THE REPUBLIC OF AZERBAIJAN

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ABSTRACT

of the dissertation for the degree
of Doctor of Philosophy

DEVELOPMENT AND OPTIMIZATION OF METHODS FOR THE CHEMICAL-TOXICOLOGICAL ANALYSIS OF STEROID SAPONINS IN PUNCTUREVINE- *TRIBULUS TERRESTRIS L.*

Speciality:	3400.02 – Pharmaceutical chemistry, pharmacognosy
Field of science:	Pharmaceutics
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GENERAL CHARACTERISTICS OF THE WORK

Relevance and development of the topic

Many of the plants which are abundant in the flora of the Republic of Azerbaijan contain various biologically active substances, such as alkaloids, cardiac glycosides, saponins and etc. They serve not only as valuable sources of raw materials for the production of highly effective medicinal compounds but also may exhibit toxic effects under specific conditions. Consequently, the different organs of these plants, their biologically active constituents, and the medicinal preparations derived from them are often subjected to chemical-toxicological analysis, particularly when used as material evidence in chemical -toxicological investigations. To determine the cause of plant poisoning and conduct laboratory evaluations, a comprehensive chemical-toxicological study is essential[1,2].

One of such poisonous plants is puncturevine *Tribulus terrestris L.*, which is widely distributed across various regions of our country[3]. This plant has been responsible for numerous cases of poisoning in domestic animals, particularly under grazing conditions, often resulting in fatalities and significant agricultural losses[4,5] Furthermore, dairy products derived from poisoned animals may pose a serious risk to human health. Consequently, a thorough

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[1] İskəndərov, Q.B. Saponinlərin kimyəvi-toksikoloji tədqiqinin müasir problemləri // Azərbaycan Əczaçılıq Jurnalı, – 2004. №1, – s. 65-67.

[2] Искендеров, Г.Б. Исследование сапонинов лекарственных препаратов и их метаболитов в биологических средах: / Автореферат дис. на соискание ученой степени доктора фармацевтических наук. / – Москва, – 1992. – 48 с.

[3]Флора Азербайджана: [в 8 томах]. – Баку: Академия наук Азербайджанской ССР. Ин-т ботаники им. В. Л. Комарова, – т. 6. – 1955. –74 с.

[4] Вильнер, А.М. Кормовые отравления сельскохозяйственных животных / А.М.Вильнер. – Москва; Ленинград : Сельхозгиз, – 3-е изд., перераб. и доп., – 1959. – 439 с.

[5] Трескина, Н.Н. Лекарственные и ядовитые растения: методические указания / Н.Н. Трескина, О.В. Кукурузов // Тирасполь, – 2014. –144 с.

chemical and toxicological investigation of this plant is of critical importance[6].

On the other hand, the corresponding medicinal preparations produced from that plant in different countries hold a notable position in the pharmaceutical market of our republic[7,8]. In certain circumstances, improper storage conditions or incorrect administration of these drugs can lead to cases of poisoning, underscoring the necessity for ongoing chemical-toxicological studies. Additionally, this plant is extensively employed in traditional medicine for the treatment of various ailments[9,10]. All these considerations underscore the critical necessity of conducting comprehensive chemical-toxicological investigations of this plant, its medicinal derivatives, and its biologically active compounds.

Given these factors, isolating and characterizing the biologically active constituents of *puncture vein plant,* elucidating their chemical properties, and developing advanced methods for chemical-toxicological analysis are of significant relevance to pharmaceutical science and practice. These efforts also hold particular value for forensic chemical analysis, laying a foundation

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[6] İskəndərov, Q.B. Sürünən dəmirtikan *Tribulus terrestris* L. və onun tədqiqi perspektivləri / Q.B. İskəndərov, K.F. Hüseynquliyeva // Azərbaycan Əczaçılıq və Farmakoterapiya jurnalı, – 2014. №2, – s. 39-44.

[7] Rzayev, T. Tribestan: problemləri unudun // Konsilium, – 2008. №1(37). – s. 20-21.

[8] Бутова, С.Н., Сальникова, В.А., Иванова, А.А. Исследование антибактериальных свойств сапонинов с целью их применения в составе косметических продуктов // Общеуниверситетская научная конференция молодых ученых и специалистов «День науки», Сборник материалов конференций: МГУПП, – Москва, – 2016. часть VI. – с. 135-136

[9] Adaikan, P.G. History of herbal medicines with an insight on the pharmacological properties of *Tribulus terrestris* / P.G. Adaikan, R.N.V. Gauthaman and Prasad // Journal The Aging Male, –2001. Vol.4(3). – p.163-169

[10] Al-Bayati, F.A. Antibacterial and antifungal activites of different parts of Tribulus terrestris L. growing in Iraq / F.A. Al-Bayati, H.F. Al-Mola // Journal Zhejiang University Science B, – 2008. Vol.9(2). – p.154-159.

for advancing this field through the introduction of novel research methodologies.

The object and subject of the research

The study utilized the raw material of *Tribulus terrestris* L., a plant widely distributed in the flora of Azerbaijan, along with various biological materials as research object. The aerial parts (herb) of the plant were collected from different locations in the Barda region, subsequently dried, crushed, and processed. Model test samples (a total of 90) were prepared, comprising mixtures of varying doses of individual saponins with internal organs of cattle, including liver, stomach, heart, kidneys, and intestines, which served as biological material.

The subject of the research included trillin, ruscogenin monoside, dioscin, and dioscinin isolated from the plant through specialized method, along with their corresponding sapogenins (diosgenin and ruscogenin), monosaccharides (D-glucose and L-rhamnose), and their methyl derivatives.

The purpose and the tasks of the research

The objective of this study is to develop effective chemical-toxicological analysis methods for steroid saponins derived from *Tribulus terrestris* L. (puncturevine). To achieve this aim, the following **tasks** were undertaken:

1. Isolation of individual steroid saponins from plant raw materials;
2. Comprehensive chemical characterization of the isolated saponins and elucidation of their chemical structures;
3. Development of a scalable method for extracting biologically active compounds of industrial significance from the plant raw material;
4. Investigation of the influence of various factors on the extraction process of saponins from biological materials and the development of an optimized isolation method;
5. Identification and quantification of saponins isolated from biological material;

6. Detection of saponin content across different biological matrices.

Research methods

The isolation of individual steroid saponins from *Tribulus terrestris L.* (puncturevine) was achieved using sequential fractional extraction and tube chromatography techniques. The chemical structures of the isolated saponins were elucidated through a combination of classical chemical methods and modern physicochemical approaches, including nuclear magnetic resonance (NMR) spectroscopy, column chromatography, infrared (IR) spectroscopy, mass spectrometry, and polarimetry. For the chemical-toxicological analysis of the saponins, traditional techniques such as isolation, characterization, and quantitative determination were employed, alongside biological testing on various biological matrices. The identification and characterization of the compounds were further supported by biological, physical, chemical, and advanced physicochemical methodologies. Quantitative analysis of the saponins extracted from biological materials was conducted using spectrophotometric techniques.

The main provisions of the defense:

- Proposing a method for individually obtaining steroid saponins from the creeping puncturevine plant;
- Identification of individual substances and determination of their chemical structures based on modern analysis methods;
- Development of efficient methods for obtaining biologically active substances of industrial importance;
- Development of a method for isolating the target substance based on optimal conditions determined as a result of investigating the effect of various factors on the isolation of saponin from biological material;
- Qualitative and quantitative evidence of saponin isolated from biological material;
- Method for isolating saponin from various internal organs, purifying it from foreign impurities, and determining it.

The scientific novelty of the research

For the first time, comprehensive chemical and chemical-toxicological studies of steroid saponins from *Tribulus terrestris L.* (puncturevine), a plant widely distributed in Azerbaijan's flora, have been conducted, contributing novel insights and materials to the field. A sequential fractional extraction method has been newly proposed for isolating saponins from raw plant materials. Additionally, certain saponin glycosides have been obtained from the plant for the first time. Innovative methods for the extraction of biologically active compounds of industrial significance have been developed, resulting in patents: "A New Method for the Extraction of Dioscinin from Puncturevine" (No. 026930, May 31, 2017) and "A Method for the Extraction of Steroid Sapogenins" (I 2017 0024, June 9, 2017).

For the first time, the effects of various factors on the isolation of dioscinin – characterized by its complex structure – have been systematically investigated. Novel approaches to the isolation, characterization, and quantitative determination of saponins have been devised for chemical-toxicological analysis. These methods have been rigorously tested and validated, demonstrating their efficiency and practicality for chemical-toxicological applications across various biological matrices and organs.

Theoretical and practical significance of the study

Since puncturevine is classified as a poisonous plant, the proposed methods for chemical-toxicological analysis hold significant importance for forensic medical practice and the laboratory investigation of acute poisoning. These methods are intended for application in these fields and for integration into the educational process for undergraduate students (as part of graduation projects) and master's students at the Faculty of Pharmacy. A novel raw material source has been identified, characterized by a richer reserve and convenient methods for industrial extraction of biologically active substances, including dioscinin, diosgenin, and ruscogenin which can be effectively applied in various fields as needed.

During the course of the dissertation work, the developed methods for the chemical-toxicological analysis of saponins, individually isolated from plant raw materials, along with their results, are incorporated into the teaching curriculum. These methods are utilized in the bachelor's level course *Pharmaceutical Toxicology* offered by the Faculty of Pharmacy, as well as in master 's level course *Chemical-Toxicological Analysis of Toxic Substances of Plant and Animal Origin* within the specialization of Toxicological Chemistry.

Personal involvement of the author

All the results reflected in the dissertation work - the formulation of the problems, the conduct of preliminary tests and model experiments, the investigation, systematization and generalization of the obtained results - were carried out with the personal participation of the author.

Approbation and application

The main results obtained during the scientific research work were presented at the scientific conference dedicated to the 110th anniversary of the birth of the Prominent statesman and scientist, Prof. Aziz Mammadkarim oglu Aliyev (Baku-2007), at the scientific conference dedicated to the 95th anniversary of the birth of the Honored Scientist, Prof. Hasan Musa oglu Isazade (2007), at the conference dedicated to the 115th anniversary of the birth of Aziz Mammadkarim oglu Aliyev (2012), at the Chemistry of Coordination Compounds - V Republican Scientific Conference (2012), at the I International Scientific Conference of Young Researchers dedicated to the 90th anniversary of the birth of the National Leader of the Azerbaijani people Heydar Aliyev (2013), at the conference of Professor A.A. Verdizadeh's 100th anniversary (2014), the scientific conference on "Actual problems of medicine" dedicated to the results of scientific research work of the employees of the Azerbaijan Medical University (2014), the Chemistry of coordination compounds: the VI Republican scientific conference dedicated to the 80th anniversary of the "Analytical chemistry" department (2015), the scientific and practical conference dedicated to the 90th

anniversary of the birth of Corresponding Member of the Azerbaijan National Academy of Sciences, Honored Scientist, Doctor of Economic Sciences, Professor, Agil Alirza oglu Aliyev (2016), the International scientific conference dedicated to the 110th anniversary of the birth of the founder of the anatomical school in Azerbaijan, Honored Scientist, Professor Kamil Abdul-Salam oglu Balakishiyev (2016), "Development, research and marketing of new pharmaceutical products" - Collection of scientific works (Pyatigorsk, 2016), Public health and healthcare (2016), II Azerbaijan Science Festival (Poster – 2016), Scientific and practical conference held at AMU on the occasion of the 120th anniversary of the birth of Aziz Aliyev (2017), "Actual Problems of Medicine" scientific and practical conference dedicated to the 25th anniversary of the restoration of Azerbaijan's state independence (2017), Public health and healthcare (2017), Chemistry of coordination compounds: Actual problems of analytical chemistry – International scientific conference dedicated to the 85th anniversary of Academician Rafiga Alirza gizi Aliyeva (2017), Current Problems of Medicine: Scientific and practical conference dedicated to the 100th anniversary of the Azerbaijan Democratic Republic (2018), Modern achievements of pharmaceutical science in the creation and standardization of drugs and dietary supplements containing components of natural origin – Materials of the III International Scientific and Practical Internet Conference (Kharkov-2021) and the V International Scientific Congress on "Modern Problems of Pharmacy" dedicated to the 90th anniversary of the establishment of Azerbaijan Medical University and the 80th anniversary of Higher Pharmaceutical Education in Azerbaijan.

The initial discussion of the dissertation work was held at the interdepartmental meeting of the Azerbaijan Medical University on 25.05.2019. The discussion at the scientific seminar was held on 08.09.2025 at the meeting of the scientific seminar operating under the BFD 4.18 One-time Dissertation Council at AMU (protocol No. 02).

The results of the dissertation work have been published in 31 scientific works, of which 2 are patents, 10 are articles, and 19 are theses and conference proceedings.

The method "Determination of steroid saponin - dioscinin in various biological materials" is applied during forensic chemical analysis and chemical expertise, and an application act has been received for this by the Republican Scientific-Experimental and Educational Union of Forensic Medical Expertise and Pathological Anatomy (03.12.2018). The method is also used in teaching at the bachelor's and master's levels in the specialty of pharmaceutical toxicology and toxicological chemistry at the Department of Pharmaceutical Toxicology and Chemistry of AMU, as well as in the performance of master's and dissertation theses, and an application act has been obtained for this (19.11.2021).

Name of the organization where the dissertation work is performed

The dissertation work was carried out at the Department of Pharmaceutical Toxicology and Chemistry of Azerbaijan Medical University according to the plan of scientific research work (State registration No. 01114106).

The scope and structure of the dissertation

The dissertation consists of a 205-page computer manuscript, including an introduction, 5 chapters, conclusion, practical recommendations, a list of references and appendices. The dissertation contains 60 tables, 18 shems, 25 figures and 12 formulas. A total of 190 literature sources (20 in Azerbaijani, 110 in Russian, 60 in English) were used in writing the dissertation.

Chapter I of the dissertation provides a literature review on the botanical description, distribution, chemical composition, application in scientific and folk medicine, preparations, and toxicological properties of the creeping thorn *Tribulus terrestris* L. plant.

Chapter II of the dissertation provides information about the research objects, methods, devices, and reagents used in conducting research, and also presents a method for statistically processing the experimental results.

Chapter III of the dissertation describes the results of the individual extraction and chemical study of saponins from the creeping barberry plant.

Chapter IV of the dissertation presents information on the development of a convenient method for obtaining practically important biologically active substances from raw materials.

Chapter V of the dissertation reflects the results of the chemical-toxicological study of saponin.

Volume of structural divisions of the dissertation with separate signs, except for pictures, tables, appendices and bibliography: introduction 12311, Chapter I – 63632, Chapter II – 11371, Chapter III – 59382, Chapter IV – 26309, Chapter V – 48603, final part 16348, results 1415, practical recommendations 620 signs. The total volume of the dissertation contains 239991 signs.

RESEARCH MATERIAL AND METHODS

The above-ground parts of the creeping ironthorn plant *Tribulus terrestris* L., collected from various areas of the Barda region, were used as raw materials. The plant raw materials were dried in a shaded area where the air changed and the sunlight could not fall on them. After being completely dried, they were crushed to a size of 3-4 mm. Although the steroid saponin content of raw materials collected from different areas differed slightly in quantity, since they were identical in number and composition, the plant raw materials were used without any distinction for the relevant experimental studies.

It is a rather abstract problem to choose or propose a single method for isolating all of the individual glycosides from the same raw material, which contain a large number of steroid glycosides and are also close to each other in terms of sapogen content, but whose carbohydrate chain is represented by different monosaccharides and therefore has different physicochemical properties. Even if this is achieved with difficulty, separating the resulting saponin complex

into its constituent components is a very complicated process. Therefore, we had to apply a fractional extraction method to isolate individual glycosides with different properties from the raw material. As a result, three fractions with different degrees of polarity - low-polarity, medium-polarity, and high-polarity glycoside fractions - were obtained separately. Adsorption tube chromatography was used to separate multicomponent fractions into their constituent elements.

The TLC method was used to detect individual steroid saponins and their sapogenins in the raw material, and to determine their number composition, specificity, and purity. The same method was also used to check the presence of substances in in various extracts and relevant studies were conducted. Standard silufol (Czech Republic) and sorbfil (RF) plates were used as stationary phases, and various solvent systems (s.s.) were used as mobile phases.

Chemically pure solvents manufactured by various companies were used to prepare the s.s. These highly purified extragents have been used the performance of chemical-toxicological studies.

Each of the chromatography chambers with s.s. was individually saturated for a day.

Sanye's reagent was used to detect natural steroid saponins and their corresponding transformation products on either a chromatography plate or a piece of chromatography paper: after the plate was removed from the chamber and dried in the open air, it was sprayed with a 1% alcohol solution of vanillin, completely dried in a "GOLD TERM F-40" drying cabinet at a temperature of 90^0C for 2 minutes, then the plate was sprayed with a mixture of concentrated sulfuric acid and acetic anhydride (1:12), and the plate was dried again under the same conditions for 1-2 minutes. Steroid saponins were detected on the plate as egg yolk-colored spots.

Detection of spots belonging not only to steroid saponins, but also to sapogenins, progenins, and fully methylated glycosides was performed using Sanye's reagent. Spots belonging to monosaccharides were detected with concentrated sulfuric acid or o-toluidine-salicylate. In the latter case, the chromatogram belonging to monosaccharides was heated in a plate drying oven at a temperature

of 100-110^{0}C for 3-5 minutes. Spots belonging to monosaccharides were detected in dark brown color.

The melting temperatures of the relevant substances were determined using the “Kofler device” at the Academician Yusif Mammadaliyev Institute of Petrochemical Processes, and the rotation angles of the polarization plane were determined using the “Poliarmetro Modelo Polar, OPTIC IVYEMEN SYSTEM” polarimeter based on the pharmacopoeial method. Drying of chromatograms and substances was carried out in a special drying cabinet, strictly adhering to the appropriate temperature regime.

Various classical chemical research methods were used to study the chemical structure of glycosides, such as multivariate acid hydrolysis (complete, analytical, partial), complete methylation, hydrolysis of the methylated product, study of both phases of the hydrolysate, etc.

The hydrolysis process under analytical conditions was also used to determine the molecular mass of glycosides, and the molecular mass of glycosides was determined based on the exact mass of diosgenin and ruscogenin obtained.

The numerical composition of monosaccharides, which represent the carbohydrate chain of glycosides, was determined based on both the determination of their molecular mass and the results of parallel studies of corresponding artificial mixtures of sugars prepared in equimolar ratios.

To determine the nature of the carbohydrate chain, i.e. whether it has a linear or branched structure, the operations of complete methylation of individual glycosides and hydrolysis of the resulting product and examination of the hydrolysate were used. It is worth noting that there are numerous methods known for performing the complete methylation operation, and although all of them have been tested, the Hakomori method is the most suitable for our studies. Therefore, methylation of glycosides was carried out using this method. The functions of individual monosaccharides in the carbohydrate chain have also been revealed through the complete methylation of glycosides, hydrolysis of the derivative, and

investigation of the hydrolysate products. Thus, it has been proven that monosaccharide residues carry intermediary, branching center, and terminal functions, depending on the nature and structure of the carbohydrate chain.

In the study of the carbohydrate chain of glycosidic biologically active substances, a partial, i.e. incomplete hydrolysis process was used to determine the nature of the monosaccharide that forms a direct bond with the gene and the sequence of combination of monosaccharide residues.

The configuration of glycosidic bonds was determined by the method proposed by Klyne W., which is based on comparing the molecular rotation indices of substances[11].

Identification of substances and recording of IR spectra were performed in the laboratory of the Analytical Expertise Center of the Ministry of Health using a TENSOR 37, BRUKER (USA) IR spectrometer.

Mass, ^{1}H and ^{13}C NMR spectra of the substances were recorded at the Spectropole Scientific Research Center of the University of Aix-Marseille, France. Measurements were performed on a Bruker AVANCE III HD-600 NMR spectrometer (Bruker Co., USA) using signals given by a certain solvent (CD_3OD: δH /δC; δ1H 3.31 ppm, δ13C 49.00 ppm, DMSO: δH /δC; δ1H 2.50 ppm, δ13C 39.52 ppm). The temperature during the experiment was -300K and the analysis vessel was carried out with a Wideband Fluorescence observation tube with a 5 mm probe. Structural assignments were based on ^{1}H NMR, ^{13}C NMR, COSY (correlation spectroscopy), HSQC (heteronuclear single quantum coherence), and HMBC (heteronuclear multiple-bond correlation HMBC) spectra.

For chemical-toxicological studies, model tests were developed using the addition method based on urine, blood, and various internal organs of cattle. The study of the effect of various chemical-

[11]Klyne, W. “Determination of Organic Structures by Physical Methods”. Optical Rotation, in Braude A.F. and Nachod F.C. / – New York: Academic Press. New York, – 1955. – vol.1, – p.73-130.

toxicological factors on the isolation of saponin from biological material was carried out on model experiments based on liver.

Isolation of dioscinin from other internal organs and liberation from extraneous impurities was performed as in the liver model test samples. The parameters of the extraction process in the water/organic solvent system used to isolate dioscinin from biological fluids - from blood and urine - and purify it from other impurities were determined and these parameters were used in conducting experimental studies. The qualitative determination of dioscinin was carried out using classical chemical and modern physicochemical methods, and the quantitative determination was carried out using spectrophotometry.

The numerical values of the indicators obtained during the study were statistically processed using the parametric method - Anova test, and non-parametric methods - Kruskal-Wallis and Mann-Whitney tests.

RESEARCH RESULTS AND THEIR DISCUSSION

This section reflects the results of experimental studies: isolation of steroid saponins from raw materials in the form of three fractions differing in their degree of polarity and their separation into constituent components, chemical study of individually separated less polar (substances A and B), medium polar (substance C) and more polar (substance D) glycosides – chapter III; extraction of industrially important biologically active substances – steroid sapogenins and dioscinin – chapter IV; isolation of biologically active substance – dioscinin obtained from plants – from different biological objects, investigation of the effect of various parameters on the isolation process, its characterization and quantitative determination – chapter V.

Based on the research conducted, we preferred to use a more convenient option to separate steroid saponins of different polarities from the raw material - sequential fractional extraction with 3

different solvents: first, we had to extract saponins with low polarities with 95% ethanol, then from the dried raw material residue, we had to extract medium polar saponins with 90% ethanol, and finally, saponins with high polarities with 50% ethanol.

Physicochemical characteristics of glycoside A: composition – $C_{33}H_{52}O_8$, molecular weight – 576, melting point – 260-262^{0}C, specific rotation $-[\alpha]_D^{20}$ – 102^0 (0.08; 95% ethanol).

To investigate the chemical structure of glycoside A and identify the sapogenin and the monosaccharide residue representing the carbohydrate chain, it was hydrolyzed in an acidic medium.

After appropriate operations, the chemical composition of the obtained sapogenin was identified as $C_{27}H_{42}O_3$, molecular mass - 414, melting point 202-204^{0}C, specific rotation $[\alpha]_D^{20}$– - 120^0 (0.1; chloroform). It was identified as diosgenin by the TLC method. The IR spectrum matches exactly the IR spectrum of diosgenin (figure 1). The absorption bands at 850, 900, 920, 980 cm^{-1} in the IR spectrum belong to spirostane-series steroid sapogens, and the fact that the band at 900 cm^{-1} is several times more intense than the band at 920 cm^{-1} once again proves that the substance belongs to isosapogenins, i.e., the 25R-series. The broad absorption band at 3300-3400 cm-1 observed in the IR spectrum corresponds to the valence vibration of the hydroxyl group. Thus, based on the studies conducted, the sapogenin of glycoside A was identified as diosgenin.

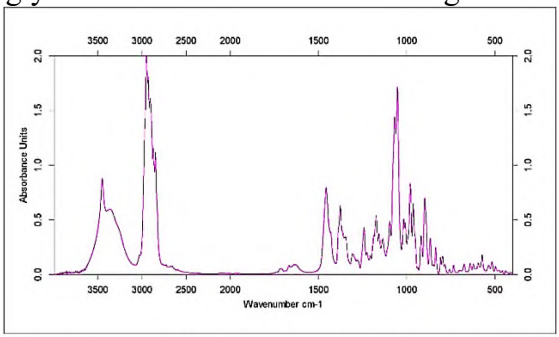
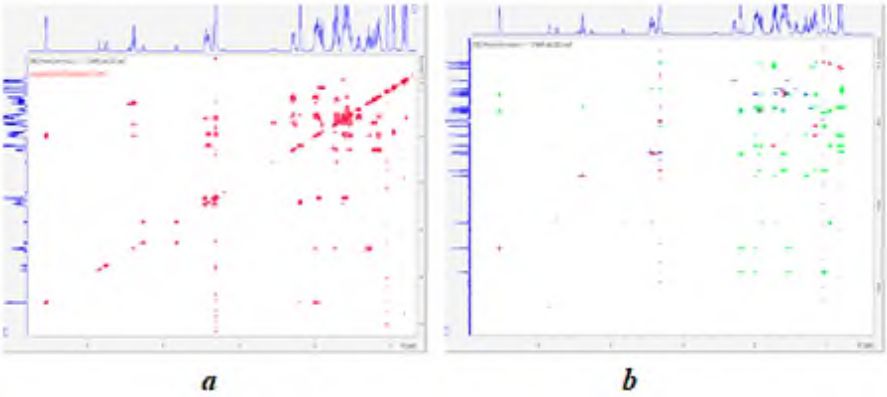


Figure 1. IR spectrum of diosgenin

Only one monosaccharide, D-glucose, was detected in the hydrolysate of glycoside A by the TLC method. To determine the number of monosaccharides in glycoside A, it was hydrolyzed and the molecular mass of the glycoside was determined. Glycoside A was proven to be a monoside.

The complete chemical structure of glycoside A was determined by determining that the glycosidic bond in the molecule has a β configuration.

^{1}H NMR, ^{13}C NMR, COSY, HSQC and HMBC spectra were used to clarify the structural assignments of glycoside A. The interpretation of the recorded spectra is given in figure 2 (a and b) and table 1.



a *b*

Figure 2. 2D NMR spectra of glycoside A: a – COSY, b – HSQC and HMBC

Thus, glycoside A was chemically characterized as diosgenin-3-O-β-D-glucopyranoside:

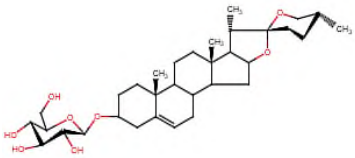


Table 1.
Interpretation of glycoside A

Atome	Glycoside A	
	$\delta_{13}C^1$	$\delta_{1H}{}^1$
=C-H	140.80	5.54; 1H; s
-	125.75	
-	110.61	
-	82.47	
-C-H	82.17	4.38; 1H; m
-C-H	78.98	3.31; 1H; t
-	68.96	3.40; 1H; m
$-CH_2$	67.85	3.44; 3.33; 2H; m
-	65.81	
-	64.07	
-C-H	63.94	1.74; 1H; m
-C-H	57.83	1.16; 1H; m
-C-H	52.13	1.15; 1H; m
$-CH_2$	49.56	2.19; 2H; d, 7.7 Hz
-	44.10	
-	43.24	
-C-H	42.92	1.90; 1H; t, 7.3 Hz
-	42.85	
$-CH_2$	42.34	1.96; 1.60; 2H; m, m
$-CH_2$	41.30	1.71; 1.22; 2H; m, m
-	41.05	
-CH	33.90	1.58; 1H; m
$-CH_2$	32.97	1.97; 2H; m
$-CH_2$	32.90	1.56; 1.29; 2H; m, m
$-CH_2$	32.43	1.70; 1.57; 2H; m, m
-CH	31.43	1.60; 1H; m
$-CH_2$	29.88	1.64; 1.41; 2H; m, dt
-	29.46	
$-CH_2$	24.81	2.29; 1.53; 2H; dd, m
$-CH_3$	17.47	0.79; 3H; d, 6.42 Hz
$-CH_3$	16.90	0.81; 3H; s
$-CH_3$	14.86	0.96; 3H; d, 6.96 Hz
$-CH_3$	13.78	1.03; 3H; s

The results of experimental studies show that glycoside A is chemically trilline glycoside. It should be noted that trilline glycoside was first isolated and identified from this plant by Moldovan researchers[12].

Physicochemical characteristics of glycoside B: composition – $C_{33}H_{52}O_8$, molecular weight –576, melting point – 272-274^{0}C, specific rotation $[\alpha]_D^{20}$– 108^0 (0.05; 95% ethanol).

To determine the chemical structure of glycoside B, acidic hydrolysis was performed and the obtained products were studied.

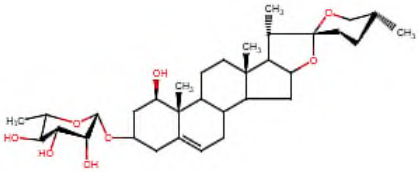
Physicochemical characteristics of the obtained sapogenin: chemical composition – $C_{27}H_{42}O_4$, molecular mass – 430, melting point 202-203^{0}C, specific rotation $[\alpha]_D^{20}$–117^0 (0.1; chloroform).

In the IR spectrum, absorption band was detected, characteristic of the spiroketal nucleus (850, 900, 920, 970 cm^{-1}) and hydroxyl groups (3300-3400 cm^{-1}) of steroid sapogenins. The sapogenin was proven to be ruscogenin by TLC and IR spectroscopy.

As a result of the study of the hydrolysate of glycoside B, it was proven that the carbohydrate chain contains only L-rhamnose.

Based on the molecular mass determined by hydrolysis of glycoside B, it was proven to be a monoside. Based on the molecular rotation data of glycoside B, it was confirmed that the glycosidic bond has a β-configuration.

As a result, it was determined that the B glycoside ruscogen has a 3-O-α-L-rhamnopyranoside structure:



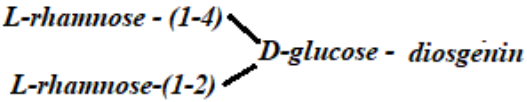
It should be noted that this glycoside was first obtained by us from the *Tribulus terrestris* L. plant.

[12]Перепелица, Э.Д. Химическое изучение стероидных гликозидов *Tribulus terrestris* IV. Стероидные сапонины / Перепелица Э.Д., Кинтя П.К. // Химия природных соединений, – 1975. №2. – с. 260-261.

Physicochemical characteristics of glycoside C: composition – $C_{45}H_{72}O_{16}$, molecular weight – 868, melting point – 298-300^{0}C, specific rotation – $[\alpha]_D^{20}$ – 101^0 (0.21; 90% ethanol).

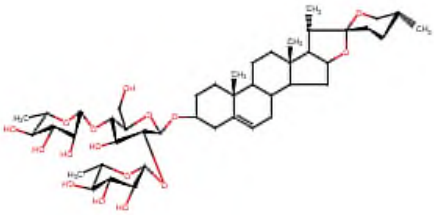
The chemical structure of glycoside C was determined in a similar manner to that of glycosides A and B. After appropriate operations, the chemical composition of the genin obtained from the solid phase of the hydrolysate was determined as $C_{45}H_{72}O_{16}$, molecular weight 868, melting point 298-300 ^{0}C, specific rotation $[\alpha]_D^{20}$– 101^0 (0.21; 90% ethanol). It was identified as diosgenin by IR spectrum and also by TLC method. Then we determined the monosaccharide composition of the C glycoside. As a result of the experiments, D-glucose and L-rhamnose were discovered as the monosaccharide components of the carbohydrate chain of C glycoside. The C glycoside was hydrolyzed under analytical conditions, and the molecular mass of the C glycoside was determined based on the amount of diosgenin. The number of monosacharide molecules was determined based on the molecular mass of the C glycoside. It was found that the carbohydrate chain consists of 1 mole of D-glucose and 2 moles of L-rhamnose.

To determine the structure of the carbohydrate chain of the C glycoside and the sequence of the monosaccharide patterns, partial hydrolysis, complete methylation, and hydrolysis of the methylated glycoside were performed, and the structure of the monosaccharide chain was determined:



The configuration of the glycosidic bonds in the C glycoside was determined based on the Klyne method[11].

Thus, the chemical structure of glycoside C was characterized as 3-O-α-L-rhamnopyranosyl-(1→4)–[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside diosgenin (dioscinin):



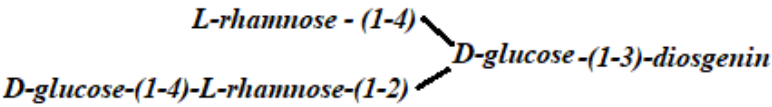
Dioscinin was first isolated and studied from this plant material by Moldovan researchers[13].

Physicochemical characteristics of D glycoside: composition – $C_{51}H_{82}O_{21}$, molecular weight –1030, melting point 208-210^{0}C, specific rotation – $[\alpha]_D^{20}$– 88^0 (0.1; 50% ethanol).

The investigation of the chemical structure of D glycoside was carried out as for other glycosides. D-glucose and L-rhamnose were detected in the carbohydrate chain of D-glycoside. D-glycoside was hydrolyzed under analytical conditions, and the molecular mass of D-glycoside was determined based on the amount of diosgenin obtained.

The number of monosaccharide molecules was determined based on the molecular mass of the D glycoside. The carbohydrate chain was found to consist of 2 moles of D-glucose and 2 moles of L-rhamnose.

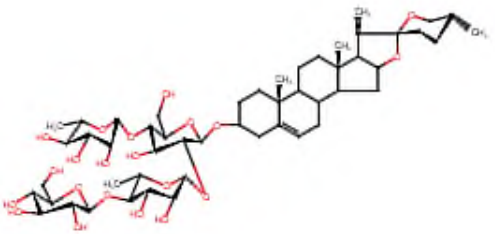
The structure of the carbohydrate chain of D-glycoside, the sequence of the monosacharide patterns, was determined, and it was found that it contains dioscin, which contains 1 mole of D-glucose:



The configuration of the glycosidic bonds in the D glycoside was determined by the Klyne method, and chemically the D glycoside was proven to be diosgenin 3-O-β-D-glucopyranosyl-

[13]Перепелица, Э.Д. Диосцин - стероидный гликозид из *Tribulus terrestris* / Э.Д. Перепелица, П.К. Кинтя // Известия АН Молдов. ССР. Сер. биол. и хим. Наук, – 1974. №6, – с. 76-80.

(1→4)-O-α-L-rhamnopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→4)]-O-β-D-glucopyranoside:



Thus, the D glycoside is dioscinin, which is the tetraoside of diosgenin. Dioscinin was first isolated from the *Dioscorea* plant by Russian researchers[14].

A new raw material source with a very large reserve and efficient, affordable methods for their extraction have been proposed for the industrially important biologically active substances diosgenin, ruscogenin, and dioscinin. The proposed method for obtaining diosgenin and ruscogenin has been granted a Patent of the Republic of Azerbaijan, and the method for obtaining dioscinin has been granted a Eurasian Patent.

As is known, the most important stage of chemical-toxicological research is the maximum isolation of the toxic substance from biological material. This depends on various chemical-toxicological factors[15]. First, the effect of various factors on the isolation of dioscinin from biological material was investigated.

Based on the experimental studies conducted, optimal conditions were identified (table 2).

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[14] Мадаева, О.С. Сапонины *Dioscorea polystachya* XIV. Диосцинин / О.С. Мадаева, В.К. Рыжкова, В.В. Панина // Химия природных соединений, – 1967. №3, – с. 155-158.

[15] 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.

Table 2.

Optimal conditions for isolating dioscinin from bio. material

№	Indicators (parameters)	Optimal conditions
1	extractive solvent	90% ethanol
2	biological material and extraction solvent ratio	1:3
3	contact time of the solvent with the biological material	6 hour
4	number of extract	3 times
5	number of extracts pH of the medium	neutral
6	temperature regime	20-25^{0}C

Taking into account these determined optimal parameters, a method for isolating dioscinin from biological material was proposed. The method is schematically presented in Scheme 1.

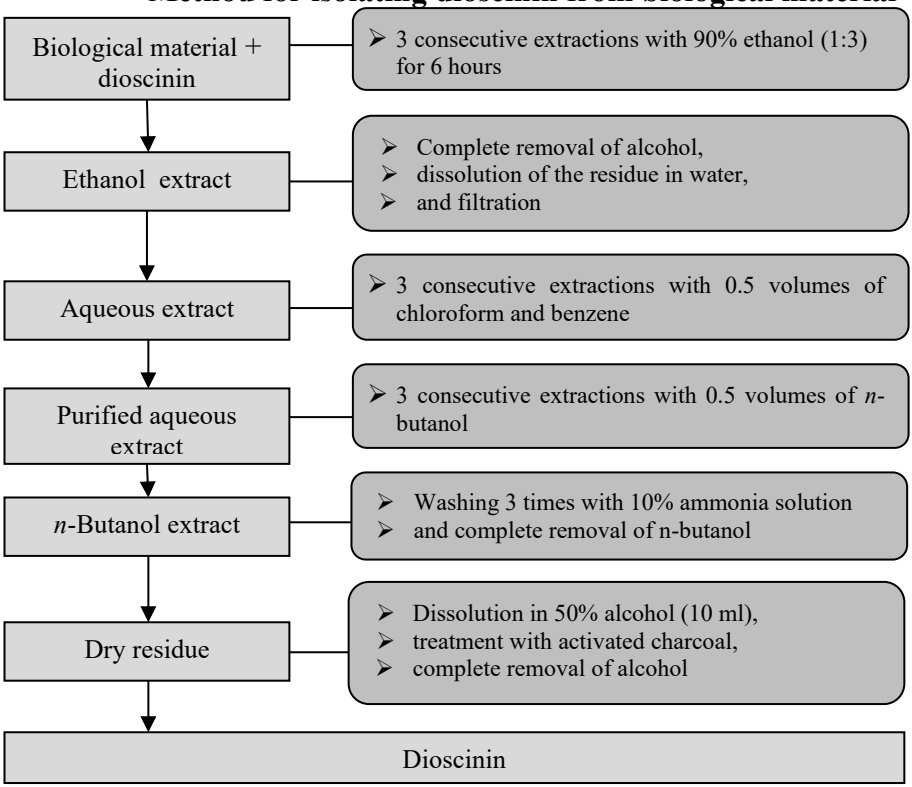
One of the most important stages of chemical-toxicological analysis is the qualitative and quantitative identification of the target substance isolated from biological material. To achieve this, we used classical chemical and modern physicochemical research methods. Dioscinin was detected by classical and modern methods. The presence of dioscinin was confirmed by color reactions conducted with Sanye's reagent.

TLC and IR spectroscopy, were also used. The detection of dioscinin by chromatography was confirmed based on a standard sample. This indicates that the natural glycoside did not undergo metabolism in the model test. The IR spectrum once again confirms that the substance belongs to the spirostane series of steroids.

We developed favorable conditions for the quantitative determination of isolated dioscinin by spectrophotometric method.

In order to test the developed chemical-toxicological analysis method for dioscinin, the internal organs of cattle - liver, kidneys, heart, stomach and intestines - were taken as the research object. Each of the studies was conducted on the basis of model samples prepared with separate internal organs. The glycosides were isolated, purified, characterized and quantified from different internal organs, and the results were compared.

Scheme 1.
Method for isolating dioscinin from biological material



Studies have shown that the percentage of dioscinin isolated varies depending on the nature of the research object. The percentage of glycoside yield depends on both the amount of the object and the main substance itself.

The degree of isolation of dioscinin from different internal organs, i.e., is not the same and depends on the nature and character of the research object (Table 3). The percentage of dioscinin extracted from liver is slightly higher than from the gastrointestinal tract. If 0.25-2 mg of dioscinin in 100 g of liver is determined to 77%, then in the same amount of stomach 0.5-2 mg of this substance is determined to 72% (Figure 3). The quantitative limit of evidence for dioscinin in liver, heart, and kidneys is 0.25 mg, and the

qualitative limit of evidence is 0.1 mg. In the gastrointestinal tract, the quantitative limit of evidence was 0.5 mg, and the qualitative limit of evidence was 0.25 mg (Figure 4).

Table 3.

Comparison of the same amount of saponin based on results obtained from different organs using the Anova test

Organs	Metrological indicators					Anova test [a]			
	$\bar{x}$	S	$S\bar{x}$	$\triangle\bar{x}$	A	Min.	Maks.	F	P
Liver	76,86	0,44	0,20	0,55	0,72	76,2	77,4	235,145	< 0,001
Kidney	77,9	0,46	0,21	0,46	0,74	77,2	78,4		
Heart	77,92	0,36	0,16	0,45	0,58	77,4	78,4		
Stomach	72,16	0,73	0,32	0,90	1,25	71,2	72,8		
Intestine	71,36	0,17	0,07	0,208	0,29	71,2	71,6		

a.Saponin = 2 mg
F- Fisher coefficient
P – correctness

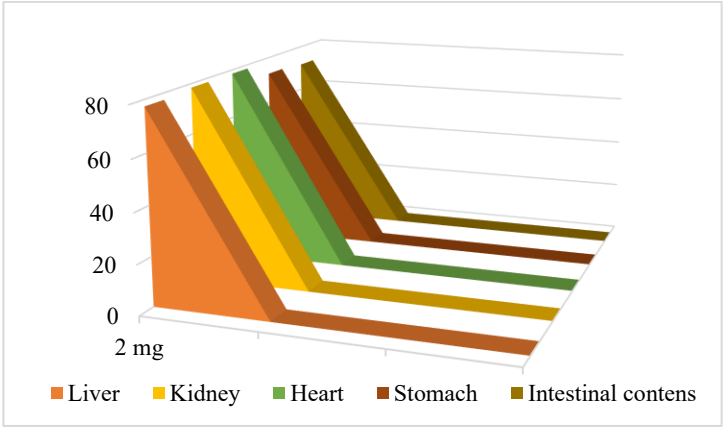


Figure 3. Degree of isolation of 2 mg dioscinin in 5 organs

Also, the degree of isolation of dioscinin extracted from the same amount of biological material was not constant, this indicator also depends on the amount of the substance in the object. When 100 g of liver contains 2 mg of dioscinin, the percentage of yield is 76.86%, while when its amount is 0.25 mg, the percentage of yield is

67.44%. This type of dependence is typical for all of the internal organs studied.

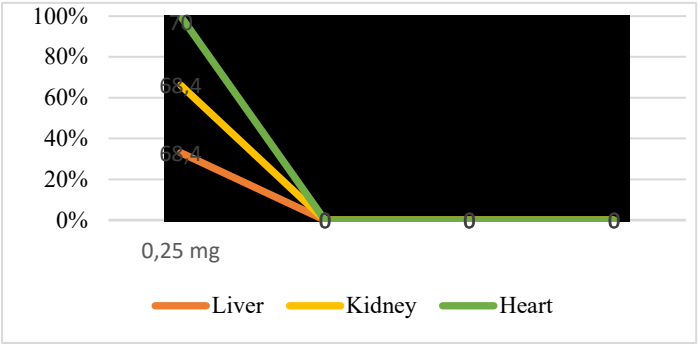


Figure 4. Degree of isolation of 0.25 mg dioscinin in 3 organs

Table 3.
Comparison of the results obtained based on 5 organs at 1 mg of saponin using the Kruskal-Wallis test

Ranks [a,b]				***Statistics*** [c]	
Organs		***n***	***Middle rank***	***Df***	***PH***
Amount, mg	*Liverr*	*5*	*21,2*	*4*	*< 0,00034*
	Kidney	*5*	*18*		
	Heart	*5*	*14,8*		
	Stomach	*5*	*7,9*		
	Intestine	*5*	*3,1*		
a. *Saponin =1 mg* ***b.*** *Variable grouping: organ* ***c.*** *Kruskal-Wallis test*			***Df*** *– degree of freedom* ***P*** *– correctness*		

The amount of dioscinin isolated by our proposed method in model test samples was statistically verified for accuracy (Tables 3, 4). The fact that the reliability coefficient is $p<0.05$ once again proves the reliability of the method.

Table 4.
Comparison of the results obtained in the intestine with different amounts of saponin (2 mg, 1 mg and 0.5 mg) using the Mann-Whitney test

Ranks[a,b]		Statistics[c]	
		P_{U1}	P_{U2}
Amount, mg	2	-	-
	1	0,01208	-
	0,5	0,01208	0,01208
a. Organ: intestine b. Variable grouping: s. quantity c. Mann-Whitney test		P – correctness P_U – <0,05	P_{U1} – With 2 group indicators P_{U2} – With 1 group indicators

Thus, the results obtained prove the efficiency and usefulness of the method proposed by us.

RESULTS

1. For the first time, steroid saponins were isolated from raw plant materials using a specialized method involving solvents of varying concentrations. This process yielded six individual glycosides (designated as substances A, B, C, D, E, and F) across three distinct fractions;
2. The chemical structures of the isolated glycosides were studied: glycoside A was identified as diosgenin, glycoside B as ruscogenin monoside, glycoside C as a diosgenin triside, and glycoside D as a tetraside (dioscinin). From this raw material, glycoside B and dioscinin were obtained for the first time and their chemical structure was completely established;
3. A scalable and industrially significant method was developed for extracting highly biologically active dioscinin, as well as diosgenin and ruscogenin, from a novel raw material. This innovation led to the issuance of a Eurasian patent and a patent of the Republic of Azerbaijan;

4. The impact of various factors on the extraction of saponins from biological materials was systematically investigated. Optimal and favorable conditions were identified, leading to the development of an efficient and reliable isolation method for the first time;
5. For the first time, sensitive, reliable, and straightforward chemical-toxicological analysis methods have been developed for the qualitative and quantitative determination of saponins isolated from various research objects;
6. The method was successfully applied to analyze saponin content in various internal organs. Results showed that 0.25–2 mg of saponin could be detected in 100 g of biological material, with degree of isolation ranging from 62.80% to 78.40%. The detection limit for saponin was established at 0.25 mg, with a qualitative detection limit of 0.1 mg. This proposed method represents a significant advancement in chemical-toxicological analysis, offering high sensitivity, convenience and practicality. Its application is purposeful and ensures reliable results for use in both research and practical settings.

PRACTICAL RECOMMENDATIONS

1. *Tribulus terrestris* L. (puncturevine), a plant widely distributed in Azerbaijan flora and containing the industrially significant biologically active compound dioscinin, can serve as a novel raw material for the production of pharmaceuticals. These include medications previously utilized successfully in the treatment of atherosclerosis but whose manufacturing was halted due to the scarcity of raw materials.
2. Diosgenin and ruscogenin, recognized as indispensable and industrially significant precursors for the synthesis of steroid hormone preparations, can be efficiently extracted from the newly identified local raw materials and can be utilized for their established pharmaceutical applications.

3. The proposed method can be successfully applied in chemical-toxicological analysis experiments and other practical domains.

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LIST OF ABBREVIATIONS AND SYMBOLS

BAS	– biologically active supplements
e.s.	– extractive solvent
m	– minute
°C	– degrees Celsius
m.p.	– melting point
s.s.	– solvent system
$[\alpha]_D^{20}$	– specific rotation
IR	– infrared spectroscopy
m/z	– mass to charge ratio
MS (MS)	– mass spectrometry (mass spectroscopy)
g	– gramme
l	– liter
μg	– microgram
mg	– milligram
TLC	– thin layer chromatography
α	– rotation angle of the plane of polarization
n	– refractive index
UV	– ultraviolet

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