MEDICAL UNIVERSITY – PLOVDIV
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CHEMICAL AND PHARMACOLOGICAL STUDY OF INDIVIDUAL AND COMBINED EXTRACTS FROM *ECHINACEA PURPUREA* AND *ONOPORDUM ACANTHIUM*

ABSTRACT

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The dissertation paper contains 108 pages, illustrated with 25 figures, 18 tables, and 2 appendices. The bibliography includes 191 sources.

The doctoral student works as an assistant at the Department of Bioorganic Chemistry at the Faculty of Pharmacy of the Medical University, Plovdiv.

The experimental studies were conducted at the Department of Bioorganic Chemistry, Faculty of Pharmacy, Medical University of Plovdiv, Department of Pharmacology and Clinical Pharmacology, Faculty of Medicine, Medical University of Plovdiv, Department of Medical Microbiology and Immunology, Faculty of Medicine, Medical University of Plovdiv and Institute of Organic Chemistry with a Center for Phytochemistry - Bulgarian Academy of Sciences, Plovdiv.

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The dissertation work was discussed by the Council of the Department of Bioorganic Chemistry, Faculty of Pharmacy, Medical University of Plovdiv on 17.03.2025, and is scheduled for public defence at an open session of a Scientific jury consisting of:

1. Prof. Ginka Antova, PhD
2. Assoc. Prof. Maria Angelova – Romova, PhD
3. Assoc. Prof. Nadezhda Petkova - Ognyanova, PhD
4. Prof. Plamen Stoyanov, PhD
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Reserve members:
1. Assoc. Prof. Zhana Petkova, PhD
2. Assoc. Prof. Iliya Kostadinov, MD, PhD

The public defense of the PhD dissertation will take place on ***May 29th***, at ***11:00*** h in the ***Auditorium hall II*** of the Medical University of Plovdiv.

All materials related to the procedure of the defense are available at the Department of Bioorganic chemistry and the Department of Science and Research and are published on the website of the Medical University of Plovdiv.

ABBREVIATIONS LIST

AO	Antioxidant
AOA	Antioxidant activity
CE 1	Combined extract 1 (1:1)
CE 2	Combined extract 2 (3:1)
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
b.w.	Body mass
AP-1	Protein-1
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
DPPH	2,2-diphenyl-1-picrylhydrazine
ELISA	Enzyme-linked immunosorbent assay
EP	*Echinacea purpurea*
GAE	Gallic acid
HORAC	Hydroxyl Radical Averting Capacity
HPLC	High-performance liquid chromatography
IFN-γ	Interferon gamma
IL-10	Interleukin ten
iNOs	Inducible NO synthase
LOD	Limit of detection
LOQ	Limit of determination
MAPK	Mass-activated protein kinase
NF-κβ	Transcription nuclear factor kappa beta
NK	natural killer cells
NO	Nitric oxide
OA	*Onopordum acanthium*
ORAC	Oxygen Radical Absorbance Capacity
PGE	Prostaglandin
RNS	Active forms of nitrogen
ROS	Active oxygen species
RSD	Coefficient of variation
TGF-β	Transforming growth factor
Th1	Cell-mediated immunity
Th2	Humoral-mediated immunity
TNF-α	Tumor necrosis factor alpha

INTRODUCTION

Oxidative stress is defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to disruption of redox signaling and control and/or molecular damage." The consequences of oxidative stress can range from mild to very severe (including oxidative damage to biomolecules, disruption of signaling transduction, mutation, and cell death), depending on the balance between the generated reactive species and the antioxidant defense of the organism. Oxidative stress is also associated with the development of anxiety-depressive states and elevated levels of pro-inflammatory cytokines, which underlie the connection between oxidative stress and inflammatory processes in the body. On one hand, pro-inflammatory cytokines stimulate the release of free radicals to counteract pathogenic agents, while on the other, oxidative stress leads to inflammation and increases the release of pro-inflammatory cytokines. Therefore, excessive production of free radicals often leads to the initiation of inflammatory processes. Oxidative stress disrupts cellular signaling and impairs the metabolism of arachidonic acid, thereby stimulating systemic inflammation.

It is well-known that several medicinal plants used in traditional medicine for their pharmacological effects also possess immunomodulatory properties. These plants have been shown to maintain immune homeostasis by regulating signaling pathways involved in oxidative stress responses and inflammatory processes, often leading to chronic inflammation, autoimmune diseases, and cancer. The immunomodulatory properties of medicinal plants are attributed to the ability of their bioactive compounds to interact with B and T lymphocytes, macrophages, dendritic cells, cytokines, transcription factors, and their downstream signaling pathways responsible for the immune response. Furthermore, the immunomodulatory properties of active compounds and extracts from traditional plants correlate with their antioxidant, anti-allergic, anti-proliferative, anti-inflammatory, and anti-tumor activities. Some of the most extensively studied plants from this perspective are *Curcuma longa* L., *Panax ginseng CA Meyer*, and *Moringa oleifera* Lam. In addition, plants from the Asteraceae family, such as *Echinacea purpurea* and *Onopordum acanthium*, are also widely used in traditional medicine. Several *in vitro* and *in vivo* studies demonstrate the broad range of effects they possess. Regarding *E. purpurea*, the extracts have been shown to exhibit anti-inflammatory, antifungal, antibacterial, antiviral, immunomodulatory, as well as anti-tumor, hepatoprotective, and neuroprotective effects. Extracts from *O. acanthium* exhibit anti-inflammatory, anti-tumor, and cardiostimulatory effects, as well as antibacterial, analgesic, and antipyretic activities. Undoubtedly, these effects are attributed to the bioactive compounds that these plants synthesize during their development. *Echinacea* species are characterized by polyphenols (derivatives of caffeic acid, flavonoids, alkylamides, polysaccharides), while *Onopordum* species contain polyphenols (flavonoids, lignans, phenolic acids), terpenes, and steroids.

It is interesting to note that while previous studies have shown that the use of Echinacea may reduce the severity and duration of respiratory infections, concerns arose during the COVID-19 pandemic regarding its potential to contribute to or enhance the risk of a cytokine storm due to its ability to stimulate the immune system. Furthermore, some adverse reactions to *Echinacea* are known, including anaphylaxis, angioedema, abdominal discomfort, and potential interactions with conventional medications, among others. On the other hand, *O. acanthium* is considered a major agricultural and wild-growing weed, tolerant of various developmental conditions and thus readily accessible, but rich in bioactive compounds. This provoked our interest and determined the aim of the present study, which is to combine *E. purpurea* with *O. acanthium*. In this regard, two combinations with different ratios of *E. purpurea* and *O. acanthium* (1:1, Combination 1, and 3:1 Combination 2) were proposed, and their *in vitro* antioxidant and *in vivo* immunomodulatory and anxiolytic effects were investigated.

AIMS AND OBJECTIVES

The aim of this dissertation is to investigate the polyphenolic composition of individual and combined extracts of *Echinacea purpurea* and *Onopordum acanthium* with different ratios of the plant materials, and to explore their antioxidant properties *in vitro,* as well as their pharmacological effects *in vivo*.

To achieve this aim, the following objectives were set:

1. Preparation of individual and combined extracts from the aerial parts of *E. purpurea* and flowers of *O. acanthium*.
2. Determination of the total polyphenol and flavonoid content of the extracts.
3. Development and validation of HPLC methods for the determination of characteristic compounds of *E. purpurea* - echinacoside, cynarin, cichoric acid, chlorogenic acid, and caffeic acid, quercetin, and apigenin, and characteristic compounds of *O. acanthium* chlorogenic acid, caffeic acid, scutellarin, quercetin, arctigenin, and apigenin.
4. Application of the developed methods for the identification of bioactive compounds in the prepared extracts and commercial products.
5. Determination of the antioxidant activity (AOA) of the obtained extracts using three methods: ORAC, HORAC, and electrochemical.
6. Investigation of the acute toxicity of the combined and individual extracts of *E. purpurea* and *O. acanthium.*
7. Study of the immunomodulatory effects of the individual and combined extracts in a rat model of induced inflammation by measuring serum levels of pro-inflammatory cytokines TNF-α, INF-γ, and the anti-inflammatory cytokine IL-10.
8. Investigation of the anxiolytic effects of the studied extracts through behavioral tests and monitoring serum levels of TNF-α, INF-γ, and IL-10.

MATERIALS AND METHODS

1. Materials

1.1. Plant Material

1.1.1. *Echinacea* species

The cultivated *Echinacea* species were grown in the experimental field of the Institute of Roses and Aromatic Plants, Kazanlak, Bulgaria. The plant material was collected from whole plants at three developmental phases: vegetation, mass flowering, and seed formation. Voucher specimens for Sample 1 (179 217 SOM), Sample 2 (179 218 SOM), and Sample 3 (179 219 SOM) have been deposited at the Institute of Biodiversity and Ecosystem Research, Sofia, Bulgaria.

Sample 1 was harvested from two-year-old plants from a population of *E. purpurea* from the institute Genofund, introduced in Bulgaria 16 years ago.

Sample 2 was harvested from one-year-old plants of *E. purpurea*, grown from seeds obtained from Pharmasaat, Germany (cat. № Epuk63006).

Sample 3 was harvested from four-year-old plants of *E. pallida*, grown from seeds obtained from the Botanischer garden der Tech Universitat Braunschweig, Germany (cat. № 1833).

1.1.2. *E. purpurea* and *O. acanthium*, purchased from the commercial market

For the pharmacological studies, dried plant material was purchased from the Herbal Pharmacy 36.6, accompanied by a quality certificate from MediHerb-83 Ltd, Plovdiv, Bulgaria. Aerial parts of *E. purpurea* (batch number: 39397, expiration date: 22.11.2022) and flowers of *O. acanthium* (batch number: 39398, expiration date: 18.02.2022) were used for the preparation of individual and combined extracts with a ratio of *E. purpurea : O. acanthium* 1:1 (Combination 1) and *E. purpurea : O. acanthium* 3:1 (Combination 2).

1.1.3. Commercial Phytopharmaceutical Products

EchinActive (StaEx Pharma, Bulgaria), Echinacea tincture (Bioherba, Bulgaria), Echinacea with propolis (Bioherba, Bulgaria), Echinacea Herb Tincture (Life Brand, Canada), Echinacea purpurea herb (Nature's Way, USA), Ultimate Echinacea herbal extract, Echinacea (*E. purpurea*), Organic Throat Coat, Lemon Echinacea (Traditional Medicinals, USA), Organic Echinacea Plus Elderberry (Traditional Medicinals, USA).

1.2.Experimental Animals

A total of 246 male Wistar rats weighing 180-200 g were used in the experiments. The animals were housed under standard laboratory conditions: a 12:12-hour light-dark cycle, 45% relative humidity, temperature of 26.5°C, and free access to food and water. The experiments were approved by the Scientific Ethics Committee of the Bulgarian Food Safety Agency with permit number

№299/15.04.2021 and by the Ethics Committee of the Medical University of Plovdiv with protocol number №3/20.05.2021.

2. Methods

2.1. Extraction and Spectrophotometric Determination of Total Polyphenol and Flavonoid Content

To 0.5 g of dried plant material, 40 ml of extractant (60% acetone solution in 0.5% formic acid) was added. The samples were then centrifuged (6000 × g), and the supernatants are used for further analysis of total polyphenols and flavonoids.

2.2. Extraction and HPLC analysis for determination of individual compounds

2.2.1. Extraction

The plant material was dried and ground into powder. From each sample, 0.2 g was weighed and extracted with 10 ml of 60% ethanol. The extraction was performed with 60% ethanol, as it has been established that water-ethanol mixtures optimally extract phenolic compounds (Rezaei et al., 2017; Koffi et al., 2010), and through maceration for 72 hours at 25°C, as higher temperatures may degrade chicoric acid (Kreft, 2005). The extracts were filtered through a microfilter (0.45 μm) and injected into the HPLC system.

The liquid phytopharmaceuticals were diluted from 3 to 20 times with methanol. Capsules containing *E. purpurea* extract were dissolved in 5 ml of 60% ethanol. Tea bags were used to prepare a 2% ethanol extract (60%). Before chromatographic analysis, the obtained solutions were filtered through a microfilter (0.45 μm).

For pharmacological studies, the obtained water-ethanol extracts were filtered and concentrated by evaporation under vacuum to completely remove the ethanol.

For the extraction of carbohydrates and organic acids, 1 g of dried plant material was subjected to extraction with 30 ml of 3% meta-phosphoric acid in a water bath with intensive shaking for 1 hour at 30°C. The samples were then centrifuged (6000 × g), and the supernatants are used for HPLC analysis.

2.2.2. HPLC Conditions

Chromatographic determination was carried out on a Varian HPLC system consisting of a ProStar 230 pump and a PDA 335 detector. Method I was used to identify characteristic compounds of *E. purpurea* such as echinacoside, cichoric acid, cynarin, caffeic acid, chicoric acid, quercetin, and apigenin. For the determination of chlorogenic acid, caffeic acid, scutellarin, quercetin, apigenin, and arctigenin, characteristic of *O. acanthium*, Method II was developed, which is a modification of the previously mentioned method. Rutin, myricetin,

epicatechin, neochlorogenic acid, and ferulic acid were determined by the method described by Teneva et al. (2022) using the Nexera-i LC-2040C Plus UHPLC system equipped with a UV detector.

For the determination of the carbohydrate composition, an Agilent 1220 chromatographic system with a binary pump and refractive index detector was used. Separation was carried out on an Aminex HPX-87H column (300 × 7.8 mm, BioRad) with a mobile phase of 4 mM H_2SO_4, a flow rate of 0.5 ml/min, at a temperature of 25°C. The identification of substances was performed based on the retention times of the standards: fructose, glucose, galactose, and xylose. The results are expressed as g/100 g dry weight.

The organic acid content is determined using an Agilent 1220 chromatographic system with a binary pump and UV-Vis detector. Detection was carried out at a wavelength of 210 nm. For the separation of organic acids, an Agilent TC-C18 column (5 µm, 4.6 mm × 250 mm) was used with a mobile phase of 25 mM phosphate (K_2HPO_4 / H_3PO_4) buffer (pH 2.4) at a flow rate of 0.8 ml/min. The identification of substances was performed based on the retention times of the standards: oxalic acid, formic acid, malic acid, shikimic acid, and citric acid. The results are expressed as mg/100 g dry weight.

2.2.3. Validation of HPLC Method I and Method II

The validation procedure for the HPLC methods included the following parameters: linearity, limit of detection (LOD), limit of quantification (LOQ), selectivity, accuracy, and precision.

2.3. Methods for determining antioxidant activity *in vitro*

2.3.1. Application of the oxygen radical absorbance capacity – ORAC Method

The method developed by Ou et al. (2001) with some modifications was used. This method measures the ability of an antioxidant to neutralize peroxide radicals. The method is based on the inhibition of the fluorescence decline of fluorescein when oxidized in the presence of an antioxidant. The peroxide radicals are generated by the thermal decomposition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). The results are expressed in µmol Trolox equivalents per gram of extract (µmol Trolox/g). Measurements are performed on a FLUOstar OPTIMA fluorimeter (BMG LABTECH, Offenburg, Germany). The excitation wavelength used is 485 nm, and the emission wavelength is 520 nm.

2.3.2. Application of Hydroxyl Radical Averting Capacity – HORAC method

The method was developed by Ou et al. (2002) and measures the complex-forming ability of a given antioxidant (AO) under Fenton reaction conditions, triggered by the interaction between Co(II) and H_2O_2. The results are expressed in µmol equivalents of gallic acid per 1g extract (µmol GAE/g). Measurements

are performed on a FLUOstar OPTIMA fluorimeter (BMG LABTECH, Offenburg, Germany). An excitation wavelength of 485 nm and an emission wavelength of 520 nm were used.

2.3.3. Application of electrochemical method for determining antioxidant activity

Antioxidant activity (AOA) was determined using an electrochemical method (Korotkova et al., 2002). The experimental procedure involves recording the voltammogram of cathodic electro-reduction of oxygen with the "AOA Analyzer" (RU.C.31.113.A N28715), connected to a personal computer. The AOA of the examined samples is assessed based on the kinetic parameter K (μmol/l.min), which reflects the amount of oxygen species reacting with the sample over time. Using this, the AOA is calculated:

$$AOA = K_{Sample} / K_{Trolox}$$

2.4. Enzyme-Linked Immunosorbent Assay for Cytokine Levels

The cytokines TNFα, IL-10, and TNF-α in rat serum were analyzed by the ELISA method using commercially available kits – Rat IL-10 ELISA Kit (DiaClone), Rat TNF-α ELISA Kit (DiaClone), Rat INF-γ ELISA Kit (DiaClone), in strict accordance with the manufacturer's instructions.

3. Pharmacological effects of individual and combined extracts of *E. purpurea* and *O. acanthium* on experimental animals

3.1. Experimental design for investigating acute toxicity of single extracts and combinations of *E. purpurea* and *O. acanthium*

The experimental animals (male Wistar rats) were divided into 9 groups of 6 animals (total of 54 animals). They were orally treated with the extracts using a probe at doses of 5 and 10 g / kg. Survival was assessed 24 hours after the administration of the extracts.

3.2. Experimental design for investigating immunomodulatory effects with a lipopolysaccharide-induced inflammation model and cytokine level measurement

For the experiment, 48 male Wistar rats were randomly assigned into six groups of eight animals. One group was the control, treated with physiological saline. The experimental groups were treated repeatedly (14 days) with the investigated extracts, after which the animals were decapitated and blood was collected for analysis. Four hours before blood sampling, lipopolysaccharide (LPS) from *E. coli* O55 was injected intraperitoneally at a dose of 250 μg/kg in the control group and the groups treated with plant extracts. The levels of TNF-α, IFN-γ, and IL-10 were measured using serum separated from the collected blood after coagulation at 37°C for 60 minutes and centrifugation at 3000 rpm.

3.3. Experimental design for investigating anxiolytic effects with an acute cold stress model and cytokine level measurement

Forty-eight male Wistar rats were used, divided into six groups. For 14 days, the animals from the different groups were treated daily orally using a probe with distilled water, both single extracts, and combinations thereof at a dose of 500 mg/kg body weight. On the 15th day, the experimental animals (except for the control group without stress) were placed in a refrigerator at 5 °C for 1 hour (acute cold stress model). Blood samples were taken from the animals 15 minutes after the stress-inducing exposure in the groups with acute stress. The levels of IFN-γ, IL-10, and TNF-α were measured in the serum using the ELISA method.

3.4. Behavioral tests for investigating anxiolytic effects of individual and combined extracts from *E. purpurea* and *O. acanthium*

3.4.1. Elevated cross maze test

The studies were conducted using an apparatus consisting of two open arms and two closed arms arranged in a cross shape, placed 50 cm above the floor. Forty-eight male Wistar rats were divided into six groups. The experimental animals were placed in the center of the cross maze and allowed to choose whether to spend time in the open or closed arms. They were observed for five minutes. Increased time spent in the open arms and an increased number of entries into them correlated with anxiolytic effects. The following parameters were measured: number of entries into the open arms, time spent in the open arms; number of entries into the closed arms, time spent in the closed arms; total number of entries into the arms; the ratio of entries into the open arms to the total number of entries.

3.4.2. Social interaction test

This test is used to assess anxiety levels and is performed in an "open field" apparatus. Forty-eight experimental animals were divided into six groups. Each rat was tested with an unfamiliar partner. The rats from each pair were placed simultaneously in opposite corners of the field. Their behavior was observed for five minutes. The time spent on social interaction with the partner was recorded. The longer the interaction time, the lower the anxiety level. A reduced social interaction time indicates anxious behavior, while increased interaction time indicates an anxiolytic effect.

4. Statistical analysis of results

Statistical analysis of the chemical analysis results was performed using SPSS (ver. 17.0) with a significance level of $p < 0.05$. Variance analysis (ANOVA) was used, and comparisons between groups were made with the Tukey-Kramer test. The results are presented as mean ± standard deviation (mean ± SD) for $n = 3$.

RESULTS AND DISCUSSION

1. Development, validation, and application of HPLC methods for determining bioactive compounds in extracts of *E. purpurea* and *O. acanthium*

E. purpurea and *O. acanthium*, belonging to the Asteraceae family, are widely used as medicinal plants in traditional medicine. Numerous *in vitro* and *in vivo* studies demonstrate the broad range of effects they possess. Undoubtedly, these effects are attributed to the biologically active compounds that the plants synthesize during their development. The genus *Echinacea* is characterized by polyphenols (derivatives of caffeic acid), flavonoids, alkylamides, and polysaccharides (Ahmadi et al., 2024), while the genus *Onopordum* is characterized by polyphenols (flavonoids, lignans, phenolic acids) (Shekarchi et al., 2012), terpenes, and steroids (Angelova et al., 2019).

1.1. Development of HPLC Methods

Chromatographic methods are among the most widely used for the analysis of biologically active compounds. For the analysis of phenolic compounds, HPLC systems with PDA detectors are commonly employed due to their availability (Pellati et al., 2011). In order to efficiently separate and identify the substances under investigation, such a system was used, equipped with the polar Hitachi LaChrom C18 AQ column and a suitable combination of solvents in gradient mode (Section 2. Materials and Methods).

To determine the characteristic compounds of the *Echinacea* genus, such as echinacoside, caffeic acid, cynarin, caffeic acid, chicoric acid, quercetin, and apigenin, in *E. purpurea* extract, Method I was developed. The retention times of the compounds were as follows: 4.95 min, 5.68 min, 6.26 min, 6.77 min, 8.73 min, 10.45 min, and 11.57 min. The chromatogram was recorded at 330 nm, at which wavelength the investigated compounds exhibit optimal absorption. Figure 1 presents the chromatogram of a model mixture with a concentration of 10 μg/ml.

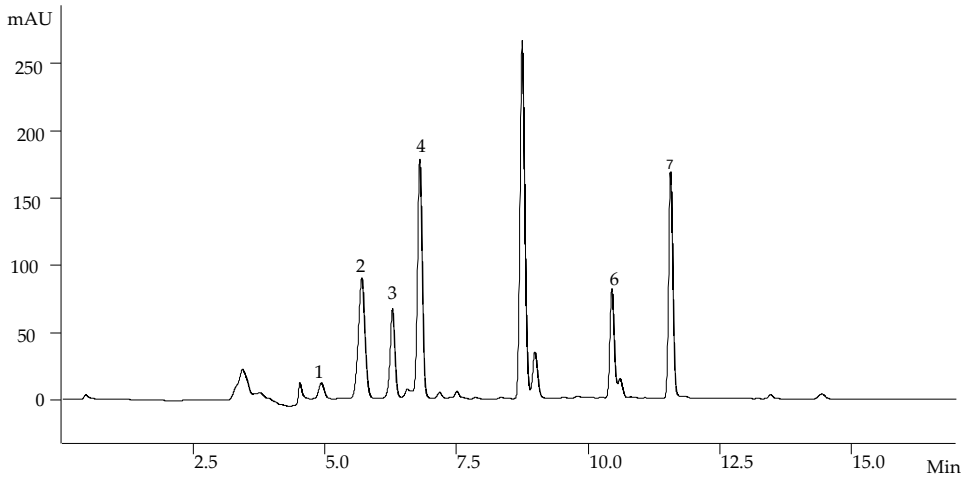


Figure 1. Chromatogram of a model mixture of 1 - echinacoside, 2 -caftaric acid, 3 - cynarin, 4 - caffeic acid, 5 - chicoric acid, 6 - quercetin, 7 - apigenin

The biologically active components in extracts of *O. acanthium* were analyzed using Method II, a modification of Method I. This method was used to determine chlorogenic acid, caffeic acid, scutellarin, quercetin, arctigenin, and apigenin, with retention times (min) as follows: chlorogenic acid – 9.25, caffeic acid – 11.00, scutellarin – 14.11, quercetin – 18.68, arctigenin – 20.30, and apigenin – 21.19 (Figure 2). The chromatogram was recorded at 330 nm until the 19th minute, after which it was recorded at 275 nm, the wavelength corresponding to the maximum absorption of arctigenin. Figure 2 presents the chromatogram of a model mixture with a concentration of the substances of 10 µg/ml..

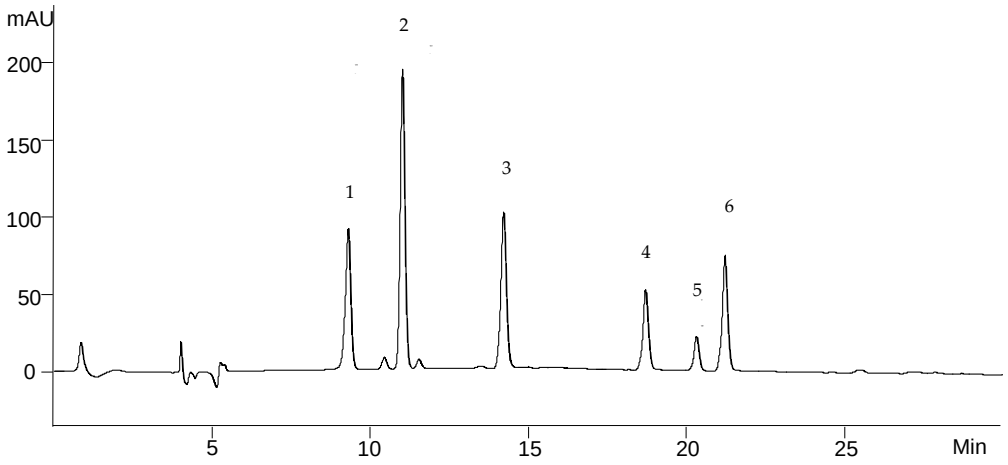


Figure 2. Chromatogram of a model mixture of 1 – chlorogenic acid, 2 – caffeic acid, 3 – scutellarin , 4 - quercetin, 5 - arctigenin, 6 – apigenin

1.2. Validation of HPLC Methods

The validation procedure for the HPLC methods includes the following parameters: linearity, LOD, LOQ, selectivity, accuracy, and precision. The results were obtained after the analysis of three series of pure standards within the following concentration ranges: from 5 to 30 µg/mL for echinacoside, cynarin, caffeic acid, quercetin, and apigenin, from 2 to 25 µg/mL for scutellarin, arctigenin, and chlorogenic acid, from 10 to 35 µg/mL for caftaric acid, and from 20 to 70 µg/mL for chicoric acid. The regression equations of the calibration curves, the coefficients of determination (r^2), RSD, as well as the LOD and LOQ, are presented in Tables 1 and 2. A linear relationship was observed between the selected concentration levels and the area of the chromatographic peak, as r^2 ranged from 0.9966 to 0.9995 with RSD values from 1.79 to 6.08%.

Table 1. Parameters of calibration curves, RSD, LOD, and LOQ for HPLC HPLC method I validation

Analyte	λ (nm)	RT (min)	Regression Equation	r^2	RSD (%)	LOD (µg/ml)	LOQ (µg/ml)
Echinacoside	330	4.95	y=6.9251e+004x	0.9975	5.58	0.660	2.178
Caftaric acid	330	5.68	y=5.3147e+005x	0.9995	1.79	0.172	0.568
Cynarin	330	6.26	y=3.4593e+005x	0.9973	5.46	0.129	0.426
Caffeic acid	330	6.77	y=8.5994e+005x	0.9980	2.73	0.126	0.416
Chicoric acid	330	8.73	y=6.4960e+005x	0.9994	2.66	0.394	1.300
Quercetin	330	10.45	y=4.4909e+005x	0.9990	2.81	0.103	0.340
Apigenin	330	11.57	y=7.4460e+005x	0.9984	2.46	0.080	0.264

Table 2. Parameters of calibration curves, RSD, LOD, and LOQ for HPLC method II validation

Analyte	λ (nm)	RT (min)	Regression Equation	r^2	RSD (%)	LOD (µg/ml)	LOQ (µg/ml)
Caffeic acid	330	11.00	y=1.0314e+006x	0.9996	2.05	0.075	0.248
Quercetin	330	18.68	y=4.2344e+005x	0.9993	3.12	0.093	0.307
Apigenin	330	21.19	y=6.0419e+005x	0.9992	2.56	0.126	0.416
Chlorogenic acid	330	6.68	y=5.2826e+005x	0.9966	6.08	0.160	0.538
Scutellarin	330	7.69	y=8.3621e+005x	0.9988	4.76	0.191	0.531
Arctigenin	275	5.91	y=1.6224e+005x	0.9986	4.86	0.228	0.752

The correlation coefficients calculated between the spectra of the standards of echinacoside, caftaric acid, cynarin, caffeic acid, chicoric acid, quercetin, apigenin, chlorogenic acid, scutellarin, arctiin, and the spectra of these compounds in the tested samples (extracts of *E. purpurea*, *O. acanthium*, and combined extracts 1 and 2) are between 0.9977 and 0.9997 (Table 3). Thus, the purity of the peaks of the investigated substances was determined, which serves as evidence of the selectivity of the developed methods.

Table 3. Correlation coefficients (r) of ten target compounds: echinacoside, caftaric acid, cynarin, caffeic acid, chicoric acid, quercetin, and apigenin

Extracts / Analyte	EP	OA	CE 1	CE 2
Caffeic acid	0.9995	0.9998	0.9979	0.9987
Caftaric acid	0.9990	-	0.9986	0.9991
Chicoric acid	0.9985	-	0.9982	0.9994
Cynarin	0.9987	-	0.9994	-
Echinacoside	0.9979	-	-	-
Chlorogenic acid	-	0.9991	0.9991	0.9989
Quercetin	0.9997	0.9982	0.9986	0.9984
Apigenin	-	0.9981	0.9984	0.9986
Scutellarin	-	0.9977	-	-
Arctigenin	-	0.9994	0.9988	0.9988

Experiments were conducted to determine the accuracy of the methods. Three samples with known concentrations of each of the investigated compounds were analyzed. Reproducibility values between 96.1% and 105.5% (R^a) were obtained. Good reproducibility was also achieved when the samples were loaded with a concentration of 10 μg/mL for echinacoside, caftaric acid, cynarin, caffeic acid, chicoric acid, quercetin, apigenin, chlorogenic acid, scutellarin, arctigenin, and it ranged from 97.5% to 103.5% (R^b). The results from both experiments indicate the accuracy of the methods. The results are presented in Table 4. The precision results of the methods are shown in Tables 5 and 6. The repeatability of the results within the same day (at low, medium, and high concentrations) is in the range from 0.056% to 0.953%, and the inter-day (intermediate) precision is from 0.089% to 0.948% (expressed as percentage RSD), respectively. The obtained values for all concentrations are significantly lower than the allowable 5% (Pinto et al., 2017). The brief analysis, available equipment, and the accuracy of the developed methods allow their use for routine work.

Table 4. Parameters related to accuracy for HPLC methods validation

Analyte	Real concentration (µg/ml)			Observed concentration (µg/ml) ±SD			R^a (%±SD)			C_u (µg/ml)	C_f (µg/ml)	R^b (%±SD)
Method I												
Echinacoside	7	17	27	7.20 ± 0.041	16.58 ± 0.033	27.52 ± 0.048	102.9 ± 0.5	97.5 ± 0.3	101.9 ± 0.4	-	9.89	98.9 ± 0.2
Caftaric acid	12	22	32	12.40 ± 0.037	22.96 ± 0.055	33.42 ± 0.042	103.3 ± 0.4	104.4 ± 0.3	104.4 ± 0.5	12.33	22.12	97.9 ± 0.7
Cynarin	7	17	27	7.08 ± 0.038	17.29 ± 0.035	27.58 ± 0.040	101.1 ± 0.4	101.7 ± 0.2	102.1 ± 0.4	traces	10.22	102.2 ± 0.9
Caffeic acid	7	17	27	7.03 ± 0.026	16.74 ± 0.032	26.80 ± 0.023	100.4 ± 0.2	98.5 ± 0.3	99.3 ± 0.5	traces	10.35	103.5 ± 0.8
Chicoric acid	25	45	65	25.28 ± 0.041	43.42 ± 0.038	62.43 ± 0.054	101.1 ± 0.4	96.5 ± 0.3	96.1 ± 0.4	31.70	41.53	98.3 ± 0.3
Quercetin	7	17	27	7.39 ± 0.028	16.87 ± 0.037	26.65 ± 0.033	105.6 ± 0.6	99.2 ± 0.2	98.7 ± 0.4	traces	9.75	97.5 ± 0.4
Apigenin	7	17	27	7.33 ± 0.036	17.65 ± 0.042	28.41 ± 0.035	104.7 ± 0.6	103.8 ± 0.5	105.2 ± 0.6	-	9.78	97.8 ± 0.2
Method II												
Chlorogenic acid	4	18	28	3.92 ± 0.25	17.74 ± 0.028	27.50 ± 0.045	98.0 ± 0.3	98.6 ± 0.5	98.2 ± 0.3	10.32	20.45	101.3 ± 0.5
Scutellarin	3	12	22	2.88 ± 0.11	12.66 ± 0.039	21.77 ± 0.032	96.0 ± 0.4	105.5 ± 0.6	98.9 ± 0.2	-	9.76	97.6 ± 0.7
Arctigenin	3	12	22	3.04 ± 0.19	12.28 ± 0.033	22.09 ± 0.047	101.0 ± 0.4	102.3 ± 0.5	100.4 ± 0.5	10.06	20.34	102.8 ± 0.4

Results are presented as mean value ± SD (n = 5).

Table 5. Intraday precision of the proposed HPLC methods

Analyte	Real concentration (µg/ml)			Observed concentration (µg/ml) ±SD			RSD (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Echinacoside	7	17	27	7.20 ± 0.041	16.58 ± 0.033	27.52 ± 0.048	0.098	0.340	0.154
Caftaric acid	12	22	32	12.40 ± 0.037	22.96 ± 0.055	32.22 ± 0.042	0.057	0.184	0.219
Cynarin	7	17	27	7.08 ± 0.038	17.29 ± 0.035	27.58 ± 0.040	0.794	0.163	0.077
Caffeic adis	7	17	27	7.03 ± 0.026	16.74 ± 0.032	26.80 ± 0.023	0.501	0.588	0.263
Chicoric acid	25	45	65	25.28 ± 0.041	43.42 ± 0.038	62.43 ± 0.054	0.196	0.130	0.056
Quercetin	7	17	27	7.39 ± 0.028	16.87 ± 0.037	26.65 ± 0.033	0.571	0.334	0.159
Apigenin	7	17	27	7.33 ± 0.036	17.65 ± 0.042	28.41 ± 0.035	0.480	0.479	0.199
Chlorogenic acid	4	18	28	3.92 ± 0.029	17.74 ± 0.033	27.50 ± 0.042	0.739	0.186	0.153
Scutellarin	3	12	22	2.88 ± 0.022	12.66 ± 0.041	21.77 ± 0.039	0.763	0.324	0.179
Arctigenin	3	12	22	3.04 ± 0.029	12.28 ± 0.034	22.09 ± 0.044	0.953	0.277	0.199

Results are presented as mean value ± SD (n = 5)

Table 6. Interday precision of the proposed HPLC methods

Analyte	Real concentration (µg/ml)			Observed concentration (µg/ml) ±SD									RSD (%)		
	Low	Medium	High	Day 1			Day 2			Day 3			Low	Medium	High
Echinacoside	7	17	27	7.15 ± 0.055	17.11 ± 0.020	27.12 ± 0.050	7.20 ± 0.059	17.09 ± 0.020	27.02 ± 0.054	7.08 ± 0.060	17.14 ± 0.025	27.03 ± 0.053	0.843	0.147	0.203
Caftaric acid	12	22	32	12.14 ± 0.050	22.22 ± 0.052	32.06 ± 0.059	12.25 ± 0.052	22.11 ± 0.057	32.14 ± 0.051	12.04 ± 0.053	22.13 ± 0.056	32.16 ± 0.053	0.438	0.264	0.164
Cynarin	7	17	27	7.15 ± 0.036	17.10 ± 0.043	27.23 ± 0.032	7.10 ± 0.033	17.13 ± 0.045	27.28 ± 0.036	7.16 ± 0.035	17.25 ± 0.040	27.22 ± 0.031	0.450	0.236	0.118
Caffeic adis	7	17	27	7.08 ± 0.059	17.14 ± 0.058	27.14 ± 0.020	7.14 ± 0.051	17.09 ± 0.054	27.19 ± 0.022	7.04 ± 0.050	17.19 ± 0.053	27.17 ± 0.021	0.710	0.292	0.093
Chicoric acid	25	45	65	25.28 ± 0.043	44.42 ± 0.044	63.43 ± 0.061	25.20 ± 0.048	44.28 ± 0.041	63.55 ± 0.068	25.22 ± 0.049	45.20 ± 0.044	64.85 ± 0.060	0.165	0.089	0.093
Quercetin	7	17	27	7.18 ± 0.043	17.15 ± 0.060	27.11 ± 0.052	7.22 ± 0.047	17.27 ± 0.068	27.05 ± 0.054	7.18 ± 0.041	17.21 ± 0.061	27.16 ± 0.055	0.557	0.349	0.203
Apigenin	7	17	27	7.20 ± 0.052	17.22 ± 0.050	27.06 ± 0.053	7.12 ± 0.058	17.12 ± 0.051	27.08 ± 0.055	7.10 ± 0.053	17.18 ± 0.049	26.98 ± 0.058	0.741	0.293	0.195
Chlorogenic acid	4	18	28	3.91 ± 0.038	17.88 ± 0.042	27.91 ± 0.039	4.01 ± 0.045	17.94 ± 0.061	27.85 ± 0.059	4.02 ± 0.035	18.10 ± 0.063	28.09 ± 0.055	0.948	0.633	0.394
Scutellarin	3	12	22	2.98 ± 0.047	12.26 ± 0.036	21.88 ± 0.033	3.05 ± 0.047	12.34 ± 0.044	21.86 ± 0.062	2.99 ± 0.048	12.22 ± 0.066	22.15 ± 0.043	0.514	0.497	0.717
Arctigenin	3	12	22	3.04 ± 0.029	12.08 ± 0.052	22.09 ± 0.062	3.01 ± 0.036	11.94 ± 0.053	22.21 ± 0.069	3.08 ± 0.043	12.18 ± 0.058	22.12 ± 0.051	0.690	0.999	0.282

Results are presented as mean value ± SD (n = 5).

1.3. Application of the developed HPLC methods for determining bioactive compounds characteristic for *E. purpurea* and *O. acanthium*

1.3.1. Application of Method I for determining bioactive compounds in cultivated species of *Echinacea*

Method I was applied for the quantitative determination of the characteristic compounds of *E. purpurea*, namely chicoric acid, caftaric acid, and echinacoside for *E. pallida*, according to the European Pharmacopoeia-8th edn. In addition to the specified compounds, other substances such as caffeic acid, cynarin, quercetin, and apigenin were also identified using the developed method, as they are also present in extracts from some species of the *Echinacea* genus according to the literature (Temerdashev et al., 2022). Ethanol extracts from the aerial parts and roots of two *Echinacea* species – *E. purpurea* (Sample 1 and Sample 2) and *E. pallida* (Sample 3) – were subjected to chromatography (see Materials and Methods section). Extraction was performed using 60% ethanol, as it has been found that water-ethanol mixtures extract phenolic compounds most effectively (Rezaei et al., 2017). Maceration was chosen for 72 hours at 25°C, as higher temperatures lead to the loss of chicoric acid (Kreft, 2005). Figure 3 shows the chromatogram of the aerial part extract of *E. purpurea* (Sample 1), where, in addition to the peaks of chicoric acid and caftaric acid, those of cynarin, caffeic acid, and quercetin were also identified. However, their quantities were below the limit of quantification. The chromatograms of Sample 2 and Sample 3 were similar.

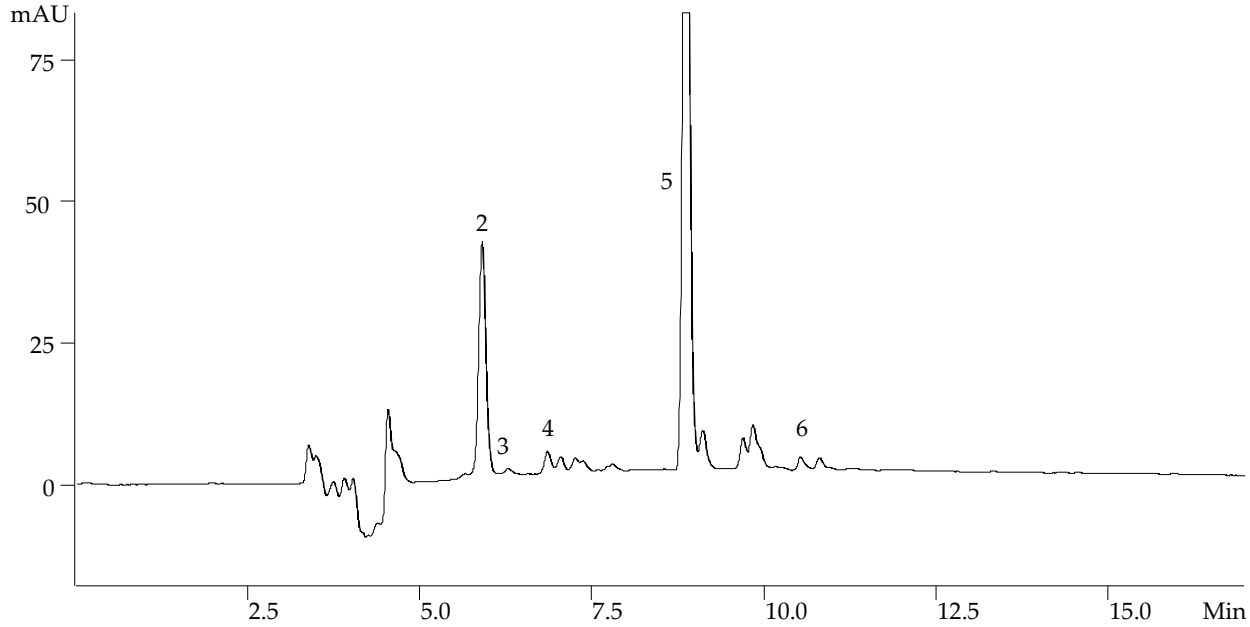


Figure 3. Chromatogram of an extract of the aerial part of *E. purpurea* (Sample 1) in mass flowering phase 2- caftaric acid, 3 – cynarin, 4 – caffeic acid, 5 – chicoric acid, 6 - quercetin

Figures 4, 5 and 6 display concentrations of chicoric and caftaric acids present in *Echinacea* extracts. These were tracked across different phases of plant development, vegetative, mass flowering and seed forming, as well as in different parts - aerial and roots, respectively.

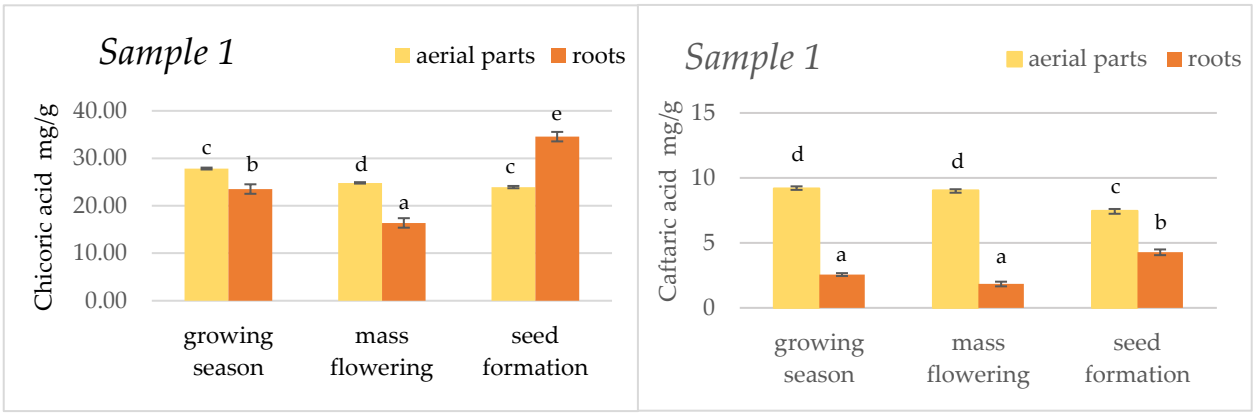


Figure 4. Chicoric and caftaric acids concentrations in extracts of aerial parts and roots of Sample 1 at different stages of development

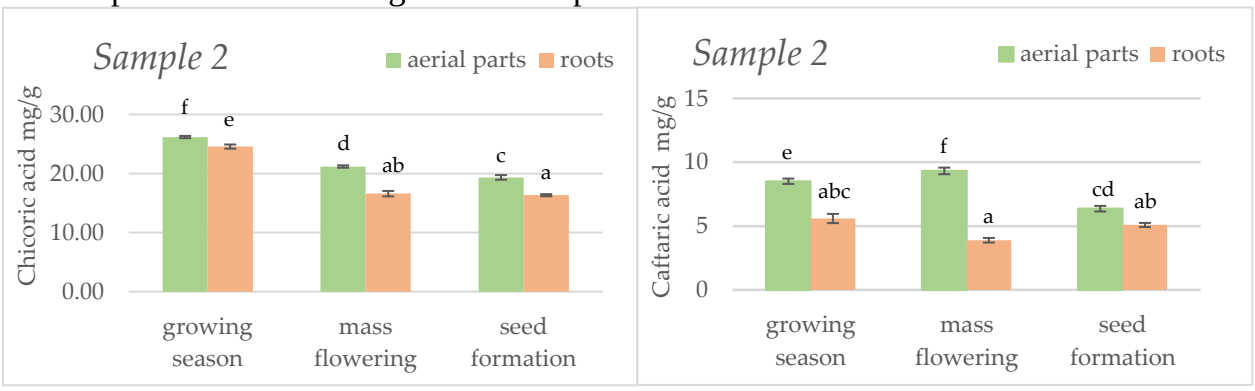


Figure 5. Chicoric and caftaric acids concentrations in extracts of aerial parts and roots of Sample 2 at different stages of development

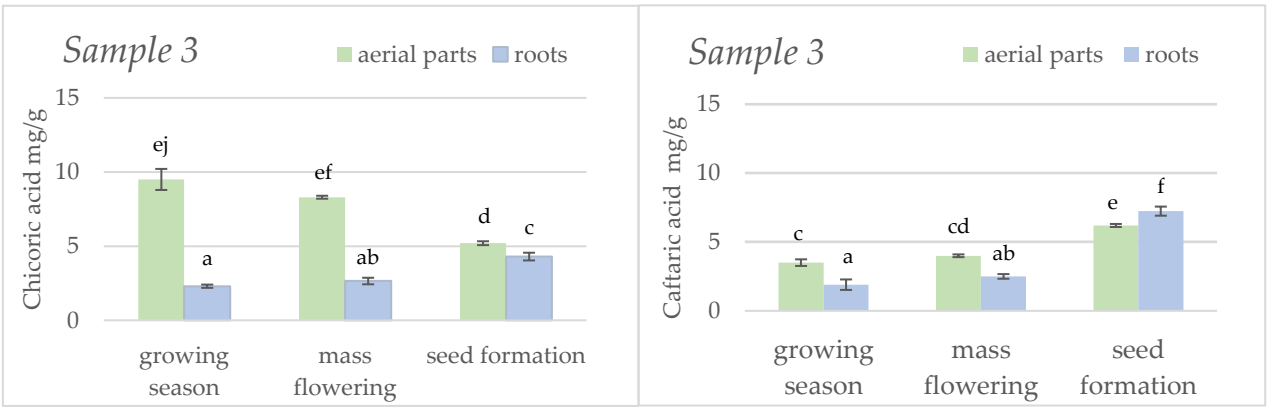


Figure 6. Chicoric and caftaric acid concentrations in extracts of aerial parts and roots of Sample 3 at different stages of development

Results are presented as mean values ± SD. Different lowercase letters indicate significant differences at the $p < 0.05$ level.

In all three Samples, chicoric acid accumulated to the greatest extent in the vegetative phase in the aerial parts but slightly decreased in the mass-flowering and seed-formation phases. In Sample 1 and 2 (E. purpurea), it peaked at 26–28 mg/g, while in Sample 3 (E. pal-lida) it reached about 8 mg/g. Chicoric acid concentration in the roots followed the same relationship only in Sample 2. In Sample 1 and 3, however, they peaked in the seed-formation phase at 34 and 4.3 mg/g, respectively.

Concentrations of caftaric acid in the aerial parts in Samples 1 and 2 were about 9 mg/g in the vegetative and mass-flowering phases and decreased to about 6–7 mg/g dur-ing seed formation. In Sample 3, however, concentrations of caftaric acid were highest during seed formation at 6 mg/g. Caftaric acid concentrations in the roots of Samples 1 and 2 were lower than those of the aerial parts, at 2 mg/g in the vegetative and flowering phases and 4 mg/g during seed-formation in Sample 1, but in Sample 2 values were the same in all three phases at about 5 mg/g. Relationships differed in Sample 3, where caftaric acid concentrations were 2 mg/g in the first two phases but reached 7 mg/g during seed formation.

Production of chicoric acid was about three times greater than that of caftaric acid in all tested samples, and Samples 1 and 2 (*E. purpurea*) in turn were richer in both acids than was Sample 3 (*E. pallida*).

In all the samples we examined, the amounts of both acids are higher in the aerial parts compared to the roots, with the exception of Samples 1 and 3, where during the seed formation phase, the amounts in the roots are greater (chicoric acid in Sample 1 reaches 34 mg/g, and caftaric acid in Sample 3 reaches 7 mg/g). The ratio of chicoric/caftaric acid is 2.8 for Sample 1, 3.0 for Sample 2, and 2.4 for Sample 3, which could serve as an identifier for the quality and authenticity of the plant material (Temerdashev et al., 2022).

The age of the plants and the season in which the plant material is collected also affect the quantity of phenolic compounds. In their study, Shekarchi et al. (2012) found that the harvest of *E. purpurea* is of the best quality when the aerial parts of two-year-old plants are collected in the spring. Such a pattern is also observed in our results, as the quantities of both acids in the extracts from Sample 1 and Sample 2 are highest during the vegetation phase.

When comparing our results for the content of chicoric acid (around 26-28 mg/g) in the aerial parts of *E. purpurea* with those obtained by Kreft (2005), it is clear that they are comparable only to the highest result for its content in the leaves (around 15 mg/g), and only in one of the observed regions in Slovenia. Regarding caftaric acid, we report a content of around 9 mg/g, while they obtain an average of 11 mg/g. In the study of cultivated *E. purpurea* in Slovenia, Senica et al. (2019) found chicoric acid quantities of around 12-16-17 mg/g and caftaric acid of 6-4-4 mg/g in leaves, flowers, and roots, respectively. Fu et al. (2021) conducted similar extraction with 70% ethanol on laboratory-grown *E. purpurea* and reported chicoric acid content of 42 mg/g and caftaric acid of 17 mg/g in the aerial parts,

and chicoric acid of 17 mg/g and caftaric acid of 7 mg/g in the roots. They also compared these values with those of *E. pallida* – 16 mg/g chicoric acid and 10 mg/g caftaric acid in the aerial parts, and 2.5 mg/g of both acids in the roots. We confirm this fact as we also report smaller amounts of the desired acids in the extract from *E. pallida* compared to *E. purpurea*. Additionally, we compared our results with those obtained by Petrova et al. (2023), who studied an extract from the roots of *E. purpurea*, also cultivated in Bulgaria. In our samples, the quantities of chicoric and caftaric acids in the roots are approximately 2 to 5 times higher with the same 60% ethanol extraction.

It is well known that the genetic features, and edaphic and climatic conditions are crucial for the ability of plants to synthesize biologically active compounds. From our analyses of extracts of *E. purpurea* cultivated in the Kazanlak region, it is clear that concentrations of chicoric and caftaric acids significantly exceed the requirements of European Pharmacopoeia 8.0, indicating that such material might serve as a reliable new feedstock for the production of bioactive phytopreparations.

1.3.2. Application of Method I for determining bioactive herbal preparations and tonic drinks containing *Echinacea* from the commercial sources

The method was also applied for evaluating the quality of commercially available phytopharmaceuticals containing *Echinacea* (See Materials and Methods section) in terms of the content of the compounds specified in the method. The chromatogram in Figure 7 illustrates the content of active compounds in the phytopharmaceutical Ultimate Echinacea herbal extract.

Nine commercially available phytopreparations and tonic drinks based on *Echinacea* were analyzed, and the quantities of echinacoside, caffeic acid, chlorogenic acid, cynarin, chicoric acid, quercetin, and apigenin were determined. The results are presented in Table 7.

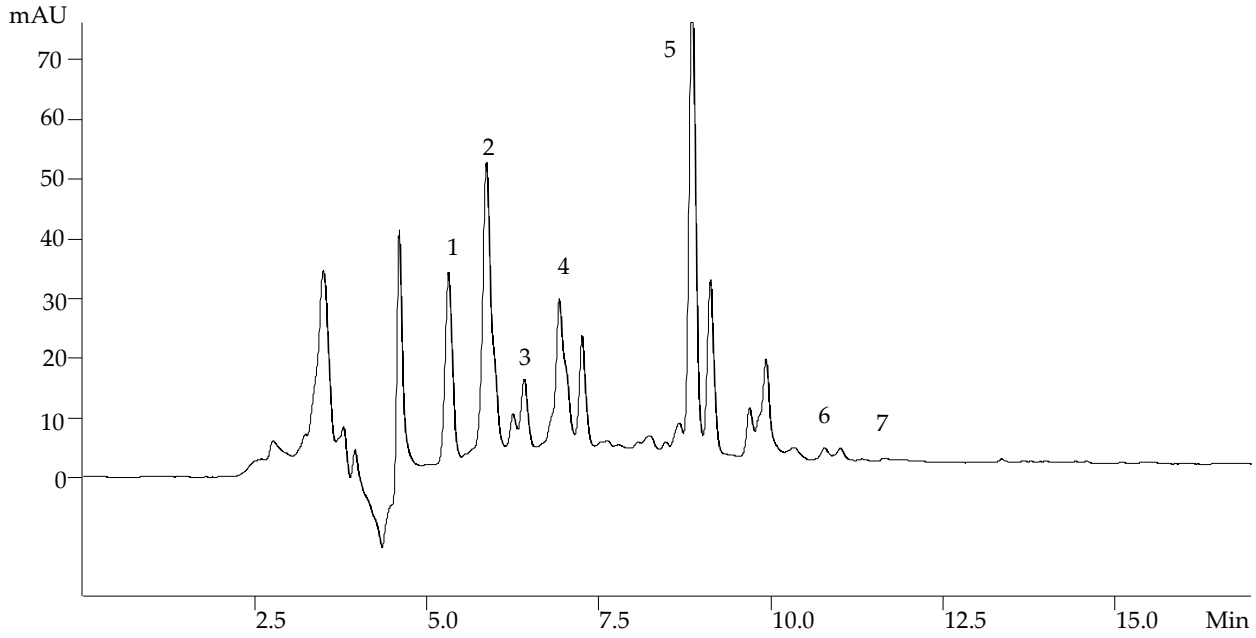


Figure 7. Chromatogram of herbal preparation Nature Sunshine, Ultimate Echinacea, 1 - echinacoside, 2 - caftaric acid, 3 - cynarin, 4 - caffeic acid, 5 - chicoric acid, 6 - quercetin, 7 - apigenin

The labels did not reflect the content of the active substances, only the amount in milligrams of the dry herb or extract (see Materials and Methods). The richest composition was found in the preparation Ultimate Echinacea herbal extract, which, according to the manufacturer's information, contains extract from the roots of *E. purpurea*, *E. pallida* and *E. angustifolia*. In addition to chicoric and caffeic acids, a significant amount of echinacoside and cynarin was also detected (Table 7), compounds typical of *E. pallida* and *E. angustifolia*. Quercetin and apigenin were also identified, but their concentrations were below the quantification limit (Figure 7). The other products mostly contain roots of *E. purpurea*, and logically, the levels of chicoric and caffeic acids were high. Apigenin was also detected in the product Echinacea with propolis tincture, most likely due to the presence of propolis, which contains phenolic acids and flavonoids (Przybyłek et al., 2018). In contrast, in Echinacea tincture (Bioherba), we detected only a low concentration of caffeic acid with no chicoric or caftaric acid, which most likely means that a poor-quality raw materials were used for this product. Composition of nutritional supplements and phytopreparations is not subject to strict control as in the case of medicines. For this reason, their composition can vary within very wide limits (Temerdashev et al., 2022). Thus, it is important to know the composition of the raw materials in order to prepare quality products (Senica et al., 2019).

Table 7. Concentrations of active substances in herbal preparations and tonic drinks containing *Echinacea* from the commercial sources

Substance (µg/ml) / Samples	Echinacoside	Caftaric acid	Cynarin	Caffeic acid	Chicoric acid	Quercetin	Apigenin
1. EchinActive,tincture	-	715.5 ± 42.9	-		2142.5 ± 127.5	-	-
2. Echinacea tincture	-	-	-	**10.93 ± 0.7**	-	-	-
3. Echinacea with propolis, tincture	-	150.7 ± 9.1	-	191.2 ± 8.9	68.0 ± 4.8	-	12.8 ± 1.1
4. Echinacea Herb, tincture	-	434.5 ± 26.1	-	50.25 ± 3.0	365.5 ± 25.2	traces	traces
5. Echinacea purpurea herb, extract	-	1274.6 ± 76.5	-	263.44 ± 18.7	1276.2 ± 107.2	-	-
6. Ultimate Echinacea herbal extract	**1954.5 ± 117.2**	**440.0 ± 26.4**	**125.5 ± 9.4**	**130.5 ± 10.4**	**562.5 ± 43.9**	**traces**	**traces**
7. Echinacea (E. purpurea), capsules	-	1817 ± 138.1	-	331.4 ± 20.5	14 365.7 ± 1034.3	-	-
8. Organic Throat Coat, Lemon Echinacea, tea bag	-	33.2 ± 2.7	-	-	395.2 ± 28.9	-	-
9. Organic Echinacea Plus, Elderberry, tea bag	-	42.6 ± 2.5	-	-	484.7 ± 36.8	-	-

1.3.3. Determination of the quantities of bioactive compounds in individual and combined extracts of *E. purpurea* and *O. acanthium*

The developed Method I and Method II were applied for the quantitative determination of active compounds in individual and combined extracts 1 and 2 from *E. purpurea* and *O. acanthium*, which were used in the pharmacological experiments. The chromatographic analysis includes characteristic individual compounds from both plants: phenolic acids – ferulic acid and caffeic acid, derivatives of caffeic acid – chicoric acid, caftaric acid, chlorogenic acid, neochlorogenic acid, cynarin, echinacoside, as well as flavonoids – quercetin, apigenin, rutin, myricetin, epicatechin, scutellarin, and additionally, arctigenin. Rutin, myricetin, epicatechin, and neochlorogenic acid were determined using the method of Teneva et al (2022). The quantities of these compounds are presented in Table 8.

Table 8. Concentrations of biologically active substances in extracts of *E. purpurea*, *O. acanthium*, Combination 1, and Combination 2

Extracts / Analyte, μg/g	EP	OA	CE 1	CE 2
Ferulic acid	770.7 ± 44.9	nd	471.9 ± 28.26	726.0 ± 43.56
Caffeic acid	1115.0 ± 55.0	265.0 ± 14.1	696.0 ± 42.9	839.0 ± 24.9
Caftaric acid	3060.0 ± 142.3	nd	1450.0 ± 74.9	2748.0 ± 147.5
Chicoric acid	12915.7 ± 773.2	nd	6505.0 ± 390.3	8350.0 ± 441.2
Cynarin	39.3 ± 2.1	nd	nd	traces
Echinacoside	55.4 ± 2.3	nd	nd	traces
Neochlorogenic acid	301.0 ± 27.3	596.0 ± 35.3	662.7 ± 49.8	443.8 ± 24.5
Chlorogenic acid	904.7 ± 54.1	661.0 ± 37.3	967.0 ± 55.5	330.6 ± 17.5
Quercetin	270.0 ± 13.3	584.6 ± 33.3	338.0 ± 3.3	98.5 ± 5.1
Apigenin	nd	280.0 ± 3.7	173.2 ± 2.7	57.5 ± 2.9
Rutin	2300.0 ± 132.1	nd	1340.0 ± 76.5	1837.0 ± 115.3
Myricetin	nd	1322.0 ± 66.3	1006.0 ± 65.7	361.0 ± 24.7
Epicatechin	142.3 ± 6.7	139.0 ± 1.2	856.0 ± 51.3	239.0 ± 15.3
Scutellarin	nd	35.0 ± 1.3	traces	nd
Arctigenin	nd	555 ± 32.7	225.2 ± 12.3	108.0 ± 13.2

Results are presented as mean value ± SD (n = 3); nd, undetected

The extract from *E. purpurea* contains the highest amounts of chicoric, caftaric, and caffeic acids. In the individual extract of *E. purpurea*, a greater number of phenolic acids and derivatives of caffeic acid have been identified, and accordingly, their quantities are higher compared to those in the extract of *O. acanthium*. The individual extract of *O. acanthium* contains a greater number of the examined flavonoids, but their quantities are lower than those in the *E.*

purpurea extract. The flavonoid content in this extract is represented by myricetin, quercetin, and apigenin, with the highest quantities present. Arctigenin is characteristic of the *Onopordum* species, and its quantity in the studied extract is 555.0 μg/g. Logically, in the combined extracts, Combination 1 and Combination 2, almost all the sought-after compounds are present, and their quantities are proportionally distributed.

2. Determination of carbohydrates and organic acids in dry plant material from *E. purpurea* and *O. acanthium* and their combinations

Primary metabolites are essential for proper plant and microorganism growth and development, while secondary metabolites form around the stationary growth phase and do not directly participate in growth, reproduction, or development (Salam et al., 2023). Primary metabolites include carbohydrates, organic acids, and tocopherols. Organic acids participate in several biochemical pathways, including energy production and the formation of precursors for amino acid biosynthesis (Lopez-Bucio et al., 2000). Mono- and oligosaccharides with low molecular mass and their derivatives play a fundamental role in the structure and function of living cells (Hernandez-Marin et al., 2012). They have been proven to influence primary and secondary metabolism, development, and gene expression (Gibson, 2000).

Information on primary metabolites specifically in *E. purpurea* and *O. acanthium* is not as extensive as on secondary metabolites. HPLC analysis has determined the content of certain monosaccharides and organic acids in the plant biomass of *E. purpurea* and *O. acanthium*. Table 9 presents the results for the content of fructose, glucose, galactose, and xylose, while Table 10 shows the levels of oxalic, formic, malic, shikimic, and citric acids.

Table 9. Carbohydrate content in dry plant material of *E. purpurea u O. acanthium*

Carbohydrates, g/100g / Sample	Fructose	Glucose	Galactose	Xylose	Total
EP	1.63±0.04	3.40±0.13	2.44±0.01	nd	7.47
OA	3.56±0.05	3.79±0.01	2.93±0.11	1.17±0.06	11.45
CE 1	2.62±0.27	3.24±0.38	3.01±0.31	0.61±0.04	9.48
CE 2	2.21±0.15	3.69±0.29	2.56±0.19	0.29±0.01	8.75

Results are presented as mean value ± SD (n = 3); nd, undetected

The total carbohydrate content in the plant material from *O. acanthium* (11.45%) is higher compared to that of *E. purpurea* (7.47%), with glucose being present in the largest quantity in both plants, followed by fructose in *O. acanthium* and galactose in *E. purpurea*. Glucose and fructose are also the main

monosaccharides found by Pires et al. (2016) in the aerial parts of *E. purpurea*, as well as by Kouki et al. (2024) and Petkova et al. (2016) in the flowers of *Onopordum* species. We find xylose in the biomass of *O. acanthium*, similar to the findings of Petkova et al. (2016) and Kouki et al. (2024), but we do not detect sucrose. Pires et al. (2016) report the presence of arabinose in the aerial parts of *E. purpurea*, which we did not detect.

Table 10. Organic acids content in dry plant material of *E. purpurea u O. acanthium*

Organic acids, mg/100g / Sample	Oxalic acid	Formic acid	Malic acid	Shikimic acid	Citric acid	Total
EP	135.9±80.3	nd	1228.9±21.2	93.9±8.1	646.8±17.5	2105.6
OA	189.2±2.6	469.9±10.9	298.9±13.7	8.1±0.1	1812.8±24.8	2778.9
CE 1	156.4±11.4	231.7±10.5	759.8±18.1	51.0±1.4	1220.8±18.2	2419.7
CE 2	148.8±10.2	117.1±11.3	996.1±12.2	72.9±8.3	975.3±17.8	2308.2

Results are presented as mean value ± SD (n = 3); nd, undetected

Among the acids investigated, the highest amount was found for malic acid in *E. purpurea* (1228.9 mg/100g dw) and citric acid in *O. acanthium* (1812.8 mg/100g dw). Additionally, the amounts of oxalic acid, shikimic acid, and formic acid were determined. Pires et al. (2016) also analyze these acids in the dry and fresh biomass of the aerial parts of *E. purpurea*, finding that the acid content in fresh biomass is higher compared to the dry one. The quantities of the investigated carbohydrates and acids are proportionally distributed in the selected combinations, as seen in Tables 13 and 14. It is important to know the composition of carbohydrates and organic acids, as they are not only the basis for the synthesis of secondary metabolites but also contribute to the taste qualities of homemade infusions from medicinal plants or dietary supplements from the commercial market. Data is showing modulatory effects of added preservatives and sweeteners on the bioavailability of active compounds in phytomedicines (Garcia-Arieta, 2018; Panakanti et al., 2012). Care must be taken not to block their effects in favor of focusing solely on the organoleptic acceptance of the plant-derived supplements.

3. Determination of antioxidant activity of extracts from *E. purpurea* and *O. acanthium* and their combinations *in vitro*

It is well known that the AOA of plant extracts is primarily attributed to phenolic compounds (Kouki et al., 2024; Petkova et al., 2016). Ethanol-water extracts are the richest in polyphenolic compounds (Vieira et al., 2022; Parzhanova et al., 2023), and for this reason, such extracts from aerial parts of *E.*

purpurea and flowers of *O. acanthium* were used in our study. The total polyphenol and flavonoid content in the extracts of *E. purpurea* and *O. acanthium* and their combinations was determined, and the results are presented in Table 11.

Table 11. Concentrations of phenolic and flavonoid compounds in extracts of *E. purpurea u O. acanthium* and their combinations

Extracts	Polyphenols, mg GAE/100g	Flavonoids, mg QE/100g
EP	3843.4 ± 34.2	891.0 ± 4.1
OA	1052.2 ± 34.4	186.8 ± 5.0
CE 1	2833.1 ± 66.4	444.8 ± 4.9
CE 2	3142.0 ± 18.4	599.1 ± 5.2

The polyphenol content determined in the *E. purpurea* extract (3843.4 mg GAE/100g) is comparable to the values obtained in a previous study by our team on *E. purpurea* (3905.4–4493.3 mg GAE/100g) (Vlasheva et al., 2024), and is twice as high as in the root extract of *E. purpurea* (1500 mg GAE/100g) (Petrova et al., 2023). As for the *O. acanthium* extract, the total polyphenols amount to 1052.2 mg GAE/100g, while Parzhanova et al. report 16800.0 mg GAE/100g (Parzhanova et al., 2023). Of course, their quantities always vary depending on the habitat and growth conditions (Vlasheva et al., 2024).

Our research team has experience in creating combined extracts that incorporate active compounds from different classes, resulting in effects that exceed those of individual plants (Kandilarov et al., 2023; Katsarova et al., 2017). The use of Echinacea-based phytopreparations has an undeniable positive effect on health in several aspects. However, data on combinations of *Echinacea* with other medicinal plants are scarce. During the Covid-19 pandemic, many efforts were made to systematize knowledge about the immunostimulatory / immunomodulatory action of *Echinacea* and its benefits in the fight against the virus (Bruno et al., 2011; Aucoin et al., 2021) or in immunocompromised conditions (Kim et al., 2021). One of the few studies on combined extracts is that of Bajrai et al. (2022), which investigates the antiviral effect of a combination of *Echinacea* and *Hypericum perforatum* against SARS-CoV-2 in vitro, and Zima et al. (2024), which addresses the anti-inflammatory, antioxidant, and antiviral properties of a combination of *E. purpurea, Aronia melanocarpa,* and *Lonicera caerulea*.

The idea to combine *E. purpurea* and *O. acanthium* arose from the fact that, despite the numerous positive effects of *Echinacea*, there are still some side effects associated with it (Williams, 2021). On the other hand, *O. acanthium* is considered a major agricultural and wild-growing weed, not demanding in terms of growth conditions, and thus easily accessible (Dewey, 1991; Stace, 2010), but rich in bioactive compounds (Oueslati et al., 2019; Bruno et al., 2011). Therefore,

two combinations with different ratios of *E. purpurea* and *O. acanthium* have been proposed, which are the subject of our study.

Many studies have focused on evaluating the antioxidant strength of various substances or extracts, using various tests such as ORAC, TEAC, TMM, and others. However, there is still no universal method for measuring AOA, which makes it difficult to compare results from different studies (Gulcin, 2020). The literature indicates that phenolic compounds are antioxidants that can act through three mechanisms: chelation, hydrogen donation, and participation in electron transfer reactions (Vuolo et al., 2019). In the present study, three methods were used: ORAC, HORAC, and electrochemical.

3.1. Determination of antioxidant activity by ORAC method

The results for the AOA of the medicinal plants we studied, determined by the ORAC method, are presented in Table 12. According to this method, the most pronounced AOA is observed in Combination 2, which is a result of the high concentration of chicoric acid, caftaric acid, and rutin in the *E. purpurea* extract, and the corresponding levels of myricetin, quercetin, and arctigenin in the *O. acanthium* extract (Table 8). These compounds contribute most significantly to the activity of the combined extracts, considering the proportionate participation of each plant in the mixture.

Table 12. Determination of antioxidant activity of *E. purpurea* and *O. acanthium* extracts and their combinations, measured by ORAC Method

Екстракт	ORAC, μmol TE/g
EP	1417.2±6.2
OA	368.4±0.5
CE1	736.5±6.3
CE 2	1841.7±75.0

Fu et al. (2021) report a high concentration of chicoric acid and caftaric acid in the aerial parts of *E. purpurea* (42 mg/g and 17 mg/g, respectively) and note a high ORAC value (1300 μmol TE/g) as well as significant ABTS-radical scavenging activity. The AOA of *O. acanthium* extracts using ethanol, water, methanol, and sunflower oil was also evaluated, with values ranging from 1742.49 μmol TE/g for the ethanol extract to 11.41 μmol TE/g for the oil extract (Parzhanova et al., 2023).

3.2. Determination of antioxidant activity by HORAC method

There are not many reports in the scientific literature on the use of the HORAC method for determining the AOA of extracts from *E. purpurea* or *O. acanthium*. Parzhanova et al. (2023) analyze the same four extracts from *O. acanthium* using the HORAC method, reporting activities ranging from 716.37 μmol GAE/g for the ethanol extract to 42.13 μmol GAE/g for the methanol

extract, with no activity detected in the oil extract. In our experiments, we obtained an activity of 60 µmol GAE/g for the *O. acanthium* extract (Table 13). The highest activity by this method was observed in Combination 2 - 277.2 µmol GAE/g - which is not simply the mechanical sum of the activities of the individual extracts participating in it proportionally. The same trend is observed in Combination 1. This is likely due to the synergistic effect of the active compounds in these combinations.

Table 13. Determination of antioxidant activity of *E. purpurea* and *O. acanthium* extracts and their combinations, measured by HORAC method

Екстракт	HORAC, µmol GAE/g
EP	181.1±10.4
OA	60.0±1.0
CE 1	174.4±1.9
CE 2	277.2±4.2

3.3. *Determination of antioxidant activity by electrochemical method*

Chiorcea-Paquim et al. (2021) provide a comprehensive review in which they present detailed information about electron transfer reactions in natural polyphenols, from the perspective of their *in vitro* antioxidant and/or prooxidant modes of action, as well as their identification in complex matrices of natural products through electroanalysis. They also discuss the use of electrochemical methods for assessing the overall *in vitro* antioxidant capacity. Electrochemical methods, which are fast, simple, and sensitive, have recently been successfully applied to analyze the antioxidant potential of polyphenols in foods (Haque et al., 2021; Chiorcea-Paquim et al., 2021; Mota et al., 2025). Electrochemical methods have been used to determine the AOA of *E. purpurea* extracts and pharmaceutical forms (Banica et al., 2020; Newair et al., 2017), with the results expressed in ascorbic acid or gallic acid equivalents per gram of extract. In our experiment, AOA was determined through a kinetic criterion, which takes into account the amount of oxygen species that reacted with the sample over time, and was calculated relative to trolox. The results are presented in Table 14.

Table 14. Determination of antioxidant activity of *E. purpurea* and *O. acanthium* extracts and their combinations, measured by electrochemical method

Extract	K,µmol/l,min±SD	AOA
EP	13.953±0.838	22.007
OA	8.499±0.035	13.405
CE 1	17.124±0.991	27.009
CE 2	25.115±1.334	39.614
Trolox	0.634±0.001	1.000

Once again, it is noteworthy that the highest activity, according to this method, is exhibited by the Combined extracts 1 and 2, which confirms the fact that the active compounds exert their synergistic effect. Undoubtedly, plant extracts possess AOA due to the presence of polyphenolic compounds (Katsarova et al., 2017). It was of particular interest to us to examine the AOA of the combined extracts we created, with which pharmacological experiments were conducted.

4. Immunomodulatory effects of individual and combined extracts from *E. purpurea* and *O. acanthium*

4.1. *In vivo* study on acute toxicity of *E. purpurea* and *O. acanthium* extracts and their combinations in experimental animals

In the acute toxicity study, the animals were orally treated with individual and combined extracts at doses of 5 and 10 g/kg body weight. After 24 hours, the survival rate of the animals was 100%. Therefore, both the individual extracts and their combinations are practically non-toxic. This serves as a basis for continuing experiments to confirm the immunomodulatory and anti-stress effects of the mentioned extracts.

4.2. *In vivo* Study on the immunomodulatory effects of *E. purpurea* and *O. acanthium* extracts and their combinations in experimental animals, using a lipopolysaccharide model and cytokine level measurement

4.2.1. Changes in Serum TNF-α in LPS – Challenged Rats

The serum level of TNF-α in the LPS-treated animals was significantly increased compared to that in the untreated control animals. In all other groups, the levels of the investigated cytokines were decreased compared to the control treated with lipopoly-saccharide. The decrease in serum TNF-α concentration in the Combination 1-treated group was most pronounced (244.82 ± 80.99 pg/mL) compared to the LPS-control (574.17 ± 97.96 pg/mL), while in the Combination 2-treated group (322.46 ± 45.61 pg/mL) it was not as large. Both results were statistically significant, $p < 0.05$. The effect of single extracts on the TNF-α levels was not pronounced, although in animals treated with O. acanthium, the reduction of the studied cytokine compared to the control treated with lipopolysaccharide was statistically significant. These results are shown in Figure 8.

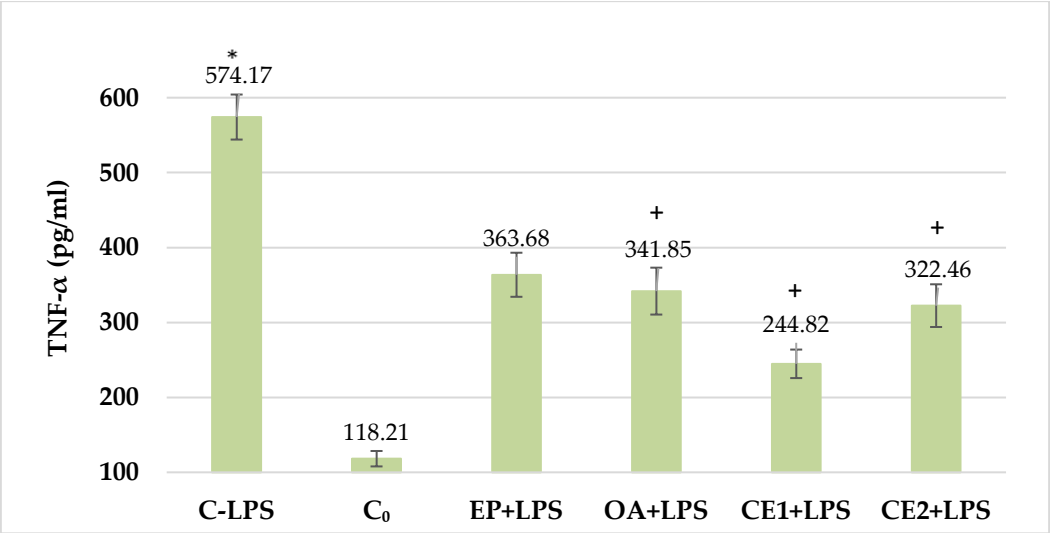


Figure 8. Serum TNF-α in LPS-challenged Wistar rats. The comparison was made using the ANOVA test, followed by LSD posthoc test; * $p < 0.05$ compared to C_0; + $p < 0.05$ compared to C-LPS

4.2.2. Changes in Serum IFN-γ in LPS – Challenged Rats

In the control group of animals treated with LPS, the level of the proinflammatory cytokine IFN-γ increased significantly compared to that of the control group not treated with LPS. In the groups treated with the studied combinations and individual extracts, the values of IFN-γ decreased. The decrease in the levels of the investigated cytokine was statistically significant in Combination 1 as well as in the Onopordum ex-tract alone, while in Combination 2 and in the Echinacea extract alone, a downward trend was observed without statistical significance. The decrease in serum levels of IFN-γ was most pronounced in the animals of the group treated with Combination 1 compared to the control group treated with LPS. If Combination 1 was compared to Combination 2 as well as to Echinacea extract alone, the decrease in IFN-γ concentra-tion was also statistically significant. These results are shown in Figure 9.

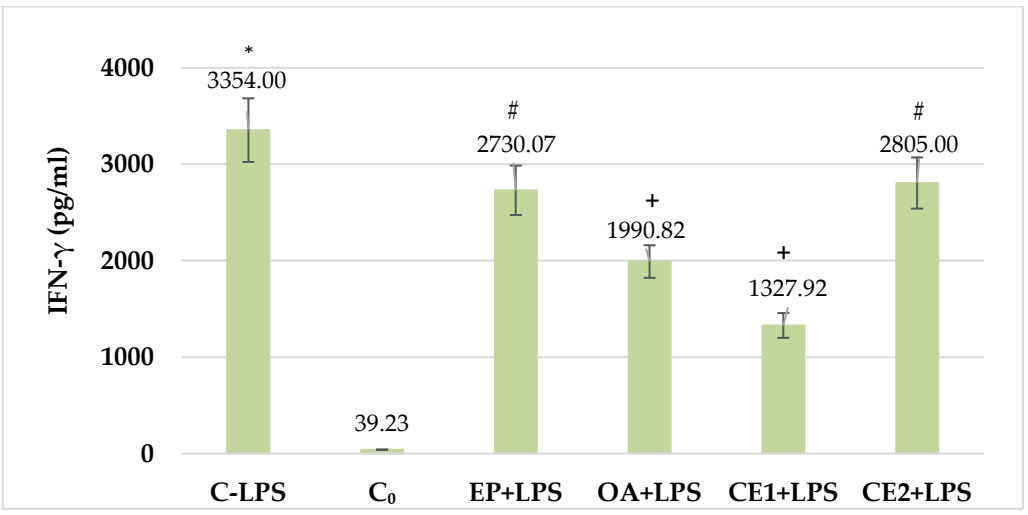


Figure 9. Serum IFN-γ in LPS-challenged Wistar rats. The comparison was made using the ANOVA test, followed by LSD posthoc test; * $p < 0.05$ compared to C0; + $p < 0.05$ compared to C-LPS; # $p < 0.05$, compared to CE1+LPS

4.2.3. Changes in Serum IL-10 in LPS – Challenged Rats

The concentration of IL-10 in the serum of rats from the control group treated with LPS increased with statistical significance compared to the untreated control group. When compared with the control group treated with LPS, IL-10 levels increased in all other groups treated with the tested extracts and combinations. In Combination 2, the difference was statistically significant. These results are presented in Figure 4.

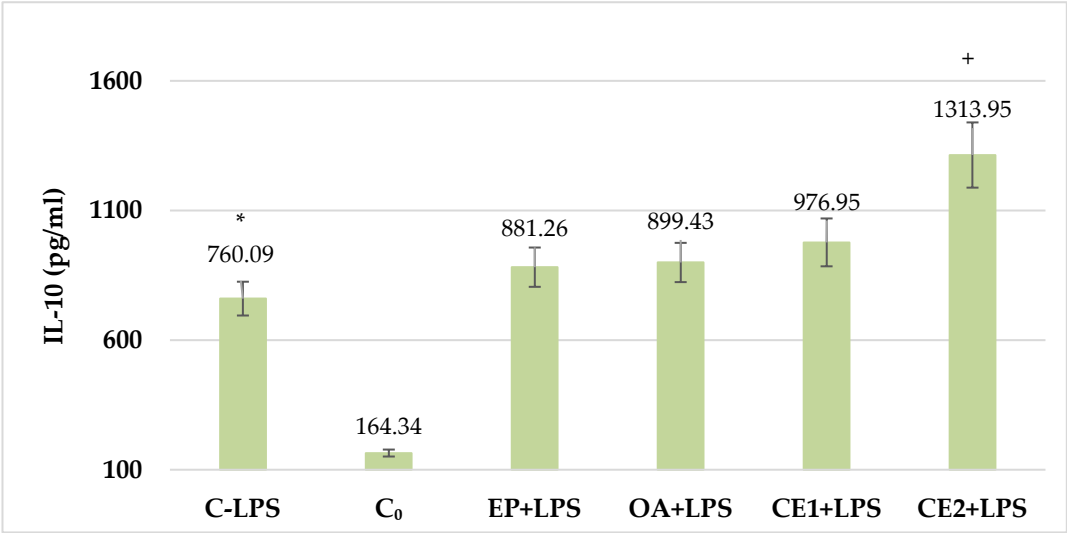


Figure 10. Serum IL-10 in LPS-challenged Wistar rats. The comparison was made using the ANOVA test, followed by LSD posthoc test; * $p < 0.05$ compared to C0; + $p < 0.05$ compared to C-LPS

4.2.4. Changes in Serum IFN-γ/IL-10 Ration in LPS – Challenged Rats

In all groups treated with the investigated extracts and in the LPS control group, an increase in the ratio of IFN-γ/IL-10 was observed when compared to the untreated control. This increase was most pronounced in the LPS-treated control group (4.51 ± 0.18). In animals from both groups treated with the combined extracts, a reduction in this ratio was observed when compared to the LPS control group ($p < 0.001$ and $p < 0.05$, respectively, for Combination 1 and Combination 2). A greater reduction was recorded in rats treated with combination 1 (1.55 ± 0.8). A statistically significant de-crease in IFN-γ/IL-10 was also observed when comparing Combination 1 with the in-dividual extract ($p < 0.05$).

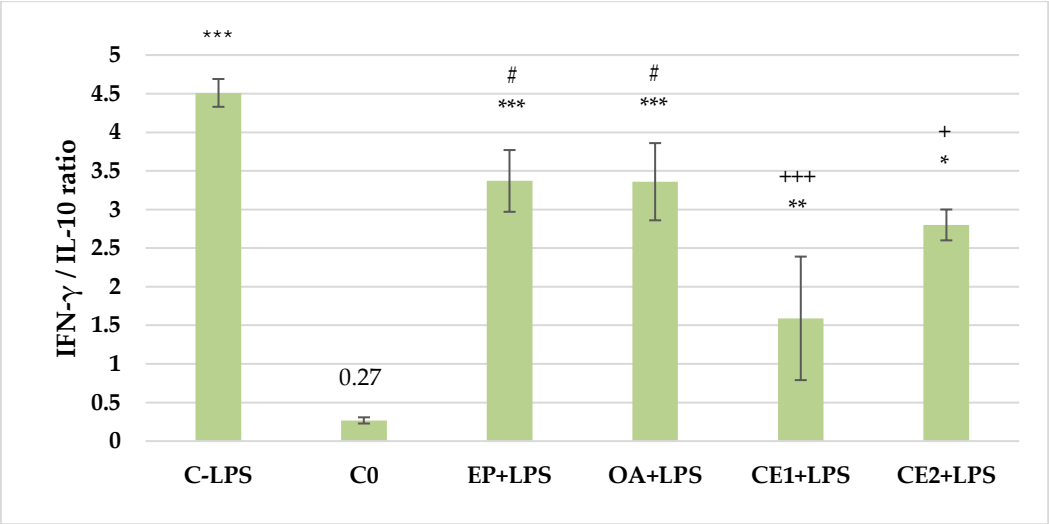


Figure 11. Serum IFN-γ/IL-10 ratio in LPS-challenged Wistar rats. The comparison was made using the ANOVA test, followed by LSD posthoc test; * $p < 0.05$ compared to C_0; ** $p < 0.01$ compared to C_0; *** $p < 0.001$ compared to C_0; + $p < 0.05$ compared to C-LPS; +++ $p < 0.001$ compared to C-LPS; # $p < 0.05$ compared to CE1+LPS

In the present study, the immunomodulatory effect of *E. purpurea*, *O. acanthium*, and their combinations was investigated in two experimental models – LPS-induced systemic inflammatory response and acute cold stress. Bacterial LPS (lipopolysaccharide) is a component of the cell wall of Gram-negative bacteria and serves as a powerful stimulant of inflammation. LPS is recognized by the toll-like receptor (TLR)-4 on the surface of phagocytes. This leads to the activation of intracellular signaling pathways, which in turn stimulate transcription factors such as nuclear factor-κβ (NF-κβ). The ultimate result of these processes is the stimulation of the release of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and others (Mazgaeen et al., 2020). LPS is one of the main triggers for TNF-α production. This cytokine plays a crucial role in the pathogenesis of inflammation and the Th1 immune response. It is synthesized not only by macrophages but also by a variety of other immune (NK cells, B cells, neutrophils, etc.) and non-immune cells (Zelová et al., 2018). Moreover, bacterial LPS stimulates the synthesis of INF-γ, which also belongs to the group of Th1 cytokines and plays a role in the development of inflammation and the immune response. INF-γ is primarily produced by NK cells and partially by T lymphocytes. LPS-induced INF-γ production is inhibited by IL-10 and stimulated by IL-12. Under LPS-induced inflammation, IL-10 is predominantly synthesized by macrophages and monocytes and plays a key role in inhibiting the inflammatory response (Varma et al., 2002).

The results of the present study demonstrate the stimulatory effect of bacterial LPS on the production of TNF-α, INF-γ, and IL-10. In the LPS control animals, a significant increase in serum levels of these molecules was observed compared to the clean control group. The increase in IL-10 is associated with its role as an anti-inflammatory molecule, which is important for preventing the

consequences of an intense inflammatory response. IL-10 suppresses the production of TNF-α, IL-1, IL-6, IL-8, which are produced in response to inflammation. The synthesis of IL-10 is inhibited by microRNA-98 (miR-98). Under LPS-induced inflammation, a decreased expression of this microRNA is observed, which may explain the increased production of this anti-inflammatory cytokine (Liu et al., 2011).

The administration of *E. purpurea* extract alone reduces serum concentrations of TNF-α and INF-γ in animals treated with LPS, but the results do not reach statistical significance. The literature on the effect of *E. purpurea* on TNF-α expression is contradictory. Earlier *in vitro* studies show that *E. purpurea* stimulates the production of pro-inflammatory cytokines, including TNF-α, from human macrophages (Burger et al., 1997; Rininger et al., 2000). Recent *in vitro* experiments on spleen lymphocytes from rats treated with *E. purpurea* (Yamada et al., 2011) found that *E. purpurea* significantly increases the production of IL-2 and INF-γ (especially in the presence of inflammatory stimulators such as LPS and concanavalin A), but not TNF-α. Under LPS stimulation of macrophages, *E. purpurea* extract leads to a significant reduction in the levels of NF-κβ and TNF-α (Stevenson et al., 2005). In an *in vivo* model of chronic inflammation, essential oil from *E. purpurea* flowers reduces the levels of pro-inflammatory cytokines (IL-2, IL-6, TNF-α) in serum (Yu et al., 2013). Although TNF-α plays a key role in the normal immune response, its overproduction leads to a damaging effect and is important for the pathogenesis of several autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel diseases, psoriasis, and others (Jang et al., 2021). Uncontrolled production of INF-γ can also lead to tissue damage and plays a role in the development of diseases (Liu et al., 2022; Kann et al., 2022). INF-γ can induce the synthesis of other inflammatory molecules, including TNF-α (Vila-del Sol et al., 2008). The application of immunostimulants, including those of plant origin, may contribute to the development of autoimmune diseases (Bax et al., 2021). Increased and inadequate secretion of TNF-α is one of the possible explanations for such an undesirable reaction (Lee et al., 2004). Literature data on the risk of such complications when using *E. purpurea*-containing preparations are scarce (Lee et al., 2004; Kocaman et al., 2007; Soon et al., 2001). Most likely, *E. purpurea* and the biologically active substances it contains have more of an immunomodulatory rather than immunostimulatory effect (Nagoor Meeran et al., 2021). The results of the present study support this thesis, as the animals treated with *E. purpurea* extract showed lower levels of TNF-α than the LPS control group. The same was found regarding the serum concentration of INF-γ, regardless of the available literature data on the stimulatory effect of *E. purpurea* on NK cell function (See et al., 1997). We can assume that under conditions of an intense inflammatory response, *E. purpurea* extract suppresses the synthesis of pro-inflammatory molecules in order to limit tissue damage. This is an expression of its immunomodulatory effect, which, at least in part, is mediated by increasing the levels of anti-inflammatory mediators

such as IL-10. Under *in vitro* conditions, Gertsch et al. (2004, 2006) found that in the presence of LPS, *E. purpurea* suppresses the synthesis of TNF-α. They attribute this effect to the alkylamides contained in the plant, which act as agonists of cannabinoid CB2 receptors. Phenolic acids (chicoric, caffeic, and chlorogenic acids) and flavonoids most likely also contribute to the immunomodulatory effect of *E. purpurea*. Chicoric acid reduces serum concentrations of TNF-α, IL-1β, and the expression of mRNA for these molecules in the brain of LPS-induced inflammatory mice (Liu et al., 2017). In a Parkinson's disease model in mice, chicoric acid reduces INF-γ levels in serum, striatum, and spleen (Wang et al., 2022). Of the three phenolic acids, we found that chicoric acid has the highest concentration in the examined extract. Quercetin, which is also found in the extract and is the subject of the current study, has the ability to inhibit TLR-4-mediated synthesis of pro-inflammatory molecules (TNF-α, IL-1β, IL-6, nitric oxide, prostaglandins, etc.) under LPS-induced inflammation conditions (Chen CY et al., 2018). There is also evidence of a stimulatory effect on INF-γ synthesis, but this has been observed in blood mononuclear cells not stimulated with a pro-inflammatory agent (Nair et al., 2002).

Surprisingly, the extract of *O. acanthium* showed a statistically significant effect in reducing serum levels of TNF-α and INF-γ. Although there is literature data supporting its anti-inflammatory action (Robertovna et al., 2019), this plant is not known as an immunomodulator. Shabsoug et al. (2008) found that *O. acanthium* stimulates the cytotoxicity of NK cells and increases the production of TNF-α and INF-γ. Recent experimental data suggest a reduction in serum levels of TNF-α in rats with streptozotocin-induced diabetes after treatment *with O. acanthium* (Mhammad et al., 2021). The reduced synthesis of TNF-α may be related to the observed inhibition of NF-κβ1 expression by *O. acanthium* (Lajter et al., 2015). The effect of the extract of this plant on the reduced serum levels of TNF-α and INF-γ observed by us can be explained by the biologically active compounds it contains. As mentioned above, quercetin has a pronounced anti-inflammatory effect. Several studies have demonstrated the ability of arctigenin to suppress the expression of TNF-α and other pro-inflammatory cytokines such as IL-1β, IL-6, and others (Gao et al., 2018). Scutellarin and apigenin also inhibit the production of pro-inflammatory cytokines and reduce the expression of molecules involved in the inflammatory response (Luo et al., 2020; Yoon et al., 2023). The combined use of the extracts significantly reduced the serum levels of TNF-α and INF-γ, with the effect being most pronounced in Combination 1. Combining *E. purpurea* and *O. acanthium* in a 1:1 ratio leads to the most optimal effect on the levels of TNF-α and INF-γ in conditions of induced systemic inflammation, due to the supplementation and enhancement of the biological activities of the compounds contained in both extracts. This combination would be useful in the treatment of infectious diseases with a lower risk of over-stimulating the immune system, especially in predisposed patients.

IL-10 suppresses the formation and release of pro-inflammatory cytokines and directly inhibits certain immune cells. In this way, this cytokine suppresses the development of the inflammatory and immune response, limiting tissue damage (Ouyang et al., 2019). Experimental *in vitro* and animal studies demonstrate that *E. purpurea* increases the expression of IL-10 (Aucoin et al., 2021). The results of the present study show that both tested extracts, as well as their combination in equal amounts, insignificantly increase the serum levels of IL-10. Statistical significance was reached only in Combination 2. This is most likely a compensatory response and is related to the high levels of INF-γ in the animals from this group. It is well-known that IL-10 is the main negative regulator of INF-γ secretion in the context of LPS-induced inflammation (Varma et al., 2002).

The combined extracts have a richer and more diverse content of bioactive compounds that interact synergistically, enhancing, complementing, or altering each other's effects. The combination of *E. purpurea* and *O. acanthium* in a 1:1 ratio appears to be more effective in reducing pro-inflammatory cytokines. This could be beneficial in situations where enhancing the immune response against an infectious agent carries the risk of excessive immune system activation, such as a cytokine storm. The combination of *E. purpurea* and *O. acanthium* in a 3:1 ratio is more effective in restoring the balance between TNF-α (Th1 cytokine) and IL-10 (Th2 cytokine). This is associated with favorable modulation of both inflammation and immune response, while minimizing the risk of immune-mediated adverse reactions.

5. Study of the anxiolytic effect of the examined extracts through behavioral tests and monitoring of serum levels of TNF-α, INF-γ, and IL-10.

5.1. Study of the anxiolytic effect through the acute cold stress model and monitoring the levels of TNF-α, INF-γ, and IL-10

The reactions triggered in the body by acute stress are often a response of its protective mechanisms, which may stimulate inflammatory processes as a result. This type of modulation may have a positive effect in the short term, but prolonged low-grade inflammation has numerous consequences on the human body. Several authors describe how the body's protective systems respond to different stress conditions to overcome them - such as increasing blood sugar, blood pressure, heart rate, and enhancing the inflammatory response by elevating cytokine levels in the blood. There is also evidence linking chronic stress to a wide range of diseases - insulin resistance, cardiovascular diseases, cancer, and others. It turns out that acute stress has various effects on several components of the immune system - such as the number and composition of circulating leukocytes, functional parameters assessed through in vitro stimulation of immune functions, and the production of various cytokines (Rohleder, 2019).

5.1.1. Changes in Serum TNF-α in Subjected to Acute Cold Stress Rats

The control stress group shows a nonsignificant trend toward an increase in plasma levels of TNF-α compared to the non-stressed control group. The groups treated with all the tested extracts and combinations lower the levels of TNF-α compared to the control group of animals subjected to acute stress, with the combined extracts showing the best result. The results are presented in Figure 12.

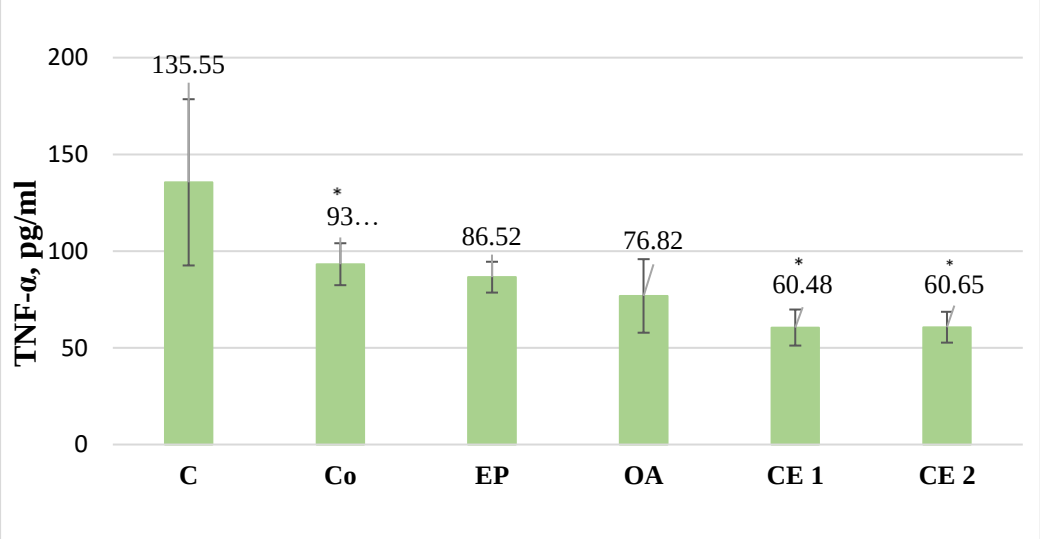


Figure 12. Serum levels of TNF-α in Subjected to Acute Cold Stress Rats

5.1.2. Changes in Serum IFN-γ in Subjected to Acute Cold Stress Rats

When comparing the two control groups, a statistically significant increase in IFN-γ levels was observed in the group subjected to acute cold stress, compared to the control group without stress. This indicates that the studied cytokine is an appropriate marker for monitoring the changes occurring in the animals as a result of acute cold stress.

All of the tested extracts and combinations reduced IFN-γ levels compared to the stress-exposed control group, with the standalone extract of O. acanthium and Combination 2 showing a significant decrease ($p<0.05$). The results are presented in Figure 13.

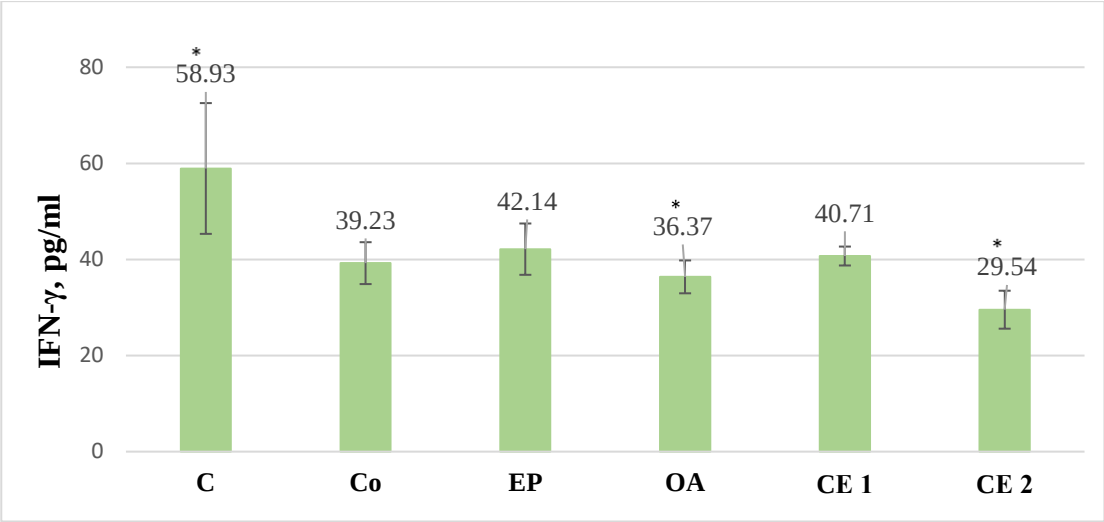


Figure 13. Serum levels of IFN-γ in Subjected to Acute Cold Stress Rats

5.1.3. Changes in Serum IL-10 in Subjected to Acute Cold Stress Rats

When comparing the tested extracts and combinations with the stress control group, it was found that all of them increased the levels of the anti-inflammatory cytokine IL-10. The increase was statistically significant in the group treated with the standalone extract of *O. acanthium* at a dose of 500 mg/kg b.w., as well as in Combination 2 at the same dose (Figure 14).

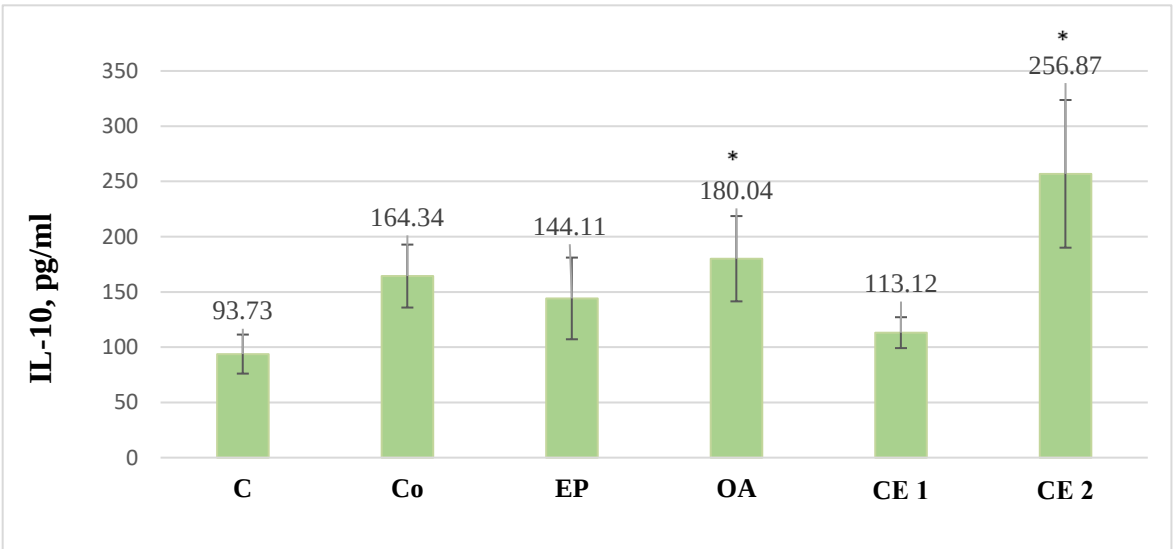


Figure 14. Serum levels of IL-10 in Subjected to Acute Cold Stress Rats

In our study, during acute cold stress, increased metabolic activity was observed. This leads to enhanced production of free oxygen radicals, while the activity of antioxidant enzymes is reduced. This imbalance results in the development of oxidative stress (Srivastava et al., 2015). The AOA of the extracts likely also plays

a role in the mechanism of the observed anxiolytic effect. Combination 2 exhibited the highest activity, which also corresponded to the most pronounced anxiolytic effect in the elevated plus maze test.

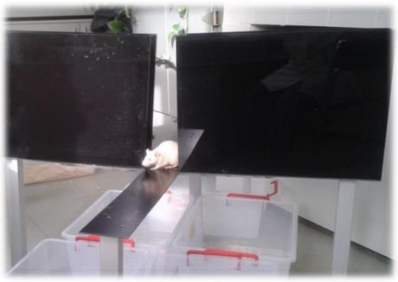
Experimental data indicate that not only chronic stress but also acute stress is characterized by immune system activation and the development of inflammation (Tang et al., 2022). A recent meta-analysis shows that acute stress is accompanied by an increase in serum levels of pro- and anti-inflammatory cytokines (Marsland et al., 2017). Since *E. purpurea* and *O. acanthium* have immunomodulatory effects, we aimed to establish their role in the observed anxiolytic effect of the extracts.

In a model of immobilization stress, cold-pressed juice from the aerial parts of *E. purpurea* reduces serum levels of IL-10, IL-6, and IL-17, as well as the mRNA expression for these cytokines in the spleen (Park et al., 2018). Our results show that in the control group exposed to cold stress, there were elevated levels of TNF-α and INF-γ, while the serum concentration of IL-10 was low compared to the non-stressed control. These results demonstrate that acute stress induces a pro-inflammatory state in the body. The increase in INF-γ was most pronounced. Studies in animals (Mandolesi et al., 2017) and humans (Gabbay et al., 2008) show that INF-γ leads to anxiety and depressive behaviors. On the other hand, it has been established that mice with a knockout gene for IL-10 exhibit anxiety-like behavior (Yang et al., 2021). In patients with major depressive disorder, low serum levels of this cytokine have been observed (Dhabhar et al., 2009).

The extract of *O. acanthium* and Combination 2 significantly reduced INF-γ and increased the serum level of IL-10 in the acute cold stress model. This result is consistent with the findings from the behavioral tests, where a significant anxiolytic effect was recorded in the same experimental groups. Likely, the inhibitory effect on the synthesis of INF-γ and the stimulation of IL-10 play an important role in the mechanism of the observed anxiolytic effect. TNF-α is not only a pro-inflammatory molecule but is also constitutively expressed in the nervous system and plays an important role in its normal functioning. However, disrupted TNF-α signaling can lead to anxiety-like behavior (Camara et al., 2013). The decreased production of this cytokine likely also contributes to the observed anxiolytic effect of the studied extracts.

5.2. Investigation of the anxiolytic effects of the tested extracts through behavioral tests

5.2.1. Elevated plus maze test



In the animals of the control group subjected to acute cold stress, a significantly lower number of entries into the open arms of the maze was observed (Figure 15), along with reduced time spent in the open arms (Figure 16), longer time spent in the closed arms, and a decreased index of the number of entries into the open arms/total number of entries into the arms ($p<0.05$) (Figure 17). In the rats treated with *O. acanthium* and Combination 2, increased time spent in the open arms and decreased time spent in the closed arms were recorded, compared to both control groups and those treated with *E. purpurea* and Combination 1 ($p<0.001$) (Figure 16). Animals from all extract-treated groups showed an increased ratio of the number of entries into the open arms/total number of entries into the arms compared to the stressed control, with the highest significance observed in Combination 2 ($p<0.01$) (Figure 17). The number of entries into the open arms of the maze was increased in all treated groups compared to the stressed control, with statistical significance observed in *O. acanthium* and Combination 1 ($p<0.05$) (Figure 15).

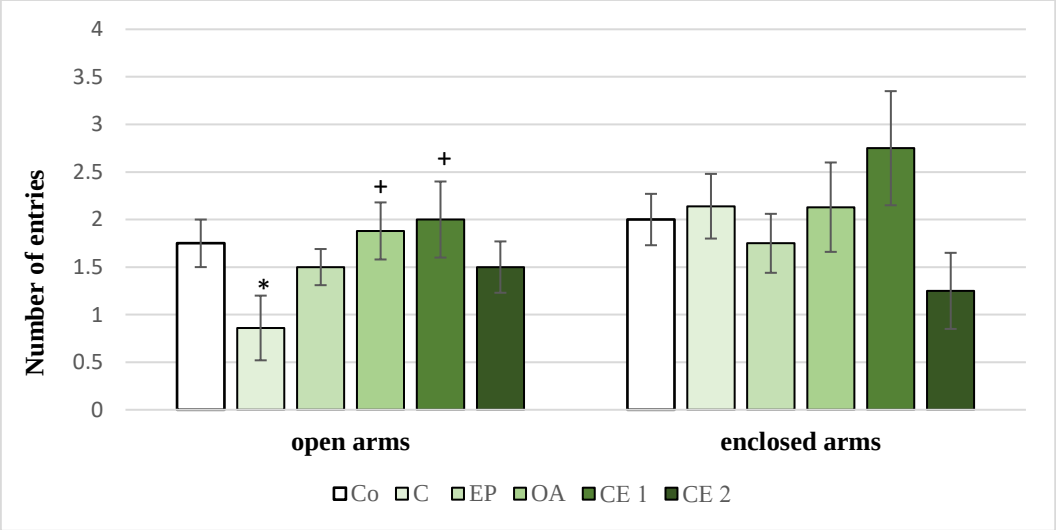


Figure 15. Effect of *E. purpurea*, *O. acanthium*, Combination 1, and 2 on the number of entries into the open and closed arms of the elevated plus-maze. The data are presented as mean±SEM. *p<0,05 compared to control; +p<0,05 compared to stress control

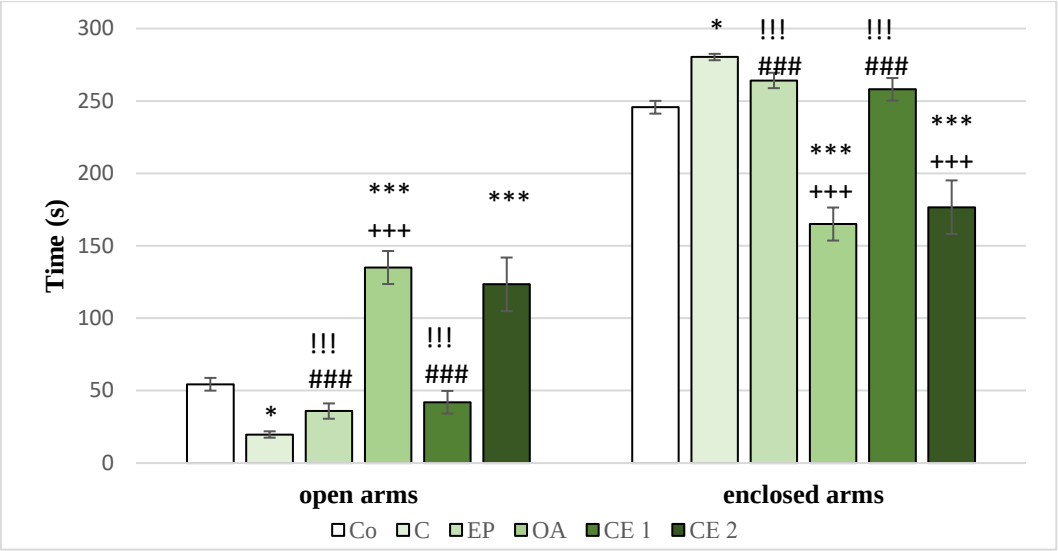


Figure 16. Effect of *E. purpurea*, *O. acanthium*, Combination 1, and 2 on the time spent in the open and closed arms of the elevated plus-maze. The data are presented as mean±SEM. *p<0,05 compared to control; ***p<0,001 compared to control; +++p<0,001 compared to stress control; !!!p<0,001 compared to OA; ###p<0,001 compared to Combination 2

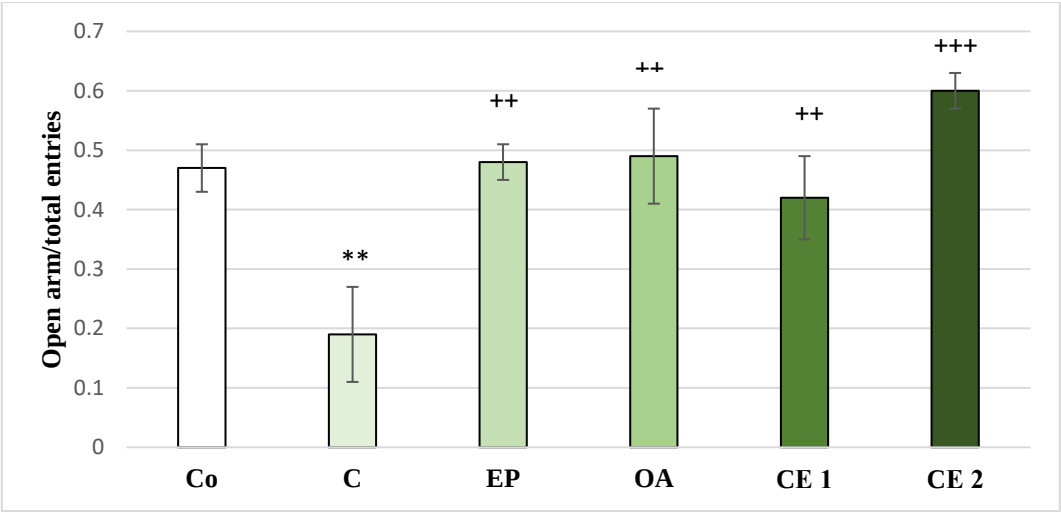


Figure 17. Effect of *E. purpurea*, *O. acanthium*, Combination 1, and 2 on the ratio of entries into the open arms to the total number of entries into the arms of the elevated plus-maze. The data are presented as mean±SEM. p<0.05 compared to C_0; ++p<0.01 compared to C; +++p<0.01 compared to C

5.2.2. Social interaction test

In the animals from the stressed control group and *E. purpurea*, a reduced social interaction time was observed compared to the non-stressed control group (p<0.001).

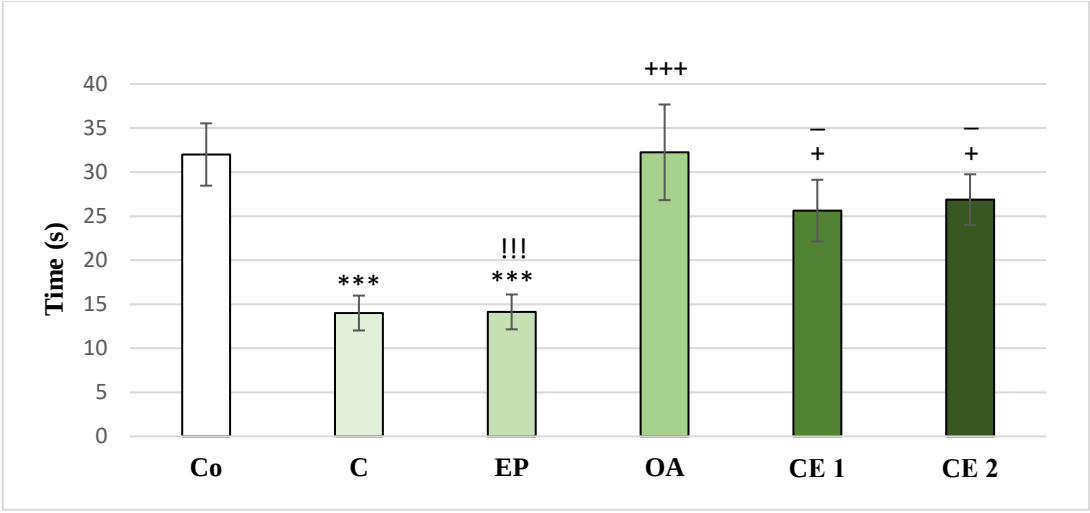


Figure 18. Effect of *E. purpurea*, *O. acanthium*, Combination 1, and 2 on the social interaction time. Data are presented as mean±SEM. $p<0.001$ comparison with C_0; +++$p<0.001$ comparison with C; +$p<0.05$ comparison with C; !!!$p<0.001$ comparison with *O. acanthium*; - $p<0.05$ comparison with *E. purpurea*

In rats from the groups treated with *O. acanthium* and the combined extracts, an increase in this parameter was observed compared to the stressed control ($p<0.001$; $p<0.05$, respectively). Animals treated with the *E. purpurea* extract showed significantly lower social interaction time compared to those treated with the other three extracts (Figure 18).

The anxiolytic effect of *E. purpurea*, *O. acanthium*, and their combinations was investigated using two behavioral methods – the elevated plus maze and the social interaction test. The first method is based on the innate instinct of rodents to prefer dark and dimly lit spaces. An increased time spent and the number of entries into the open arms of the maze are considered as signs of an anxiolytic effect, while entries into the dark arms and the total number of entries are indicators of the animals' motor activity. When considering individual parameters (number of entries or time spent in the open arms), an anxiolytic effect was observed in *O. acanthium* and the combined extracts. Using the integrated parameter of the number of entries/total number of entries into the arms of the maze, the data showed that all tested extracts exhibited an anxiolytic effect without affecting the animals' motor activity. This effect was most pronounced in Combination 2. In the social interaction test, an anxiolytic effect was observed in animals treated with *O. acanthium* extract and Combination 2. Haller et al. (2010) in a study on the anxiolytic effect of various plant extracts from the *Echinacea* genus found this effect in some of the used extracts, with only one of them showing it across a broader dose range. Literature data on the anxiolytic effect in animals and humans mostly refer to another species of the genus (*E. angustifolia*) (Haller et al., 2013; Haller et al., 2020). To date, there are no reports of an anxiolytic effect for *O. acanthium*. In the present study, this effect was observed across all registered parameters. This is likely due to the high content of chlorogenic acid in the extract,

which is absent in *E. purpurea*. This organic acid has proven anxiolytic and antidepressant effects due to its neuroprotective, anti-apoptotic, and anti-inflammatory properties (Chen et al., 2021). It has been found to also stimulate the action of γ-aminobutyric acid, which is a neurotransmitter with an inhibitory effect on the CNS (Hara et al., 2014).

CONCLUTIONS

1. Two HPLC methods were developed for the determination of characteristic compounds of the *Echinacea* genus – chicoric acid, caftaric acid, caffeic acid, echinacoside, quercetin, apigenin (Method I) and for the *Onopordum* genus – arctigenin, chlorogenic acid, caffeic acid, apigenin, quercetin, scutellarin (Method II). Both methods are accurate, precise, and sufficiently sensitive for the quantitative determination of the listed substances.
2. The plant biomasses of *E. purpurea*, cultivated in the Kazanlak, Bulgaria, were standardized by the quantities of chicoric and caftaric acids accumulated in different stages of plant development. According to the European Pharmacopoeia 8, their total content in the roots should be at least 0.5%, and in the aerial parts, at least 0.1%. Our results show that in the roots, they range from 1.8 to 3.9%, and in the aerial parts, from 2.5 to 3.7%, making *E. purpurea* a reliable raw material for the production of phytopharmaceuticals.
3. Method I is also applicable for determining chicoric acid, caftaric acid, caffeic acid, echinacoside, cynarin, quercetin, and apigenin in herbal preparations and tonic drinks containing *Echinacea* from the commercial sources, with the finding that Ultimate Echinacea is richest in these active substances.
4. Dry plant material from *E. purpurea* (cat. no. 39397) and *O. acanthium* (cat. no. 39398) was characterized according to the amounts of echinacoside, cynarin, caftaric acid, chicoric acid, caffeic acid, quercetin, and apigenin (Method I) and chlorogenic acid, caffeic acid, scutellarin, quercetin, arctigenin, and apigenin (Method II). From these plant biomasses, individual and combined ethanol extracts were obtained. The extract from *E. purpurea* contains a greater number of the examined acids, while the extract from *O. acanthium* contains a greater number of the examined flavonoids. Combined extract 1 (1:1) and Combined extract 2 (3:1) contain proportional amounts of the examined substances.
5. The AOA of the obtained individual and combined extracts was determined by three methods – ORAC, HORAC, and electrochemical methods. It was found that the activity of Combined extract 2 is the most pronounced.
6. The individual and combined extracts from *E. purpurea* and *O. acanthium* are non-toxic in *in vivo* acute toxicity tests on experimental animals at doses of 5 and 10 g/kg body weight.
7. In a model of induced inflammation with lipopolysaccharide, the combined extracts exhibited a more pronounced immunomodulatory effect compared to the individual extracts, reducing the levels of pro-inflammatory markers TNF-α and IFN-γ and significantly increasing the levels of the anti-inflammatory cytokine IL-10.

8. In the acute cold stress model, Combined extract 2 most significantly reduced the levels of pro-inflammatory cytokines TNF-α and IFN-γ and significantly increased the levels of IL-10.
9. The anxiolytic effect of the extracts was proven through two behavioral tests – the elevated plus maze and the social interaction test, as well as through the acute cold stress model. The most pronounced anxiolytic effect was observed in the animals treated with *O. acanthium* extract and Combined extract 2.

CONTRIBUTIONS

1. For the first time, species of *Echinacea* cultivated in the Kazanlak, Bulgaria have been characterized, and it was established that they are a reliable raw material for the production of phytopharmaceuticals.
2. The developed HPLC methods can be applied for the quality control of plant biomasses, extracts, and phytopharmaceuticals obtained from *E. purpurea* and *O. acanthium*, as well as combinations of these.
3. For the first time, combined extracts from *E. purpurea* and *O. acanthium* with different drug ratios – 1:1 and 3:1, respectively – were studied, and a more pronounced antioxidant, immunomodulatory, and anxiolytic effect of the combined extracts was proven in comparison with the individual extracts.
4. For the first time, the anxiolytic effect of the O. *acanthium* extract was proven through two behavioral tests.

VII. SCIENTIFIC PUBLICATIONS AND PARTICIPATION IN SCIENTIFIC CONFERENCES, RELATED TO THE DISSERTATION

I. Scientific publications

1. **Vlasheva**, M.; Katsarova, M.; Dobreva, A.; Dzhurmanski, A.; Denev, P.; Dimitrova, S. Echinacea Species Cultivated in Bulgaria as a Source of Chicoric and Caftaric Acids. *Agronomy* **2024**, 14, 2081. **Q1**

2. **Vlasheva**, M.; Katsarova, M.; Kandilarov, I.; Zlatanova-Tenisheva, H.; Gardjeva, P.; Denev, P.; Sadakova, N.; Filipov, V.; Kostadinov, I.; Dimitrova, S. *Echinacea purpurea* and *Onopordum acanthium* Combined Extracts Cause Immunomodulatory Effects in Lipopolysaccharide-Challenged Rats. *Plants* **2024**, *13*, 3397. **Q1**

3. **Vlasheva, M.;** Katsarova, M.; Dimitrova, S. Biological Activity of Medicinal Plants from the Asteraceae Family – Mini Review. Scientific Papers of the Union of Scientists in Bulgaria–Plovdiv, Series G, 2024. Medicine, Pharmacy and Dental Medicine, Vol. XX, ISSN 1311-9427.

II. Citations

1. Vlasheva, M.; Katsarova, M.; Dobreva, A.; Dzhurmanski, A.; Denev, P.; Dimitrova, S. *Echinacea* Species Cultivated in Bulgaria as a Source of Chicoric and Caftaric Acids. Agronomy 2024, 14, 2081. Q1
   Cited by:
   Ahmadi, F. Genomic Innovations and Marker-Assisted Breeding in *Echinacea* Species: Insights and Applications. Sci 2025, 7, 43.

III. Participation in scientific conferences

1. **Science and Youth 2021.** Medical University - Plovdiv, MND "Asklepius", April 9-11, 2021, online. Poster: Study on the polyphenolic composition of extracts from *Echinacea purpurea* and *Onopordum acanthium*. Authors: Maria Zgureva, Viktor Filipov, Mariyana Katsarova, Stela Dimitrova.
2. **1st International Scientific and Practical Internet Conference "Importance of Soft Skills for Life and Scientific Success",** 1-2 March 2022, Dnipro, Ukraine**.** Development and validation of hplc methods for quantitative determination of biologicaly active substances in extracts

of *Echinacea purpurea* and *Onopordum acanthium*. Autors: Maria Zgureva, Viktor Filipov, Mariana Katsarova, Stela Dimitrova.

3. **3rd International Scientific and Practical Internet Conference "Ways of science development in modern crisis conditions",** 2-3 June, 2022, Dnipro, Ukraine. Chemical composition and biological activity of extracts from *Echinacea purpurea* and *Onopordum acanthium.* Autors: Maria Zgureva, Viktor Filipov, Mariana Katsarova, Stela Dimitrova.
4. **7th Congress of Pharmacy in North Macedonia "Modern trends in pharmacy. Opportunities and challenges"** 6-8 October, 2022, Ohrid, North Macedonia. Poster: Non-toxicity and anti-inflammatory effect of extracts from *Echinacea purpurea* and *Onopordum acanthium*. Autors: Maria Vlasheva, Mariana Katsarova, Petia Gardjeva, Hristina Zlatanova, Ilin Kandilarov, Viktor Filipov, Stela Dimitrova.
5. **Scientific Conference "Days of Science 2022",** Union of Scientists in Bulgaria - Plovdiv, November 25-26, 2022, online. Poster: *In vitro* antioxidant activity and *in vivo* anti-stress effect of extracts from *Echinacea purpurea* and *Onopordum acanthium*. Authors: Maria Vlasheva, Mariyana Katsarova, Petya Gardjeva, Hristina Zlatanova, Ilin Kandilarov, Viktor Filipov, Stela Dimitrova.
6. **Sustainable Utilization of Bio-Resources and Waste of Medicinal and Aromatic Plants for Innovative Bioactive Products – ICSUMAP'23,** 27-28 March 2023г., Bulgaria. Online presentation: *Echinacea* species introduced in Bulgaria as a source of caftaric and chicoric acids. Autors: Maria Vlasheva, Mariana Katsarova, Ana Dobreva, Anatoli Dzhurmanski, Stela Dimitrova.
7. **4th International Mediterranean Congress,** 15-16 June 2023, Türkiye. *Echinacea purpurea* and *Onopordum acanthium* exert anxiolytic effect in experimental model of acute stress. Autors: Maria Vlasheva, Mariana Katsarova, Stella Dimitrova, Ilin Kandilarov, Hristina Zlatanova, Ilia Kostadinov.
8. **Anniversary Conference "Pharmacy - Science with the Future",** Medical university of Plovdiv, 17-19 November 2023. Poster: Anxiolytic effect of individual and combined extracts from *Echinacea purpurea* and *Onopordum acanthium*. Authors: Maria Vlasheva, Mariana Katsarova, Ilin Kandilarov, Kristina Zlatanova-Tenisheva, Iliya Kostadinov, Viktor Filipov, Stela Dimitrova.
9. **Scientific Conference "Days of Science 2023"**, Union of Scientists in Bulgaria – Plovdiv, November 23-24, 2023. Poster: Biological activity of medicinal plants from the Asteraceae family. Authors: Maria Vlasheva, Mariana Katsarova, Viktor Filipov, Stela Dimitrova.

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To my family and friends!