мкг/мл соответственно; время культивирования – 24 ч

Table 2. Antioxidant activity of *Glycyrrhiza glabra* L., *Salix alba* L., and *Echium vulgare* L. extract samples

Таблица 2. Антиоксидантная активность экстрактов *Glycyrrhiza glabra* L., *Salix alba* L. и *Echium vulgare* L.

| Extracts | Antioxidant activity, μmol TE/g | | |
|---|---|---|---|
| | ABTS | DPPH | FRAP |
| *Glycyrrhiza glabra* | 158.86 ± 4.74 | 37.02 ± 2.22 | 14.99 ± 0.45 |
| *Salix alba* | 634.90 ± 19.02 | 412.58 ± 24.72 | 219.79 ± 6.57 |
| *Echium vulgare* | 87.82 ± 5.22 | 74.58 ± 2.23 | 55.80 ± 2.47 |

Note: All values in the columns were significantly different from each other ($p < 0.05$, Tukey's test).

Примечание: Все значения в столбцах существенно отличаются друг от друга ($p < 0,05$, тест Тьюки).

destroying its structure. They also inhibit the activity of both singlet and triplet oxygen, as well as bind free radicals [51].

Bioactivity depends not only on the amount of phenolics, but also on the nature and structure of the phenolic compound [52–54]. According to Soobrattee *et al*. [55], the intensity of the binding reaction of free radicals by natural flavonoids, phenolic acids, and their derivatives depends directly on the number of free hydroxyl groups in their structures. These findings were confirmed by other scientists who proved the antioxidant, antiviral, antimicrobial, anti-inflammatory, anxiolytic, and analgesic properties of *E. vulgare* extracts [13].

Bioactivity also depends on the extraction method, particularly the type and nature of the extractant used [56–58]. For example, Algerian *Echium* aqueous methanol extracts with an IC50 of 110.8 μg/mL were superior to methanol extracts [57].

Spectrophotometric methods (ABTS, FRAP, DPPH) showed that the methanol extracts of *Echium* had reliable antioxidant activity. They were also rich in flavonoids (16.26 mg CE/g dry weight) [59]. Similar DPPH results were obtained by Kefi *et al*. [60], who studied the antioxidant potential of *Echium* ethyl acetate extracts. *Echium rubrum* L. and *E. vulgare* plant extracts demonstrated health-beneficial antioxidant profile, confirmed by ABTS, FRAP, and DPPH [61].

Hashemi *et al*. [62] studied the phytochemical composition and biological potential of methanol extracts of *E. vulgare*: they contained ≤42.64 QE/g flavonoids and showed an antioxidant activity of 162.3 μg/mL (DPPH).

Dresler *et al*. [63] discovered that unfavorable growing conditions contributed to the accumulation of phenolic compounds in plants and increased the antioxidant potential of their extracts. In our studies, the yield of phenolic compounds and their antioxidant activity depended on the extraction method. According to Tahmouzi [64], they also depend on the drying mode and its technological parameters.

Other authors confirmed the high antioxidant potential of *Echium italicum* L., *E. vulgare*, *Echium parviflorum* L., and *Echium angustifolium* L. plants in a series of experiments based on the FRAP and DPPH methods [65–67].

Table 3. Antibacterial activity of *Glycyrrhiza glabra* L., *Salix alba* L., and *Echium vulgare* L. extract: inhibition zone diameter, mm

Таблица 3. Антибактериальная активность экстрактов *Glycyrrhiza glabra* L., *Salix alba* L. и *Echium vulgare* L.: диаметр зоны ингибирования, мм

| Samples | Concentration, µg/disk | Test strain | | | |
|---|---|---|---|---|---|
| | | *Escherichia coli* | *Bacillus subtilis* | *Pseudomonas aeruginosa* | *Candida albicans* |
| *Glycyrrhiza glabra* | 100 | 6.5 ± 0.5 | 8.0 ± 0.5 | 7.0 ± 0.5 | 0 |
| | 500 | 7.0 ± 0.5 | 9.0 ± 0.5 | 8.5 ± 0.5 | 0 |
| *Salix alba* | 100 | 0 | 0 | 0 | 0 |
| | 500 | 0 | 0 | – | 0 |
| *Echium vulgare* | 100 | 0 | 6.5 ± 0.5 | 7.0 ± 0.5 | 0 |
| | 500 | 0 | 10.0 ± 0.5 | 10.0 ± 0.5 | 0 |
| Control (kanamycin) | 50 | 12.0 ± 0.5 | 13.0 ± 0.5 | 14.0 ± 0.5 | – |
| Control (fluconazole) | 500 | – | – | – | 10.0 ± 0.5 |
| Negative control (methanol) | – | 0 | 0 | 0 | 0 |

Note: All values in the columns were significantly different from each other ($p < 0.05$, Tukey's test).

Примечание: все значения в столбцах существенно отличаются друг от друга ($p < 0,05$, тест Тьюки).

According to Dang *et al*. [68], the plant extract of *G. glabra* contained liquiritigin and its derivatives. The content of liquiritin was 5.037 mg/g while that of its derivatives was 1.8 times less. The content of apigenin-rutinoside was 2.571 mg/g, and the content of liquiritigenin-apiosylglucoside was 2.946 mg/g. The strong antioxidant potential could be due to these compounds since liquiritin is known to have potent anti-inflammatory, antioxidant, and antimicrobial properties.

Dang *et al*. [68] also reported *G. glabra* extract to contain various isoflavones, flavonols, flavanones, and chalcones, as well as other isoflavone compounds, flavonoids, and isoflavone acids. This unique composition is responsible for the remarkable antioxidant action of the plant. Isoflavones (ispaglabridins A, B; glabridin) are especially known for their antioxidant potential [69].

The antioxidant efficacy of the *G. glabra* methanolic extract was demonstrated using the DPPH free radical scavenging method in [70]. Its antioxidant potential depended on the concentration: it was 85.47% at 50 µg/mL, and the highest antioxidant activity (79.29%) was recorded for the 1,000 µg/mL extract. In fact, the antioxidant activity of the concentrated plant extract was comparable to that of ascorbic acid (385.85 µg/mL IC50).

According to Dang *et al*. [68], saponins from the extract of *G. glabra* also exhibited strong antiviral, anti-inflammatory, and immunoregulatory properties [68].

As demonstrated by *Candida albicans*, the plant extracts of *G. glabra*, *S. alba*, and *E. vulgare* exerted no fungicidal activity (Table 3). The *S. alba* extract had no antibacterial properties. The plant extracts of *E. vulgare* and *G. glabra* had an inhibitory effect on both Gram-positive and Gram-negative bacteria.

In addition to their known antioxidant activity, *G. glabra* extracts were reported to have remarkable antibacterial effects [71, 72]. These findings are supported by both our empirical data (Table 3) and other publications [73, 74]. For instance, the extracts of *G. glabra* could inhibit the vital activity of *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* [73, 74].

Extracts of *G. glabra*, *S. alba*, and *E. vulgare* require more phytochemical study to isolate and identify their valuable bioactive components. Such studies may help to investigate the mechanisms of synergism at the molecular level [75], which may be able to reduce the denaturation and speciation of several valuable plant components.

**Conclusion**

In this research, the aqueous methanol extracts of *Glycyrrhiza glabra* L., *Salix alba* L., and *Echium vulgare* L. exhibited strong antioxidant and antimicrobial properties. The extract of *G. glabra* contained daidzein derivatives (279.1 mg/kg), apigenin (567.1 mg/kg), formononetin (21.7 mg/kg), daidzein (579.6 mg/kg), as well as genistein (160.2 mg/kg) and catechin (2,014.7 mg/kg). The *S. alba* extract contained catechin and ferulic acid. The *E. vulgare* extract was rich in rosmarinic acid (3,479.0 mg/kg), astragalin (26.6 mg/kg), quercetin derivatives (315.0 mg/kg), apigenin (264.2 mg/kg), quercetin-3D-glucoside (239.8 mg/kg), rutin (861.6 mg/kg), hyperoside (257.3 mg/kg), and some other polyphenolic compounds.

The antioxidant activity (ABTS) of *S. alba* extract was four times greater than that of *G. glabra* and more than seven times higher than that of *E. vulgare*. Similar antioxidant results were provided by the DPPH and FRAP methods. The extracts of *G. glabra* were active against Grampositive and Gram-negative bacteria while the *E. vulgare* extracts were able to inhibit Gram-negative bacteria. However, none of the extracts showed any activity against *Candida albicans*.

The extracts of *S. alba* and *E. vulgare* at 10, 50, and 100 µg/mL reduced the viability of T-lymphoblastic leukemia (Jurkat) cells and human blood mononuclear

cells after 24 h of exposure. The *G. glabra* extracts were less cytotoxic. The viability of Jurkat cells remained unaffected whereas mononuclear cells died only at the highest concentration of 100 μg/mL.

The revealed antioxidant and antibacterial effects of *G. glabra*, *E. vulgare*, and *S. alba* aqueous methanol extracts on human blood cell lines provide a better understanding of the role of plant medicines in modern medical practices.

**Contribution**

S.A. Sukhikh was responsible for data curation, research, drafting, and proofreading. S.A. Ivanova provided formal analysis, drafting, and proofreading. A.Kh. Bakhtiarova was responsible for data curation and research. S.E. Pshenichnikov developed the research methodology and performed the research. K.V. Levada developed the research methodology and performed the research. O.O. Babich designed the research methodology and concept, supervised the project, performed the research, and drafted the original manuscript.

**Conflict of interest**

The authors declared no potential conflict of interest regarding the research, authorship, and/or publication of this article.

**Критерии авторства**

С. А. Сухих – сбор данных, проведение исследований, написание статьи и редактирование. С. А. Иванова – формальный анализ, написание статьи и редактирование. А. Х. Бахтиарова – сбор данных и проведение исследований. С. Е. Пшеничников – разработка методологии, проведение исследований. Е. В. Левада – разработка методологии, проведение исследований. О. О. Бабич – разработка методологии и концепции, проведение исследований, руководство проектом, написание статьи.

**Конфликт интересов**

Авторы заявляют об отсутствии потенциальных конфликтов интересов в отношении исследования, авторства и/или публикации данной статьи.

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