

## STUDY OF PHENOLIC COMPOUNDS OF UMBELLATE WINTERGREEN HERB AND THEIR INFLUENCE ON BIOCHEMICAL INDICATORS OF BLOOD AND URINE IN THE RAT MODEL OF CHRONIC KIDNEY DISEASE

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*Diseases of the kidneys and urinary tract are a common problem in people of all ages. Kidneys filter blood, removing water-soluble waste from the body, maintain water-salt balance, stabilize blood pressure and PH level. Intoxication, hypothermia, injuries and other causes lead to problems with the kidneys – inflammatory disease, urolithiasis, etc. Therefore, the development of effective herbal remedies that affect the etiopathogenic factors of diseases is urgent.*

**The aim.** Study of the profile of phenolic compounds of umbellate wintergreen, study of antioxidant and nephroprotective properties of umbellate wintergreen extract on the model of chromate-induced renal failure (chronic kidney disease) in rats.

**Materials and methods.** Phenolic compounds were analyzed by high-performance liquid chromatography (HPLC) using a Waters e2695 Alliance HPLC system in combination with a 2998 PDA detector (Waters, Milford, MA, USA).

Study of *in vitro* antioxidant activity by HPLC method for 50 % extract of umbellate wintergreen herb. A Waters 2695 chromatograph (Waters, USA) equipped with a Waters 996 diode-matrix detector was used.

To determine the nephroprotective effect, 60 outbred sexually mature rats (males), divided into six groups, were studied. Based on the results of biochemical studies, creatinine clearance and urea clearance were calculated in experimental animals.

**Results.** 8 phenolic compounds – apigenin, hyperoside, quercitrin, rutin, quercetin, gallic acid, guaiaverine and isoquercetin – were identified and quantified in umbellate wintergreen by HPLC. Based on the results of a preliminary assessment of the antioxidant contribution of individual phenolic compounds to the overall effectiveness of the umbellate wintergreen grass extract, a significant effect of caffeic acid derivatives and quercetin was determined.

The use of *Chimaphila umbellata* extract in experimental animals was marked by the normalization of diuresis, a decrease in the total protein content of urine by 1.8 times. The effect of the use of umbellate wintergreen extract was expected to be dose dependent.

**Conclusions.** The profile of umbellate wintergreen phenolic compounds was investigated by HPLC, and their antioxidant effect was determined. In the conditions of the development of renal failure in rats, the studied extract of *Chimaphila umbellata* improved the physical condition of the animals, reduced their mortality, improved the excretory function of the kidneys, normalized nitrogen and protein metabolism, contributed to the protection of the structure of the kidney tissue, and therefore had a positive effect on the course of nephropathy

**Keywords:** *Chimaphila umbellata* (L.), phenolic compounds, antioxidant effect, chronic kidney disease, nephroprotective effect

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### 1. Introduction

Chronic kidney disease is one of the global problems of today in the medicine of the whole world [1]. Today, returning to the problem of chronic kidney disease is very important, as it concerns both socio-economic and general medical aspects. According to the results of a study conducted in 12 countries with a total of 75,058 participants, the prevalence of chronic kidney disease among adults is quite high: 14.3 % in the gener-

al population and 36.1 % in high-risk groups (hypertension, diabetes, cardiovascular diseases) [2]. Approximately two million people have end-stage renal disease, and the proportion of such diseases is increasing annually by 5–7 %. According to the World Health Organization, 5–10 million people die every year due to kidney disease [3].

Means of natural, in particular, plant origin, are an important source of new drugs. The World Health

Organization (WHO) encourages the use of medicinal herbs as tools used in the complex therapy of traditional treatment. The main attention is paid to the research of biologically active compounds, their chemical composition and the pharmacological potential of various types of plants to obtain compounds with less toxicity than existing molecules [4].

The genus *Chimaphila* is a typical representative of the Ericaceae family, which naturally grows in Bhutan, China, Japan, Korea, etc.; it includes about five species worldwide, of which three species (one of which is endemic) can be found in China. *Chimaphila umbellata* (L.) is a perennial herb with diuretic, astringent, analgesic, etc. properties. and can be used for various conditions, such as oedema, hydropsy, etc. [5].

Therefore, conducting research on expanding information on the chemical composition of the plant and studying its pharmacological properties in the direction of treating diseases of the kidneys and urinary tract is a relevant direction today.

## 2. Planning (methodology) of research

The research was carried out in several stages, consisting of the determination of phenolic compounds of the *Chimaphila umbellata* grass, the determination of the antioxidant effect and the determination of the nephroprotective effect on the model of chromate-induced renal failure (chronic kidney disease) in rats (Fig. 1).

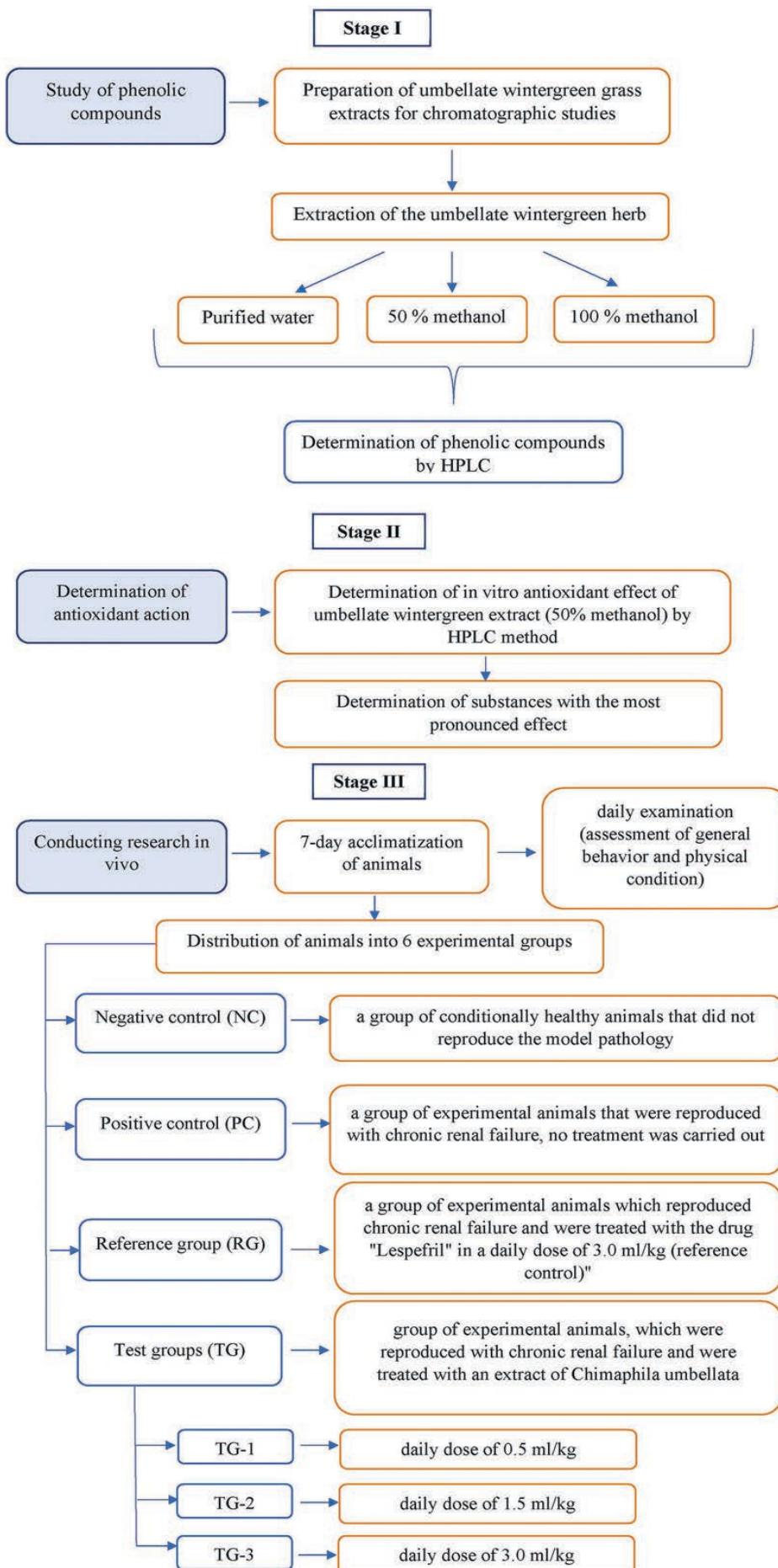


Fig. 1. Stages of planning and design of the experiment

### 3. Materials and methods

The research was conducted in September-November 2023.

*Preparation of extracts for chromatographic studies.* A weight of plant material (0.2 g) crushed to 2–3 mm particles was extracted with 10 ml of 50 % (v/v) methanol, purified water, and 100 % methanol in an ultrasonic bath (WiseClean) at 45±2 °C for 20 min. The resulting extracts were filtered through 0.45 µm syringe filters (Carl RothGmbH & Co. KG, Karlsruhe, Germany) and stored at 4 °C until analysis.

*HPLC chromatographic analysis.* Chromatographic separation of phenolic compounds in umbellate wintergreen was performed using a Waters e2695 Alliance HPLC system coupled with a 2998 PDA detector (Waters, Milford, MA, USA). Phenolic compounds were separated on an ACE Super C18 column (250×4.6 mm, with a particle size of 3 µm) (ACT, Aberdeen, UK). The column temperature was 25 °C. The mobile phase consisted of two solutions: 0.1 % aqueous solution of trifluoroacetic acid (mobile phase A) and acetonitrile P (mobile phase B). The mode of gradient elution was as follows: 0 min, 5 % B; 8–30 min, 20 % B; 30–48 min, 40 % B; 48–58 min, 50 % B; 58–65 min, 50 % B; 65–66 min, 95 % B; 66–70 min, 95 % B; 70–81 min, 5 % B. The injection volume of the test solution (comparison solution) was 10 µl, the flow rate was 1000 ml/min, and the column temperature was 25 °C. Substances were detected by comparing the UV-visible spectrum of each peak with valid reference standards and measuring their retention time. Quantification of substances was calculated using graduation graphs. Validation characteristics of the HPLC method were performed according to ICH Q2 (R1) recommendations and included linear calibration curves ( $r>0.999$ ), limits of detection (LOD) and quantification (LOQ), precision, specificity, and are outlined in [6]. When quantifying umbellate wintergreen metabolites, each sample was analyzed twice, and the average value was used for calculation.

*Study of in vitro antioxidant activity by HPLC method for 50 % extract of *Chimaphila umbellata*.* A Waters 2695 chromatograph (Waters, USA) equipped with a Waters 996 diode-matrix detector was used. The mobile phase was transferred to a reaction loop with the ABTS reagent, which was supplied by a Gilson 305 pump. The reaction loop was made of Teflon and had a length of 3 m and an inner diameter of 0.25 mm. The parameters of the ABTS solution system were set as follows: temperature – 50 °C, reagent flow rate – 0.5 ml/min. The conditions for preparing the systems are described in works [7, 8].

*Determination of the pharmacological activity of the extract of 50 % of the herb umbellate wintergreen.*

The study was performed on 60 outbred sexually mature rats (males) weighing 250±20 g, kept in the vivarium of the Educational and Scientific Training Center for Medical and Biological Research (ESTC MBR of NUPh). The animals were kept in a separate room with controlled microclimate parameters: air temperature 18–22 °C, relative humidity 50–65 %, light mode “12 hours day/night”,

in plastic cages with individual ventilation [9]. Sterilization of the premises with the help of a UV lamp was carried out daily. Animals had free access to water (pre-settled tap water from drinking fountains). Granulated balanced feed (TU.U15.7-2123600159-001:2007) was used for animal feeding. Animal care was performed in accordance with the standard operating procedures of the ESTC MBR of NUPh. All stages of the research were carried out in accordance with Directive 2010/63/EU of the European Parliament and the Council of the EU dated September 22, 2010 “On the protection of animals used for scientific purposes” [10, 11].

Before the experiment, the animals were acclimatized for 7 days. During the acclimatization period, each animal was examined daily (behaviour and general physical condition were assessed), and animals were observed for possible morbidity or mortality. Before the start of the experiment, each rat was placed for 3 hours in a metabolic cage for acclimatization [9].

During this experiment, the study of the activity of different dose levels of the extract of the umbellate wintergreen grass on the model of chronic kidney disease was carried out in comparison with the comparator - the drug “Lespefril” manufactured by PJSC “Lubnyfarm” (Ukraine), a solution for oral use in bottles of 100 ml containing the extract Lespedeza bicolor from 70.8 g of shoots [12].

After acclimatization, the experimental animals were evenly divided into 6 experimental groups of 10 animals in each according to the following design:

1. Negative control (NC) – a group of conditionally healthy animals that did not reproduce the model pathology.

2. Positive control (PC) – a group of experimental animals that were reproduced with chronic renal failure, no treatment was carried out.

3. Reference group (RG) – a group of experimental animals which reproduced chronic renal failure and were treated with the drug “Lespefril” in a daily dose of 3.0 ml/kg (reference control).

4. Test group No. 1 (TG-1) – a group of experimental animals that were treated with umbellate wintergreen extract at a daily dose of 0.5 ml/kg.

5. Test group No. 2 (TG-2) is a group of experimental animals, which were reproduced with chronic renal failure and were treated with an extract of *Chimaphila umbellata* in a daily dose of 1.5 ml/kg.

6. Test group No. 3 (TG-3) is a group of experimental animals that were treated with umbellate wintergreen extract at a daily dose of 3.0 ml/kg and were treated with chronic renal failure.

Daily doses of the test sample were chosen empirically according to the results of previous screening studies with the expectation of establishing an effective dose in this experiment; the daily dose of the reference sample for experimental animals was calculated considering the interspecies difference in mass and body surface area in accordance with commonly used FDA recommendations [13, 14].

The model of chronic kidney disease in rats was reproduced by a single subcutaneous injection of a 2.5 %

solution of potassium chromate at a dose of 0.7 ml/kg [4, 15]. After that, they started daily administration of test and reference samples for 20 days. Samples were administered intragastrically on an empty stomach once a day using a special metal probe for intragastric administration. Animals of the NC group received the corresponding amount of purified water. On the 19<sup>th</sup> day of observation, animals were placed in metabolic cages for 24 hours, spontaneous diurnal diuresis was determined, and daily urine samples were collected [16]. On the 20<sup>th</sup> day, animals were humanely euthanized in a CO<sub>2</sub> chamber, blood was taken from the inferior vena cava to obtain serum, an autopsy was performed, and the kidneys were removed to determine mass ratios. Considering the high rate of lethality of this model, the survival rate of animals was also recorded during the observation process.

Urea and creatinine were determined in blood serum and urine samples, and total protein in urine was additionally determined. Biochemical analysis was performed with the help of standard sets of reagents "Creatinine HP014.01", "Urea-U HP018.02", "Total protein UL HP010.02" (ToV NVP "Filisit-Diagnostika", Ukraine) according to the relevant instructions for use on the spectrophotometer LabAnalyt SP-V1000 (Granum, China). Based on the results of biochemical studies, creatinine clearance and urea clearance were calculated in experimental animals [17, 18].

The obtained results were expressed as arithmetic mean (M) and standard error of the mean (SEM). Comparison of experimental groups was performed using parametric methods of analysis (ANOVA, Tukey HSD test). Indicators expressed as percentages were compared using Fisher's angular transformation. The probability of differences was determined at the significance level of  $P < 0.05$ . Statistical processing was carried out using the MS Excel 2007 basic program package and IBM SPSS Statistics 22 [19]. Methods (techniques) of determining generalizing aggregate indicators were used.

#### 4. Research results

To select the optimal extract for the herb umbellate wintergreen, first a weight (0.2 g) was extracted with water, 50 % methanol and 100 % methanol and the BAS content was evaluated (Table 1, Fig. 2).

As a result, it was determined that 50 % methanol extracts the highest content of substances (Fig. 3, 4). For the preliminary screening of substances in umbellate wintergreen extracts, the HPLC method with a diode-matrix detector was used (Table 1).

To assess the antioxidant potential of individual phenolic compounds *in vitro* in umbellate wintergreen, the HPLC/ABTS method with post-column derivatization was used (Fig. 4).

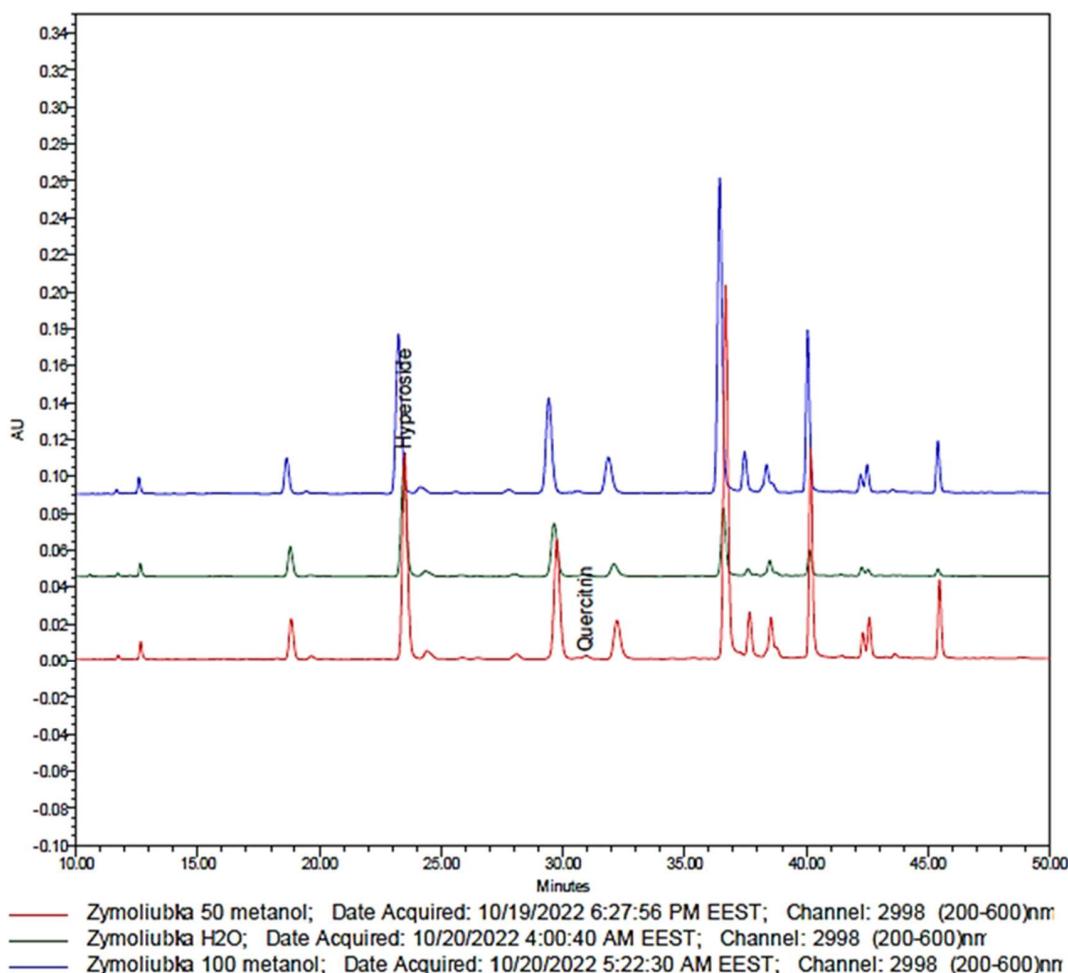


Fig. 2. General view of HPLC chromatograms of umbellate wintergreen extracts (full spectrum 200-600 nm) obtained with different solvents: red line – 50 % methanol; green line – water; blue line – 100 % methanol

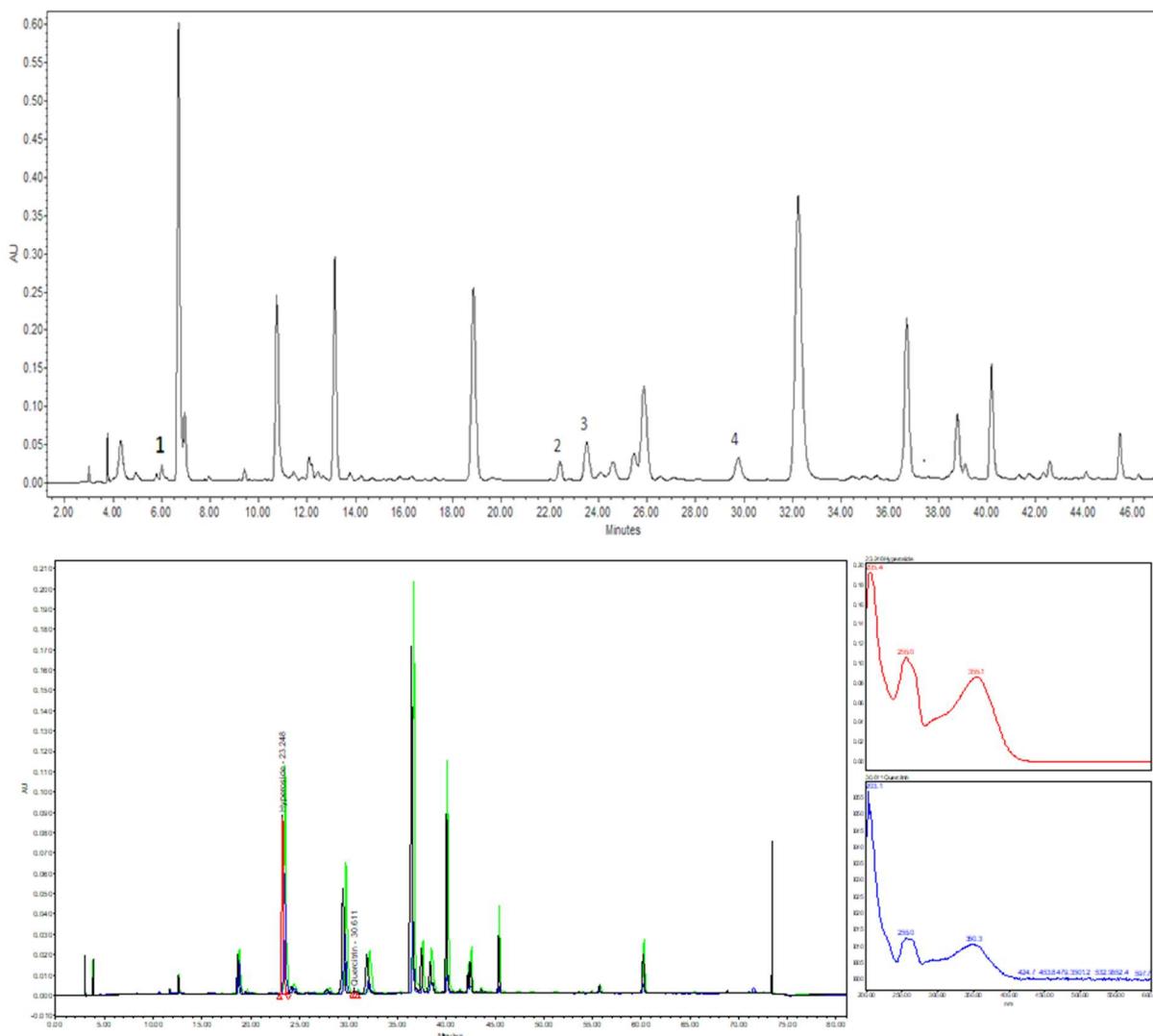


Fig. 3. HPLC chromatogram of the methanolic extract of umbellate wintergreen grass and UV spectra of two marker substances – hyperoside and quercitrin

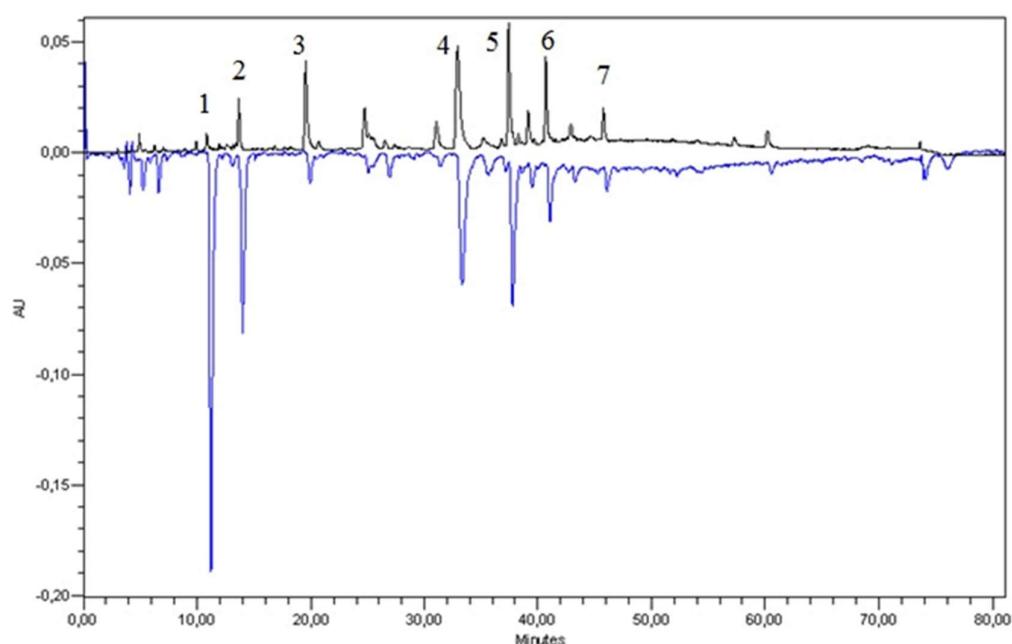


Fig. 4. HPLC/ABTS chromatogram of umbellate wintergreen extract (50 % methanol) at 247 nm (HPLC, black) and 650 nm (ABTS, blue)

Table 1  
The content of identified substances (mg/g) in *Chimaphila umbellata* extracts by HPLC

Substance	Retention time	50 % methanol	100 % methanol	Water
Apigenin	48.776	0.01±0.00	—	—
Hyperoside	23.512	3.71±0.06	2.90±0.05	2.02±0.04
Quercitrin	30.828	0.06±0.01	0.05±0.01	0.04±0.01
Rutine	23.248	4.16±0.07	3.29±0.06	2.28±0.04
Quercetin	43.643	0.03±0.01	0.02±0.01	—
Gallic acid	6.008	0.25±0.04	—	—
Guayaverine	29.422	3.99±0.08	3.25±0.06	1.83±0.03
Isoquercetin	24.746	0.05±0.01	—	—

The selected HPLC-ABTS method was used for the preliminary assessment of the antioxidant contribution of individual phenolic compounds to the overall effectiveness of the *Chimaphila umbellata* herb extract. Two peaks at 10 and 14 min (peak 1, peak 2) indicate a pronounced antioxidant activity of these compounds. These compounds were not precisely identified, but comparing the release time and the UV spectrum, it can be assumed that they are derivatives of caffeic acid.

The peak at 23.5 min (peak 3) corresponds to hyperoside, which was also identified by HPLC, but the substance showed moderate antioxidant activity.

At 33.5 min (peak 4) and at 40 min (peak 5), probably flavone derivatives, the substances were not precisely identified, but they showed an antioxidant effect.

Quercetin was identified at 43.6 min (peak 6). Even though the substance has a lower content than hyperoside, it showed a relatively greater antioxidant effect, although compared to other unidentified substances – less efficiency.

A less pronounced peak at 46.8 min (peak 7) was identified as apigenin, which showed a moderate contribution to the antioxidant effect of umbellate wintergreen.

According to the results of the study on the model of chronic kidney disease in rats, it was found that in the PC group, under the influence of chromium compounds, severe kidney failure developed. The survival rate in this group was equal to 80 % (Table 2).

Table 2  
The effect of the studied samples on the course of chromate-induced nephropathy in rats (M±SEM)

Experimental group	Spontaneous daily diuresis, ml/100 g	Total protein in urine, g/l	Kidney mass index, g/100 g (2x)	Survival rate, %
NC (n=10)	2.76±0.10	0.01±0.00	0.64±0.01	100
PC (n=8)	1.44±0.11 <sup>a</sup>	0.33±0.01 <sup>a, b</sup>	0.69±0.03	80 <sup>a</sup>
RG (n=8)	2.38±0.21 <sup>b</sup>	0.18±0.02 <sup>a, b</sup>	0.79±0.04	80 <sup>a</sup>
TG-1 (n=7)	1.52±0.14 <sup>a, c</sup>	0.27±0.03 <sup>a, c</sup>	0.75±0.04	70 <sup>a</sup>
TG-2 (n=7)	2.40±0.23 <sup>b</sup>	0.19±0.02 <sup>a, b</sup>	0.79±0.05	70 <sup>a</sup>
TG-3 (n=10)	2.81±0.19 <sup>b</sup>	0.10±0.02 <sup>a, b, c</sup>	0.76±0.06	100 <sup>b, c</sup>

Notes: a – differences are probable relative to the negative control ( $p<0.05$ ); b – differences are probable relative to the negative control ( $p<0.05$ ); c – differences are probable relative to the reference control ( $p<0.05$ ).

At the same time, diuresis was probably reduced by 1.9 times, and the content of total protein in the urine increased by 33 times, which indicated a significant proteinuria ( $p<0.05$  vs. NC). In addition, the deterioration of the functional state of the kidneys led to a decrease in the excretion of urea and creatinine by the kidneys and an increase in their content in the blood serum, due to which the indicators of creatinine clearance and urea clearance probably decreased by 7.3 times and 9.8 times, respectively, relative to similar indicators into the NC group (Tables 3, 4). It should also be noted that although there were no significant changes in kidney mass index, macroscopically, the kidneys of this group were enlarged and had a “coffee with milk” colour, in contrast to the healthy kidneys of NC group animals.

Table 3  
Creatinine clearance in rats with chromate-induced nephropathy (M±SEM)

Experimental group	Creatinine content in blood serum, $\mu\text{mol/l}$	Creatinine content in urine, $\mu\text{mol/l}$	Creatinine clearance, $\mu\text{l}/\text{min}/100 \text{g}$
NC (n=10)	67.27±1.67	3410.77±172.46	98.71±8.22
PC (n=8)	193.39±8.24 <sup>a</sup>	2658.27±104.84	13.52±1.01 <sup>a</sup>
RG (n=8)	121.65±7.91 <sup>a, b</sup>	2811.63±169.28	56.01±6.03 <sup>a, b</sup>
TG-1 (n=7)	169.12±7.45 <sup>a, c</sup>	2605.69±101.13	16.67±2.23 <sup>a, c</sup>
TG-2 (n=7)	125.73±12.54 <sup>a, b</sup>	2710.85±218.18	55.04±8.16 <sup>a, b</sup>
TG-3 (n=10)	95.28±4.26 <sup>a, b</sup>	3083.60±446.51	86.16±10.18 <sup>b</sup>

Note: a – differences are probable relative to the negative control ( $p<0.05$ ); b – differences are probable relative to the negative control ( $p<0.05$ ); c – differences are probable relative to the reference control ( $p<0.05$ ).

Table 4  
Urea clearance in rats with chromate-induced nephropathy (M±SEM)

Experimental group	Urea content in blood serum, $\text{mmol/l}$	Urea content in urine, $\text{mmol/l}$	Urea clearance, $\mu\text{l}/\text{min}/100 \text{g}$
NC (n=10)	6.64±0.19 <sup>a</sup>	168.78±22.43	49.47±7.30
PC (n=8)	20.41±0.78 <sup>a</sup>	101.19±7.24 <sup>a</sup>	5.06±0.76 <sup>a</sup>
RG (n=8)	13.47±0.73 <sup>a, b</sup>	125.00±17.20	16.42±3.44 <sup>a</sup>
TG-1 (n=7)	17.95±0.68 <sup>a, c</sup>	107.33±5.46 <sup>a</sup>	6.30±0.56 <sup>a</sup>
TG-2 (n=7)	13.79±0.96 <sup>a, b</sup>	120.94±5.47	15.22±2.15 <sup>a</sup>
TG-3 (n=10)	9.47±0.47 <sup>a, b, c</sup>	133.33±10.28	27.13±2.02 <sup>a, b</sup>

Note: a – differences are probable relative to the negative control ( $p<0.05$ ); b – differences are probable relative to the negative control ( $p<0.05$ ); c – differences are probable relative to the reference control ( $p<0.05$ ).

The use of the reference sample in experimental animals was marked by the normalization of diuresis, a decrease in the total protein content of urine by 1.8 times ( $p<0.05$  vs. PC). Along with this, there was a statistically significant increase in glomerular filtration by creatinine clearance by 4.1 times and a decrease in serum urea by 1.5 times (without a significant effect on urea clearance) compared to animals of the NC group.

The effect of using the test sample was dose-dependent, as expected. Thus, in a dose of 0.5 ml/kg, the extract

of the umbellate wintergreen was not able to influence the excretory function of the kidneys of animals against the background of the model of chronic renal failure. The test sample in a dose of 1.5 ml/kg contributed to the improvement of the functional state of the kidneys at the level of the reference agent. The best effect was demonstrated by the dose of the test sample of 3.0 ml/kg, against the background of which it was noted: a probable increase in the survival of animals to 100 % (compared to the PC and RG groups), normalization of diuresis to the NC level, a significant decrease in the content of total protein in the urine in 3.3 times compared to PC and 1.8 times compared to RG. A practical normalization of the glomerular filtration rate was also noted, which was expressed in the achievement of the creatinine clearance index of the physiological norm ( $p>0.05$  vs. NC). In addition, the nitrogen-releasing function of the kidneys was significantly improved, which was reflected in the likely increased clearance of urea by 5.4 times compared to untreated animals.

## 5. Discussion of research results

A phytochemical study of the whole plants of *Chimaphila japonica* Miq. led to the isolation of 23 compounds, including ten triterpenoids (1–10), six flavonoids (11–16), two sterols (17 and 18), two quinonoids (19 and 20), one saccharide derivative (21), one phenolic glycoside (22), and one megastigmane glycoside (23). The structures of these isolated compounds were identified using NMR spectroscopy ( $^1\text{H}$  and  $^{13}\text{C}$ ) by compari-

son with previously reported data. All compounds, except 19 and 22, were reported from *C. japonica* for the first time. Among them, 16 compounds (1–4, 6–9, 12, 13, 15, 16, 18, 20, 21, and 23) were reported from the genus *Chimaphila* for the first time, while compounds 12, 16, and 23 were isolated from the Ericaceae family for the first time. The chemotaxonomic significance of the isolates was also discussed (Fig. 5) [20].

Plant extracts were intensively tested for their antibacterial activity against Gram-negative strains, including ethanolic extract of *Chimaphila umbellata* (L.). The ethanolic extracts of *Chimaphila umbellata* (L.) W.P.C. Barton (princes pine) were found to be antimicrobial active against all tested strains including Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* at a concentration of 10,000  $\mu\text{g}/\text{ml}$  [21].

In summary, to identify compounds with potent diuretic activity, the components of *C. japonica* were separated, resulting in the identification of 12 compounds (1–12), including three previously undescribed phenols (1–3) and one new cyclohexanol (4). Bioassays demonstrated that compounds 4, 7, and 11 possess potent diuretic activity. The docking study further revealed that the diuresis potential of the active compounds could be realized by the WNK1 kinase domain. Their binding driving forces comprise hydrophobic and hydrogen bond interactions. However, to improve the bioavailability of active compounds, further structural modification and mechanism analysis are necessary in the future [20, 22].

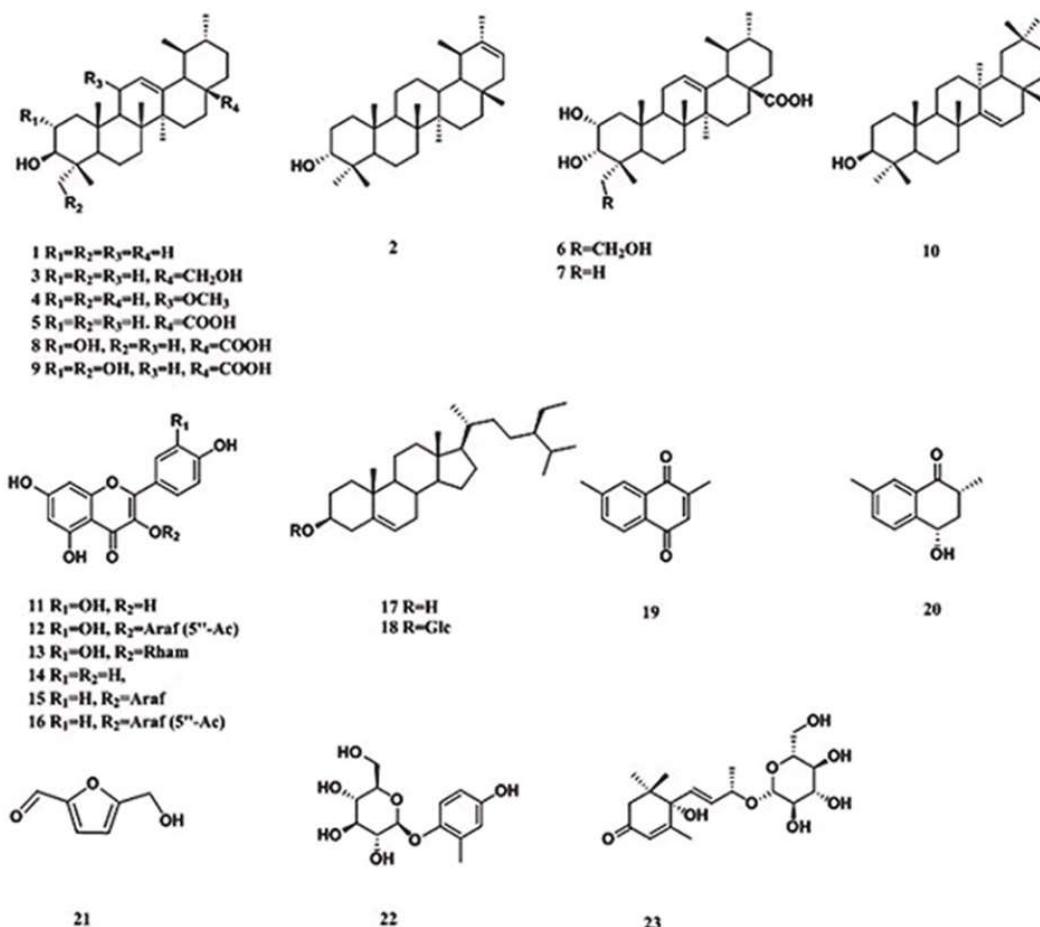


Fig. 5. The chemotaxonomic significance of the isolates of *Chimaphila umbellata*

Therefore, the obtained experimental data reflect the global trends in the study of the raw materials of umbellate wintergreen in the direction of the study of the class of phenolic compounds and their influence on the functioning of the kidneys and urinary system.

**Practical relevance.** The importance and feasibility of this study lies in the study of a potential diuretic agent of plant origin to expand the treatment group of patients with CKD who need effective, timely and safe therapy. Chronic kidney disease is a multifactorial disease, the pathogenesis of which varies depending on the primary pathology, but in any case, the most common outcome is renal failure. For this reason, an appropriate experimental model was chosen in this study. This work did not consider the problems of combined therapy of umbellate wintergreen extract with other drugs for the treatment of chronic renal failure.

**Study limitations.** In the original study, clinical and biochemical markers were determined and there were no histological data.

**Prospects for further research.** Further investigation of umbellate wintergreen extract in preclinical and clinical settings is recommended to expand the evidence base, as well as to search for possible safe and effective combinations with drugs that potentiate the effects of *Chimaphila umbellata* extract, which can be considered as a basic combination therapy for kidney pathology.

## 6. Conclusions

8 phenolic compounds – apigenin, hyperoside, quercitrin, rutin, quercetin, gallic acid, guaiaverine and isoquercetin – were identified and quantified by HPLC.

Based on the results of a preliminary assessment of the antioxidant contribution of individual phenolic compounds to the overall effectiveness of the *Chimaphila umbellata* herb extract, a significant effect of caffeic acid derivatives and quercetin was determined.

Under the conditions of the development of renal failure in rats, the studied extract of umbellate wintergreen improved the physical condition of the animals, reduced their mortality, improved the excretory function of the kidneys, normalized nitrogen and protein metabolism, contributed to the protection of the structure of the kidney tissue, and therefore had a positive effect on the course of nephropathy. So, in experimental studies, umbellate wintergreen extract had a pronounced nephroprotective and hypoazotemic effect and may be a promising tool for the treatment of chronic kidney disease.

## Conflict of interests

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research, and its results presented in this paper.

## Funding

The study was performed without financial support.

## Data availability

Data will be made available at a reasonable request.

## Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies when creating the current work.

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