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ISOLATION OF BENZOPHENANTHRIDINE ALKALOIDS FROM *MACLEAYA* LEAVES WITHOUT USING TOXIC SOLVENTS

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This study aims to develop a new method for isolation of benzophenanthridine alkaloids from Macleaya leaves, which do not use toxic or ecologically dangerous solvents or reagents. According to this method, the extraction of alkaloids from the plant material is performed with 90% ethanol by percolation at 40±2 °C until 5–8 parts (V/M) of extract is obtained. The extract is acidified with sulphuric acid to pH 4.0–4.5 for sedimentation of ballast substances. After filtration or centrifugation, sulphuric acid is added to the extract to obtain the concentration of 0.1–0.12 M. Crystallisation of bisulphates of alkaloids occurs during 14 days at room temperature, then is finished in 7 days at 2–8 °C. The crude product is separated by filtration, washed with 96% ethanol and dried.

Upon purification, alkaloids pass into a concentrated aqueous solution in the form of sulphates. A major part of impurities is removed by sedimentation, the remainder – by sorption on activated carbon. Than alkaloids are crystallised again in the form of bisulphates or other salts as required.

The procedure has been created to obtain the sum of benzophenanthridine alkaloids of Macleaya in form of salicylates. This substance, named "Sanguirisal", is proposed as an active substance for preparation of pharmaceutical forms for topic administration, being much more lipophilic than sanguiritrine.

Keywords: *Macleaya microcarpa* (Maxim.) Fedde, benzophenanthridine alkaloids, sanguiritrine, sanguirisal.

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Introduction

The development of new antimicrobial products of plant origin is continually increasing. The background of the problem is the acquired resistance of pathogenic microorganisms to most traditional used antibiotics [1, 2]. Plants from genera *Sanguinaria*, *Chelidonium*, *Bocconia*, *Macleaya* of the *Papaveraceae* family are known to be sources of benzophenanthridine alkaloids, possessing antibacterial, antimycotic [3–6] and antiparasitic properties [7]. Also, was identified that Macleaya alkaloids have antitumor activity, especially being prescribed for sanguinarine [8, 9], chelerythrine [10], as well as for the crude extract [11], anticholinesterase [12] and catabolic protease inhibitory properties [13]. Species of *Macleaya microcarpa* (Maxim.) Fedde and *Macleaya cordata* (Willd) R. Br., considered as officinal plants [14], are used in the pharmaceutical industry for the production of the sanguiritrine (active substance) as well as some extractive preparations.

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The substance "Sanguiritrine" (the sum of benzophenanthridine alkaloids, the main ones being sanguinarine and chelerythrine, in the form of bisulphates) was authorised for application to clinical practice in 1967 (minutes of the meeting of the USSR Pharmacological Committee No. 21 of 22.12.1967). It was recommended as an effective antimicrobial remedy against gram-positive and gram-negative bacteria, pathogenic fungi of *Candida* genus [15, 16].

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However, a serious obstacle in the industrial production of this substance is the use of organic solvents containing chlorine in the technological process, both for the isolation of the alkaloids from the raw material and for the purification of the raw product. This presents a risk to the staff of the producing enterprises and for the ecological system [17]. Moreover, the traditional technology is voluminous and multistage. Some authors attempted to modify the initial technological process towards selecting less toxic solvents and reducing their required amounts, but without finding a radical solution [18–20].

Thus, the aim of the study was to refuse completely the use of toxic or ecologically harmful solvents and reagents, to develop a new simple and innocuous procedure for the production of sanguiritrine from *Macleaya* leaves and to evaluate the technological factors that influence the yield and purity of the finished product. Further task was to select the optimal chemical form of *Macleaya* alkaloids for the preparation of pharmaceutical forms for topical use, and to develop the technological procedure for obtaining the corresponding substance. It was made because the traditionally used form of bisulphates is substantially insoluble in lipids and does not provide effective penetration of the active principle into biological tissues.

Experimental

Plant material. As raw material, for isolation of the sum of benzophenanthridine alkaloids, have been used leaves from plants of *Macleaya microcarpa*, dried after harvesting in various phenophases during 2014–2018 years. The plants were cultivated in the Scientific Centre for Medicinal Plants Cultivation of "Nicolae Testemitanu" State University of Medicine and Pharmacy, located in the central region of the Republic of Moldova.

Apparatus. Cylindrical extractors with internal volume of 0.5 and 2.8 litres were used for the extraction of vegetal raw material by the percolation method. They were installed in a thermostat and a peristaltic pump with adjustable speed was used for the extragent supply. Analysis of the plant material, extracted products and obtained substances was performed using the "Agilent 1260" liquid chromatograph with diode array detector (DAD). Were used the following chromatographic conditions: analytical column: Kromasil 100 C1, 5 mkm, 4.6 × 100 mm at 30 °C; mobile phase: acetonitrile – 0.05% trifluoroacetic acid solution (32 : 68) at flow rate of 1.5 ml/min; detection at 400 nm (benzophenanthridine alkaloids), 286 nm (related alkaloids) and 303 nm (salicylic acid).

Chemicals. Analytical reference substances "Sanguinarine chloride hydrate" (*Sigma*) and "Chelerythrine chloride" (*Alfa Aesar*) have been used to identify and assay the main alkaloids. Other reagents of analytical grade and solvents of grade "for HPLC" were purchased from *Sigma-Aldrich Chemie GmbH* and *Merck* (Germany).

Results and discussion

Isolation of benzophenanthridine alkaloids in the form of bisulphates. On the base of carried research studies, was the technology for the coptisine isolation from aerial parts of Great celandine, developed in the framework of one of the previous projects [21]. However, differences in the properties of individual benzophenanthridine alkaloids, as well as the phenomenon of binding of main alkaloids of Macleaya with both the insoluble and soluble components of the biological matrix [22], motivated the need of introducing essential changes at all stages of the technological process.

At the stage of plant material extraction, the extragent composition, temperature and rate of percolation, the ratio of the raw material mass and the obtained extract volume have been optimised.

Ethanol of different concentrations was used as extragent for the plant material. This establised that the yield of alkaloids increases together with the ethanol concentration up to 80%, then decreases slightly for chelerythrine (table 1). At the same time, the yield of alkaloid bisulphates, at the crystallisation stage, decreases considerably, if the ethanol concentration in the extragent is less than 90%. For these reasons, we selected ethanol of 90% concentration as the optimal extragent.

Similarly, the yield of alkaloids at the extraction stage increases together with temperature. But at high temperatures there is a tendency to decrease the yield at crystallisation, the raw product purity and, accordingly, the yield of the finished product (table 2). Therefore, the temperature of 40 °C can be considered optimal for the plant material extraction, on the condition that this material has been collected in the optimal phase (budding, till flowering). It is known that in case of tardy harvesting the binding of alkaloids with the biological matrix increases, causing the decreasing of the extraction yield [22]. In these conditions, the maximum yield of the finished product can be reached at extraction temperatures of about 45 °C.

Table 1. Content of the main alkaloids in extracts (1 : 5), obtained at 20±2 °C from a batch of *Macleaya* leaves in dependence on the ethanol concentration in the extragent

Ethanol concentration, %	Alkaloids concentration in the extract, mg/ml					
	Sanguinarine		Chelerithrine		Sum	
	Free fraction	Total amount	Free fraction	Total amount	Free fraction	Total amount
40	0.410	0.543	0.620	0.699	1.03	1.24
50	0.446	0.627	0.676	0.774	1.12	1.40
60	0.545	0.730	0.772	0.827	1.32	1.56
70	0.638	0.892	0.828	0.920	1.45	1.81
80	0.687	0.952	0.839	0.931	1.53	1.88
90	0.779	0.987	0.809	0.872	1.59	1.86

Table 2. The yield of alkaloids at different technological stages according to the extraction temperature

Extraction temperature, °C	Yield at stages, %						Total yield, %	
	Extraction		Crystallisation		Purification			
	Sang.	Cheler.	Sang.	Cheler.	Sang.	Cheler.	Sang.	Cheler.
35	67	61	77	81	89	96	46	47
40	84	77	80	85	89	93	60	61
45	92	77	62	74	86	88	49	50

Note: At each temperature from 0.5 kg Macleaya leaves, taken from a homogeneous batch, 3 litres of extract (1:6) was obtained using 90% ethanol at a percolation rate of 175 ml/h.

As an optimal variant we accepted the ethanol concentration of 90±1%, temperature 40±2 °C and percolation speed, which allows to obtain 5–8 parts (volume) of extract from each part (mass) of raw material during 20–30 hours. This regime provides the yield of benzophenanthridine alkaloids extraction about 70–85% (depending on the plant material quality). The plant material-to-extract ratio of about 1:6 has proven to be optimal for laboratory installations used to perform this study. Collection of less than 5 parts of the extract from each part of the plant material leads to a considerable reduction in the yield of the finished product, whereas the increase of this volume over 8 parts has no effect.

At the stage of crystallisation of the benzophenanthridine alkaloids bisulphates, the temperature and the time of the process, as well as the sulphuric acid concentration, have been optimised. Crystallisation of the crude product proceeds gradually, due to the binding of benzophenanthridine alkaloids with polymeric compounds of acidic nature, present in the extract. It finishes over 2–3 weeks at 20 °C – the time required for the acid hydrolysis of built forms of alkaloids. This process can be accelerated by increasing the temperature, but with decreased yield of the finished product caused by the additional polymerisation of complex agents in strong acidic medium. Therefore, we have decided to carry out the process at room temperature, decreasing it down to 2–8 °C till the final stage to finish the crystallisation.

As a result, we propose the following working technique: *Macleaya* leaves, collected in the budding phase, dried and fragmented to 3–6 mm particle size, are loaded and slightly pressed, into a cylindrical percolator, which is then thermostated at 40±2 °C. The alimentation with extragent (90% ethanol) is performed through the top of the percolator by means of a peristaltic pump at the volumetric rate of 350–450 ml/h per kilogram of plant material, with the outlet valve opened, until 6 parts (volume) of extract to each part (mass) of plant material has been obtained.

The obtained extract is acidified with concentrated sulphuric acid to pH 4.0–4.5 (potentiometric), maintained at 2–8 °C overnight, then filtered or centrifuged. This operation removes calcium ions and some polysaccharides from the extract. Concentrated sulphuric acid is added to the extract, stirring continuously, to the final concentration of 0.1–0.12 M. It is recommended, to preheat the extract up to 25–30 °C to prevent rapid crystallisation and formation of fine-crystalline precipitate. The acidified extract is left for 14 days at room temperature to carry out the hydrolysis process of the complex benzophenanthridine alkaloids. After that, it is maintained for 7 days at 2–8 °C to finish crystallisation of the bisulphates of alkaloids. The precipitate is separated on a glass filter of high porosity (100–160 μm) under weak vacuum, washed with ethanol 96% until the brown colour of the draining liquid disappears and dried at 30–35 °C. A crude product in the form of orange-brown powder is obtained with a yield of 13.5–16 g per one kilogram, or 62–68% alkaloids from their content in the plant material. This product presents a mixture of benzophenanthridine alkaloids in form of bisulfates, inorganic sulphates and low-soluble polymeric organic substances. The content of the benzophenanthridine alkaloids bisulfates is 92–93%.

Purification of the crude product is carried out in the following manner: 1 part of the crude product is dissolved in 20 parts of water on heating to 60–70 °C. 0.125 parts of sodium carbonate in form of 3–5% aqueous solution to pH 3.0–6.0 (controlled potentiometrically, or by the indicator paper) is added to the solution. It is stirred intensively, avoiding foaming. The heating of the solution is continued up to 85–90 °C, stirring until the complete dissolution of light-colour particles which may form upon addition of the sodium carbonate solution. The mixture is cooled to 2–8 °C, centrifuged and filtered through a cotton swab. To the obtained solution is added 0.1 part of activated carbon. The mixture is heated to boiling, then cooled to room temperature and filtered under weak vacuum through a paper filter placed on the Buchner funnel. The sorption procedure is repeated with 0.03 parts of activated carbon. The purified solution is heated up to 65–75 °C. 1 part (by volume) of 9 M sulphuric acid is added. Then the solution is slowly cooled for 12–24 hours to 0–4 °C. The crystalline precipitate is separated on a glass filter of high porosity (100–160 μm) under weak vacuum, washed with 96% ethanol until the draining liquid is weakly acidic (pH 2.3–2.5) and dried at 30–35 °C. The yield at the purification sage is 75–82% by weight, or about 90% by the alkaloids content. The total yield of alkaloids is 56–61% of their content in the plant raw material.

In the purification process described above, bisulphates of benzophenanthridine alkaloids are transformed into sulphates, which are more soluble in water. Inorganic sulphates and the major part of insoluble organic compounds sediment from the obtained concentrated solution in the form of resin precipitate. The rest of the organic impurities are removed by sorption on the surface of the activated carbon. Finally, the benzophenanthridine alkaloids are transformed again into bisulphates due to the excess of sulphuric acid addition.

The final product contains at least 98% of the sum of benzophenanthridine alkaloids bisulphates. It presents an orange or yellowish-orange crystalline powder without odour. It is slightly soluble in water and ethanol up to 30%, forming transparent orange solutions with pH 2.2–2.7, very slightly soluble in 70% and 96% ethanol, moderately soluble (1 : 50) in dimethylsulfoxide, practically insoluble in chloroform and hexane.

Study for obtaining various salts of benzophenanthridine alkaloids and their distribution in the n-octanol-water system. The idea of this research was to look for a chemical form of Macleaya alkaloids, more lipophilic than sanguiritrine, since it is practically insoluble in lipids and does not provide effective penetration of active principles into biological tissues from pharmaceutical forms of topical use.

To obtain various salts of benzophenanthridine alkaloids we used the solution of sulfates, obtained in the sanguiritrine purification stage, after treatment with activated carbon. This solution was heated to 70–80 °C. After that, were added aqueous solutions of corresponding acids (to obtain acid salts), or their soluble salts (to obtain neutral salts). After mixing, solutions were gradually cooled during 16–20 hours to 2–4 °C. Formed precipitates were separated on glass filters, washed with 96% ethanol (or with chilled water, if the product is soluble in ethanol) and dried at 30–40 °C.

The coefficient of distribution of obtained salts was determined in the n-octanol-water system. After the balance was established, the alkaloids concentration in both layers was determined by liquid chromatography (HPLC), after diluting (if necessary) the n-octanol layer with acetonitrile and the water layer – with a mixture of acetonitrile and water (3 : 7). The obtained values are shown in table 3.

We assume that salicylates of *Macleaya* alkaloids are an optimal chemical form for preparations of topical use from the following reasons:

– The substance has average values of the distribution coefficient and satisfactory solubility in both water and lipids, which can ensure easy penetration through the lipid membranes;

– The substance can be obtained by a simple technological procedure with sufficient yield;

– The salicylate ion possesses its own antimicrobial properties, which can be manifested by synergism or broadening of the spectrum of actions.

After optimisation of the quantitative ratio and the concentrations of the starting substances, as well as the reaction conditions, we proposed the following technological procedure to obtain the salicylates of *Macleaya* alkaloids in laboratory conditions:

1 part of the *Macleaya* alkaloids bisulphates (crude product) is passed through the procedure described for the purification of the alkaloids bisulfates until the solution, double treated with activated carbon, is obtained. This solution is added to 80 parts of water preheated to boiling. Then the solution of 0.35 parts of sodium salicylate (about 10% excess in relation to the stoichiometric amount) in 15 parts of water is added gradually. The solution is gradually cooled to 1–4 °C, maintained at this temperature for a day, then the supernatant is decanted and the precipitate is separated on a glass filter of 100 μm porosity. After that it is washed with 5–6 parts of chilled water and dried at

35 °C. The yield of the product is 0.69–0.72 parts by weight, or 72–75% based on the amount of benzophenanthridine alkaloids.

The obtained product was named "Sanguirisal". It presents a dark-brown crystalline powder with no odour. It is slightly soluble in water, slightly soluble in 96% ethanol and chloroform, soluble in 70% ethanol, freely soluble in dimethylsulfoxide, and practically insoluble in ethyl acetate and hexane.

Table 3. Values of the distribution coefficient of various salts of main Macleaya alkaloids in the n-octanol-water system

Chemical form	Values of the distribution coefficient	
	Sanguinarine	Chelerithrine
Bisulphate	0.081	0.016
Chloride	0.290	0.045
Bioxalate	0.379	0.054
Bromide	0.568	0.093
Oxalate	1.12	0.150
Iodide	1.62	0.369
Bisuccinate	9.4	1.11
Salicylate	30.1	5.11
Nonanoate	1890	1780
Palmitate	23400	11900

Conclusions

A new method has been developed to obtain sanguiritrine (sum of benzophenanthridine alkaloids in form of bisulphates) from *Macleaya* leaves, which does not use toxic or ecologically dangerous solvents or reagents. The method is based on crystallisation of alkaloids in the form of bisulphates directly from the hydroalcoholic extract, followed by their purification in the form of sulphates by sedimentation and sorption of impurities from an aqueous solution.

We propose a new pharmaceutical substance – Sanguirisal (sum of benzophenanthridine alkaloids in form of salicylates), as well as the procedure for its obtaining. This substance is destined for preparation of topical pharmaceutical forms, being much more lipophilic than sanguiritrin, that facilitates penetration into biological tissues.

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