THE REPUBLIC OF AZERBAIJAN

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ABSTRACT

of the dissertation for the degree
of Doctor of Philosophy

CHEMICAL-TOXICOLOGICAL STUDY OF *POLYGONATUM GLABERRIMUM* C. KOCH SAPONINES

Speciality:	3400.02 - Pharmaceutical chemistry, pharmacognosy
Field of science:	Pharmacy
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GENERAL CHARACTERISTICS OF THE WORK

Relevance and developing level of the topic

Treatment with plants and their preparations is usually considered harmless. Unfortunately, it is not always so at all. Some plants contain substances that damage the body's systems and many organs by causing poisoning under appropriate conditions. A group of these substances is saponins. In addition to being biologically active substances of plant origin, saponins can cause serious complications, including poisoning and even death, if ingested in large quantities. Saponin poisoning and death can occur both when using plants containing saponins as a biologically active substance for therapeutic and other purposes, as well as when using saponin-containing preparations derived from those plants. The cause of the poisoning may be accidental or intentional. Therefore, the chemical-toxicological study of saponin containing plants is very important[1,2].

Our flora differs in the composition of plural species. The number of poisonous plants in the rich flora of our republic is not small. Chemical-toxicological study of poisonous plants widespread in our flora is a necessary direction[3,4,5,6]. It is known that one of the specific features of chemical-toxicological analysis is the diversity of

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[1] İskəndərov, Q.B. Kimya-toksikoloji analizdə diossininin su/üzvi həlledici sistemində ekstraksiya parametrlərinin müəyyən edilməsi / Q.B. İskəndərov, K.F. Hüseynquliyeva // Azərbaycan Əczaçılıq və Farmakoterapiya Jurnalı, - Bakı: - 2017. - №1, - s. 20-23.

[2] İskəndərov, Q.B. *Polygonatum* Hill cinsindən olan növlərin kimyəvi-toksikoloji tədqiqi perspektivləri haqqında / Q.B. İskəndərov, S.A. Paşayeva //Azərbaycan Əczaçılıq və Farmakoterapiya Jurnalı, - Bakı: - 2013. №1, - s. 14-20.

[3] Hüseynquliyeva, K.F., İskəndərov, Q.B., İsmayılova, Ş.Y. Diossininin kimyəvi-toksikoloji tədqiqinin bəzi cəhətləri // Akademik Rəfiqə Əlirza qızı Əliyevanın 85 illik yubileyinə həsr olunmuş beynəlxalq elmi konfransın materialları, - Bakı: Bakı Universiteti nəşriyyatı, -16-17 noyabr, - 2017, - s. 178-179.

[4] Gupta, V.K. Forensic Applications of Indian Traditional Toxic Plants and their Constituents / V.K. Gupta, B. Sharma. // Forensic Research & Criminology International Journal, -2017. v. 4, no 1, p. 27-32.

[5] Kumari, K. Chromatographic Analysis of few Toxic Plant Seeds for Forensic Aid / K. Kumari, S. Bhargava, R. Yadav [et al.] // J Punjab Acad Forensic Med Toxicol, - 2018. v. 18, no 2, - p. 15-19.

[6] Gupta, R. Plants and their toxic constituent's forensic approach: A Review / R. Gupta, V. Dhingra // Eur J Forensic Sci, - 2015, v. 2, №2, - p. 15-26.

the research object. One group of the research objects is poisonous plants. *Polygonatum glaberrimum* C. Koch is also included in the list of poisonous plants and is considered to be the subject of chemical-toxicological analysis. However, it has not been chemically and toxicologically studied. According to the classification of poisonous plants, they are included in the list of plants that cause symptoms of heart damage[7,8].

P.glaberrimum is a perennial plant widespread in many regions of our republic. Attractive poisonous berries are more dangerous for children. There are also possibilities of potential poisoning with other parts (especially rhizome), as it is used in traditional medicine as a remedy[9,10,11]. Chemical-toxicological research of analytical samples (blood, urine, gastric lavage water, etc.) is very important to determine the exact cause of poisoning with this plant, to obtain objective diagnostic information and to conduct a proper examination. Taking into account the above-mentioned reasons, it is very important and actual to study chemically and toxicologically biologically active substance of *P.glaberrimum*, which is widespread in the flora of our republic[12,13].

Object and subject of the research

The object of the research was *P. glaberrimum*, which is widespread in the flora of Azerbaijan. The plant was collected from

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[7] Гусынин, И.А. Токсикология ядовитых растений / И.А. Гусынин. - Москва: Сельхозгиз, -1955, -330 с.

[8] Фруентов, Н.К. Ядовитые растения. Медицинская токсикология растений Дальнего Востока / Н.К. Фруентов, Г.Н. Кадаев. - Хабаровск: Кн. изд-во, - 1971. -256 с.

[9] Dökmeçi, İ. Toksikoloji zehirlenmelerde tanı və tedavi / İ. Dökmeçi, A. Dökmeçi. -İstanbul: nobel tıp kitapevleri, - 2005. 4. baskı., -675 s.

[10] Jin, J. Cytotoxic Steroidal Saponins from *Polygonatum zanlanscianense* / J. Jin, Y. Zhang, H. Li [et al.] // J. Nat. Prod., - 2004. v. 67, no 12, - p. 1992-1995.

[11] Zhao, P. The genus Polygonatum: A review of ethnopharmacology, phytochemistry and pharmacology / P. Zhao, C. Zhao, X. Li [et al.] // J. Ethnopharmacol., - 2018. v. 214, - p. 274-291.

[12] Илларионова, Е.А. Химико-токсикологическое определение ламивудина в биологических объектах / Е.А. Илларионова, Н.В. Чмелевская, Ю.А. Гончикова // Судебно-медицинская экспертиза, - Москва: - 2020. №1, - с. 42-46.

[13] Мельник, Е.В. Химико-токсикологическая диагностика отравлений чемерицей / Е.В. Мельник, М.В. Белова, И.А. Тюрин [и др.] // Судебно-медицинская экспертиза, - Москва: - 2020. № 4, - с. 34-38.

the territory of Guba region Qachrash village of the Republic of Azerbaijan. The fruits and rhizome of the plant have been chemically and toxicologically studied. The subject of the research were model samples of various internal organs, steroidal saponins, sapogenins, monosaccharides.

The purpose and the tasks of the research

The purpose of the research is to develop chemical-toxicological analysis methods based on steroidal saponin – the biologically active substance of *P.glaberrimum*. To do this, the following tasks were performed:

1. To get individual form of steroidal saponins from plant raw material.
2. To investigate chemically and determine the structure of individual saponins.
3. To develop and patent a convenient obtaining method of biologically active substances with practical importance from raw material.
4. Selection of optimal condition as a result of studying the effect of various factors on the isolation of saponin from biological material and based on this to develop a method of isolation.
5. To prove quantitatively and qualitatively saponin isolated from biological material.
6. To determine the saponin in different biological materials.

Research methods

Two variants of the extraction process were used in the obtaining of saponins from the studied plant raw material, various biological materials and in the purification of impurities: solid-liquid extraction and liquid extraction.

Various chromatographic methods: column chromatography, thin layer chromatography, GC-MS, IR spectroscopy, MS, spectrophotometry and classical chemical methods have been used to identify and quantify biologically active substances derived from raw material. At the same time, these methods have been used to determine the quality and quantity of saponins isolated from biological materials, respectively.

The main provisions of the defense

- Offering *P.glaberrimum* plant – a new source of raw materials for steroidal compounds with high biological activity;
- Obtaining methods of steroidal compounds - diosgenin, pennogenin and caucasosaponin from raw materials and 2 Eurasian patents related to them;
- An effective and convenient method for isolating of substance from research objects on the basis of optimal parameters determined as a result of experimental studies;
- Methods of qualitative and quantitative proof of saponin isolated from biological material and testing on the basis of various internal organs in the form of model samples;

The scientific novelty of the research

For the first time, individually obtaining, chemical research and chemical-toxicological study of steroidal saponins of *P.glaberrimum*, which is widespread in the flora of Azerbaijan, were carried out.

For the first time, two steroidal saponins were obtained from the raw material, and their chemical structure was fully determined. One of the saponins is caucasosaponin - the main active ingredient of diosponin, which was once widely used as an indispensable drug against atherosclerosis. But production of this drug has been temporary stopped due to lack of raw material reserve. The second saponin is a new steroidal saponin for the genus *Polygonatum* Mill.- pentaoside of pennogenin (the steroidal sapogenin).

For the first time, a new source of raw materials for biologically active substances with practical importance and a convenient method for obtaining thise substances were proposed. The I Eurasian Patent for the getting method of caucasosaponin from *P.glaberrimum* was obtained (№ 026863, May 31, 2017), then a new material on the getting method of steroidal sapogenins from *P.glaberrimum* was submitted and the II Eurasian Patent was obtained (№ 031707, November 30, 2018).

For the first time, the optimal parameters of various factors influencing the isolation of chemically different saponin from biological material were determined, a new isolation method was

developed and this method was proved on the basis of experimental studies that it is suitable for chemical-toxicological analysis on the example of various internal organs.

Practical and theoretical significance of the research

A new source of raw material for biologically active substances with high efficiency and a wide range of pharmacological effects and convenient methods for obtaining of these substances from raw materials are proposed. These methods are particularly importance for supplying the pharmaceutical industry with highly effective biologically active substances - caucasosaponin, diosgenin and pennogenin, and are very practical.

The developed chemical-toxicological analysis methods can be used to determine the cause of poisoning in forensic-chemical expert practice and laboratory examination of acute poisoning, if necessary, and allow to enrich the practice of chemical-toxicological analysis with new materials.

The method of chemical-toxicological analysis of saponins obtained individually from plant raw materials during the dissertation and its results are used in the teaching of "Chemical-toxicological analysis of toxic substances of plant and animal origin" on the specialty "Toxicological chemistry" at the master's level at the faculty of Pharmacy.

Approbation and application

The main results obtained during the scientific research were presented at the scientific conference dedicated to the 70th anniversary of doctor of medical sciences Azam Tayyar oglu Agayev (Baku, 2014), scientific-practical conference "Actual problems of medicine" dedicated to the 92nd anniversary of national leader Heydar Aliyev (Baku, 2015), scientific-practical conference "Actual problems of modern biology and chemistry" dedicated to the 92nd anniversary of national leader Heydar Aliyev (Ganja, 2015), III International scientific conference of young researchers dedicated to the 92nd anniversary of national leader Heydar Aliyev (Baku, 2015), VI Republican scientific conference dedicated to the 80th anniversary of the department of Analytical Chemistry (Baku, 2015), scientific-practical conference dedicated to the 90th anniversary of Agil Alirza

oglu Aliyev, corresponding member of the Azerbaijan National Academy of Sciences, honored scientist, doctor of economics, professor (Baku, 2016), international scientific conference dedicated to the 110th anniversary of the founder of the school of anatomy in Azerbaijan, honored scientist, professor Kamil-Abdul-Salam oglu Balakishiyev (Baku, 2016), scientific-practical conference on the occasion of the 120th anniversary of Aziz Aliyev (Baku, 2017), scientific-practical conference "Actual problems of medicine" dedicated to the 25th anniversary of the restoration of state independence of Azerbaijan (Baku, 2017), international scientific conference"Chemistry of coordination compounds: actual problems of analytical chemistry" dedicated to the 85th anniversary of academician Rafiga Alirza gizi Aliyeva (Baku, 2017), international scientific conference dedicated to the 85th anniversary of the honored scientist, professor Rafig Ashraf oglu Askerov (Baku, 2018). The initial discussion of the dissertation was held on 18.05.2018 at the interdepartmental meeting at AMU. The discussion at the scientific seminar was held on 21.06.2021 at AMU in the meeting of the scientific seminar operating BFD 4.02 Dissertation council

The results of the dissertation were published in 24 scientific works, including 8 journal articles, 14 conference and thesis materials, 2 patents.

Methods for the determination of saponins of *P. glaberrimum* in various biological materials can be applied during forensic chemical examinations and expertise, and a certificate of application was issued (08.05.2018). This method is also used in the teaching on "Toxicological Chemistry" specialty at the Department of General and Toxicological Chemistry of AMU and an application act was obtained (01.05.2018).

Name of the organization where the dissertation work is performed. The dissertation work was carried out at the Department of General and Toxicological Chemistry of Azerbaijan Medical University according to the plan of research work (State registration № 01114106).

Volume and structure of the dissertation. The dissertation consists of 168 pages of computer writing, including an introductory

part, 5 chapters, conclusions, practical recommendations, list of references and appendices. The dissertation contains 23 tables, 4 schemes, 20 pictures and 2 chemical formulas and 13 formulas. A total of 180 literary sources (14 in Azerbaijani, 1 in Turkish, 64 in Russian and 101 in English) were used in writing the dissertation.

In the chapter I of the dissertation has been presented a literature review on the botanical features, chemical composition, usage in scientific and traditional medicine, toxicological properties of species belonging to the *Polygonatum* Mill. genus.

Chapter II of the dissertation provides information on research object, methods, devices and reagents used in research.

In the chapter III of the dissertation has been presented the results of the chemical study of *P. glaberrimum* saponins.

Chapter IV of the dissertation contains information on a convenient method of obtaining biologically active substances of practical importance from raw materials.

In the chapter V of the dissertation has been presented the results of the study of saponins in the various biological materials.

Volume of structural divisions of the dissertation with separate signs, except for pictures, tables, appendices and bibliography: introduction 10852, Chapter I – 45316, Chapter II – 17084, Chapter III – 72982, Chapter IV – 26248, Chapter V – 42484, final part 16509, results 1576, practical recommendations 362 signs. The total volume of the dissertation contains 233413 signs.

MATERIALS AND METHODS OF RESEARCH

The rhizomes, aerial part and ripe fruits of *P.glaberrimum* collected from the territory of Qachrash village of Guba region and used for the research.

Determination of plant species was carried out with the help of employees of the herbarium department of the Institute of Botany of ANAS. The rhizomes were collected in september, cleaned, crushed to a size of 3-4 mm and dried. Ripe fruits were collected in august,

crushed to a size of 2-3 mm, and the aerial part was collected in may, cut into 5-6 mm pieces and dried.

Different variants of the extraction process were used to obtain saponin sum from the studied plant and to remove impurities. Two types of extraction were used in the experiments: a) extraction from solid material - phase; b) extraction from the liquid (solution) phase[14].

The solid phase extraction method was used to isolate saponin from plant raw materials and relevant biological material. Steroidal glycosides were isolated from the raw material with an aqueous-alcoholic solution. In the isolation of saponins from various biological materials, water-saturated n-butanol was used as an extragent. The second variant of the extraction process, ie the liquid phase extraction method, was used to purify the saponin (isolated from both plant raw material and biological material) from impurities. In the studies were used column and thin-layer chromatography methods. In general, a thin-layer chromatography method was used to determine the number of substances obtained at each stage of the study, to check their purity and to identify them. Standard silufol and sorbfil plates were taken as the stationary phase, and various solvent mixtures such as the mobile phase were taken. Sannie's reagent was used as a detection reagent to detect steroidal glycoside, its progenin, genine and methylated derivatives on the chromatogram. In order to detect stains belonging to monosaccharides and their corresponding derivatives, chromatographic plates were sprayed with a solution of o-toluidine-salicylate. In determining the chemical structure of an individual glycoside, multivariate hydrolysis (acid, analytical, partial), complete methylation, hydrolysis of methylated product and etc. classical methods, as well as chromatography on a thin layer, modern physicochemical methods such as IR spectroscopy (TENSOR 37, manufacturer - BRUKER (USA)), MS, GC-MS methods were used. Mass spectroscopy was performed on a "Bruker Esquire 2000 ESI ion source" spectrometer. Diosgenin was studied by GC-MS method on

[14] Санжиева, Д.Ю. Изучение влияния некоторых факторов экстракции на изолирование флупентиксола из растворов / Д.Ю. Санжиева, А.С. Рыбасова, А.С. Карсаева / Разработка, исследование и маркетинг новой фармацевтической продукции. Сборник Научных Трудов, -Пятигорск: ООО Принт-2, - 2016. вып.71, - с. 186-188.

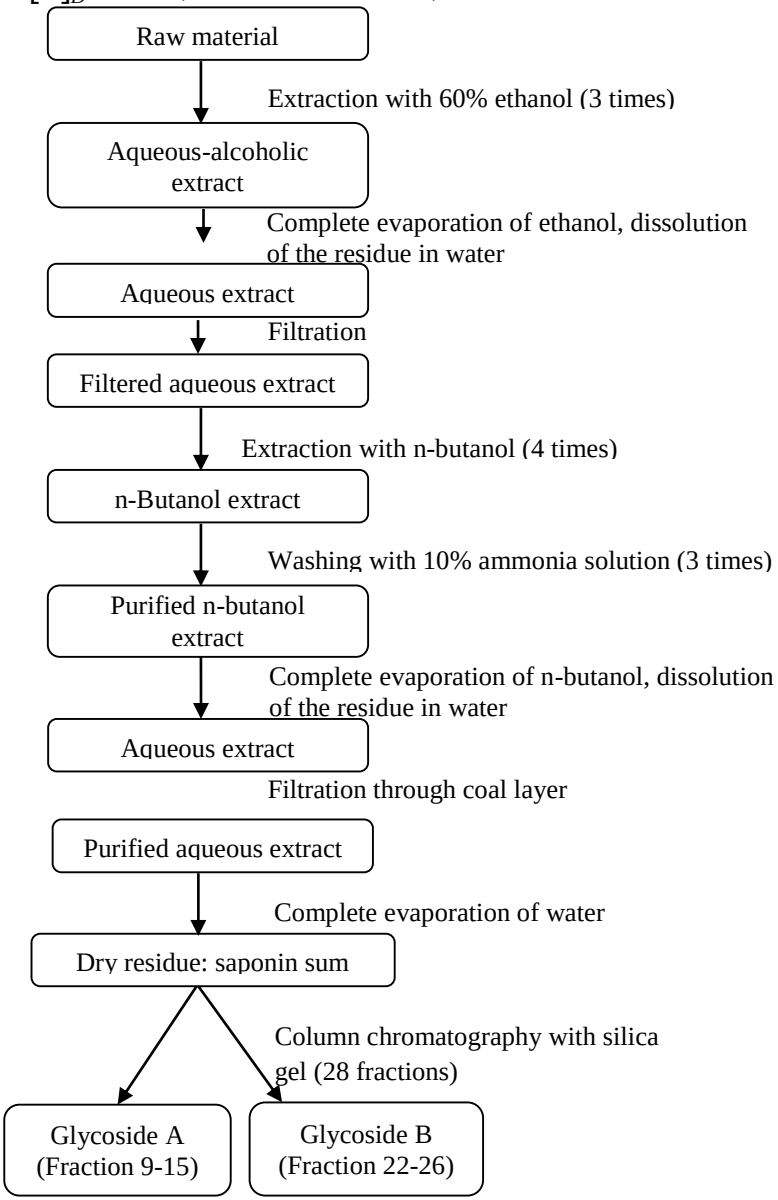
"Agilent Technologies 7890B Network GC System" chromatograph, and quantitative determination was performed on "UV-VIS spectrophotometer UV 752D" and "Jenway 7315" spectrophotometers. “Poliarmetro Model Polar, OPTIC IVYEMEN SYSTEM” polarimeter was used to determine the angle of rotation of the polarization plane of substances, and “Kofler block” was used to determine the melting temperature. Drying of individual substances, their corresponding derivatives, hydrolysis products, as well as chromatographic plates was carried out in the "GOLD TERM F-40" drying cabinet with strict adherence to the required temperature. Non-parametric methods (Anova test, Kruskal-Wallis criterion) were used for statistical processing of numerical indicators of the obtained results.

RESEARCH RESULTS AND THEIR DISCUSSION

For the purpose of chemical-toxicological study of *P.glaberrimum*, the steroidal saponin composition of the plant was studied by preliminary experiments. For this purpose, extractions were separately performed with purified water, 60% ethanol and 95% ethanol from the rhizome, aerial part and fruits of the plant. As a result of preliminary tests, 2 spirostane-type steroidal glycosides were found in the fruits and rhizome of the plant. The steroidal saponin content of rhizomes was first investigated, because all three extracts from rhizomes are more transparent and pure than fruit extracts. Based on the results of preliminary tests, 60% ethanol was used as the most suitable solvent to isolate these substances from the rhizomes. Yellowish-white saponin sum was obtained after appropriate cleaning operations. The yield of saponins from plant raw materials was 3.9%. Low-polar glycoside A and polar glycoside B were obtained individually from the sum by column chromatography method (Scheme 1).

Chemical composition of glycoside A - $C_{51}H_{82}O_{22}$, molecular weight 1046, melting point 220-222^{0}C, $[\alpha]_D^{20}$ - 69,6^0 (c 0,2; 60% ethanol). Physico-chemical parameters of glycoside B: chemical

composition $C_{56}H_{90}O_{27}$, molecular weight 1194, melting point 217-219^{0}C, $[\alpha]_D^{20}$ - 60^0 (C 0,5; 60% ethanol).



Scheme 1. Obtaining saponins from raw material

The process of acid hydrolysis was applied to study the aglycone and monosaccharide compositions of glycosides[15].

Aglycone obtained from the acid hydrolysis of glycoside A is a white crystalline substance, well soluble in 95% ethyl alcohol, chloroform, benzene, acetone, hexane and ethyl acetate, insoluble in aqueous and aqueous-alcoholic solutions. Physicochemical parameters were determined after recrystallization of aglycone with 95% ethanol. Thus, the chemical composition is $C_{27}H_{42}O_3$, molecular weight 414, melting point 201^0-203^0 C, $[\alpha]_D^{20}$ - 119^0 (c 0,2; chloroform).

Based on IR spectroscopy and thin-layer chromatography, it was determined that the IR spectrum of the substance is the same with the IR spectrum of a standard sample of diosgenin, and the numerical value of R_f on the chromatogram is the same as the standard R_f of diosgenin. Aglycone was also identified as diosgenin by mass spectroscopy $[M+H]^+$415,6; $[M+Na]^+$437,5) and GC-MS methods. To investigate the monosaccharide content of the glycoside A carbohydrate chain, the liquid phase of the hydrolyzate obtained by acid hydrolysis was chemically investigated. Based on the experiments, it was determined that the carbohydrate chain of glycoside A is represented by D-glucose and L-ramnose monosaccharide residues[16].

Aglycone obtained by hydrolysis of glycoside B is a white crystalline powder, good soluble in 95% ethanol, chloroform, benzene, acetone, hexane and ethyl acetate, but insoluble in water and aqueous-alcoholic solutions.The physicochemical properties and structure of aglycone were determined after recrystallization with 95% ethanol: chemical composition $C_{27}H_{42}O_4$, molecular weight 430, melting point 231-233 C, $[\alpha]_D^{20} -105^0$ (c 0,4; chloroform). Experimental studies of the aglycone content of glycoside B, as well

[15] Толкачева, Н.В. Стероидные гликозиды луковиц *Allium cyrillii* / Н.В. Толкачева, А.С. Шашков, В.Я. Чирва // Химия природных соединений, -Ташкент: - 2012. № 2, - с. 243-246

[16] Синтез стероидных гормональных препаратов из тигогенина интродуцированной в Грузии *Yucca gloriosa* L. и изучение химического состава растения / Э.П. Кемертелидзе [и др.]. - Тбилиси: Georgian National Academy Press, - 2018. - 231 с.

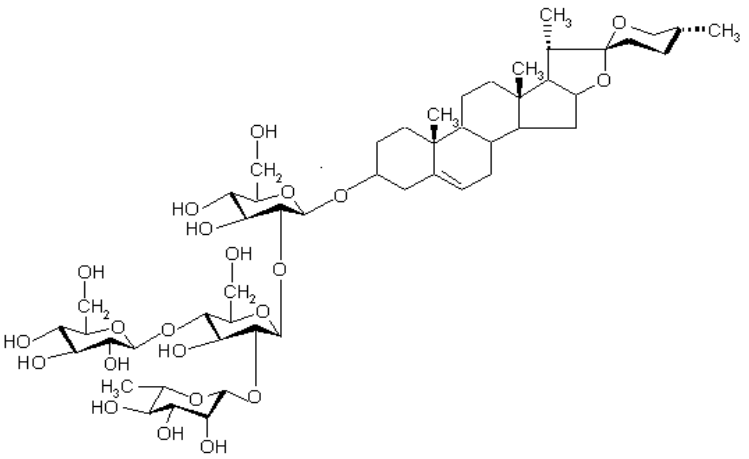
as its chemical composition, melting point, specific rotation parameters, IR-spectral data, chromatographic and mass-spectroscopic studies have shown that it is a steroidal sapogenin of pennogenin. The liquid phase of the hydrolyzate obtained from acid hydrolysis was investigated and it was determined that the monosaccharide residues of the B glycoside molecule, which are part of the carbohydrate chain, consist of D-glucose, L-ramnose and L-arabinose.

In order to determine the sequence of monosaccharides in the carbohydrate chain of glycosides A and B, the fragment directly associated with aglycone, the functions of each monosaccharide and the nature of glycoside bonds, ie the complete chemical structure of glycosides classical chemical research methods were used, including analytical hydrolysis, complete methylation of glycosides, hydrolysis of methylated derivatives, partial hydrolysis, and study of progenins[16].

Based on the results of experimental studies on the chemical structure of glycosides and their objective analytical analysis, it was determined that the glycoside A **(I)** is the diosgenin 3- O-α-L-ramnopyranosyl- (1→2)-[O-β-D-glucopyranosyl-(1→4)]-O-β-D-glucopyranosyl-(1→2)-O-β-D-glucopyranoside, polar B glycoside (**II)** is the pennogenin 3-O-β-D-glucopyranosyl-(1→2)-[O-β-D-glucopyranosyl-(1→ 4)]-O-α-L-ramnopyranozyl-(1→ 4)-[O-α-L-arabinopyranozyl-(1→ 2)]-O-β-D-glucopyranoside.

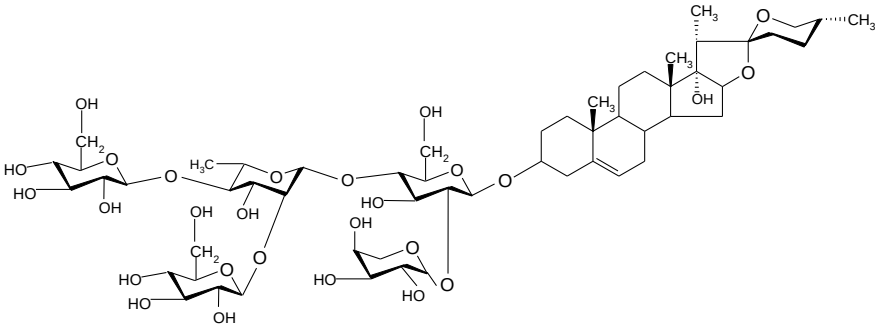
For the first time, a comparison of glycoside A, obtained from *P.glaberrimum* raw material, with caucasosaponin, revealed that both glycosides were identical. Although former Soviet scientists obtained caucasosaponin from the plant *Dioscorea caucasica* Lipsky and proved that it is a tetraozide of diosgenin, they did not determine its exact chemical structure[17]. We have achieved this and present the complete chemical structure of caucasosaponin (**I**).

[17] Мадаева, О.С. Кавказосапонин и кавказопросапогенин из *D.caucasica* XV. Сапонины *Dioscorea* / О.С. Мадаева, В.К. Рыжкова, В.В. Панина // Химия природных соединений, - Ташкент: - 1967. №4, - с. 237-241



(I)

The second saponin - glycoside B is a pentaozide of the steroidal sapogenin of pennogenin and is presented the complete chemical structure (**II)**. Comparison of the physicochemical parameters of glycoside B with the known glycosides of pennogenin proved that this glycoside is a new substance for the genus *Polygonatum* Mill. and was conventionally called glaberroside.



(II)

The fruits of *P.glaberrimum* are considered the object of chemical-toxicological analysis, since they have a very strong toxic

effect, and research in this area is extremely important. Preliminary studies have shown that both the rhizomes and the fruits of the plant contain steroidal saponins. However, due to the high content of saponins in the rhizomes and a small amount of impurities that did not have a significant effect on the release of saponins, rhizomes were chosen at the beginning of the study as a raw material for obtaining individual saponins. Individual saponins were obtained from raw materials, their physicochemical parameters and complete chemical structure were determined. Considering that most cases of poisoning occur from the fruits of *P.glaberrimum*, since the main poisonous part of the plant is the fruit, further research was carried out to obtain and identify steroidal saponins from the fruits.

According to the results of studies carried out with rhizomes, 60% ethanol, which we consider to be the most suitable solvent, was also used to isolate steroidal saponins from fruits. Before extraction with ethanol, the raw material was treated with petroleum ether three times. Then it was extracted with 60% ethanol in the same way as with the rhizomes. To remove a number of impurities from the saponin sum, filtration on filter paper, treatment with chloroform, and sequential extraction of saponins with n-butanol were carried out, and for the complete removal of colored substances, a number of operations were carried out, including treatment with 10% ammonia and filtration of the saponin fraction through activated coal. As a result, a white saponin sum with a yellowish tint was obtained. The yield from plant raw material was 1.8%. The saponin sum was examined by thin layer chromatography and defined two substances. Separation of the saponin sum into components was carried out by column chromatography with silica gel. The purity and individuality of glycosides obtained by combining all fractions of the same composition and passing them through a layer of activated coal were confirmed by thin layer chromatography. These glycosides are conventionally named A1 and B1. Both glycosides are white amorphous powders, well soluble in water, dilute solutions of alcohols, water-saturated n-butanol, insoluble in 95% ethanol, hexane, benzene, acetone, ether, chloroform and other organic solvents.

In order to study the composition of these substances, as in the case of rhizomes, we used such classical chemical methods as different hydrolysis (acidic, analytical, partial), complete methylation, hydrolysis of the methylated product, etc., as well as thin layer chromatography and IR spectroscopy. The melting point and specific rotation of individual substances were determined. Studies have shown that the steroidal saponins of the fruits are completely identical to the steroidal saponins of the rhizomes, and caucasosaponin and glaberroside are exactly the same substances.

An affordable method has been developed for obtaining biologically active substances of industrial importance from raw materials - caucasosaponin, diosgenin and pennogenin, which were first discovered in the raw material of *P.glaberrimum*. We have developed and patented the materials "Methods for obtaining caucasosaponins from *P.glaberrimum*" and "A method for obtaining steroidal sapogenins from *P.glaberrimum*" and obtained Eurasian patents for these methods (No. 026863, May 31, 2017; No. 031207, November 30, 2018).

The rhizomes of the *D. caucasica* are considered to be the first natural raw material of caucasosaponin as a very valuable and irreplaceable biologically active substance. The method of obtaining caucasosaponin from D. *caucasica* rhizomes was proposed by former Soviet scientists.

However, proposed by us "Method of obtaining caucasosaponins from *P.glaberrimum*" has a number of advantages: as a suitable raw material for obtaining caucasosaponin, in contrast to *D. caucasica* (which currently has no raw material reserves), *P.glaberrimum* with wide distribution area, rich raw material, as well as contains the required amount of caucasosaponin is recommended; washing of n-butanol extract with 10% ammonia solution allows complete release of phenolic compounds, dyes, pectin and other mixtures, as well as passing of the aqueous-alcoholic solution of caucasosaponin through the activated coal layer ensures the high degree of purity and individuality of the substance, these processes ensure maximum output of full-purpose substance from raw materials; precipitation of more polar glycosides from an aqueous-alcoholic

solution with 95% ethanol significantly accelerates the method of obtaining caucasosaponin in its pure form; highly toxic, carcinogenic, health hazardous solvents are not used.

Developed and patented by us, the "Method of obtaining steroidal sapogenins from smoothies" reflects the development of a more suitable method of obtaining steroidal sapogenins - diosgenin and pennogen from more suitable plant raw materials, which are valuable raw materials for the production of steroid hormones and is characterized by a number of advantages: as a starting material for the production of steroidal sapogenins, P. *glaberrimum* is offered, in contrast to the species *Tribulus terrestris* and P. *stenophyllum*, from which only one steroidal sapogenin can be obtained, thus, highly toxic, carcinogenic, health-hazardous solvents (dichloroethane, methanol) are not used to expand the supply of raw materials and to obtain not one, but two steroidal sapogenins (diosgenin and pennogenin) thus ensuring the safety and harmlessness of the method; the degree of purity of the obtained products is increased, because the hydrolysis is carried out without benzene, which dissolves well other impurities; the method is very simple, because there is no need for column chromatography, eluation and other long-term processes.

It is known that when the source of poisoning is a plant, chemical-toxicological analysis is carried out on the main biologically active substance contained in the plant[13]. We also continued our research on the basis of the glaberrozide - pentaozide pennogenin, which we obtained from *P.glaberrimum*, which is the object of our research. In order to develop methods for the main stages of chemical and toxicological analysis, first of all, model samples were prepared by the method of addition based on various biological materials - the internal organs of cattle. Our experimental studies were performed with these model samples. First, the effect of some chemical-toxicological factors on the isolation of saponin from liver tissue was studied. For this purpose, first of all, the good solubility of saponin in one or another solvent is taken into account. Nine different solvents were tested and the most suitable was selected. Water-saturated n-butanol provided more excretion of saponin from the liver tissue (55.24%), and a minimum consumption of extractives. The other 8

solvents tested were found to be unsuitable for this purpose. The optimal ratio of biological material and extracting solvent was determined by experiments to study the second factor. One of the another factors (that increase the yield of the target substance from the biological material) is the contact time of the extracting solvent with the object. Based on the research, the most favorable contact time was found, which ensures the excretion of saponin from the liver (yield - 67.04%). Taking into account that the extraction, ie the number of extracts, had a significant effect on the isolation of the substance from the biological material, as a result of research in this direction, it was found that 3 consecutive extractions of the research object provided the desired result (yield - 84, 84%). These 4 parameters are the main parameters in proposing a method for the isolation stage of chemical-toxicological analysis. Studies of the last two parameters, temperature regime and environmental pH, have shown that the optimal conditions are a neutral environment for pH and normal room temperature, in this case the glycoside substance does not undergo any transformation and is natively isolated. Thus, as a result of experimental studies, favorable conditions (optimal parameters) were determined for maximum isolation of saponins and minimum isolation of impurities (Table 1)

Table 1.

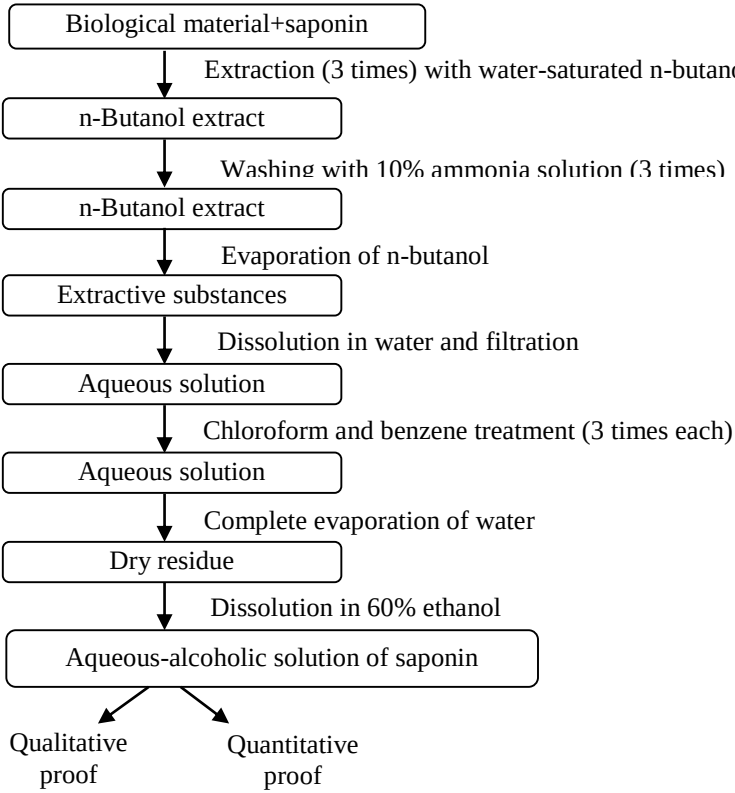
Optimal parameters for saponin isolation method

№	Optimal parameters	Indicators
1	Extracting solvent	Water-saturated n-butanol
2	Biological material and solvent ratio	1:2,5
3	The contact time of the solvent with the biological material	6 hours
4	Number of extracts	3 times
5	pH of the environment	Neutral
6	Temperature mode	Normal room conditions

Determining the optimal conditions provides extensive opportunities for the development of a suitable isolation method,

which is important in chemical-toxicological analysis[18]. Therefore, taking into account these optimal conditions, we have proposed a simple and easy, cost-effective method that does not require expensive reagents and equipment to isolate the saponin from the biological material and thus purificate it from impurities. The essence of the method is presented in Scheme 2.

As can be seen, in order to purificate the saponin from undesirable substances, a purification stage was carried out washing by 10% ammonia solution, filtration, chloroform and benzene extraction.



Scheme 2. Isolation of saponin from biological material

[18] Шорманов, В.К. Особенности изолирования 4-нитроанилина из биологического материала / В.К. Шорманов, Д.А. Герасимов, В.А. Омельченко // Судебно-медицинская экспертиза, - Москва: - 2014. №3, - с. 34-38.

The next direction of our research was to prove the quality and quantity of saponin isolated from biological material and completely separated from mixtures. A number of known methods have been used to prove the quality of the isolated and purified saponins: biological testing, physical testing, chemical reactions, physico-chemical methods. Color reactions (with Sannie's reagent) are characterized by higher sensitivity and specificity. The results of classical chemical qualitative methods have been confirmed by physico-chemical methods, which are more accurate, evidential and have advantages. Spectrophotometry was chosen to prove quantity of saponin. When the saponin spectrum was plotted in the UV field, it was found that the maximum absorption was at a wavelength of 229 nm. Therefore, the quantitative determination was performed on the basis of a specific absorption parameters at that wavelength. Lambert-Buger-Behr law proves itself in the range of 1.50-3.46 mcg/ml of concentration. To determine the specific absorption index ($E_{1sm}^{1\%}$), five standard saponin solutions, satisfying the Lambert-Buger-Ber law were prepared and the optical densities were measured. This process was repeated twice more, and the average numerical value of the optical density for each consentration was found. The obtained optical densities (A) were divided by the corresponding concentrations (C%) and the specific absorption was calculated ($E_{1sm}^{1\%} = 5000$). The following formula was used to directly calculate the percentage of saponin ($C_{\%\ relative}$) in the amount of saponin mixed with the biological material in different extract samples isolated from the biological material:

$$C_{\%relative} = \frac{A \cdot V}{E_{1sm}^{1\%} \cdot m}$$

here

V - total volume of solution in ml, the density of which is measured;

m - is the mass in grams of saponin mixed with biological material.

The methods developed by us have been tested on the basis of 5 different internal organs and proved to be suitable for the practice of chemical-toxicological analysis. The model samples, which were prepared by the addition method on the example of these 5 different

internal organs (liver, kidneys, heart, stomach and intestines with content), were successively studied as in the method we proposed. 20 g of biological material was taken each time to prepare the model sample.

As a result of the research, it was determined that the yield percentage of the target substance with the same mass from the same amount of model samples of different internal organs was not the same, depending on the type and nature of the biological material (Table 2). According to the amount of yield of saponin, the first place belongs to the heart and kidneys, the second place to the liver, and the last place to the stomach and intestines (with content) (Pictures 1-3).

Table 2

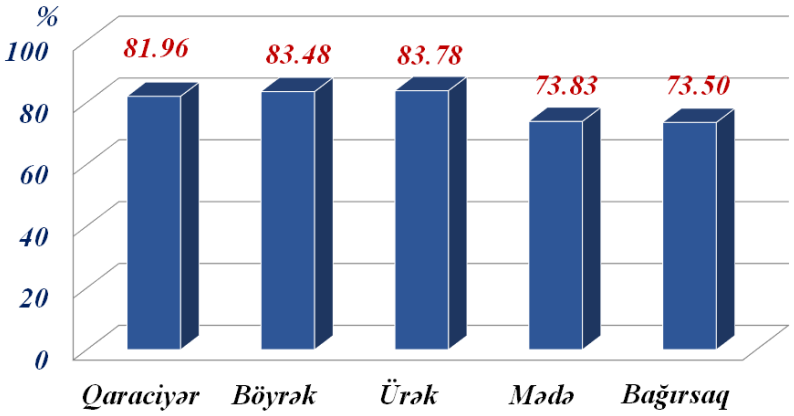
Comparison of the results obtained on the basis of 5 organs with the Anova test at 5 mg saponin

Organs	Isolated amount , %	Metrological indicators					Test Anova	
		$\bar{x}$	S	$S\bar{x}$	$\Delta\bar{x}$	A	F	P
Liver	81,10-82,50	81,96	0,61	0,27	0,75	0,92	313, 275	0, 000
Kidneys	82,50-84,24	83,48	0,75	0,34	0,94	1,12		
Heart	83,20-84,24	83,78	0,53	0,24	0,66	0,78		
Stomach	72,86-74,50	73,83	0,69	0,31	0,86	1,16		
İntestine	72,86-74,50	73,50	0,68	0,30	0,85	1,15		
F- Fisher coefficient P- correctness								

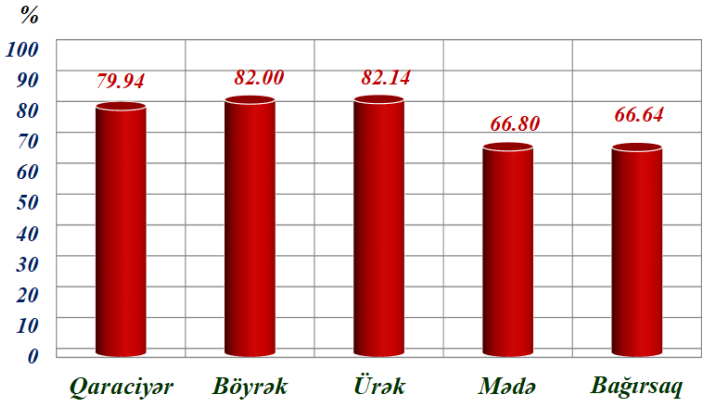
If up to 80.32-83.78% of 0.25-5 mg of the substance in the model samples of the heart and kidneys is isolated by the proposed method, from the same amount of stomach and intestines (with content) model samples only 66.64-73.83% of 1-5 mg of the same substance was isolated, ie about 10-15% less.

In model samples prepared from the stomach and intestines (with content), the amount of 0.25 mg of saponin was found only qualitatively, not quantitatively determined. The amount of 0.1 mg saponin added to the liver, kidney and heart model samples was only proved qualitatively, not quantitatively determined. The reason for this can be explained by the fact that the contents of the stomach and

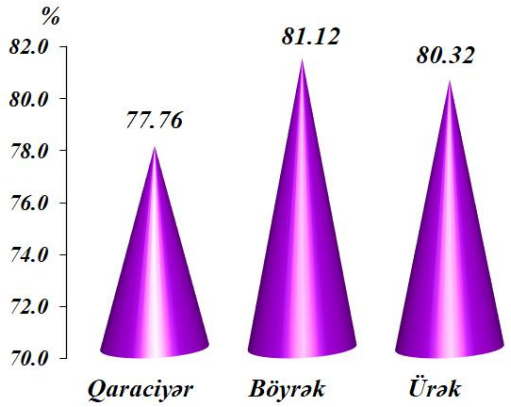
intestines adsorb more saponin due to their more complex composition and soft mass, and therefore the percentage of quantitative yield is lower than in other organs.



Picture 1. The isolation degree of 5 mg saponin from different organs



Picture 2. The isolation degree of 1 mg saponin from different organs



Picture 3. The isolation degree of 0.25 mg saponin from different organs

Quantitative and qualitative proof boundaries of saponin for model samples of different internal organs are not the same. The quantitative proof boundary for saponin in liver, kidney, and heart muscle tissue is 0.25 mg, and qualitative proof boundary is 0.1 mg. For model samples based on the stomach and intestines (with content), the quantitative limit is 1 mg and qualitative limit is 0.25 mg.

Statistical analysis of the results of quantitative determination of saponins isolated from model samples of 5 different internal organs by the proposed method once again proved that the numerical value of the relative error (A) was less than 3.17% ($p < 0.05$). This is another indicator of the statistical reliability of the method.

Thus, the results of our experimental studies prove once again that the isolation, purification and determination methods we propose for the chemical-toxicological analysis of *P. glaberrimum* are very convenient and useful and can be used successfully in appropriate practical areas if necessary.

RESULTS

1. Two steroidal saponins (substances A and B) of spirostane sequence were individually obtained from the rhizomes and fruits of *P.glaberrimum*, physicochemical parameters and chemical structures were determined [14,16,19,20].
2. Substance A is a tetraozide of diosgenin and represents 3 molecules of D-glucose and 1 molecule of L-rhamnose in the carbohydrate chain. Substance A has been identified as caucasosaponin, and was first found and obtained from *P.glaberrimum*. Substance B is a pentaozide of the steroidal sapogenin pennogenin, and the carbohydrate chain contains 3 molecules of D-glucose, 1 molecule of L-rhamnose and 1 molecule of L-arabinose. This chemically structured glycoside is a new substance for the *Polygonatum* Mill. genus and was first discovered and obtained from *P.glaberrimum*. [3,4,5,6,7,8,10,13].
3. From the new and different source of raw materials - a completely different obtaining method of practical and industrially important caucasosaponin of *P.glaberrimum*, a new obtaining method of steroidal sapogenins - diosgenin and pennogenin were proposed and Eurasian patents for both methods were obtained [2,11,12,21,24].
4. As a result of studying the effect of various chemical-toxicological factors on the isolation of saponin from biological material, optimal conditions were determined and on this basis for the first time the effective method for isolating saponin from biological material was proposed [9,15,17,18,22].
5. For the first time, convenient, evidence-based, sensitive and easy-costing chemical-toxicological analysis methods have been proposed to prove quantitatively and qualitatively saponins isolated from biological material [23].
6. The proposed method has been tested for the determination of saponins in various biological materials and the desired positive result has been obtained. Therefore, it is guaranteed to be used in practical areas as a very convenient and useful method for the practice of chemical-toxicological analysis [23].

PRACTICAL RECOMMENDATIONS

1. The raw material of *P.glaberrimum* can be used as a source of caucasosaponin in the future.
2. This raw material can be used to obtain industrially important steroidal sapogenins - diosgenin and pennogenin.
3. The proposed method can be applied with high confidence in the practical areas of chemical-toxicological analysis, if necessary, to determine the cause of poisoning.

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LIST OF ABBREVIATIONS AND SYMBOLS

$[\alpha]_D^{20}$– special rotation
İR – infrared
MS – mass-spectrometry
GC-MS – gas chromatografy-mass spectrometry
UV – ultraviolet

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