DEVELOPMENT OF PHENOLIC COMPOUNDS CHROMATOGRAPHIC IDENTIFICATION IN BILBERRY SHOOTS

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Summary: the methodology of bilberry shoots official herbal raw material identification by the method of thin layer chromatography was developed. It was proposed to perform the identification by the means of the appearance of chromatographic profile of the raw material methanol withdrawal comparison with the standard zones of rutin position, chlorogenic acid, hyperoside and quercitrin. The formic acid – water – ethyl acetate (6: 9: 90) mixture was proposed as the aimed mobile phase.

Key words: bilberry shoots, identification, TLC, HPLC, quercitrin, chlorogenic acid, rutin, hyperoside.

Introduction. Bilberry shoots have hypoglycaemic properties and are used for easy forms of diabetes as in the form of plant raw materials and as part of various charges, such as Arfazetyn and others [1-4]. Hypoglycaemic activity of bilberry fruit extract, rich in phenolic compounds, was shown on the model of streptozotocin diabetes in mice [5]. A number of other types of activity, such as antiradical, antiinflammatory and anti-inflamatory are well studied concerning bilberry fruit and its extracts [5-7]. Hypoglycaemic properties of fruit are associated with phenolic compounds content, including anthocyanins [5, 8].

Shoots of bilberries are considered as herbal substance that contains tannins, although their presence is not associated with mild hypoglycaemic effect. It is known that the leaves of bilberry contain tannins, hydroxycinnamic acids, flavonoids, triterpene acids and vitamins. The highest content of phenolicarboxylic acids and flavonoids is observed for shoots harvested in summer, while the content of catechins and tannins is higher for raw materials harvested in autumn [9]. Qualitative and quantitative composition of flavonoids and hydroxycinnamic acids of bilberry shoots was investigated in numerous works [8, 10-13].

Standardization of bilberry shoots as herbal substance is still going to be important because it is still little studied and in the State Pharmacopoeia of Ukraine there is no corresponding monograph. Manufacturers of bilberry shoots official herbal raw material perform their identification by means of a quality reaction on tannins with ammonium iron (III) sulphate, which is a nonspecific method. Moreover, taking into consideration the literature data and a various biological activity hydroxycinnamic acids and flavonoids it is necessary to study their composition for raw materials growing within the territory of Ukraine.

The objective of our work was investigation of bilberry shoots phenolic compounds with the help of chromatographic methods, development of methodology of their identification, selection of identification markers.

Investigation methods. The qualitative composition of four bilberry shoots samples that were harvested in summer in the following regions: 1 – Transcarpathia, 2 – Ternopil, 3 – Volyn, 4 – Ivano-Frankivsk, and five samples of “Shoots of bilberries” therapeutic agent, produced in PLC “Liktravy”, Zhytomyr city was investigated.

The qualitative composition of phenolic compounds was studied by the methods of thin layer and high performance liquid chromatography. Chromatographic plates Silica gel 60 F254 (‘Merck’, Germany), chromatographic chamber ‘CAMAG’, an instrument for spotting Linomat 5 (‘CAMAG’, Switzerland), lamp for observing chromatograms in ultraviolet light ‘CAMAG’ were used for investigations by TLC method. Agilent 1200 liquid chromatograph with diode array detector (‘Agilent’, the USA) was used for HPLC investigations.

Standard samples of caffeic and chlorogenic acids (Fluka), rutin, hyperoside, quercitin, isoquercitin, quercetin, kaempferol, luteolin, naringenin, isorhamnetin, myricetin and apigenin (Sigma, Fluka) were used for identification of phenolic compounds. Exact dispensing (3 mg of acids or aglycones and 5 mg of flavonoids glycosides) of standard samples were dissolved in 10 ml of methanol.

The tested solutions for TLC and HPLC investigations of glycoside forms of flavonoids were being prepared by boiling of 1 g of the powdered raw material with 25 ml of methanol under reflux on a water-bath for 1 hour.

While investigating flavonoids glycosides the TLC chromatograph – was performed in two solvent systems: 1 – formic acid – water – ethyl acetate (6:9:90); 2 – formic acid – glacial acetic acid – water – ethyl acetate (7.5: 7.5: 17: 67.5). 5 μl for standards and 15 μl for the tested solutions were applied as 7.5 mm band. Solvent front passed over a path of 15 cm from the start.

The tested solutions for the same content of aglycone investigations were prepared by the following algorithm:
The flavonoids aglycone content studying of nine bilberry shoots samples allowed to identify aglycones: quercetin as a zone of very intense orange fluorescence and kaempferol as a zone of weak yellow-blue fluorescence. In the described sample processing conditions there are other fluorescent zones on the TLC chromatogram. Thus, the main bilberry shoots aglycones flavonoids, which grows within the territory of Ukraine and is industrially stored up, is quercitin.

As described for HPLC chromatographic conditions nine selected samples of bilberry shoots were investigated. Examples of chromatograms for the tested solutions of some regional and industrial samples are shown in Figures 1 and 2.

The results of TLC about the presence of quercetin, hyperoside, traces of rutin, as well as chlorogenic and traces of caffeic acid in significant amount in all studied samples of bilberry shoots were confirmed by HPLC analysis. HPLC investigation of bilberry shoots samples after hydrolysis confirms the presence of quercetin as a dominant aglycone and traces of kaempferol.
### Table 1. Results of TLC-investigation of bilberry shoots flavonoid glycosides and hydroxycinnamic acids

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<thead>
<tr>
<th>Ref. sol. 1</th>
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<td><strong>Mobile phase 1. formic acid – water – ethyl acetate (6:9:90)</strong></td>
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<td><strong>Mobile phase 2. formic acid – glacial acetic acid – water – ethyl acetate (7.5: 7.5: 17: 67.5)</strong></td>
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**Note.**
1. Ref. sol. 1: isoquercitrin.
2. Ref. sol. 2: rutin, chlorogenic acid, hyperoside, quercitrin, caffeic acid.
Table 2. Chromatographic properties of aglycone in different solvent systems

<table>
<thead>
<tr>
<th>Aglycone</th>
<th>The color zones</th>
<th>Dimension of $R_f$ in mobile phase</th>
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<td>myricetin</td>
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<td>quercetin</td>
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<td>0,20</td>
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<td>luteolin</td>
<td>yellow</td>
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<tr>
<td>apigenin</td>
<td>yellow-green</td>
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<tr>
<td>kaempferol</td>
<td>yellow-blue</td>
<td>0,39</td>
</tr>
<tr>
<td>isorhamnetin</td>
<td>yellow-blue</td>
<td>0,41</td>
</tr>
<tr>
<td>naryngenin</td>
<td>blue</td>
<td>0,48</td>
</tr>
</tbody>
</table>

Fig. 1. HPLC-chromatogram of Ternopil region raw material test solution ($\lambda=330$ nm).

Fig. 2. HPLC-chromatogram of industrial series of raw materials (sample 7) test solution ($\lambda=330$ nm).
Analysis of drugs

The obtained data from investigations of phenolic compounds qualitative content of Ukrainian bilberry shoots samples indicate a difference from raw material samples that were investigated and described earlier [12, 13]. The main representative of bilberry shoots flavonoids according to [12] is hyperoside, and according to [13] – rutin. In the investigated Ukrainian samples six flavonoids glycosides are presented two of which (unidentified) are with intense fluorescence zones between the rutin and chlorogenic acid zones – hyperoside and quercitrin, and the other two – (unidentified) that are placed under and over the quercitrin zone. Conditions of TLC-identification of bilberry shoots were proposed by [10] authors; caffeic acid and quercetin-3-O-β-D-xylopyranoside have been identified among 4 defined phenol compounds.

The difference between Ukrainian samples analysis results and data of [10-13] authors is obviously caused by not only differences in sample preparation and sensitivity of the methods used, but also indicates the quality different from bilberry shoots phenolic compounds that grow in different conditions.

Therefore, TLC identification method of bilberry shoots was developed for medicinal plants growing in Ukraine.

**Method of bilberry shoots phenolic compounds identification.**

**Test solution.** Place 1.0 g of the powdered drug in a 50 ml flask and add 25 ml of methanol. Heat under a reflux condenser on a water-bath for 1 hour. Allow to cool and filter.

**Reference solution.** Dissolve 3 mg of the chlorogenic acid, 5 mg of rutin, 5 mg of hyperoside, 5 mg of quercitrin in 10 ml of methanol.

**Plate:** TLC silica gel plate.

**Mobile phase:** formic acid, water, ethyl acetate (6:9:90 V/V).

**Application:** 15 μL, as 7,5 mm bands.

**Development:** over a path of 15 cm.

**Drying:** in air.

**Detection:** heat at 100º C for 3 min; spray the plate whilst still hot with a 10 g/l solution of diphenylboric acid aminoethyl ester in methanol and then with a 50 g/l solution of macrogol 400 in methanol; allow to dry in air for about 30 min; examine in ultraviolet light at 365 nm.

**Results:** see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, others faint zones may be present in the chromatogram obtained with the test solution.

**Conclusions.**

All bilberry shoots analyzed samples contain the rutin, chlorogenic acid, hyperoside, quercitrin, that have been identified with the help of TLC and HPLC methods.

TLC identification method of bilberry shoots was developed and these compounds were proposed as identification markers.

Caffeic acid and isoquercitrin are contained in the studied samples of raw materials in small quantities, so they aren’t recommended as obligatory identification markers.
Аналіз лікарських препаратів

Literature
1. Державний реєстр лікарських засобів України http://www.drlz.kiev.ua

ВИВЧЕННЯ МОЖЛИВОСТІ ІДЕНТИФІКАЦІЇ ПАГОНІВ ЧОРНИЦІ ХРОМАТОГРАФІЧНИМ МЕТОДОМ ЗА СКЛАДОМ ФЕНОЛЬНИХ СПОЛУК
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Тернопільський державний медичний університет імені І. Я. Горбачевського
Ключові слова: пагони чорници, ідентифікація, ТШХ, ВЕЖХ, кверцитрин, кислота хлорогенова, рутин, гіперозид.

ИЗУЧЕНИЕ ВОЗМОЖНОСТИ ИДЕНТИФИКАЦИИ ПОБЕГОВ ЧЕРНИКИ ХРОМАТОГРАФИЧЕСКИМ МЕТОДОМ ПО СОСТАВУ ФЕНОЛЬНЫХ СОЕДИНЕНИЙ
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Тернопольский государственный медицинский университет имени И.Я. Горбачевского
Ключевые слова: побеги черники, идентификация, ТСХ, ВЭЖХ, кверцитрин, кислота хлорогеновая, рутин, гиперозид.

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