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Phytochemical Profiling and Biological Activity Screening of *Anemone nemorosa* L. Extracts with Anti-Inflammatory and Hepatoprotective Effects

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ABSTRACT: This study represents a preliminary phytochemical and pharmacological screening of *Anemone nemorosa* L. collected in Ukraine. The aim was to characterize the phytochemical composition of the herb, rhizomes, and extracts obtained using solvents of different polarity, and to perform an initial evaluation of their toxicity, anti-inflammatory, hemostatic, and hepatoprotective activities. UPLC–MS/MS analysis identified 12 phenolic compounds, including flavonoids and hydroxycinnamic acids, with the highest level observed in AN-40 extract (obtained using 40% ethanol). Their content varies depending on the plant organ, location, and year of collection. For the first time, amino acid profiling identified 14 amino acids, complementing the phytochemical characterization of *A. nemorosa* herb and its extracts. The composition of phenolic compounds and amino acids depended on the extraction solvent. The highest total phenolic content was observed in AN-40 extract, whereas the highest flavonoid content was obtained with 70% ethanol. Toxicological evaluation demonstrated that *A. nemorosa* extracts may be classified as low-toxic substances (toxicity class IV), with no mortality observed at a dose of 5000 mg/kg. Preliminary pharmacological screening using single-dose evaluations in established experimental models indicated anti-inflammatory and hepatoprotective effects of *A. nemorosa* extracts. The extracts exhibited only weak hemostatic activity. Among the investigated extracts, AN-40 showed the most pronounced anti-inflammatory and hepatoprotective activities under the experimental conditions used. These preliminary findings warrant further dose–response, mechanistic, and bioactivity-guided studies.

KEYWORDS: *Anemone nemorosa*; wood anemone; herb; extract; phenolic substances; hepatoprotective activity

1 Introduction

The genus *Anemone* L. is one of the largest genera in the *Ranunculaceae* Juss. family, comprising up to 200 species according to various authors [1,2]. The geographical distribution of the genus *Anemone* encompasses North and South America, the Mediterranean region, Eastern Europe, and North Africa and Asia [1]. In the flora of Ukraine, five species have been identified: *A. nemorosa* L., *A. ranunculoides* L., *A. sylvestris* L., *A. narcissiflora* L., and *A. laxa* (Ulbr.) Juz. [3]. All of these species belong to the spring flora and, from

an ecological perspective, are predominantly light-demanding mesophytes (*A. nemorosa*, *A. ranunculoides*), whereas only *A. sylvestris* is classified as a xerophyte.

A. nemorosa is widely distributed, and its raw material is readily available; however, it remains insufficiently studied and underutilised in scientific medicine, highlighting the need for further research in this field. The aerial parts of *A. nemorosa* have been reported to contain alkaloids, glycosides (protoanemonin, anemonin, ranunculin), saponins, tannins, ascorbic acid, resins, organic acids (including chelidonic acid), coumarins, and flavonoids [4–6]. However, representatives of the Ukrainian flora remain poorly studied.

Species of the genus *Anemone* have long been used in traditional medicine as anti-spasmodics, sedatives, anti-inflammatories, antibacterials, antifungals, and expectorant agents, among others [4,5]. The presence of triterpenoids and saponins contributes to the anticancer potential of *A. nemorosa*, particularly through cell cycle arrest and the induction of apoptosis [7]. In addition, antioxidant activity has been confirmed, which is attributed to the presence of caffeic acid, quercetin glycosides, isorhamnetin derivatives, and anemonin, highlighting the potential relevance of this species for applications in regenerative medicine [8]. The antimicrobial activity of *A. nemorosa* has also been investigated and is attributed to the presence of phenolic compounds [9]. Notably, no toxic effects of *A. nemorosa* extracts have been reported [10]. Currently, pharmaceutical products derived from raw materials of the genus *Anemone* are not available on the Ukrainian pharmaceutical market.

Despite its widespread distribution and traditional use, *A. nemorosa* remains insufficiently characterized from both phytochemical and pharmacological perspectives. In particular, information regarding the relationship between its chemical composition and biological activities is limited. Therefore, the present study was designed as a preliminary phytochemical and pharmacological screening of *A. nemorosa* extracts from the raw material collected in Ukraine. The study included characterization of phenolic compounds and amino acids in extracts obtained using solvents of different polarity. Amino acid profiling was included as a complementary component of the phytochemical characterization because information on the amino acid composition of *A. nemorosa* raw materials and extracts remains limited. Although amino acids are not considered chemotaxonomic markers in the present study, their composition may be relevant to the overall physicochemical and biopharmaceutical properties of multicomponent plant extracts, potentially affecting interactions, solubility, and bioavailability of other constituents. Therefore, characterization of the amino acid profile may provide additional information on extract composition and support future investigations of interactions between primary and secondary metabolites. Their acute toxicity, as well as anti-inflammatory, hepatoprotective, and hemostatic activities, was subsequently evaluated. This integrated approach was intended to provide a comprehensive initial assessment of the species and to identify the most promising directions for future pharmacological investigations.

2 Methods

2.1 Plant Raw Material

The aerial parts of *Anemone nemorosa* were collected during the flowering period, whereas rhizomes were harvested in early spring at the onset of vegetation. Plant material was obtained from four locations in western Ukraine (Table 1) [3]. The herb was harvested by cutting the aerial parts several centimetres above ground level using scissors, whereas the rhizomes were carefully excavated, washed, and rinsed with water. All collected materials were air-dried under ambient conditions in a well-ventilated attic until constant weight was achieved. Each sample yielded approximately 100–150 g of dried plant material. Botanical identification was performed by Svitlana Danyliv, PhD (Biology), Associate Professor at the Department of Pharmaceutical Management, Drug Technology and Pharmacognosy, Ivano-Frankivsk National Medical

University (Ukraine), using the Plant Identifier of Ukraine as the taxonomic reference [3]. Voucher specimens and corresponding herbarium materials (Nos. 782–786) have been deposited in the departmental herbarium, where they are stored under dry, well-ventilated, and light-protected conditions [11].

Table 1: Collection data of *Anemone nemorosa* plant materials used in the study.

Plant Material	Year	Collection Site	Geographical Coordinates	Voucher Specimen No.
Herb	2024	Outskirts of Kliubivtsi village, Ivano-Frankivsk region, Ukraine	48°54′38.6″ N, 24°56′21.4″ E	782
Herb	2023	Mochary tract, Ivano-Frankivsk region, Ukraine	48°50′40.2″ N, 24°34′45.1″ E	783
Herb	2024	Mochary tract, Ivano-Frankivsk region, Ukraine	48°50′40.2″ N, 24°34′45.1″ E	784
Herb	2024	Outskirts of Slyvky village, Ivano-Frankivsk region, Ukraine	48°46′58.6″ N, 24°12′47.9″ E	785
Herb	2024	Outskirts of Ozeriany village, Ternopil region, Ukraine	48°53′36.8″ N, 25°56′19.0″ E	786
Rhizomes	2024	Outskirts of Slyvky village, Ivano-Frankivsk region, Ukraine	48°46′58.6″ N, 24°12′47.9″ E	785

2.2 Extract Preparation

For phytochemical profiling by UPLC–MS/MS, each individual *A. nemorosa* sample was extracted separately with 70% ethanol using a raw material-to-solvent ratio of 1:20 (w/v). The resulting extracts were filtered prior to chromatographic analysis.

Following the phytochemical characterization of the individual samples, all herb materials collected from the different locations were pooled and thoroughly homogenized to obtain a representative composite sample. This composite material was subsequently used for extract preparation intended for the preliminary pharmacological investigations. The use of a pooled sample was intended to minimize the influence of local environmental variability while providing sufficient plant material for all biological assays.

For preparation of the aqueous extract (AN-0), 100 g of dried herb was extracted with 1000 mL of purified water. The suspension was heated to 100°C for 15 min and then left to macerate overnight at room temperature. The extract was subsequently filtered.

To obtain the hydroethanolic extracts, 100 g of the dried herb was extracted separately with 1000 mL of either 40% or 70% ethanol (extracts AN-40 and AN-70, respectively). Maceration was carried out for 24 h at ambient temperature. The extraction procedure was repeated twice using fresh portions of the corresponding solvent, after which the filtrates from each extraction cycle were combined.

Each extract was concentrated individually under reduced pressure using a Büchi B-300 rotary evaporator (Büchi AG, Flawil, Switzerland) operated at 150 mbar, a rotation speed of 50 rpm, and a water-bath temperature of 85°C until viscous concentrates were obtained. The concentrates were subsequently freeze-dried with a SCANVAC COOLSAFE 55-4 Pro lyophilizer (LaboGene ApS, Lillerød, Denmark).

2.3 Assay of Polyphenolic Compounds by UPLC–MS/MS

The polyphenolic composition of *A. nemorosa* was determined using an Acquity H-Class UPLC system (Waters, Milford, MA, USA) coupled to a Xevo TQD triple-quadrupole mass spectrometer, following a previously validated analytical procedure [12,13]. Chromatographic separation was carried out on a YMC Triart C18 column (100 × 2.0 mm, 1.9 µm) thermostated at 40°C. The mobile phase consisted of 0.1% formic acid in water (phase A) and LC–MS-grade acetonitrile (phase B), delivered at a constant flow rate of 0.5 mL/min. The elution profile, expressed as the percentage of mobile phase B, was as follows: 0–1 min, 5% B; 1–5 min, 5–30% B; 5–7 min, 30–50% B; 7–7.5 min, 50–100% B; 7.5–8 min, 100% B; and 8–10 min, re-equilibration to the initial conditions (5% B). Mass spectra were acquired in negative electrospray ionization (ESI) mode. The capillary voltage was maintained at –2 kV, while the cone voltage was set to 30 V. Nitrogen served as the desolvation gas at 400°C with a flow rate of 700 L/h, whereas the curtain gas flow was maintained at 20 L/h. The ion source temperature was 150°C. Target phenolic constituents were identified by comparing both chromatographic retention times and MS/MS fragmentation profiles with those of authentic reference standards analyzed under identical experimental conditions. Only compounds verified using authentic standards were included in the final results. The analytical method was designed specifically for targeted determination of phenolic compounds represented by available reference substances; consequently, other classes of secondary metabolites, including triterpenoid saponins, were not investigated. Quantification was based on external calibration using linear regression equations obtained from standard solutions [14].

The limits of detection (LOD) and quantification (LOQ) for polyphenolic compounds ranged from 0.8 to 5.1 ng/mL and from 2.8 to 17.5 ng/mL, respectively. Intra-day and inter-day precision (RSD%) ranged from 2.9% to 6.8% and from 3.6% to 7.6%, respectively. The analytical procedure was based on previously validated UPLC–MS/MS methods [14,15] and the corresponding validation characteristics have been reported in the cited studies.

During this study, analytical-grade standards were used. The purity of the polyphenolic standards ranged from 89% to 99%. 3,4-Dihydroxybenzoic acid was purchased from Sigma Aldrich (97%, BCBF4090V), neochlorogenic acid from Phytolab (98%, 150950), chlorogenic acid from AlfaAesar (F30X080, 95%), ferulic acid from Sigma Aldrich (99%, 102494616), caffeic acid from Extrasynthese (99%, 05-0718/0), rosmarinic acid CRS was from EDQM (Y0000786 batch 4, 89%), rutin from Phytolab (1000379, 99%), hyperoside from Rotichrom (99%, 7932.1), isoquercetin from HWI Analytic (94%, HWI03431), luteolin-7-O-rutinoside from Sigma Aldrich (SMB00200, 98%), kaempferol-3-O-glucoside from Extrasynthese (99%, 14091015), kaempferol-3-O-rutinoside (Phytolab, 98%, 316231), and amino acid standard solution of 2.5 mmol L^{–1} was purchased from Sigma Aldrich-Amino Acids Mix Solution (high purity quality) TraceCert, 79248.

2.4 Assay of Amino Acids by UPLC–MS/MS

The amino acid profile of *A. nemorosa* was analysed using an Acquity H-Class UPLC system (Waters, Milford, MA, USA) interfaced with a Xevo TQD triple-quadrupole mass spectrometer (Waters, Milford, MA, USA), following a previously validated analytical procedure [16]. Chromatographic separation was performed on a BEH Amide column (150 × 2.1 mm, 1.7 µm; Waters) maintained at 25°C. Each analysis was carried out with an injection volume of 1 µL. The chromatographic system employed a binary mobile phase consisting of 10 mmol ammonium formate supplemented with 0.125% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). Elution was performed at a flow rate of 0.6 mL/min using the following gradient: 95% B during the first minute, decreasing to 70% B between 1.0 and 3.9 min, then to 30% B from 3.9 to 5.1 min. The column was subsequently washed with 70% A until 6.4 min and re-equilibrated to the initial chromatographic conditions from 6.5 min onward. The total run time for each analysis was 10

min. Mass spectrometric detection was carried out in positive electrospray ionisation (ESI+) mode. The capillary voltage was set to +3.5 kV and the cone voltage to 30 V. Nitrogen was used as the desolvation gas at 400°C with a flow rate of 800 L/h, while the source temperature was maintained at 120°C. Individual amino acids were assigned by comparison of chromatographic retention times and MS/MS fragmentation spectra with those obtained from authentic analytical standards. Quantitative determination was based on external calibration using linear regression equations generated from standard solutions [17]. The linearity range for amino acids was 0.8–4.4 µg/mL. The LOD and LOQ ranged from 80 to 240 ng/mL and from 300 to 790 ng/mL, respectively, while recovery ranged from 81% to 116%. Intra-day and inter-day precision ranged from 4.5% to 8.5% and from 5.5% to 21.7%, respectively. The analytical procedure was based on previously validated UPLC–MS/MS methods [16], and the corresponding validation characteristics have been reported in the cited studies.

2.5 Toxicity and Pharmacological Activity Studies

Toxicological and pharmacological experiments were performed using laboratory animals maintained at the Clinical and Biological Experimental Base of Ivano-Frankivsk National Medical University (IFNMU). Prior to the experiments, the animals were standardized according to physiological and biochemical characteristics and housed under controlled environmental conditions with free access to a standard laboratory diet. All *in vivo* procedures complied with the national General Ethical Principles for Animal Experiments (Ukraine, 2001) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) [18–20]. The experimental protocol was reviewed and approved by the Ethics Committee of IFNMU (Protocol No. 153/25, 17 September 2025). Biochemical and hematological analyses were carried out at the Bioelementology Centre of IFNMU (Certificate of Technical Competence No. 191/24, issued 05 July 2024; valid until 04 July 2029). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using the standardized Reitman–Frankel dinitrophenylhydrazine method with commercial diagnostic reagent kits (Filisit-Diagnostics, Ukraine). Alkaline phosphatase (ALP) activity was measured using standardized commercial kits supplied by the same manufacturer. Hematological parameters were determined with an automated 5-DIFF hematology analyzer (Swelab Lumi), whereas the erythrocyte sedimentation rate (ESR) was assessed according to the Panchenkov method.

2.6 Acute Toxicity Study

Acute oral toxicity was evaluated in adult non-linear white rats of both sexes according to the methodological approach described by Stefanov [21]. The animals were randomized into four groups (n = 6 per group). Groups I–III received the AN-0, AN-40, and AN-70 extracts, respectively, as a single intragastric administration of 5000 mg/kg body weight, whereas group IV consisted of untreated intact animals. The selected dose corresponds to the upper limit specified for toxicity class IV (low-toxicity substances) in the methodological recommendations of the State Expert Centre of the Ministry of Health of Ukraine. According to these recommendations, further dose escalation is not required when administration of 5000 mg/kg does not result in mortality. Following treatment, the animals were observed daily for 14 consecutive days. Mortality, overall clinical status, and manifestations of toxicity were recorded throughout the observation period. The acute toxicity category of the investigated extracts was subsequently assigned according to the classification system described by Stefanov [21].

2.7 Anti-Inflammatory Activity Study

Anti-inflammatory effects of the *A. nemorosa* extracts were assessed using a formalin-induced paw oedema model of acute aseptic inflammation [21]. Adult non-linear white rats weighing 130–240 g were randomized into six groups (n = 6 per group). Groups I–III were assigned to treatment with the AN-0, AN-40, and AN-70 extracts, respectively. Diclofenac sodium (Darnitsa Pharmaceutical Company, batch No. SK10224) and quercetin (Borshchahivskiy Chemical Pharmaceutical Plant, batch No. 1861221) served as reference treatments in groups IV and V, respectively, while group VI was maintained as the untreated control. Acute inflammation was initiated by injecting 0.1 mL of 2% formalin into the plantar aponeurosis of the hind paw. The AN-0, AN-40, and AN-70 extracts were administered intragastrically at 100 mg/kg body weight 2 h before induction of inflammation and once again immediately after formalin injection. The reference treatments were administered according to the same schedule at doses of 8 mg/kg for diclofenac sodium and 5 mg/kg for quercetin, whereas control animals received drinking water. The 100 mg/kg dose was chosen for screening on the basis of previous pharmacological studies of medicinal plant extracts, including earlier investigations of *A. nemorosa* [14,22]. This single-dose design was intended to enable an initial comparison among the extracts and to identify candidates for subsequent dose–response studies rather than to determine pharmacological potency or an optimal therapeutic dose. Paw volume was measured plethysmometrically before formalin administration and at 1, 3, and 5 h after induction of inflammation using a digital plethysmometer (Ugo Basile, Gemonio, Italy). Anti-inflammatory activity was evaluated from the reduction in formalin-induced paw oedema relative to the untreated control group.

2.8 Hepatoprotective Activity Study

The hepatoprotective effects of the *A. nemorosa* extracts were assessed in a rat model of acute liver injury induced by carbon tetrachloride (CCl₄), a hepatotoxic agent known to cause biochemical and morphological alterations resembling acute toxic hepatitis [14]. Adult non-linear white rats (130–240 g) were randomized into six groups (n = 6 per group). Animals in groups I–III were administered the AN-0, AN-40, or AN-70 extract intragastrically at 25 mg/kg body weight, respectively. Group IV received Silibor (Pharmaceutical Company “Zdorovye”, batch No. 10924), used as the reference hepatoprotective preparation, at the same dose of 25 mg/kg. Each Silibor tablet contained a dry extract of *Silybum marianum* (drug-to-extract ratio 22–27:1; extraction solvent, 95% acetone), equivalent to 35 mg of silymarin. The 25 mg/kg dose was selected to match that of the reference preparation in this screening model [14], thereby enabling direct comparison of the investigated extracts with Silibor under identical experimental conditions. Acute hepatic injury was induced in groups I–V by two subcutaneous administrations of a 50% oily CCl₄ solution (0.8 mL per 0.1 kg body weight) at a 24-h interval. The AN-0, AN-40, and AN-70 extracts and Silibor were administered 1 h before and 2 h after each CCl₄ exposure. Group V represented the untreated pathological control. Group VI was maintained as the intact control and received purified water following the corresponding administration schedule. All animals were euthanized by decapitation on the third day after the initial CCl₄ administration. Hepatoprotective effects were assessed from animal survival and biochemical indices of hepatic injury. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities were determined in serum and liver homogenates as biochemical markers of liver injury.

2.9 Hemostatic Activity Study

Local hemostatic effects of the *A. nemorosa* extracts were assessed in adult guinea pigs according to the Duke bleeding-time procedure [23,24]. An experimental wound was created in the avascular area of the ear, approximately 5–7 mm from its edge, using a sterile 26 G (0.45 mm) needle. Bleeding time was

recorded with a stopwatch from puncture until fresh blood was no longer detectable on a gauze pad [25,26]. The animals were randomly distributed into five groups ($n = 7$ per group). Following ear puncture, groups I–III received topical application of sterile gauze pads saturated with freshly prepared 1% (w/v) solutions or suspensions of the AN-0, AN-40, and AN-70 extracts, respectively. In group IV, the wound was treated with a gauze pad containing the reference preparation, a liquid extract of *Persicaria hydropiper* (Fitofarm, Ukraine; batch No. 10222). Group V served as the control and received a gauze pad moistened with 0.9% sodium chloride solution. The resulting bleeding times were used to compare the local hemostatic effects of the investigated extracts with those of the control and reference preparation.

2.10 Statistical Analysis

Quantitative results are expressed as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of the mean (SEM), depending on the type of experiment. Phytochemical measurements were carried out in at least three independent determinations, while pharmacological experiments comprised no fewer than six biological replicates per experimental group. Confidence intervals were estimated using the corresponding critical values of Student's *t*-distribution. Statistical processing was performed using Statistica 10.0 (StatSoft Inc., Tulsa, OK, USA). Differences among experimental groups were assessed by one-way analysis of variance (ANOVA) with Tukey's *post hoc* multiple-comparison test. Differences were considered statistically significant at $p \leq 0.05$.

3 Results

3.1 Phytochemical Research

Dry extracts were prepared from the herb of *A. nemorosa*. The yields of extracts AN-0, AN-40 and AN-70 were $16.00 \pm 0.80\%$, $16.67 \pm 0.72\%$ and $14.46 \pm 0.70\%$, respectively.

Phenolic compounds and amino acids (AA) in the raw materials and extracts of *A. nemorosa* were analysed using a UPLC–MS/MS system (Table 2 and Figs. S1–S13).

A total of 12 phenolic compounds were identified in the herb of *A. nemorosa* collected from different locations and their extracts, whereas 10 compounds were detected in the rhizomes. The content of phenolic compounds in the aerial parts ranged from 262 to 481 $\mu\text{g/g}$, while in the underground parts it was 144 $\mu\text{g/g}$. The total content of phenolic compounds in the extracts ranged from 945 to 1581 $\mu\text{g/g}$. The highest content of phenolic compounds was observed in AN-40 extract (1581 $\mu\text{g/g}$). Flavonoid extraction was most efficient when using 70% ethanol (654 $\mu\text{g/g}$). A dependence of the quantitative content of phenolic compounds in the extracts on the type and concentration of the extraction solvent was established.

In all analysed samples, the content of chlorogenic acid in both the herb and rhizomes was found to be within comparable ranges (18–72 $\mu\text{g/g}$ and 31–59 $\mu\text{g/g}$, respectively), indicating similar levels of accumulation. In contrast, the contents of rosmarinic acid (7–23 $\mu\text{g/g}$ in the herb and 38–133 $\mu\text{g/g}$ in the rhizomes) and neochlorogenic acid (7–10 $\mu\text{g/g}$ in the herb and 11 $\mu\text{g/g}$ in the rhizomes) were notably higher in the underground parts. Conversely, ferulic and caffeic acids were present at higher concentrations in the herb of *A. nemorosa*. Caffeic acid was identified as the dominant phenolic acid in the herb (42–170 $\mu\text{g/g}$), whereas rosmarinic and chlorogenic acids predominated in the rhizomes. Among flavonoids, kaempferol-3-O-glucoside (astragalin) and isoquercetin were the predominant compounds in the herb (31–45 $\mu\text{g/g}$ and 19–37 $\mu\text{g/g}$, respectively), while rutin and hyperoside dominated in the rhizomes (7 $\mu\text{g/g}$ each). The total flavonoid content in the herb exceeded that in the rhizomes by 4–7-fold. The highest content of phenolic compounds was observed in the herb of *A. nemorosa* collected in the vicinity of the Mochary tract (Bohorodchany district, Ivano-Frankivsk region) in 2024 (481 $\mu\text{g/g}$), which is 1.84 times higher than that recorded for samples collected from the same location in 2023.

Table 2: Content of phenolic compounds and amino acids in the herb and rhizomes of *A. nemorosa* collected from different locations.

No.	Compound	Content, µg/g								
		Herb (Kli- ubivtsi, 2024)	Herb (Mochary, 2023)	Herb (Mochary, 2024)	Herb (Slyvky, 2024)	Herb (Ozeryany, 2024)	Rhizomes (Slyvky, 2024)	Dry Extracts of the Herb		
								AN-0	AN-40	AN-70
Phenolic acids										
1	3,4-Dihydroxybenzoic acid	25 ± 2	25 ± 2	27 ± 1	26 ± 2	21 ± 1	-	122 ± 7	124 ± 8	157 ± 9
Hydroxycinnamic acids										
2	Neochlorogenic acid	7 ± 1	7 ± 0	10 ± 1	8 ± 0	8 ± 0	11 ± 1	59 ± 10	42 ± 0	40 ± 3
3	Chlorogenic acid	59 ± 5	20 ± 1	72 ± 2	18 ± 2	31 ± 1	59 ± 2	142 ± 1	180 ± 6	189 ± 5
4	Ferulic acid	22 ± 2	25 ± 2	44 ± 3	30 ± 1	54 ± 6	-	16 ± 0	92 ± 8	72 ± 7
5	Caffeic acid	42 ± 4	59 ± 3	155 ± 7	170 ± 13	167 ± 4	13 ± 1	266 ± 23	543 ± 27	318 ± 14
6	Rosmarinic acid	8 ± 1	8 ± 0	23 ± 1	7 ± 1	8 ± 0	38 ± 2	43 ± 1	36 ± 3	36 ± 2
Flavonoids										
7	Rutin	11 ± 1	11 ± 1	14 ± 1	16 ± 1	13 ± 1	7 ± 1	43 ± 3	48 ± 4	57 ± 4
8	Hyperoside	8 ± 1	6 ± 0	9 ± 0	9 ± 1	9 ± 0	7 ± 1	43 ± 3	39 ± 3	46 ± 4
9	Isoquercetin	30 ± 3	19 ± 1	37 ± 3	23 ± 1	30 ± 2	3 ± 0	57 ± 6	149 ± 1	186 ± 13
10	Luteolin-7-O-rutinoside	8 ± 1	27 ± 1	22 ± 1	20 ± 0	18 ± 1	4 ± 0	48 ± 4	74 ± 2	102 ± 7
11	Kaempferol-3-O-glucoside	40 ± 4	31 ± 2	45 ± 1	32 ± 0	39 ± 1	1 ± 0	67 ± 5	188 ± 9	223 ± 15
12	Kaempferol-3-O-rutinoside	6 ± 0	24 ± 3	22 ± 2	19 ± 3	16 ± 2	-	39 ± 6	66 ± 7	40 ± 14
Essential amino acids										
13	Histidine	4 ± 0	6 ± 0	2 ± 0	7 ± 0	7 ± 0	4 ± 0	272 ± 17	68 ± 1	52 ± 3
14	Isoleucine	20 ± 0	35 ± 1	20 ± 0	29 ± 1	27 ± 0	1 ± 0	52 ± 5	114 ± 11	131 ± 6
15	Leucine	8 ± 0	21 ± 0	17 ± 1	18 ± 1	23 ± 1	5 ± 1	37 ± 1	71 ± 4	82 ± 2
16	Lysine	5 ± 0	6 ± 0	5 ± 0	7 ± 0	11 ± 0	1 ± 0	194 ± 16	50 ± 3	33 ± 2
17	Phenylalanine	21 ± 1	40 ± 3	19 ± 0	22 ± 0	23 ± 0	2 ± 0	117 ± 13	144 ± 7	178 ± 12
18	Threonine	25 ± 0	41 ± 1	21 ± 2	18 ± 1	38 ± 4	1 ± 0	299 ± 16	180 ± 7	240 ± 25
19	Valine	21 ± 1	30 ± 2	18 ± 1	25 ± 1	29 ± 1	1 ± 0	58 ± 7	140 ± 7	171 ± 15
Conditionally essential amino acids										
20	Tyrosine	1 ± 0	1 ± 0	2 ± 0	1 ± 3	2 ± 0	1 ± 0	16 ± 2	10 ± 2	8 ± 1
21	Arginine	11 ± 0	10 ± 1	27 ± 0	8 ± 1	25 ± 0	57 ± 6	749 ± 34	71 ± 4	57 ± 5

Table 2: *Cont.*

No.	Compound	Content, µg/g								
		Herb (Kli- ubivtsi, 2024)	Herb (Mochary, 2023)	Herb (Mochary, 2024)	Herb (Slyvky, 2024)	Herb (Ozeryany, 2024)	Rhizomes (Slyvky, 2024)	Dry Extracts of the Herb		
								AN-0	AN-40	AN-70
22	Proline	56 ± 2	67 ± 4	32 ± 1	46 ± 3	26 ± 2	19 ± 1	10 ± 1	307 ± 5	374 ± 25
23	Serine	20 ± 1	22 ± 0	15 ± 1	18 ± 2	21 ± 1	2 ± 0	64 ± 10	122 ± 8	117 ± 6
24	Aspartic acid	10 ± 1	36 ± 2	15 ± 2	10 ± 1	21 ± 2	10 ± 1	737 ± 42	88 ± 3	125 ± 6
25	Glutamic acid	19 ± 1	20 ± 1	20 ± 0	20 ± 1	30 ± 2	9 ± 1	1160 ± 161	133 ± 8	102 ± 3
Non-essential amino acids										
26	Alanine	14 ± 1	10 ± 1	31 ± 2	20 ± 2	37 ± 4	7 ± 0	881 ± 28	70 ± 3	75 ± 9

Note: Dry extracts of *A. nemorosa* herb: AN-0, obtained using purified water; AN-40, obtained using 40% aqueous ethanol; AN-70, obtained using 70% aqueous ethanol; “-” — not detected.

A total of 14 AAs were identified, including 7 essential, 6 conditionally essential, and 1 non-essential. The total AA content in the herb of *A. nemorosa* ranged from 235 to 345 µg/g, including 42.03–52.86% essential and 34.72–50.08% of conditionally essential AAs. In the rhizomes, the total AA content was 112 µg/g, of which 8.59% essential ones and 81.66–85.52% corresponded to non-essential AAs. In the herb samples of *A. nemorosa*, essential and conditionally essential AAs predominated, depending on the location of raw material collection. The dominant AAs in the herb were proline (26–67 µg/g), threonine (18–41 µg/g), isoleucine (19–35 µg/g), phenylalanine (19–40 µg/g), and valine (18–30 µg/g). In the rhizomes of *A. nemorosa*, conditionally essential AAs predominated, particularly arginine (57 µg/g), proline (19 ± 1 µg/g), aspartic acid (10 µg/g), and glutamic acid (9 µg/g). The AA content in the herb collected from the Mochary tract (Bohorodchany district, Ivano-Frankivsk region) in 2023 was 1.47-fold higher than that in the herb collected near Kliubivtsi village (Tysmenytsia district, Ivano-Frankivsk region), which exhibited the lowest AA content.

3.2 Acute Toxicity of the Extracts

Acute toxicity studies of the extracts were conducted on non-linear white rats of both sexes following the method of Stefanov [21]. The studied extracts were administered to the animals intragastrically at a dose of 5000 mg/kg. The animals were observed for 14 days. The toxicity class was determined according to the generally accepted classification [21]. Immediately after administration of the extracts and throughout the experimental period, the animals' general condition remained normal. The animals were clean and active, exhibited appropriate responses to auditory and light stimuli, and maintained normal physiological functions, including urination and defecation. No signs of respiratory distress or convulsions were observed. Reflex excitability remained within physiological limits. Food and water intake were also within normal ranges. Previous studies have also shown that oral administration of alcoholic extracts of *A. nemorosa* herb at doses of 5000 mg/kg and higher does not result in signs of toxicity or mortality in experimental animals [22]. Therefore, the median lethal dose (LD50) of the extracts exceeds 5000 mg/kg. According to Sidorov's classification, the investigated extracts may be classified as low-toxic substances (toxicity class IV).

3.3 Anti-Inflammatory Activity

The anti-inflammatory activity of *A. nemorosa* extracts was evaluated using a model of aseptic formalin-induced inflammation [21,27]. The effects of the studied extracts were evaluated based on their ability to inhibit the development of formalin-induced paw oedema in rats compared with the control group. The results of the conducted studies (Tables 3 and 4) indicate that the investigated extracts exhibit anti-inflammatory activity.

The development of the inflammatory response in the control group was observed as early as 1 h after administration of the phlogogenic agent, with the maximum response recorded at 3 h. The studied extracts exhibited anti-exudative activity throughout the entire experimental period. The most significant inhibition of edema was observed at 5 h after administration of the 2% formalin solution. At 5 h, the anti-exudative effects of the AN-0 (51.22%), AN-40 (43.66%), and AN-70 (39.58%) extracts were numerically greater than those observed for diclofenac sodium (29.62%) and quercetin (38.37%) under the same experimental conditions. These findings suggest that the observed anti-inflammatory effects may be related to the presence of phenolic compounds, which have previously been reported to modulate inflammatory pathways, including prostaglandin-mediated responses [14]. However, no direct mechanistic investigations were performed in the present study. At 1 and 3 h, the highest anti-exudative activity was demonstrated by AN-40 extract, reaching 46.58% and 37.49%, respectively. The activity of AN-0 extract was 38.09% and 31.46%, while that of AN-70 extract was 28.33% and 18.63%, respectively. The maximum effect of the extracts was observed at 5 h, with anti-exudative activity

reaching 51.22% ($p < 0.05$) for AN-0, 43.66% ($p < 0.05$) for AN-40, and 39.58% ($p < 0.05$) for AN-70. The reference drugs, diclofenac sodium and quercetin, also exhibited anti-exudative activity throughout the experiment. Quercetin inhibited the inflammatory response by 38.25% ($p < 0.05$) at 1 h, 34.54% ($p < 0.05$) at 3 h, and 38.37% ($p < 0.05$) at 5 h. The anti-inflammatory activity of the reference compound Diclofenac Sodium was 40.11% ($p < 0.05$) at 1 h, 18.38% ($p < 0.05$) at 3 h, and 29.62% ($p < 0.05$) at 5 h.

Table 3: Effect of *A. nemorosa* extracts on paw volume in the model of aseptic formalin-induced inflammation.

Animal Group	Investigated Substance	Dose, mg/kg	Increase in Paw Volume, %, $\bar{x} \pm \Delta\bar{x}$, n = 6		
			1 h	3 h	5 h
I	AN-0	100	21.2 $\pm$ 3.57 [#]	33.3 $\pm$ 4.39	19.74 $\pm$ 3.6 [#]
II	AN-40	100	18.3 $\pm$ 3.11 [#]	30.3 $\pm$ 3.1	22.8 $\pm$ 3.28
III	AN-70	100	24.5 $\pm$ 2.52	39.5 $\pm$ 2.88	24.4 $\pm$ 2.68
IV	Diclofenac sodium	8	21.2 $\pm$ 2.75 [#]	31.8 $\pm$ 4.02	24.9 $\pm$ 4.39
V	Quercetin	5	20.5 $\pm$ 3.57 [#]	39.6 $\pm$ 4.19	28.4 $\pm$ 3.82
VI	Intact animal group	-	34.3 $\pm$ 4.48	48.6 $\pm$ 7.7	40.4 $\pm$ 7.43

Note: [#]—statistically significant difference compared to the intact animal group ($p \leq 0.05$).

Table 4: Anti-exudative activity of *A. nemorosa* extracts and reference drugs.

Time	AN-0	AN-40	AN-70	Diclofenac Sodium	Quercetin
1 h	38.09 $\pm$ 10.40	46.58 $\pm$ 9.07	28.33 $\pm$ 7.34	40.11 $\pm$ 10.41	38.25 $\pm$ 8.02
3 h	31.47 $\pm$ 9.02	37.50 $\pm$ 6.38	18.64 $\pm$ 5.93	18.38 $\pm$ 8.61	34.54 $\pm$ 8.26
5 h	51.22 $\pm$ 8.89	43.66 $\pm$ 8.11	39.58 $\pm$ 6.62	29.62 $\pm$ 9.45	38.37 $\pm$ 10.85

Note: Data are presented as mean $\pm$ SEM (n = 6). Comparisons were made with the intact animal group.

3.4 Hepatoprotective Activity

The hepatoprotective activity of the extracts was evaluated using a screening model of acute carbon tetrachloride-induced hepatitis. The toxic agent, carbon tetrachloride, induces morphological and biochemical changes in the liver of experimental animals that are comparable to those observed in acute toxic hepatitis in humans [21,27]. To assess the degree of hepatoprotective effect during pharmacological screening, the activities of ALT, AST, and ALP were determined in blood serum and liver homogenate, as these enzymes are established markers of liver function. One criterion for evaluating the hepatoprotective activity of the studied extracts was the survival rate of the animals. During the experiment, mortality was observed in the control group and in animals treated with extracts AN-0 (16.7%) and AN-70 (33.3%). The corresponding survival rates were 83.3% in the control group, 83.3% in the AN-0 group, 66.7% in the AN-70 group, and 100% in both the AN-40 and Silibor-treated groups. Due to the small group size, these data should be interpreted descriptively and primarily as supportive indicators of hepatoprotective potential. In the remaining experimental groups, all animals survived until the end of the study, indicating the feasibility of further investigation of the hepatoprotective potential of the studied substances. The results of biochemical analyses of blood serum and liver homogenate, as well as hematological parameters, are presented in Table 5.

Table 5: Effect of *A. nemorosa* extracts on biochemical parameters in blood serum and liver homogenate in acute hepatitis.

Biochemical and Hematological Parameters	Objects					
	AN-0	AN-40	AN-70	Silibor (Reference Drug)	50% CCl ₄ Oil Solution	Intact Animals (Untreated Control)
Dose, mg/0.1 kg	2.5	2.5	2.5	2.5	0.8 mL	2.5
Blood serum						
ALT, $\mu\text{mol/h}\cdot\text{mL}$	$3.6 \pm 0.44^{\#/\ddagger}$	$1.51 \pm 0.12^{\#/\ddagger/\dagger}$	$3.66 \pm 0.48^{\#/\ddagger}$	$2.32 \pm 0.45^{\dagger}$	$3.39 \pm 0.47^{\#}$	2.47 ± 0.35
AST, $\mu\text{mol/h}\cdot\text{mL}$	$1.89 \pm 0.33^{\#/\ddagger/\dagger}$	$1.56 \pm 0.40^{\dagger/\ddagger}$	$3.83 \pm 0.49^{\#}$	$4.19 \pm 0.44^{\#}$	$4.29 \pm 0.51^{\#}$	1.21 ± 0.19
ALP, $\text{nmol/s}\cdot\text{L}$	$12,084.60 \pm 1415.16^{\#/\ddagger/\dagger}$	$6164.17 \pm 805.65^{\#/\ddagger/\dagger}$	$7946.5 \pm 731.73^{\#/\ddagger/\dagger}$	$8402.33 \pm 906.95^{\#/\ddagger/\dagger}$	$10,071.00 \pm 1152.23^{\#}$	3554.67 ± 587.81
Liver homogenate						
ALT, $\mu\text{mol/h}\cdot\text{mL}$	5.83 ± 0.59	5.77 ± 0.56	$4.48 \pm 0.32^{\#/\ddagger/\dagger}$	$5.44 \pm 0.79^{\dagger}$	$6.35 \pm 0.3^{\#}$	5.76 ± 0.34
AST, $\mu\text{mol/h}\cdot\text{mL}$	$3.67 \pm 0.73^{\#/\ddagger/\dagger}$	$4.18 \pm 0.75^{\#/\ddagger/\dagger}$	$3.49 \pm 0.50^{\#/\ddagger/\dagger}$	$3.47 \pm 0.58^{\#/\ddagger/\dagger}$	$5.30 \pm 0.97^{\#}$	1.64 ± 0.25
ALP, $\text{nmol/s}\cdot\text{L}$	$432.60 \pm 58.94^{\#/\ddagger/\dagger}$	$1794.83 \pm 268.10^{\#/\ddagger/\dagger}$	$1088.00 \pm 112.06^{\#/\ddagger/\dagger}$	$754.83 \pm 104.88^{\#/\ddagger/\dagger}$	520.4 ± 60.95	570.00 ± 122.88
Whole blood						
Leukocytes, $\times 10^3/\mu\text{L}$	$9.48 \pm 1.03^{\#/\ddagger/\dagger}$	$10.68 \pm 1.34^{\#/\ddagger/\dagger}$	$9.3 \pm 0.92^{\#/\ddagger/\dagger}$	$7.32 \pm 0.68^{\#/\ddagger/\dagger}$	$15.18 \pm 1.28^{\#}$	6.05 ± 0.53
Erythrocytes, $\times 10^6/\mu\text{L}$	$6.77 \pm 0.59^{\#/\ddagger/\dagger}$	$6.46 \pm 0.36^{\dagger}$	$6.49 \pm 0.55^{\dagger}$	$6.37 \pm 0.5^{\dagger}$	$7.28 \pm 0.79^{\#/\ddagger/\dagger}$	6.9 ± 0.64
Platelets, $\times 10^3/\mu\text{L}$	292.4 ± 55.43	$345.5 \pm 31.8^{\dagger}$	$399.25 \pm 56.38^{\#/\ddagger/\dagger}$	$313.17 \pm 22.14^{\dagger}$	258.2 ± 41.56	312.17 ± 70.82

Notes: $\#$ —statistically significant difference compared to the intact animal group ($p \leq 0.05$); $\dagger$ —statistically significant difference compared to the control group ($p \leq 0.05$); $\ddagger$ —statistically significant difference compared to the reference drug group ($p \leq 0.05$).

3.5 Hemostatic Activity

The hemostatic properties of the extracts (Table 6) were evaluated by measuring bleeding time using the Duke method in a model of ear bleeding in experimental animals [25]. Bleeding was induced by a rapid puncture in the avascular zone of the ear, 5–7 mm from the edge, using a 26 G injection needle (0.45 mm). The endpoint of bleeding was determined by the absence of fresh blood on a gauze pad. Bleeding time was measured using a stopwatch [25,26].

Table 6: Effect of *A. nemorosa* extracts on bleeding time.

Animal Group	Test Substance	Bleeding Time, s, $\bar{x} \pm \Delta\bar{x}$, n = 7	Reduction in Bleeding Time Relative to the Control Group, %
I	AN-0	53.43 $\pm$ 2.37 [#]	15.58
II	AN-40	55.29 $\pm$ 1.80 ^{#/†}	12.61
III	AN-70	58.14 $\pm$ 1.68 ^{#/†}	8.14
IV	Liquid extract of <i>Persicaria hydropiper</i>	50.57 $\pm$ 3.82 [#]	20.10
V	0.9% Sodium chloride solution	63.29 $\pm$ 3.15	-

Note: [#]—statistically significant difference compared with the control group ($p \leq 0.05$); [†]—statistically significant difference compared with the reference drug group ($p \leq 0.05$).

4 Discussion

In all analysed samples, hydroxycinnamic acids were quantitatively predominant (120–305 $\mu\text{g/g}$), with less pronounced differences between the aerial and underground parts of the plant. The total flavonoid content in the herb exceeded that in the rhizomes by 4–7-fold. It was reported that summer moisture conditions, particularly precipitation during the warmest quarter, play a critical role in the survival and development of *A. nemorosa*, as drought stress can negatively affect rhizome growth and bud formation despite the species' summer dormancy [28], as well as the chemical composition of the plant. Previous studies have reported that caffeic acid derivatives and quercetin glycosides, including isorhamnetin derivatives, are the major polyphenolic constituents of *A. nemorosa* extracts; however, these findings were primarily based on TLC analysis [8]. In contrast, the present study employed UPLC–MS/MS, allowing for more precise identification and quantification of phenolic compounds, thereby providing a more detailed characterisation of the phytochemical profile. The observed variability in phenolic composition among samples collected from different locations is consistent with previous reports demonstrating that environmental conditions, harvest year, and ecological factors may substantially influence the accumulation of secondary metabolites in medicinal plants [29]. Such variability further justifies the use of a representative composite sample for the preliminary pharmacological screening performed in the present study. It should be emphasized that AN-0, AN-40, and AN-70 were prepared from the same pooled and homogenized composite herb material and therefore do not represent independent batches. Consequently, the differences in their chemical profiles primarily reflect the extraction selectivity of water, 40% ethanol, and 70% ethanol rather than variability among plant batches. The use of the same composite starting material also minimized the influence of geographical and inter-sample variability when comparing the biological activities of the three extracts. Nevertheless, reproducibility of the chemical composition and biological activity across independently collected batches and different harvest years was not evaluated in the present study and requires further investigation.

The identified amino acids further expand the phytochemical characterization of *A. nemorosa*. Some of these amino acids have been reported to participate in metabolic and antioxidant processes [30,31]; however, their contribution to the biological activities observed in the present study was not investigated. According to Table 2, the total AA content in the extracts of *A. nemorosa* herb ranged from 1745 to 4656 µg/g. A pronounced dependence of the AA composition of the extracts on the nature of the extraction solvent was established. The extract obtained using purified water exhibited the highest total AA content, with a predominance of conditionally essential AAs (58.76%). In contrast, extracts obtained using 70% and 40% ethanol were characterized by a predominance of essential AAs (50.85% and 49.02%, respectively). Some non-polar AAs showed an increase in content with increasing ethanol concentration. In particular, the content of isoleucine in AN-70 extract was 2.2- and 2.5-fold higher than in extracts AN-40 and AN-0, respectively. A similar trend was observed for valine (2.4–3.0-fold higher content) and phenylalanine (1.2–1.5-fold higher content). Conversely, highly polar AAs demonstrated an opposite trend. For example, the content of arginine in AN-0 extract reached 749 µg/g, compared to 71 µg/g and 57 µg/g in AN-40 and AN-70 extracts, respectively. A similar pattern was observed for glutamic acid, the content of which in AN-0 extract was 8.7–11.3-fold higher than in ethanol-derived extracts. This is the first study to demonstrate the amino acid composition of *A. nemorosa* raw materials and their extracts. Although the present study did not investigate interactions between amino acids and secondary metabolites, characterization of the amino acid fraction may also be relevant to future pharmaceutical studies, since components of the extract matrix can potentially influence the physicochemical properties and bioavailability of bioactive constituents. Further studies are required to determine whether such interactions contribute to the biological effects of *A. nemorosa* extracts.

The results of this preliminary screening study indicate anti-inflammatory activity of *A. nemorosa* extracts, with the most significant effect observed at 5 h of the experiment. The highest anti-exudative activity was shown by extracts AN-0 and AN-40. The anti-inflammatory potential of the extracts was assessed using only one experimental inflammation model, which limits the generalizability of the findings. Further investigations employing multiple complementary inflammatory models are required to confirm and better characterize the observed pharmacological effects. In addition, only a single dose level (100 mg/kg) was evaluated for each extract. Therefore, the pharmacological potency of the extracts and their dose-response relationships could not be determined. Further studies employing multiple dose levels are required to identify the optimal therapeutic range and better characterize the anti-inflammatory potential of the investigated extracts. Previous studies have demonstrated that aqueous extracts of *A. nemorosa* exhibit anticancer potential by inducing apoptosis through cell cycle arrest, mitochondrial dysfunction, caspase activation, and reactive oxygen species generation [7]. These mechanisms, particularly the modulation of oxidative stress and inflammatory pathways, may also contribute to the anti-inflammatory activity observed in the present study. In contrast to previous findings obtained using the carrageenan-induced inflammation model [22], where certain extracts of *A. nemorosa* showed no or minimal anti-exudative activity. The glycosides anemonin and ranunculin, present in representatives of the *Anemone* genus, are considered potent anti-inflammatory compounds, the mechanism of action of which is associated with the inhibition of nitric oxide (NO) synthesis [32]. In addition, anemonin is used in cosmetology as a hypopigmenting agent due to its ability to inhibit tyrosinase activity and modulate protein and mRNA expression levels in human melanocytes, thereby suppressing melanin synthesis [33]. Furthermore, anemonin has been shown to promote the restoration of the intestinal mucosa, enhance enterocyte proliferation and restitution, and support the regeneration of the mucosal epithelium by increasing the expression of the epidermal growth factor receptor (EGFR) gene and reducing the levels of pro-inflammatory cytokines (TNF-α, IL-1β,

IL-6, and IL-8) [34]. Although triterpenoid and butenolide constituents such as anemonin have previously been reported in *Anemone* species, they were not identified in the present study because the analytical method was specifically designed for targeted determination of phenolic compounds and amino acids using authentic reference standards. Since an authentic reference standard of anemonin was not available, its presence could not be unequivocally confirmed under the analytical conditions employed.

The observed anti-inflammatory effects may be associated with the presence of phenolic compounds identified in the extracts [35], which are known to modulate inflammatory pathways, including the inhibition of cyclooxygenase activity and reduction of oxidative stress [36,37]. Among the identified constituents, caffeic acid, chlorogenic acid, rosmarinic acid, rutin, isoquercetin, and kaempferol glycosides have previously been reported to possess anti-inflammatory, and antioxidant properties. Therefore, the biological effects observed in the present study may result from the combined action of these phenolic compounds rather than from a single active constituent. In particular, the relatively high content of caffeic acid and flavonoid glycosides in AN-40 extract may contribute to its pharmacological activity. The anti-inflammatory activity observed in the present study should be interpreted within the context of a screening investigation. Because only one experimental model and one dose level were employed, the results primarily identify biologically active extracts and provide a rationale for future pharmacological characterization rather than establishing definitive therapeutic efficacy.

In the screening model of acute CCl₄-induced hepatitis, administration of *A. nemorosa* extracts was associated with improvements in several biochemical markers of liver injury. Treatment with the studied preparations resulted in reduced ALT and AST activities in both blood serum and liver homogenate compared with the CCl₄ control group. Among the investigated extracts, AN-40 demonstrated the most pronounced effects. Compared with the CCl₄ control group, serum ALT activity decreased from 3.39 ± 0.47 to 1.51 ± 0.12 $\mu\text{mol/h}\cdot\text{mL}$ (55.5% reduction), serum AST activity decreased from 4.29 ± 0.51 to 1.56 ± 0.40 $\mu\text{mol/h}\cdot\text{mL}$ (63.6% reduction), and serum ALP activity decreased from $10,071 \pm 1152$ to 6164 ± 806 $\text{nmol/s}\cdot\text{L}$ (38.8% reduction). Notably, the serum ALT activity in the AN-40 group was lower than that observed in intact animals (1.51 ± 0.12 versus 2.47 ± 0.35 $\mu\text{mol/h}\cdot\text{mL}$), whereas serum AST activity remained slightly elevated compared with the intact group (1.56 ± 0.40 versus 1.21 ± 0.19 $\mu\text{mol/h}\cdot\text{mL}$). The effects of AN-40 were comparable to, and for some parameters numerically greater than, those observed for the reference drug Silibor. Compared with the CCl₄ control group, the AN-40 extract reduced serum ALT, AST, and ALP activities by 55.5%, 63.6%, and 38.8%, respectively, whereas the corresponding changes observed with Silibor were 31.6%, 2.3%, and 16.6%, respectively. These changes indicate partial normalization of biochemical markers of liver injury under the experimental conditions used. A significant decrease in transaminase levels was also observed following administration of AN-70 extract, although the effect was less pronounced than that of AN-40. Hematological parameters indicated an inflammatory response in the control group, whereas leukocyte levels were significantly reduced in groups I–III. Thus, the results of this preliminary screening study suggest hepatoprotective potential of AN-40 extract, whereas AN-0 and AN-70 extracts showed less pronounced effects. It should be noted that the assessment of hepatoprotective activity in the present study was based on biochemical markers (ALT, AST, and ALP) and animal survival data. Histopathological examination of liver tissue was not performed; therefore, the observed effects should be interpreted as preliminary evidence of hepatoprotective potential that requires confirmation in future studies incorporating histological evaluation. Nevertheless, the hepatoprotective effect of *A. nemorosa* extracts has been investigated for the first time. The observed hepatoprotective effects may be associated, at least in part, with the antioxidant and anti-inflammatory properties of phenolic constituents identified in the extracts. Polyphenols are known to attenuate oxidative stress and preserve hepatocellular integrity, mechanisms that are particularly relevant to chemically induced liver injury [37]. Several constituents identified in the present

study, including caffeic acid, chlorogenic acid, rosmarinic acid, rutin, isoquercetin, and kaempferol derivatives, have also been reported to possess antioxidant and anti-inflammatory properties [34–36]. These literature data provide a plausible pharmacological context for the effects observed in the present study; however, they do not establish that these individual compounds are responsible for the hepatoprotective activity of the extracts. Overall, these findings support further investigation of the anti-inflammatory and hepatoprotective potential of *A. nemorosa* extracts.

The observed differences in biological activity among the extracts may be partially explained by their distinct phytochemical profiles. AN-40 extract, which demonstrated the strongest anti-inflammatory and hepatoprotective effects, contained the highest total concentration of phenolic compounds (1581 µg/g), including particularly high levels of caffeic acid and several flavonoid glycosides. In contrast, although AN-70 extract exhibited the highest total flavonoid content, its overall phenolic content was lower than that of AN-40. AN-0 extract was characterized by the highest total amino acid content but lower levels of several phenolic constituents. Notably, the differences in total amino acid content among the extracts did not parallel their pharmacological activity: AN-0 contained the highest total amino acid content, whereas AN-40 generally demonstrated the most pronounced pharmacological profile, particularly in the hepatoprotective screening. Therefore, the present results do not support a direct association between total amino acid content and the observed biological effects. Because only three extracts were included in the biological screening, formal correlation analysis between individual phytochemical constituents and pharmacological endpoints was not considered statistically robust; therefore, the observed composition–activity patterns are interpreted descriptively and should be regarded as hypothesis-generating. Nevertheless, differences in the amino acid composition may contribute to the overall physicochemical properties of the multicomponent extract matrix and could potentially influence interactions, solubility, stability, or bioavailability of other constituents. Such effects were not investigated in the present study and should therefore be regarded as a hypothesis requiring experimental verification. Overall, the observed biological activities may depend on interactions among multiple extract constituents rather than on the concentration of a single chemical class. Further bioactivity-guided fractionation, interaction studies, and mechanistic investigations are required to clarify the contribution of individual constituents and extract-matrix effects. For further pharmacological development, standardization of *A. nemorosa* extracts will also be necessary to ensure reproducible chemical composition and biological activity. The phenolic constituents identified in the present study, particularly abundant hydroxycinnamic acids and flavonoid glycosides, may represent potential candidates for future standardization markers. However, selection of definitive marker compounds should be based on the analysis of multiple independently collected batches and confirmation of relationships between marker content and relevant biological activity.

The results of the study indicate that local application of *A. nemorosa* extracts resulted in only a slight reduction in bleeding time. Extracts AN-40 and AN-70 reduced bleeding time by 12.61% and 8.14%, respectively, compared to the control group. The greatest reduction in bleeding time was observed for AN-0 extract, which reduced bleeding time by 15.58%. The reference preparation, *Persicaria hydropiper* liquid extract, reduced bleeding time by 20.10% compared to the control group. Compared with the reference drug, the hemostatic activity of *A. nemorosa* extracts was less pronounced. The hemostatic activity of *A. nemorosa* extracts has been screened for the first time.

The present study provides a preliminary phytochemical and pharmacological screening of *A. nemorosa* and identifies AN-40 as the most active extract under the experimental conditions used, warranting further dose-dependent and mechanistic investigations.

The limitations of the study. The present study should be considered a preliminary screening investigation of the phytochemical composition and biological activity of *A. nemorosa* extracts. Some compounds could not be definitively identified due to the lack of reference standards. Moreover, the targeted UPLC–MS/MS approach was restricted to compounds represented by the available authentic reference standards and therefore did not provide comprehensive coverage of the secondary metabolome. Future studies should employ complementary untargeted high-resolution approaches, such as LC–HRMS/MS, to characterize additional potentially bioactive constituents, including triterpenoid saponins and other secondary metabolites not investigated in the present study. A major limitation of the pharmacological screening was the use of a single dose level for each investigated extract. Consequently, dose–response relationships, pharmacological potency, ED50 values, and an effective therapeutic dose range could not be established. The observed effects should therefore be interpreted as preliminary evidence of biological activity rather than as evidence of an optimized pharmacological effect. In addition, although the use of pooled and homogenized plant material minimized inter-sample variability in the comparative screening of the extracts, reproducibility across independently collected batches and different harvest years was not evaluated and remains to be established. Future studies should evaluate multiple dose levels, preferably including at least three doses, to establish dose dependency and better characterize the pharmacological profile of the most promising extracts. Furthermore, hepatoprotective activity was assessed using biochemical and survival endpoints only, without histopathological examination of liver tissue. Therefore, future studies should incorporate histopathological examination of liver tissue together with dose–response and mechanistic investigations. Such studies should include evaluation of relevant inflammatory mediators and signalling pathways, including TNF- α , IL-6, IL-1 β , COX-2, iNOS, and NF- κ B, as well as oxidative stress biomarkers, to clarify the mechanisms underlying the anti-inflammatory and hepatoprotective effects of *A. nemorosa* extracts.

5 Conclusions

This study provides a preliminary phytochemical and pharmacological screening of *Anemone nemorosa* L. extracts obtained from the herb and rhizomes collected within the flora of Ukraine. UPLC–MS/MS analysis expanded the phytochemical characterization of *A. nemorosa* and delivered the first detailed data on its amino acid composition, contributing novel information for this insufficiently investigated species. The examined extracts demonstrated low acute toxicity and exhibited anti-inflammatory and hepatoprotective activities under the experimental conditions used, particularly AN-40 (40% ethanol extractant). Overall, the observed pharmacological effects should be regarded as preliminary and require confirmation in further dose-dependent and mechanistic studies. These findings provide a scientific basis for subsequent bioactivity-guided investigations and more comprehensive pharmacological characterization of the most promising extracts.

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Availability of Data and Materials: The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary Materials.

Ethics Approval: The pharmacological properties of *A. nemorosa* extracts were studied according to the “European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes” (Strasbourg, 1986). The research was approved by the Bioethics Commission of the Ivano-Frankivsk National Medical University (protocol No. 153/25, dated 17 September 2025). According to the national regulations of Ukraine, approval by the institutional ethics committee is sufficient for conducting the described animal experiments, and no additional national permit was required. All animal experiments were performed in accordance with institutional ethical standards and are reported following the ARRIVE guidelines [38].

Conflicts of Interest: Given his role as Guest Editor of this journal, Ain Raal and Oleh Koshovyi had no involvement in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to another journal editor. The authors declare no other conflicts of interest.

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