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The determination of the phytochemical composition of the Altabor substance

The development of medicines based on alder cone extracts led to the introduction of Altan and Altabor medicines into medical practice. The technology of extraction cake from cones has made it possible to obtain extracts with different therapeutic properties.

Aim. To develop an effective method for studying the qualitative composition of the Altabor substance and determine the quantitative content of its components.

Results and discussion. The Altabor substance is a complex mixture of ellagitannins containing more than 70 components. The main components of the extract (or their isomers) were determined by mass spectrometry and by comparing the retention times with the literature data. Gallic, ellagic, valoneic acids dilactone were conclusively determined by adding reference standards of these acids to the extract. The substance contains the following compounds: 2,3-hexahydroxydiphenoyl-(α/β)-glucose t_{r1} = 0.55 min, t_{r2} = 0.89 min (α and β isomers), 4,6-O-[(S)-valeonyl]-D-glucose (isomer) t_r = 0.64 min, gallic acid t_r = 1.198 min, pedunculagin t_{r1} = 3.63 min, t_{r2} = 4.62 min (α and β isomers), proecoxin A (isomer) t_r = 4.78 min, valoneic acid dilactone t_r = 6,19 min, ellagic acid pentoside (isomer) t_r = 7.07 min, ellagic acid t_r = 7.335 min.

Experimental part. The composition analysis was performed using an Agilent 1200 chromatograph with a UV detector, a G6140 mass detector, an Alltech 3300 light scattering detector (ELSD), as well as the Agilent ChemStation Rev.B.04.03 software. The molecular weights of the compounds in the extract were determined using the method of mass spectrometry of ESI-electrospray ionization. The determination of the components was performed using an ultraviolet detector at a wavelength of 280 nm. The column was Rapid Resolution HT Cartige, 4.6 × 30 mm, 1.8 μm, Zorbax SB-C18.

Conclusions. A new effective method of analysis of the Altabor substance has been developed; it allows determining the qualitative and quantitative content of its structural components. The method gives the possibility to control the process of obtaining the Altabor substance, study the dependence of its composition on the conditions of its obtaining, batch number, place, time of the natural raw material collection, and study the composition of other pharmaceutical substances, the plant raw material containing tannins. The advantage of the method is the short time (up to 10 min) of analysis using high-performance liquid chromatography at high resolution.

Key words: gallotannins; ellagitannins; Altabor; chemical composition; structure; HPLC (high-performance liquid chromatography)

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Визначення фітохімічного складу субстанції Альтабор

Розробка лікарських засобів на основі екстрактів суплідь вільхи вперше завершилась впровадженням у медичну практику препаратів Альтан та Альтабор. Особливості технології екстрагування підготовленого шроту суплідь дозволило отримувати екстракти з різними терапевтичними властивостями.

Мета. Розробити ефективний метод вивчення якісного складу субстанції Альтабор із визначенням кількісного вмісту компонентів.

Результати та їх обговорення. Субстанція Альтабор становить собою складну багатокомпонентну суміш елаготанінів, що містить більше 70 компонентів. Основні компоненти екстракту визначено за допомогою мас-спектрометрії, а також шляхом порівняння часів утримування з літературними даними. Галову, елагову, валонову кислоти визначено остаточно шляхом додавання в екстракт стандартів цих кислот. До його складу входять ідентифіковані сполуки: 2,3-гексагідроксидифеноїл-(α/β)-глюкоза t_{r1} = 0,55 хв, t_{r2} = 0,89 хв (α та β ізомери), 4,6-O-[(S)-валеоніл]-D-глюкоза (ізомер) t_r = 0,64 хв, галова кислота t_r = 1,198 хв, педункулагін t_{r1} = 3,63 хв, t_{r2} = 4,62 хв (α та β ізомери), праекоксін А (ізомер) t_r = 4,78 хв, дилактон валонової кислоти t_r = 6,19 хв, пентозид елагової кислоти (ізомер) t_r = 7,07 хв, елагова кислота t_r = 7,335 хв.

Експериментальна частина. Аналіз складу проводили за допомогою Agilent 1200 хроматографа з УФ-детектором, мас-детектором G6140 та детектором світлорозсіювання (ELSD) Alltech 3300. Програмне забезпечення Agilent ChemStation Rev.B.04.03. Молекулярні маси сполук, які входять до складу екстракту, визначено за допомогою методу мас-спектрометрії іонізацією розпиленням в електричному полі (ESI-electrospray ionization). Визначення компонентів проводили із застосуванням ультрафіолетового детектора за довжини хвилі λ 280 нм. Колонка: Rapid Resolution HT Cartige 4,6 × 30 мм, 1,8 мкм, Zorbax SB-C18.

Висновки. Розроблено новий ефективний метод аналізу фармсубстанції Альтабор, який дозволяє вивчати її якісний склад та визначати кількісний вміст компонентів. Метод дозволяє контролювати процес виробництва субстанції Альтабор, вивчати залежність її складу від умов отримання, номеру партії, місця, часу збору природної сировини, вивчати склад інших фармсубстанцій, природної сировини, що містить таніни. Перевага методу – короткий час (до 10 хвилин) проведення аналізу за допомогою високоефективної рідинної хроматографії за високої роздільної здатності.

Ключові слова: галотаніни; елаготаніни; Альтабор; хімічний склад; структура; ВЕРХ (високоефективна рідинна хроматографія)

European alder (*Alnus glutinosa*) and grey alder (*Alnus incana*) are found almost on the whole territory of Ukraine, especially on the forest lowland wetlands and floodplains of many small and large rivers and firstly in the Polissia area [1].

Since ancient times, the interest of folk medicine to this plant has been associated with the healing properties of its bark and cones. The high content of tannins, namely gallo- and ellagitannins, reveals the therapeutic effect of tinctures and decoctions from the parts of this plant [2].

The development of medicines based on alder cone extracts has led to the introduction of such medicines as Altan and Altabor into medical practice. The technology of extraction cake from cones has made it possible to obtain extracts with different therapeutic properties [3, 4].

Thus, the use of 75% aqueous ethanol under the conditions of filtration extraction of European alder (*Alnus glutinosa*) infructescence and grey alder (*Alnus incana*) allows obtaining a dry extract for the preparation of Altan medicine prescribed for diseases of the digestive tract of different localization – for the complex treatment of gastric ulcers and various sections of the stomach and small and large intestine, as well as infections of the digestive tract [5–9]. The hepatoprotective effect of Altan medicine in the case of acute and chronic liver lesions is quite pronounced and manifests itself even when using the drug in low doses [10]. The drug tablet contains 10 mg of the Altan extract calculated with reference to dried substance, and the content of ellagitannins amounts to 60% in it.

Altabor medicine is produced on the basis of the aqueous extract of European alder (*Alnus glutinosa)*. The active ingredients of the dry extract – gallo- and ellagitannins in the composition of the drug have a marked antiviral effect against influenza viruses and herpes. This drug, which tablet contains 20 mg of the dry extract, is used in the treatment of influenza, acute respiratory viral infections, and for the prevention of these diseases [3, 4, 11].

The active agents of both substances of the dry extract of the European alder infructescence exhibit a pronounced antioxidant, anti-inflammatory and membrane-stabilizing action, which complements their pharmacological effects [3, 4].

The technological processes of obtaining these dry extracts in the production of both drugs may alter their true phytochemical composition, and this may be due to their ability to easily hydrolyze gallo- and ellagitannins, constituents of alder cones (*Fructus Alni*).

In connection with this, we should further study the natural phytochemical composition of biologically active substances of European alder, in particular tannins, which, in fact, has not been studied with some exceptions [12]. It is also important to examine the substance from the alder raw material – Altabor since its constant composition of active ingredients, namely tannins, will enable to achieve a guaranteed permanent pharmacological effect of drugs from this substance. The paper [12] presents the isolation of three substances – tannins in the extract from cones by four preparative HPLC methods. The substances were obtained in very small amounts of 1–8 mg. The qualitative HPLC chromatograms of alder cone extracts, which would determine their component composition, are not described in the literature. Moreover, in Ukraine, the studies to determine the phytochemical composition of the natural raw material containing hydrolyzable tannins, as well as pharmaceuticals manufactured based on this raw material, have not been carried out at all. Only the total tannin content was determined. Altabor and Altan have been manufactured since 1990s by the PJSC SIC "Borshchahivskiy CPP". The company previously made attempts to obtain high-quality HPLC chromatograms, which would allow studying the qualitative composition of these substances, as well as the raw material, and determine the quantitative content of components, but all of them were unsuccessful [13]. The phytochemical composition of the Altabor substance is unknown. To date, the quality of the Altabor substance is determined by measuring the total amount of tannins by spectrophotometry. The raw material, i.e. the alder cones, has been studied similarly. It is important to find the phytochemical composition of the raw material since its composition can be influenced by such factors as soil properties, the time of the raw material collection, climatic and other conditions under which alder grows [14]. This can affect the manufacturing process. To separate a mixture containing more than 70 substances with the similar chromatographic properties is a complicated task [15]. Therefore, the results presented in this paper are important.

Thus, the aim of this work was to develop an effective method for studying the qualitative composition of the Altabor substance and determine the quantitative content of its components.

To determine the composition, the crushed sample of the Altabor substance (batch No. 301217) was extracted with methyl alcohol. As a method of analysis of the extract obtained the method of high-performance liquid chromatography using an Agilent 1200 chromatograph was proposed.

The extract is a complex multicomponent mixture of tannins with very similar chromatographic properties, which is difficult to separate. In order to select the conditions for successful separation of the extracted ellagitannins, a suitable chromatographic column and solvent systems for elution, as shown in the ex-

perimental part, were selected. On the way to achieve the desired result, namely the creation of the HPLC method that would allow determining the Altabor substance composition, a number of isocratic solvent systems were tested, for example: H_2O:CH_3CN in the ratio of 90:10; H_2O:CH_3CN:THF:HCOOH in the ratio of 70:5:25:0.2; H_2O:CH_3CN:THF:HCOOH in the ratio of 90:5:5:0.2; 0.1N H_3PO_4:0.1N KH_2PO_4:CH_3CN in the ratio of 42.5:42.5:15; H_2O:CH_3CN:THF:HCOOH in the ratio of 80:5:15:0.2, as well as gradient systems, for example: H_2O:CH_3CN with various linear gradients – 0–30; 0–50; 0–100 and others. However, the systems presented in the article turned out to be much better.

For the analysis of such polar substances as tannins it is better to use the chromatographic columns filled with C18 modified silica gel. One should choose the smallest size of particles among available – 1.8 μm. Resolution decreases when 5 μm columns are used.

According to the results of the chromatographic and mass spectrometric studies the Altabor substance is a complex mixture of ellagitannins containing more than 70 components (see chromatogram, Fig. 1). The main components of the substance (or their isomers) were determined by mass spectrometry and by comparing the retention times with the literature data [16–18]. Gallic (**3**), ellagic (**8**), valoneic acid dilactone (**6**) were conclusively determined by adding reference standards to the substance. The reference standard of valoneic acid dilactone with a purity of more than 98% was obtained by the method described in the experimental part. Its ^{1}H NMR spectrum and HPLC are shown in Fig. 14, 15. To determine gallic and ellagic acids, commercially available Sigma-Aldrich samples were used.

The HPLC-MS experiments revealed that the Altabor substance contains the following compounds (Fig. 1, 2): 2,3-hexahydroxydiphenoyl-(α/β)-glucose (**1**), t_{r1} = 0.55 min, t_{r2} = 0.89 min (α and β isomers) (Fig. 3); 4,6-O-[(S)-valeonyl]-D-glucose (an isomer) (**2**), t_r = 0.64 min (Fig. 4); gallic acid (**3**), t_r = 1.198 min; pedunculagin (**4**), t_{r1} = 3.63 min, t_{r2} = 4.62 min (α and β isomers) (Fig. 5)[a]; praecoxin A (**5**) (an isomer), t_r = 4.78 min (Fig. 6); valoneic acid dilactone (**6**),

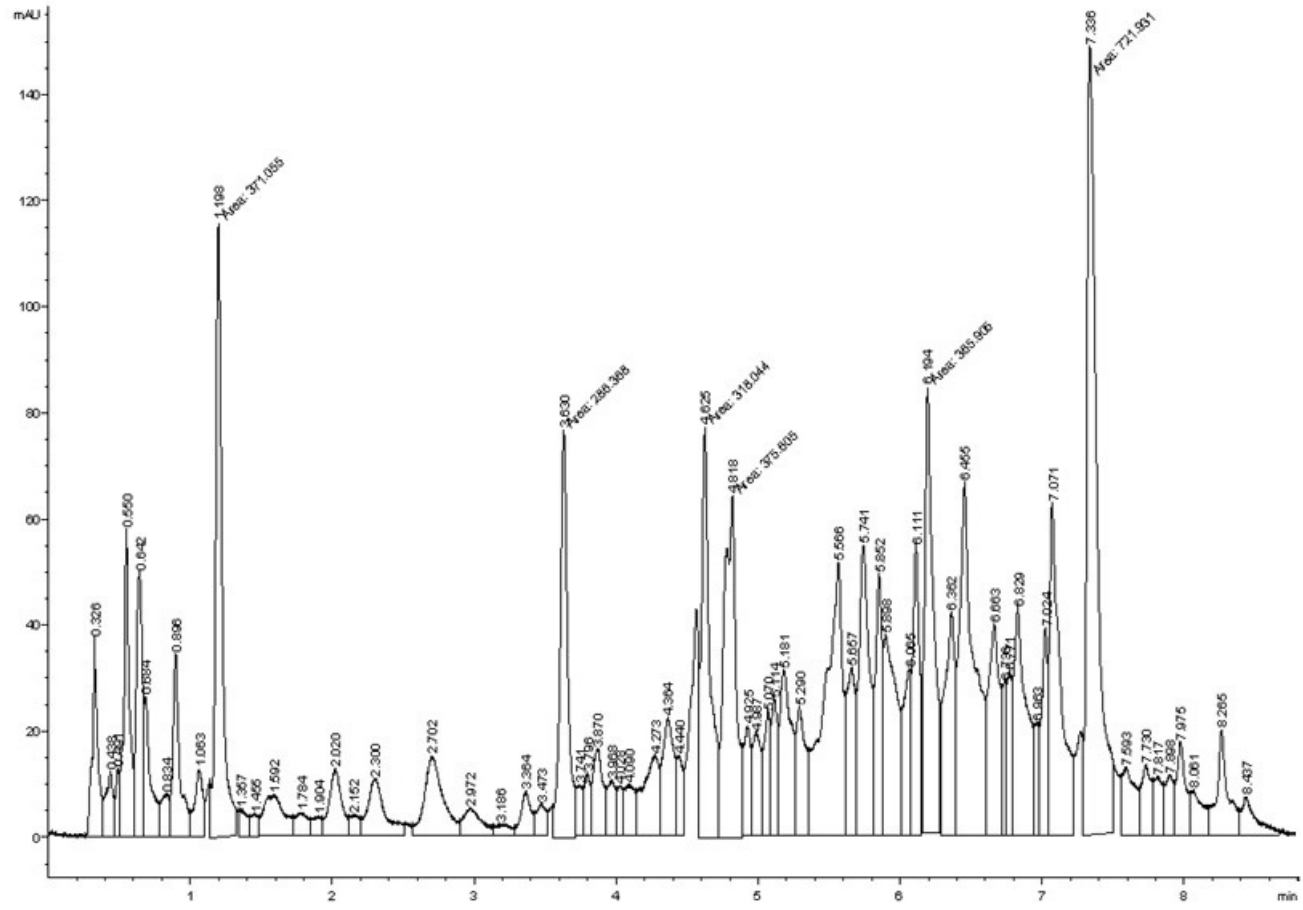

Fig. 1. The chromatogram of the Altabor substance (Batch No. 301217). Methanol Extract. System I. (A) 0.1% HCOOH (aqueous solution), (B) CH_3CN. 0-1.5 min, 100% A (isocratic mode); 1.5–7.5 min, 0-20% B in A (linear gradient); flow-rate – 1 mL/min, 280 nm

[a] Mass spectra of the compounds with t_r =3.63 min and 4.62 min are the same. The areas of two peaks with t_r = 3.63 min and t_r = 4.62 min are almost identical. Having compared the retention times with the literature data we can conclude that two peaks on the chromatogram probably belong to the same substance – α and β pedunculagine isomers (**4**).

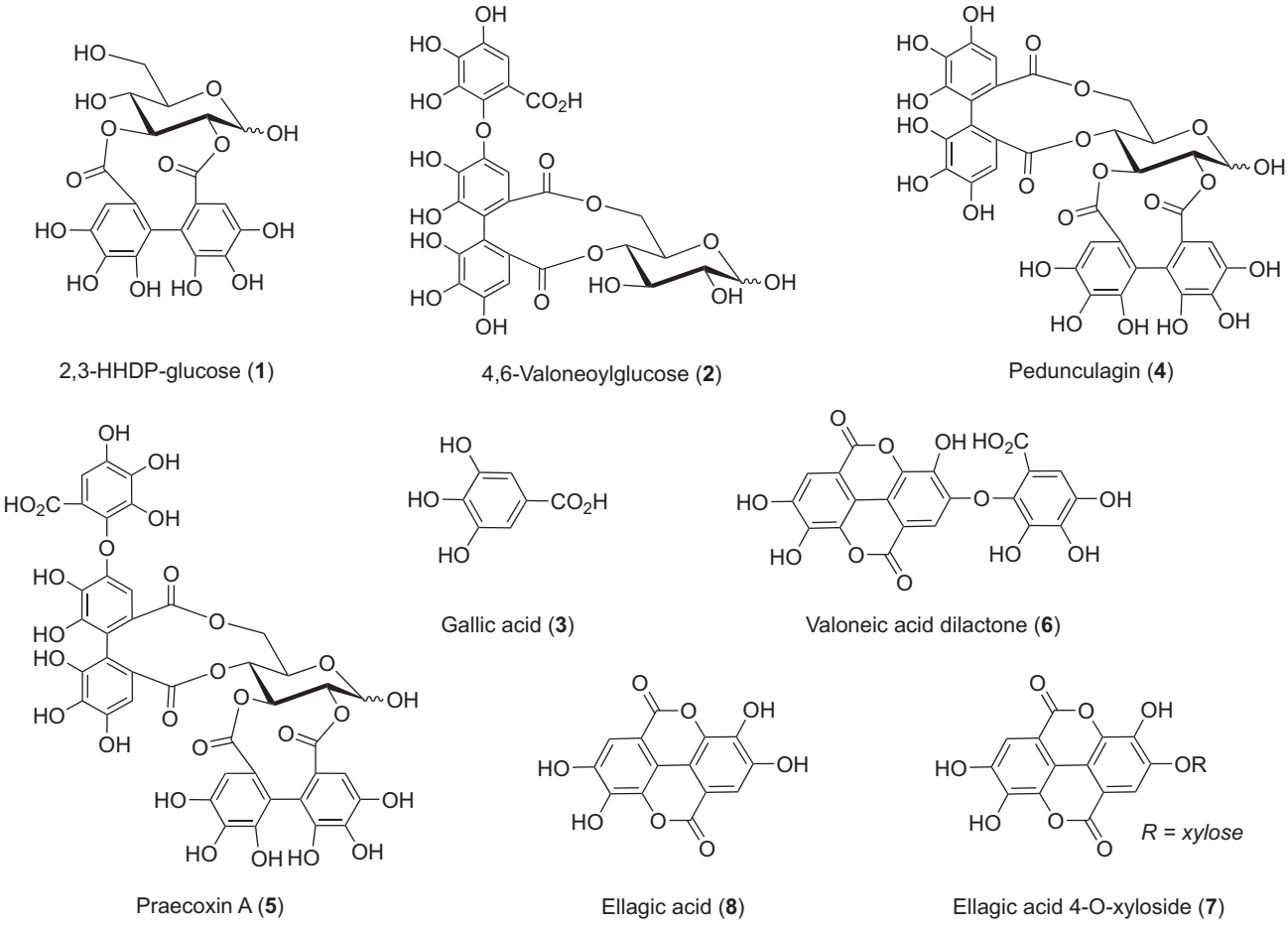

Fig. 2. The main components of the Altabor substance

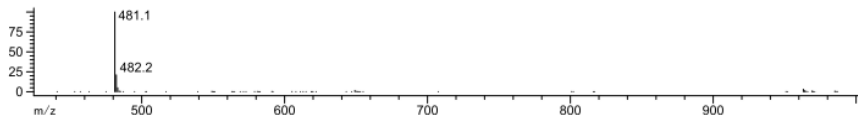

Fig. 3. The negative ion mass spectrum of the compound **1**

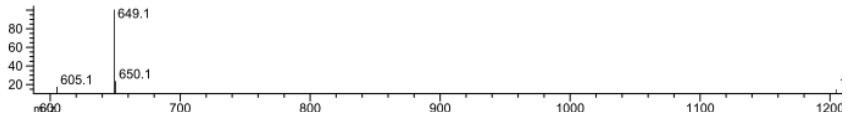

Fig. 4. The negative ion mass spectrum of the compound **2**

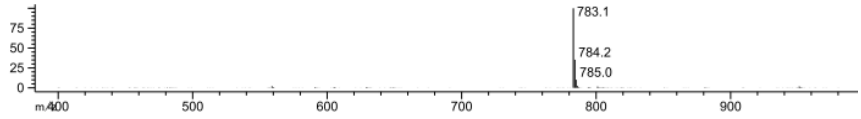

Fig. 5. The negative ion mass spectrum of the compound **4**

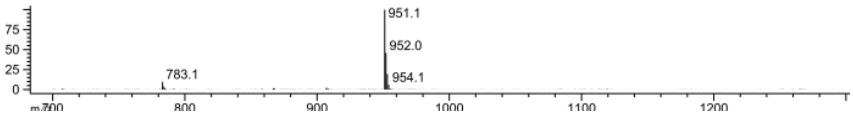

Fig. 6. The negative ion mass spectrum of the compound **5**

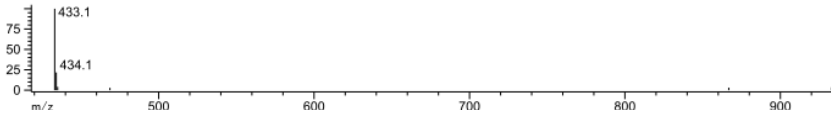

Fig. 7. The negative ion mass spectrum of the compound **7**

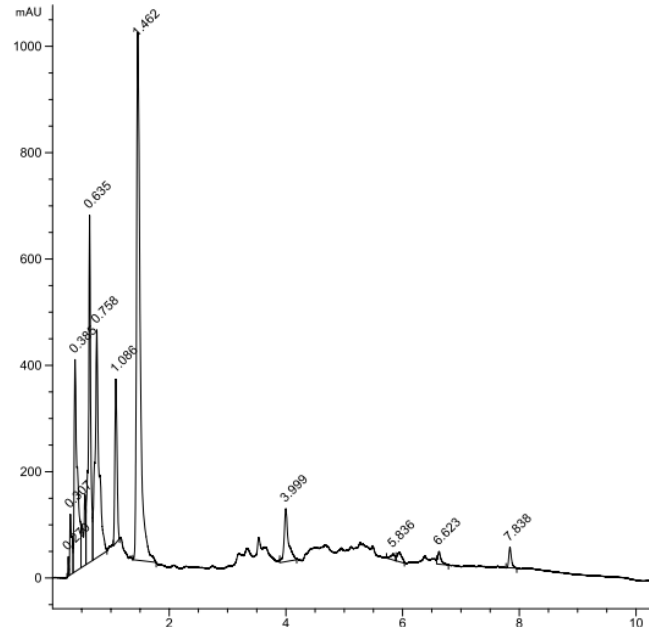

Fig. 8. The chromatogram of the Altabor substance fraction 1 (Diaion HP-20, pure water) (system I)

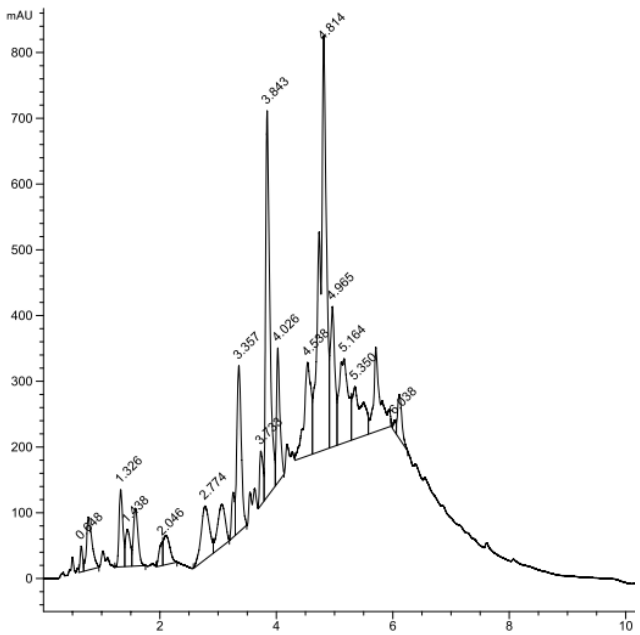

Fig. 9. The chromatogram of the Altabor substance fraction 2 (Diaion HP-20, 10% EtOH) (system I)

t_r = 6.19 min; ellagic acid pentoside (most probably xyloside, an isomer) (**7**), t_r = 7.07 min (Fig. 7); ellagic acid (**8**), t_r = 7.335 min. The Altabor extract also contains a mixture of hexoses and possibly pentoses, which are not fixed by the UV detector and are not retained by the C-18 column phase. These compounds can be detected on a light scattering detector at the beginning of the chromatograms with t_r = 0.3 min in the form of a mixture.

The Altabor substance was additionally fractionated by column chromatography with a Diaion HP-20 sorbent. While fractioning the starting substance gave fractions containing much less number of compounds than the raw material. Thus, it is easier to analyze such mixtures. It becomes possible to obtain the minor compounds characteristics, which small peaks are hidden by other peaks on the raw material chromatogram. Moreover, re-determination of the molecular masses in fractions is an additional proof of the presence of the compounds previously found.

As an eluent the aqueous solution of ethyl alcohol of different concentrations was used. The concentration of ethyl alcohol was increased stepwise. As the result 5 fractions were obtained. The fraction analysis was performed by HPLC, in particular fraction 1 was obtained by elution of the Altabor substance with water

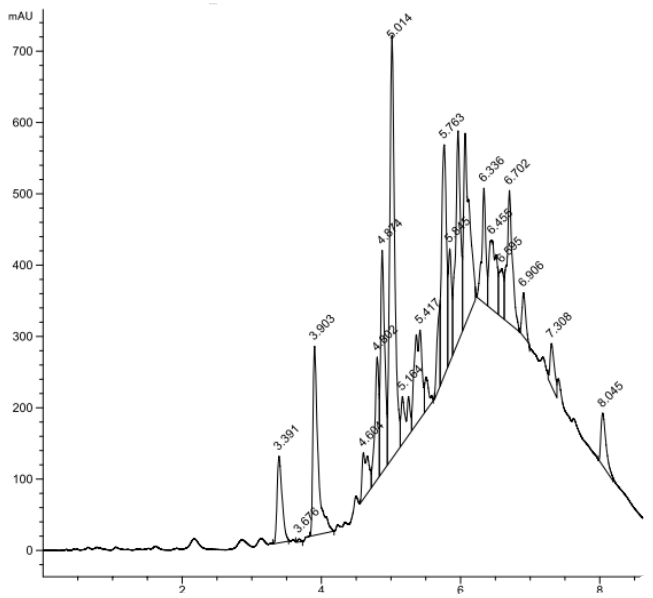

Fig. 10. The chromatogram of the Altabor substance fraction 3 (Diaion HP-20, 20% EtOH) (system I)

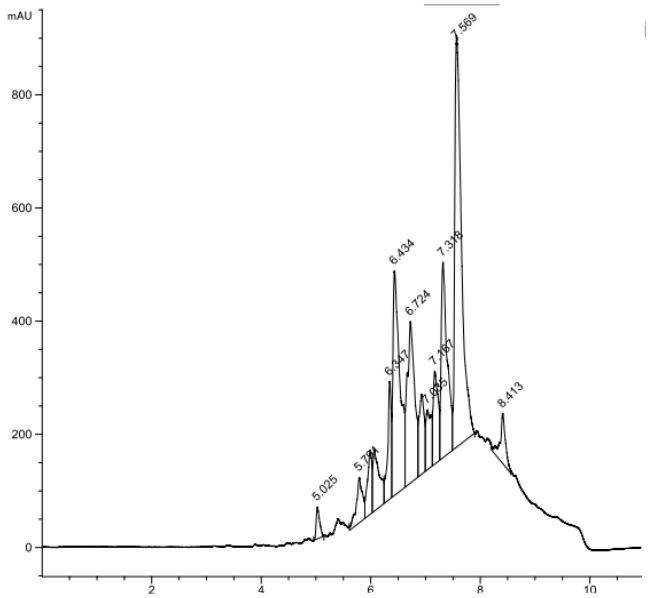

Fig. 11. The chromatogram of the Altabor substance fraction 4 (Diaion HP-20, 40% EtOH) (system I)

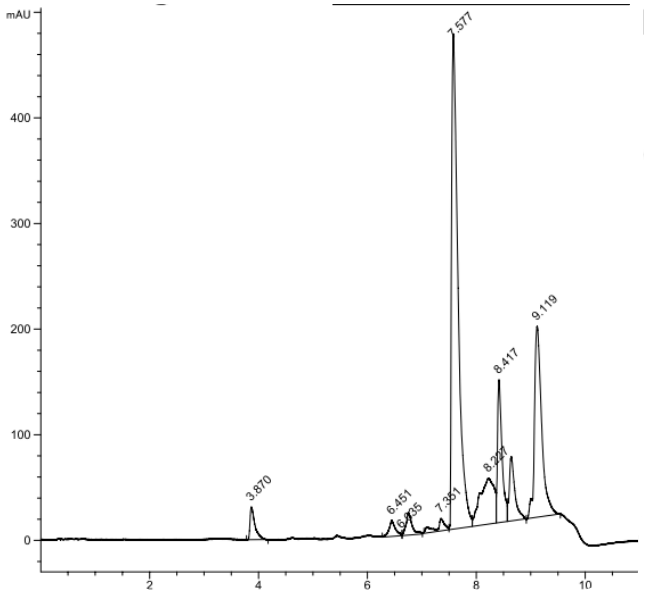

Fig. 12. The chromatogram of the Altabor substance fraction 5 (Diaion HP-20, 96% EtOH) (system I)

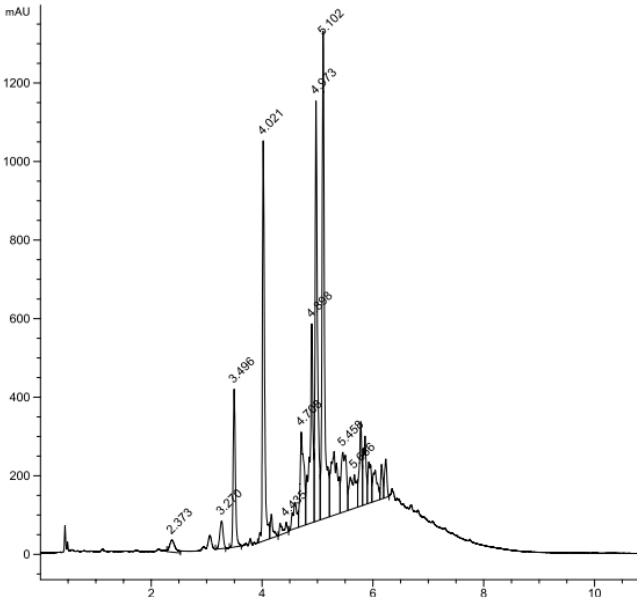

Fig. 13. HPLC repeated chromatographic (Diaion HP-20, 20% EtOH) (system I) separation of fraction 3 (elution with 20% EtOH)

(Fig. 8), fractions 2, 3, 4, 5 by elution with 10%, 20%, 40% and 96% ethyl alcohol, respectively (Fig. 9–12).

The order of the substance release (ellagitannins from the column filled with Diaion HP-20) was similar to the order of the substance release from the HPLC column filled with a sorbent – C18 modified silica gel. The main components of the fraction 1 (Fig. 8) were 2,3-hexahydroxydiphenoyl-(α/β)-glucose (**1**), t_{r1} = 0.63 min, t_{r2} = 1.08 min (α and β isomers), 4,6-O-[(S)-valeonyl]-D-glucose (**2**) (an isomer), t_r = 0.76 min, gallic acid (**3**), t_r = 1.46 min; the fractions 2 and 3 (Fig. 9 and Fig. 10, respectively) – pedunculagin (**4**), t_{r1} = 3.84 min, t_{r2} = 4.81 min (α and β isomers), praecoxin A (**5**), t_r = 4.96 min; fractions 4 and 5 – ellagic acid (**8**) and

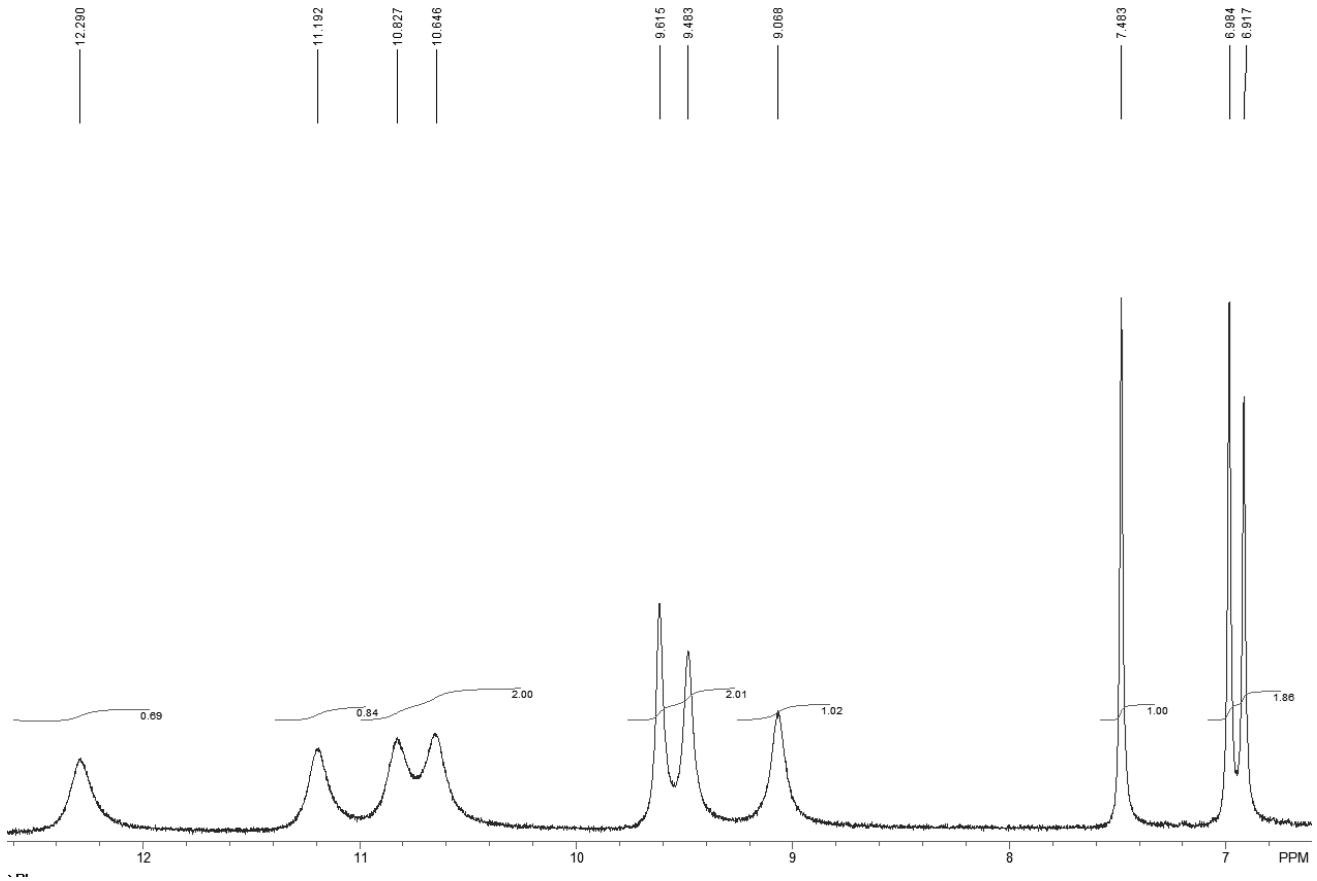

Fig. 14. ^{1}H NMR spectrum of valoneic acid dilactone in DMSO-d_6

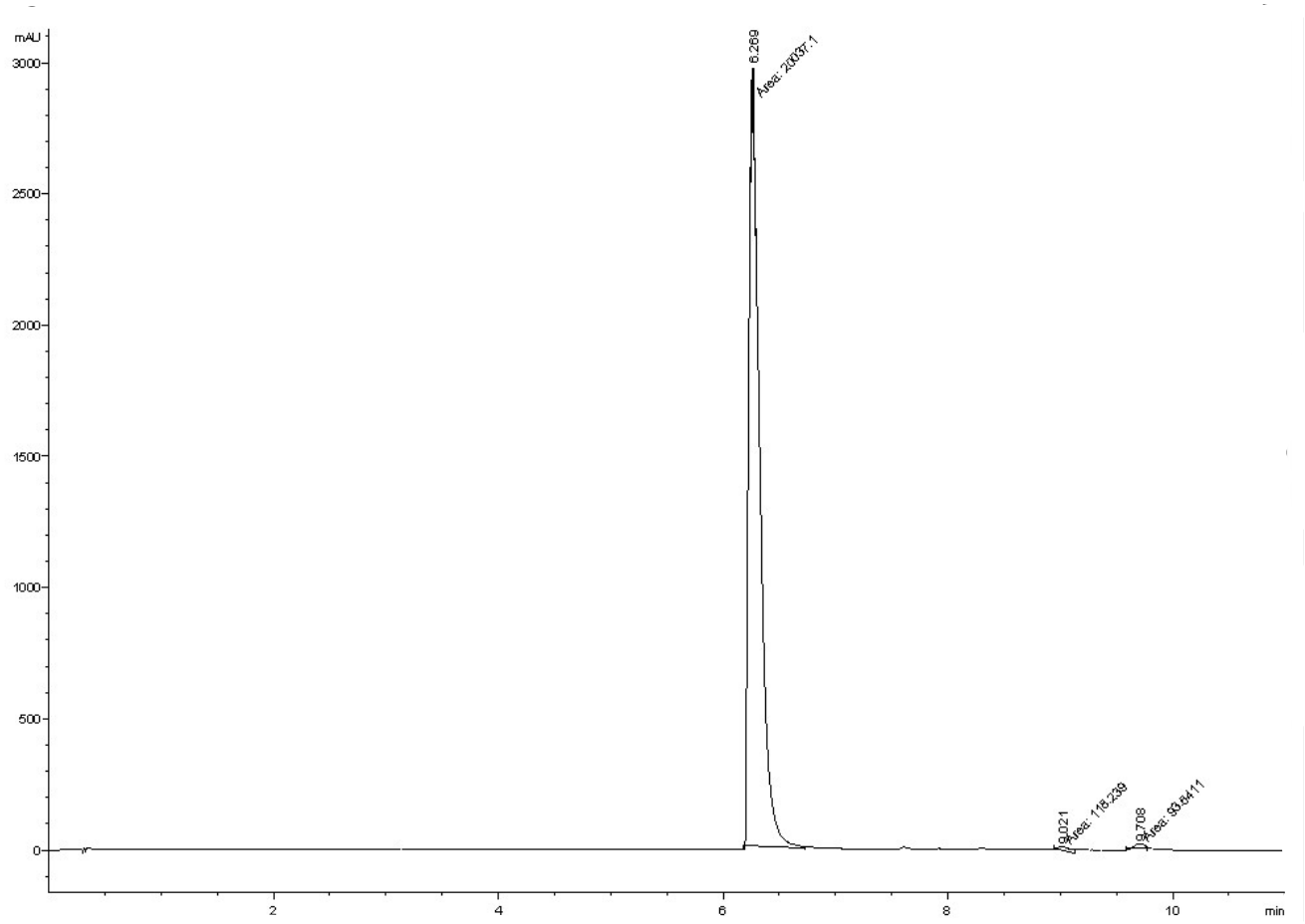

Fig. 15. HPLC of the valoneic acid dilactone reference standard

valoneic acid dilactone (**9**), ellagitannins with molecular weights of 934 Da and 936 Da.

The main components of the purified fraction 3 of tannins were pedunculagin (**4**) and praecoxin A (an isomer) (**5**) (Fig. 13). The resulting concentrate is promising for further isolation of these compounds required as standards for the study of their quantitative content in the Altabor substance.

A preparative method for valoneic acid dilactone isolation from the Altabor substance hydrolyzed in acidic conditions was also developed. It can be used to obtain a reference standard of this compound. The ^{1}H NMR spectrum of the valoneic acid dilactone isolated and its HPLC are shown in Fig. 14 and 15, respectively.

Experimental part

Equipment

The composition analysis was performed using an Agilent 1200 chromatograph with a UV detector, a G6140 mass detector, an Alltech 3300 light scattering detector (ELSD), as well as the Agilent ChemStation Rev.B.04.03 software.

The molecular weights of the compounds in the extract were determined using the method of mass spectrometry of ESI-electrospray ionization – a gentle ionization method that allowed obtaining molecular ion peaks of complex ellagitannin molecules without destroying them. Formic or trifluoroacetic acid should be used to ionize the molecules in the extract, as their use improves the resolution. One can also use ammonium acetate or ammonium formate.

The determination of the components was performed using an ultraviolet detector at a wavelength of 280 nm.

The column was Rapid Resolution HT Cartige, 4.6 × 30 mm, 1.8 μm, Zorbax SB-C18.

^{1}H NMR spectrum was taken with a Varian VNMRS 400 MHz spectrometer, using TMS as an internal standard in DMSO-d_6 solution.

Mobile phase systems

System I: (A) 0.1% HCOOH (aqueous solution), (B) CH_3CN. 0–1.5 min, 100% A (isocratic mode); 1.5–7.5 min, 0–20% B in A (linear gradient); flow-rate – 1 mL/min.

System II: (A) 0.05% CF_3COOH (aqueous solution), (B) CH_3CN. 0–1.5 min, 100% A (isocratic mode); 1.5–7.5 min 0-20% B in A (linear gradient); flow-rate – 1 mL/min.

System III: (A) 0.1% HCOOH, (B) CH_3CN. 0–1.5 min, 100% A (isocratic) 1.5–3 min, 0-5% B in A (linear

gradient); 3–4.5 min, 5% B (isocratic); 4.5–9 min, 5–20% B in A (linear gradient); flow-rate – 1 mL/min.

System IV: (A) 0.1% HCOOH (aqueous solution), (B) CH_3CN. 0–3 min, 100% A (isocratic); 1.5–7.5 min, 0-20% B in A (linear gradient); flow-rate – 1 mL/min.

Preparation of the methanol extract of the Altabor substance. The sample of of the Altabor substance (6.0 g) was mixed with 120 mL of methyl alcohol for 3 h. The dark solution was filtered, and the solvent was evaporated in vacuum at a temperature below 40°C. A dark solid product was obtained. The yield was 4.2 g.

Fractionation of the Altabor substance with Diaion HP-20 sorbent. A column: inner diameter d = 1.5 cm, total volume v = 70 mL, the height of the sorbent column h = 25.5 cm, weight of the resin – 20 g. The Altabor substance (700 mg) was dissolved in 1.5 mL of distilled water with stirring. The solution was filtered and applied to the top layer of the sorbent. Elution of the column was carried out at a rate of 1.5 mL per minute. The first elution was carried out with pure water. Fraction 1 was collected in a volume of 110–120 mL. Then elution by 10%, 20%, 40%, 96% ethyl alcohol was successively carried out. Thus, fractions 2, 3, 4, 5 in volumes of 110–120 mL were collected. All fractions were evaporated in vacuum at 20 mmHg on a water bath with a temperature below 40°C.

Valoneic acid dilactone (reference standard). The Altabor substance (2 g) was dissolved in the ethyl alcohol-water mixture (1:2). Then 7 mL of the concentrated hydrochloric acid were added to the solution, and it was refluxed for 4 h. After the resin was separated, the solvents were evaporated. As the result, a crystalline product, being preferably a mixture of valoneic acid dilactone and ellagic acid, was obtained. The resulting product (900 mg) was refluxed for 30 min in 10 mL of dioxane. The hot solution was filtered, while valoneic acid dilactone remained in the solution. The filtrate was left to crystallize. The precipitate was filtered. The procedure was repeated 3–4 times to obtain valoneic acid dilactone with a purity of more than 98% (HPLC control). The yield was 50 mg.

Conclusions

A new effective method of analysis of the Altabor substance has been developed; it allows determining the qualitative and quantitative content of its structural components. The method gives the possibility to control the process of obtaining the Altabor substance, study the dependence of its composition on the conditions of its obtaining, batch number, place, time of the natural raw material collection, and study the composition of other pharmaceutical substances, the plant raw material containing hydrolysable tannins. The advantage of the method is the short time (up to 10 min) of analysis using high-performance liquid chromatography at high resolution.

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