Hedera helix is widely used as a remedy to treat the respiratory infections and cold accompanied with cough due to its anti-inflammatory effect. In addition, the antioxidant activity of its extracts has been confirmed. It is explained by the high content of flavonoids and phenolic acids among all phytochemicals of H. helix leaves. However, it remains uncertain which exactly components are responsible for the antioxidant activity and what is the best way to perform extraction.

**Aim.** To determine the antioxidant profile of different extracts from H. helix leaves using the in vitro HPLC method combined with the ABTS reagent.

**Materials and methods.** Extraction of H. helix leaves was conducted with different solvents (from 20 % methanol to 100 % methanol using an ultrasound bath); the method described in the Pharmacopeia was also used. A Waters chromatograph was used to determine the antioxidant profile.

**Results.** About 90 % of the components responsible for the antioxidant activity were determined using the HPLC method proposed. Among them, chlorogenic acid and 3,5-caffeoylquinic acid showed the highest activities. Other components, such as neochlorogenic acid, hyperoside and 3,4-caffeoylquinic acid were revealed as components with the antioxidant scavenging activities in H. helix extracts.

**Conclusions.** The results obtained indicate that extracts from H. helix leaves possess the high antioxidant scavenging capacity. In addition, the in vitro HPLC method proposed can be used for the primary screening of components in the plant raw material.

**Key words:** Hedera helix leaves; antioxidant activity; HPLC; screening

I. V. Bezruk, V. O. Grudko, V. A. Georgiyants, L. Ivanauskas*

National University of Pharmacy, Ukraine
Lithuanian University of Health Sciences, Republic of Lithuania*

SCREENING OF THE ANTIOXIDANT ACTIVITY OF EXTRACTS FROM **HEDERA HELIX** LEAVES USING THE HPLC/ABTS METHOD

Hedera helix is widely used as a remedy to treat the respiratory infections and cold accompanied with cough due to its anti-inflammatory effect. In addition, the antioxidant activity of its extracts has been confirmed. It is explained by the high content of flavonoids and phenolic acids among all phytochemicals of H. helix leaves. However, it remains uncertain which exactly components are responsible for the antioxidant activity and what is the best way to perform extraction.

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I. V. Безрук, В. О. Грудько, В. А. Георгіянц, Л. Іванаускас*

Національний фармацевтичний університет, Україна
Литовський університет наук здоров'я, Литовська республіка*

Скринінг профілю антиоксидантної активності екстрактів листя **Hedera helix** за допомогою ВЕРХ/ABTS методу

Hedera helix широко використовується для лікування респіраторних інфекцій та застуди, що супроводжується кашлем, завдяки протизапальним властивостям. Крім цього, була підтверджена антиоксидантна активність його екстрактів. Це пояснюється високим вмістом флавоноїдів та фенольних кислот серед фітохімічного профілю листя плюща. Проте залишається незрозумілим, які саме речовини відповідають за антиоксидантну активність та яким чином краще проводити їх екстракцію.

**Мета дослідження.** Визначити профіль антиоксидантів різних екстрактів листя плюща з використанням in vitro ВЕРХ методу, об’єднаного з ABTS реагентом.

**Матеріали та методи.** Екстракцію листя плюща проводили за допомогою різних розчинників від 20 % до 100 % метанолу з використанням УЗ-бани, а також застосовували фармаконепійний метод екстракції. Для визнання антиоксидантного профілю використовували хроматограф Waters.

**Результати.** За допомогою запропонованого методу було визначено майже 90 % основних речовин, що відповідають за антиоксидантну активність. Серед них найбільшу активність мали хлорогенова кислота та 3,5-кафеоїлохінова кислота. Також до речовин з антиоксидантною активністю в листі плюща можна віднести неохлорогенову кислоту, гіперозид, 3,4-кафеоїлохінову кислоту.

**Висновки.** Отримані результати свідчать про високу антиоксидантну активність екстрактів листя плюща.

Крім того, запропонований in vitro ВЕРХ метод може використовуватися для попереднього скринінгу антиоксидантної активності речовин у рослинній сировині.

**Ключові слова:** листя плюща; антиоксидантна активність; ВЕРХ; скринінг

I. В. Безрук, В. А. Грудько, В. А. Георгіянц, Л. Іванаускас*

Национальный фармацевтический университет, Украина
Литовский университет наук здоровья, Литовская республика*

Скрининг профиля антиоксидантной активности экстрактов листьев **Hedera helix** с использованием ВЭЖХ/АВТС метода

Hedera helix широко применяется для лечения респираторных инфекций и простуды, что сопровождается кашлем, благодаря своим противовоспалительным свойствам. Кроме того, антиоксидантная активность его экстрактов была подтверждена. Это объясняется высоким содержанием флавоноидов и фенольных кислот среди фитохимического профиля листьев плюща. Однако остаётся непонятным, какие именно вещества отвечают за антиоксидантный эффект и каким образом лучше проводить их экстракцию.

И. В. Безрук, В. А. Грудько, В. А. Георгиянц, Л. Иванаускас*
Herbal medicines are in high demand due to some of their benefits over synthetic drugs [1]. Pharmaceuticals containing the H. helix extract are required all around the world. Different pharmacological studies confirmed the anti-inflammatory, antibacterial [2] and antioxidant [3] activities of H. helix extracts. To assess the quality of medicines containing H. helix, the determination of hederacoside C is required [4]. However, it remains uncertain which exactly components are responsible for the antioxidant activity. Thus, the implementation of methods that can separately determine the activity of each substance in the mixture is essential to choose the correct biomarkers.

Guidelines for ethical conduct when using animals in research require the application of alternative methods for pharmacological studies of pharmaceutical products [5]. According to the guidelines, at the stage of the primary screening of drug pharmacological activities, it is preferable to replace the experimental animals with other types of models, such as in vitro analysis, cell cultures, computer modeling. Docking provides all required information about biological activities using no animal in the research [6]. Also, the implementation of alternative procedures for pharmacological activities remains in high demand. This research is aimed to perform the HPLC method for the in vitro antioxidant studies of H. helix samples.

Materials and methods

Plant raw material

The leaves of H. helix for this experiment were sampled in different European countries, such as Lithuania (Naujoji Akmenė), Ukraine (Kharkiv), Czech Republic (Prague), Austria (Vienna).

After collection, the samples were air-dried at the ambient temperature with protection from direct sunlight.

Reagents and solvents

Methanol, acetonitrile, potassium persulfate, 2,2-azinobis (ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), Trolox, neochlorogenic acid, chlorogenic acid, hyperoside 3,5-cafeoylquinic acid and 3,4-cafeoylquinic acid were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Water for chromatography was obtained using the Millipore (Burlington, MA, USA) water purification system. All chemicals and analytical standards used were of HPLC grade.

Sample preparation

The samples were prepared according to Bezruk et al. [7]. Briefly, 1.0 g of accurately weighted powder of H. helix leaves were extracted thrice with 15 ml of methanol (or 20%, 50%, 70% methanol) on an ultrasound bath for 15 minutes at ambient temperature. All supernatants were mixed and diluted to 50.0 ml with the solvent used for extraction.

The samples of H. helix were prepared according to the European Pharmacopoeia [8]. Concisely, 1.0 g of accurately weighted powdered leaves were extracted with 50 mL of the mixture of 80% methanol under reflux on a water bath at 80 °C for 1 hour; after that a cooled solution was filtered through cotton. The cotton used and the residue were extracted with 30 mL of the same solution for 30 minutes. The extracts were mixed and diluted to the volume 100.0 mL with the same solution.

Chromatographic conditions

HPLC-PDA and HPLC-ABTS were performed as described in Bezruk et al. [8]. A Waters Alliance 2695 (Waters, Milford, USA) separation system coupled with Waters 2487 UV/Vis and Waters 996 PDA diode-array detector (DAD). The separation of components was performed with the ACE C18 column (250 mm × 4.6 mm, particle size – 5 µm). Using the PDA detector the mobile phase containing phytochemicals was mixed with the ABTS solution in the reaction coil, as described in the previous papers [9-11]. The ABTS post-column chromatograms were registered at 650 nm. The calibration curve was constructed with a Trolox standard.

Results and discussion

The components were identified compared to standard retention times and their UV-spectra. Twenty substances were found in the studies (Fig.). However, only five of them showed the antioxidant scavenging activity. Chlorogenic acid and 3,5-cafeoylquinic acid were the dominant components and explained from 58% up to 93% of the total
antioxidant activity in all samples. Other substances were also revealed as possible markers; neochlorogenic acid, hyperoside and 3,4-caffeoylquinic acid were among them.

Sample collected in Naujoji Akmene possessed the highest total antioxidant scavenging capacity among the samples analyzed from 23.373 up to 43.024 µmol TE/g DM for all extracts (Table). On the other hand, leaves from Prague showed the lowest concentration among the samples studied; however, its values were relatively significant from 5.317 up to 9.257 µmol TE/g DM for all extracts.

Among all solvents, extracts of 100 % methanol showed the highest antioxidant activity. This data was shown in our previous research [8]. Mostly, components of methanol extracts were in the following decreasing order: 3,5-caffeoylquinic acid > chlorogenic acid > hyperoside > 3,4-caffeoylquinic acid.

Table

Antioxidant scavenging capacities of *H. helix* compound expressed in Trolox equivalent values (µmol TE/g DM) using the HPLC-ABTS post-column assay

| Sample (solvent)            | Neochlorogenic acid | Chlorogenic acid | Hyperoside | 3,5-caffeoylquinic acid | 3,4-caffeoylquinic acid |
|-----------------------------|---------------------|------------------|------------|-------------------------|-------------------------|
|                             | 1                   | 2                | 3          | 4                       | 5                       |
| Naujoje Akmene (20 % methanol) | 0.330±0.015         | 2.395±0.107      | 1.059±0.051| 24.776±1.235            | 0.776±0.035             |
| Kharkiv (20 % methanol)     | 1.124±0.056         | 4.197±0.183      | 1.825±0.076| 12.170±0.573            | 0.905±0.042             |
| Prague (20 % methanol)      | 1.027±0.051         | 2.299±0.096      | 1.497±0.066| 2.097±0.093             | 0.731±0.034             |
| Vienna (20 % methanol)      | 1.596±0.072         | 4.785±0.032      | 1.420±0.052| 3.566±0.152             | 0.959±0.036             |
| Naujoje Akmene (Pharmacopeia method) | 0.479±0.022         | 3.669±0.158      | 1.232±0.052| 16.764±0.829            | 1.227±0.058             |
| Kharkiv (Pharmacopeian method) | 1.289±0.059         | 7.991±0.352      | 0.832±0.038| 8.009±0.381             | 2.238±0.109             |
| Prague (Pharmacopeian method) | 0.956±0.042         | 1.116±0.043      | 1.282±0.062| 1.714±0.082             | 0.249±0.012             |
| Vienna (Pharmacopeian method) | 1.639±0.075         | 5.461±0.263      | 1.465±0.068| 0.751±0.028             | 0.063±0.002             |

Fig. A typical chromatogram of the antioxidant evaluation [1 – HPLC chromatogram, 2 – HPLC/ABTS chromatogram, A – neochlorogenic acid, B – chlorogenic acid, C – hyperoside, D – 3,5-caffeoylquinic acid, E – 3,4-caffeoylquinic acid]
acid > neochlorogenic acid. The same order could be found almost in each sample. However, leaves collected in Vienna had a higher amount of neochlorogenic acid than 3,4-caffeoylquinic acid. Phenolic components depended on the structural characteristics and showed a correlation between their concentration in the raw material and antioxidant scavenging capacities. The HPLC/ABTS method proposed (post-column assay) possessed the possibility to determine components with substantial activities and fast initial reactions. Consequently, the procedure is suitable for the detection and quantification of fast-acting high-capacity antioxidants to determine the main markers. Hence, this method is worth considering for the primary screening of antioxidants instead of using animals. Another point to consider is that the antioxidant activity markers can be advantageous to provide the quality and effectiveness of the plant raw material with promising health effects.

CONCLUSIONS
The extracts from H. helix leaves have shown the high antioxidant scavenging capacity; the main biomarkers responsible for the activity have been found. The method proposed provides the acceptable determination of every component, as well as the measurement of their activity. Thus, the procedure mentioned can be applied for screening of antioxidant effects in complex mixtures of the plant raw material and herbal medicines.

Conflict of interests: authors have no conflict of interests to declare.

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Information about authors / Відомості про авторів / Сведения об авторах

Bezruk I. V., postgraduate student of the Department of Pharmaceutical Chemistry, National University of Pharmacy (https://orcid.org/0000-0002-1212-1649). E-mail: vania.bezruk@gmail.com

Безрук И. В., аспирант кафедры фармацевтической химии, Національний фармацевтичний університет (https://orcid.org/0000-0002-1212-1649). E-mail: vania.bezruk@gmail.com

Grudko V. O., Candidate of Pharmacy (Ph.D.), associate professor of the Department of Pharmaceutical Chemistry, National University of Pharmacy (https://orcid.org/0000-0003-2221-7887). E-mail: 431230@ukr.net

Грудько В. О., кандидат фармацевтичних наук, доцент кафедри фармацевтичної хімії, Національний фармацевтичний університет (https://orcid.org/0000-0003-2221-7887). E-mail: 431230@ukr.net

Georgiyants V. A., Doctor of Pharmacy (Dr. habil.), professor, head of the Department of Pharmaceutical Chemistry, National University of Pharmacy (https://orcid.org/0000-0001-8794-8010). E-mail: vgeor@ukr.net

Георгіянц В. А., доктор фармацевтичних наук, професорка, завідувачка кафедри фармацевтичної хімії, Національний фармацевтичний університет, (https://orcid.org/0000-0001-8794-8010). E-mail: vgeor@ukr.net

Ivanauskas L., Doctor of Pharmacy (Dr. habil.), professor, head of the Department of Analytical and Toxicological Chemistry, Lithuanian University of Health Sciences (https://orcid.org/0000-0001-5390-2161). E-mail: liudas.ivanauskas@lsmuni.lt

Иванаускас Л., доктор фармацевтичных наук, профессор, заведующий кафедрой аналитической и токсикологической химии, Литовский университет наук здоровья (https://orcid.org/0000-0001-5390-2161). E-mail: liudas.ivanauskas@lsmuni.lt

Mailing address: 4, Valentynivska str., Kharkiv, 61168, Department of Pharmaceutical Chemistry, NUPh. +380668376642. 

E-mail: vania.bezruk@gmail.com

Адреса для листування: 61168, м. Харків, вул. Валентинівська, 4, кафедра фармацевтичної хімії НФаУ. +380668376642. 

E-mail: vania.bezruk@gmail.com

Адрес для переписки: 61168, г. Харьков, ул. Валентиновская, 4, кафедра фармацевтической химии НФаУ. +380668376642. 

E-mail: vania.bezruk@gmail.com

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