4.2 Total phenolic and flavonoid contents and antioxidant activity of callus cultures Delphinium elatum L.

Research that provides the development of drugs based on natural raw materials, including medicinal plants, is an important task for modern medicine. The search for promising plants among the representatives of the Ukrainian flora is an urgent task for pharmacies today. The flora of the Carpathians is extremely rich and diverse. Many representatives of the Carpathian flora have useful and healing properties. That is why for many centuries used in folk medicine for decoctions, tinctures, and ointments for the treatment and prevention of many diseases. Among the plants common in the Carpathians, there are relics, endemics, and rare and endangered species that need protection and conservation measures. Plants of the *Ranunculaceae* family, in particular *Delphinium elatum* L. in Fig.1, are promising objects for research [214].

Figure 1. *Delphinium elatum* L.

Delphinium elatum L. (*alphine delphinium* [215], *candle larkspur* [216], dauphinelle élevée [217], pied d'alouette élevé [218]) – unique, extremely rare, one of the most popular medicinal plants of the Ukrainian Carpathians, which is actively used in ethnomedicine.

Delphinium elatum L. – perennial herb, stem 80-200 cm high, hollow, branched; root system - rod-fibrous, leaves 8-9 cm long and 15-16 cm wide, rounded or rounded-heart-

shaped, at the base - deep-heart-shaped, their plate is cut into 3 parts. The flowers are blue and purple. Flowering period - June-July. Reproduction - generative, vegetative. Grows on neutral, moist, humus, mostly carbonate soils [219].

The plant's distribution area in Ukraine is the territory of the Carpathians within the Ivano-Frankivsk and Zakarpattia regions (Borzhava massif, Marmaros massif, Gorgan and Chivchyn mountains) in Fig.2 [220].

Figure 2. Distribution of *Delphinium elatum* L. on the territory of Ukraine

Delphinium elatum L. is listed in the Red Data Book of Ukraine (III category - rare species). Natural populations suffer from continuous deforestation and cattle grazing, natural reproduction is weak.

Medicinal plant raw materials are herbs and seeds, which have long been used in ethnomedicine to treat toothache and headache, cystitis, conjunctivitis, pneumonia, pleurisy, and skin diseases. The plant has pronounced antiparasitic and hemostatic properties, anti-inflammatory, wound healing, immunosuppressive, analgesic, antitumor, cardiotonic, antihypertensive, vasodilator, and other actions [221,222].

There are no products based on *Delphinium elatum* L. on the Ukrainian market of medicines. Raw material stocks of medicinal plant raw materials are very limited and are not subject to commercial use [220].

An alternative promising source of biomass is the use of biotechnological methods for growing *Delphinium elatum* L. *in vitro*. The resulting biomass may contain the full range of biologically active compounds inherent in this plant [223].

The literature describes various methods of introduction into the culture of *in vitro Delphinium elatum* L. and variants of culture media, obtaining a culture of isolated roots, and micro-dilution of the plant [224-226].

The study aimed to introduce *Delphinium elatum* L. *in vitro* culture, obtain extracts of *Delphinium elatum* L. biomass, and study the content of phenolic compounds, flavonoids, and antioxidant activity.

Delphinium elatum L. seeds were harvested on the slopes of Igrovets Mountain (Gorgany Massif, Ivano-Frankivsk Region) in July-August 2019-2020.

Performed pre-sowing treatment of *Delphinium elatum* L. seeds to improve their germination. The most optimal scheme of pre-sowing treatment and sterilization of seeds for planting in the Murashige-Skuga medium is the following: machining by damaging the seed coat, wetting with cold sterile water, and refrigeration for 48 hours - 70% ethyl alcohol (2–3 min) - 10% sodium hypochlorite solution (20 min) – sterilized bidistilled water (triple). Under such conditions, the yield of viable explants was 84%. Then the seeds were transferred to agar medium Murashige-Skuga (MS) and incubated at 16 hours photoperiod and a temperature of + 24 ± 1 ° C, with a light of 2000 lux and relative humidity of 70%. Determined the sterilization efficiency (percentage of aseptic seeds relative to the total number of original seeds) in each of the samples for 7 days and germination efficiency (percentage of seeds that formed viable after treatment with sterilizing agents sprouts).

The next step was the further cultivation of *Delphinium elatum* L., which was performed on Murashige-Skuga (MS) medium [227] with the addition of growth regulators. Explants to initiate callusogenesis were roots, meristematic apices and hypocotyl. The main factors influencing the induction of callusogenesis include the composition of the culture medium and cultivation conditions [223].

Kinetin, 6-benzylaminopurine, 2,4-dichlorophenoxyacetic acid, and naphthylacetic acid in various concentrations were used for callusogenesis. The carbon

source was glucose (30 g / l). Cultivation was performed for 35 days. The explants were cultured under the following conditions: photoperiod 16/8 h (light/dark), illumination 2000 lux, temperature 25 ° C (± 2° C), and relative humidity 70%. The frequency of callusogenesis was defined as the ratio of the number of explants with callus to their total number [227]. Variants of the nutrient medium of MS with different content of growth regulators are presented in table.1.

Table 1.

The content of growth regulators in the nutrient medium, mg / l

Growth regulators	MS environment					
	MS 1	MS 2	MS 3	MS 4	MS 5	MS 6
6-benzylaminopurine	1,0	10,0	5,0	10,0	10,0	10,0
naphthylacetic acid	0,2	–	0,6	–	1,0	–
2,4-dichlorophenoxyacetic acid	–	0,1	–	0,5	–	1,0

During cultivation, significant differences were observed in the intensity of growth processes, which depended on the variant of the nutrient medium. The lowest values of the growth index were characteristic of callus culture of *Delphinium elatum* L., which was cultured on nutrient medium MS 1 with the addition of 1.0 mg/l 6-benzylaminopurine and 0.2 mg/l naphthylacetic acid. The highest value of the growth index was observed on MS 6 medium with the addition of 10 mg/l 6-benzylaminopurine і 1,0 мг/л 2,4-dichlorophenoxyacetic acid.

The doubling time of *Delphinium elatum* L. biomass, which was cultivated on different variants of nutrient medium (MS 1 - MS 6) up to 35 days of cultivation, was studied. It was found that the optimal among the tested media is MS 6, which had the highest values of growth index and, accordingly, the lowest doubling time of callus biomass. We received callus biomass - a porous mass, relatively hard, with an uneven surface. Particles 0.5-1.3 cm in size, indeterminate, yellow, with a specific odor and sweet and sour, tart taste. The weight of callus biomass is 94 g per 1 liter of cultural medium.

Analysis of callus biomass was performed on the following indicators: total ash, ash insoluble in HCl and the content of extractives in accordance with the requirements of the State Pharmacopoeia of Ukraine [228].

Determination of total ash was performed according to the requirements of Article 2.4.16 of the SFU: porcelain crucible was calcined for 30 minutes, after cooling in a desiccator weighed. A sample of 1.00 g was added to the cooled desiccator, dried in an oven for 1 h at 100-105 ℃, and then burned in a muffle furnace at about 600 ℃ to constant weight, cooling the crucible in the desiccator before each weighing [229]. The total ash content was 9.12%.

Determination of insoluble hydrochloric acid ash was carried out in accordance with the requirements of Article 2.8.1 of the SFU: to the residue, after the determination of total ash was added 15 ml of water and 10 ml of the hydrochloric acid solution, the mixture was boiled in a water bath for 10 min, then cooled.

The contents of the crucible were filtered using ashless filter paper, after which the filter residue was washed with hot water to achieve a neutral reaction of the filtrate. The filter was placed in a porcelain crucible, dried, burned and the mass was determined after pre-cooling [230]. The content of insoluble hydrochloric acid ash was 0.47%.

The results of experimental studies show that callus biomass grown on agar medium, the main indicators meet the requirements for medicinal plant raw materials as a source of biologically active compounds and can be used as an alternative source. The extracts of callus biomass *Delphinium elatum* L. were obtained by maceration. Aqueous ethanol solutions in concentrations of 20% (D1 extract), 40% (D2 extract), 70% (D3 extract) and 90% (D4 extract) were used as extractants. The ratio of raw material and extractant was 1:20 [229].

The content of biologically active compounds (phenolic compounds and flavonoids) in the extracts was determined.

Determination of total phenolic content. The determination was performed using a spectrophotometric analysis using a modified Folin-Ciocalteu method. 1 ml of Folin reagent, 20 ml of distilled water and 3 ml of 20% Na2CO3 solution were added to 1

ml of the analyzed solution, diluted in a ratio of 1:20. The prepared mixture was shaken for 10 min, then kept in a water bath at 40 ℃ for 20 min. The solution was cooled and the optical density of the resulting solution was measured at 760 nm. The conversion was performed per gallic acid according to a calibration curve that was constructed under similar conditions, replacing the analyte with the gallic acid solution used as standard. A 3-fold measurement was performed for data validity [231].

The amount of flavonoids was determined by a modified spectrophotometric method by the complexation reaction of flavonoids with AlCl3. For this purpose, a 5% solution of NaNO2, a 0.1M solution of sodium hydroxide NaOH and a 10% solution of AlCl3 were prepared. 0.2 ml of the obtained extract was taken into a test tube and dissolved in 0.8 ml of ethyl alcohol. 0.06 ml of 5% sodium nitrite solution was added and mixed together. After that, the tube was kept for 5 min in a dark place. 0.06 ml of a 10% solution of aluminum chloride was added and kept for 5 min in the dark place until the reaction was complete. Then 0.4 ml of 0.1 M sodium hydroxide solution and 0.275 ml of ethyl alcohol of appropriate concentration were added. The measurements were performed at a wavelength of 510 nm. For calibration, a standard curve was constructed using the solution of quercetin as standard, and the content of flavonoids was determined in terms of quercetin. A 3-fold measurement was performed for the accuracy of the data [232].

The total content of phenolic compounds in the investigated extracts was determined, the result is expressed in mg of gallic acid per g of plant material.

The total content of flavonoids was determined, the result is expressed in mg of quercetin per g of plant material. The results are presented in Table 2.

Table 2.

Total phenolic and flavonoid content extracts of callus biomass *Delphinium elatum* L.

	Sample			
	D1	D2	D3	D4
Total phenolic content (mg gallic acid/g) $\bar{x}\pm\Delta\bar{x}, n = 3$	1,3513	2,1049	1,6517	1,4025
Total flavonoid content (mg quercetin/g) $\bar{x}\pm\Delta\bar{x}, n = 3$	1,6981	2,6230	2,1404	1,5913

The obtained results show that the maximum content of phenolic compounds and flavonoids was observed in 40% of aqueous-ethanolic extracts of callus biomass *Delphinium elatum* L.

There were used DPPH radical cation assays for the sake of appreciation of free radical scavenging properties of callus biomass *Delphinium elatum* L. extracts.

DPPH radical scavenging effect. The DPPH method of measuring the antioxidant effect of the extract was used with some modifications. Freshly prepared solution of DPPH was about 0.1 mM (0.2 g DPPH in 500 mL of ethanol). 4.5 mL of solution of DPPH and 500 μL of the extract were mixed in a test tube, which was incubated for 30 minutes in the dark at room temperature. A UV-VIS spectrophotometer was used for measuring the decrease in absorbance (at 517 nm). The following formula was used for calculating percentage of inhibition of the radicals:

$$\%_{\text{inhibition}} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100\%$$

where Acontrol is the absorbance of DPPH solution without extract and Asample is the absorbance of the sample with the added DPPH solution. A 3-fold measurement was performed for the accuracy of the data [232].

For the evaluation of antioxidant activity of single compounds has been widely used relatively stable organic radical DPPH as well as the different callus biomass *Delphinium elatum* L. extracts. A rapid decrease in the optical density at 517 nm was induced by the addition of extracts to the DPPH solution. The effect callus biomass

Delphinium elatum L. extracts of different concentrations in comparison with quercetin and vitamin C on the inhibition of DPPH radical is shown in Table 3.

Table 3.

DPPH radical scavenging activity of callus biomass *Delphinium elatum* L.

Sample	% inhibition of DPPH, $\bar{x}\pm\Delta\bar{x}, n = 3$
D1	40,7491
D2	*79,1724*
D3	70,0469
D4	61,0866
Vitamin C	78.5060
Quercetin	89.2298

The study of radical-absorbing activity of extracts of callus biomass of *Delphinium elatum* L. showed that 40% of water-ethanol extracts have the maximum activity. Our investigation shows that free radical scavenging ability of D2 extracts was better than vitamin C.

The research done into the chemical composition of callus biomass of *Delphinium elatum* L. ethanol extracts discovered the quantitative content (strength) of phenol compounds and flavonoids as well as examined their antioxidant effects. Sufficient content of phenol compounds and flavonoids as well as revealed antioxidant effects of the extracts under study allow to consider *Delphinium elatum* L. a promising medical herb for developing herbal preparations and to conduct further studies of the plant.