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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.

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* lextracts.

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

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Скринінг профілю антиоксидантної активності екстрактів листя Hederahelix за допомогою ВЕРХ/ABTS методу

Hederahelix 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-бані, а також застосовували фармакопейний метод екстракції. Для визначення 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-кофеоїлохінова кислота. Також до речовин з антиоксидантною активністю в листі плюща можна віднести неохлорогенову кислоту, гіперозид, 3,4-кофеоїлохінову кислоту.

Висновки. Отримані результати свідчать про високі антиоксидантні властивості 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

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Скрининг профиля антиоксидантной активности экстрактов листьев *Hederahelix* с использованием ВЭЖХ/ABTS метода

Hederahelix 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.

Цель исследования. Изучение профиля антиоксидантов различных экстрактов листьев плюща с использованием *in vitro* ВЭЖХ метода, объединенного с ABTS реагентом.

Материалы и методы. Экстракцию листьев плюща проводили с помощью различных растворителей от 20 % до 100 % метанола с использованием ультразвуковой бани, а также применяли метод экстракции, описанный в фармакопее. Для определения антиоксидантного профиля использовали Waters хроматограф.

Результаты. С помощью предлагаемого метода было определено почти 90 % веществ, отвечающих за антиоксидантный эффект, среди которых наибольшую активность имели хлорогеновая кислота и 3,5-кофеоилхиновая кислота. Также к веществам, которые обладают антиоксидантными свойствами, можно отнести неохлорогеновую кислоту, гиперозид и 3,5-кофеоилхиновую кислоту.

Выводы. Полученные результаты свидетельствуют о высоких антиоксидантных свойствах экстрактов листьев плюща. Кроме того, предложенный *in vitro* ВЭЖХ метод может быть использован для предварительного скрининга веществ в растительном сырье.

Ключевые слова: листья плюща; антиоксидантная активность; ВЭЖХ; скрининг

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 Akmene), 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-caffeoylquinic acid and 3,4-caffeoylquinic 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-caffeoylquinic acid were the dominant components and explained from 58 % up to 93 % of the total

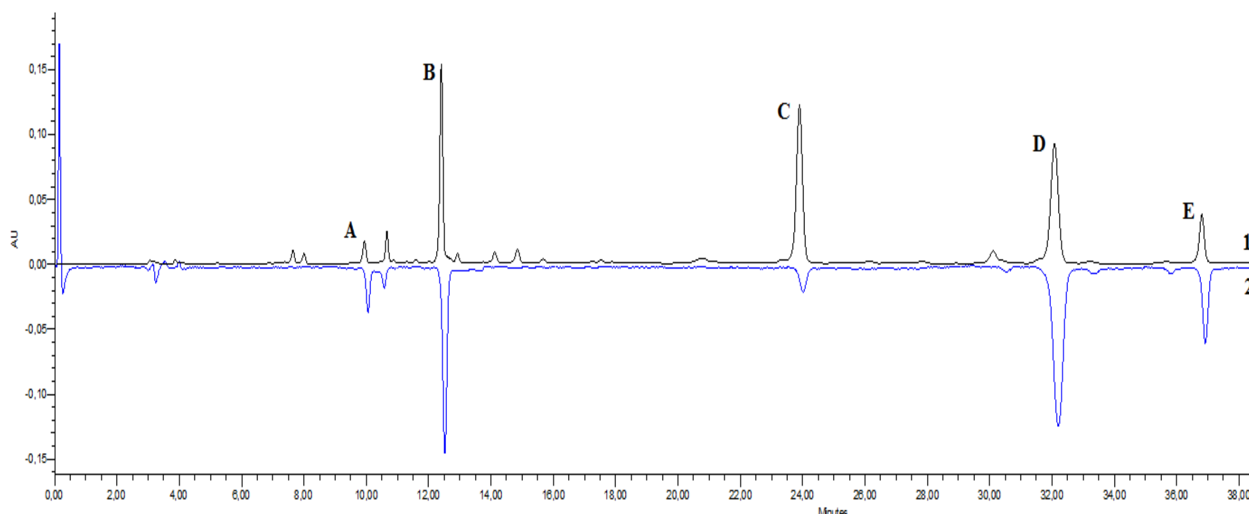

Fig. A typical chromatogram of the antioxidant evaluation (1 – HPLC chromatogram, 2 – HPLC/ABTS chromatogram, A – neochlorogenic acid, B – chlorogenic acid, C –hyperoside, D – 3,5-caffeoylquinic acid, E – 3,4-caffeoylquinic acid

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

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	6
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

Continuation of Table

1	2	3	4	5	6
Naujoje Akmene (50 % methanol)	0.136±0.003	2.513±0.114	1.202±0.057	24.545±1.218	14.628±0.729
Kharkiv (50 % methanol)	1.010±0.048	9.313±0.442	1.713±0.068	14.142±0.683	10.661±0.528
Prague (50 % methanol)	0.719±0.032	2.414±0.117	1.912±0.059	0.960±0.047	0.049±0.002
Vienna (50 % methanol)	1.238±0.049	2.606±0.117	1.054±0.037	3.779±0.185	0.291±0.012
Naujoje Akmene (70 % methanol)	0.367±0.016	3.061±0.149	1.103±0.053	25.095±1.247	39.141±1.839
Kharkiv (70 % methanol)	1.161±0.037	11.927±0.486	1.886±0.073	15.991±0.783	27.851±1.369
Prague (70 % methanol)	1.202±0.054	2.982±0.137	1.877±0.086	2.228±0.093	2.763±0.129
Vienna (70 % methanol)	1.609±0.078	4.216±0.207	1.165±0.052	3.863±0.157	0.704±0.036
Naujoje Akmene (100 % methanol)	0.411±0.017	2.985±0.149	1.128±0.052	26.082±1.283	1.963±0.095
Kharkiv (100 % methanol)	1.352±0.052	11.992±0.591	1.976±0.095	16.094±0.795	4.834±0.227
Prague (100 %methanol)	1.319±0.062	3.180±0.142	1.744±0.075	2.273±0.110	0.751±0.032
Vienna (100 % methanol)	1.889±0.091	5.674±0.271	1.403±0.067	3.814±0.186	1.008±0.043

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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