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OPTIMAL CONDITIONS FOR CLEANING AND DRYING FUROSTANOL SAPONINS FROM *TRIBULUS TERRESTRIS*

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Furostanol saponins from *Tribulus terrestris* may be separated from ballast substances by liquid-liquid extraction with changing polarity of the extractant, depending on the dynamics of the process. Treatment with chloroform removed the maximum number of lipid-like compounds and showed minimal losses of furostanol saponins, and subsequent treatment with ethyl acetate retrieved low-polarity substances. Fourfold extraction with butanol was used to extract furostanol saponins from an aqueous solution. Optimal conditions were developed for spray drying of furostanol saponins. The greatest yield of "Dry extract Tribulus" was obtained with a solution feed rate of 80 l/h, a spray head rotation speed of 8000 rpm, and a heat carrier velocity of 2000 kg/h. Technology was developed for obtaining "Dry extract Tribulus" from *Tribulus terrestris* that contained at least 45% furostanol saponins. The reproducibility of the developed technology was shown with the obtaining of 5 series of substances that meet the requirements of regulatory and technical documentation on the lines of the Scientific and Technological Center for GMP Requirements the Institute of Chemistry of Plant Substances.

Keywords: *Tribulus terrestris*, furostanol saponins, extraction, cleaning and drying extract, technology.

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Introduction

Tribulus terrestris L. from the *Zygophyllaceae* family [1] is often used in folk and modern medicine. Various pharmacological properties of *Tribulus terrestris* have been identified, including diuretic, tonic, immunomodulatory, antidiabetic, vasodilatory, hypotensive, hypolipidaemic, antitumour, anti-inflammatory, analgesic, antioxidant, antimicrobial, hepatoprotective, and cardiotonic effects [2–9].

Using the saponins of this plant, more than ten drug analogues have been introduced that are used to increase the levels of certain hormones, including testosterone, luteinizing hormone, follicle-stimulating hormone, and oestradiol [10–12]. The preparations created based on *Tribulus terrestris* saponins must meet the requirements of the British Pharmacopoeia, and the content of furostanol saponins must be at least 45% in terms of protodioscin ($C_{51}H_{84}O_{22}$, MM 1049.2: 26-O-beta-D-Glycopyranosyl-22-hydroxyfurost-5-en-3β, 26-diol-3-O-β-diglucorhamnoside). Proceeding from this, we conducted a study on the development of technology for obtaining the substance "dry extract Tribulus" with a furostanol saponin content of at least 45% in terms of protodioscin from *Tribulus terrestris* growing in the Republic of Uzbekistan.

The literature provides data on the acquisition of saponins from *Tribulus terrestris*, according to which the extraction of saponins was carried out with ethyl alcohol at concentrations ranging from 60% to 90% [2, 13, 14]. Our experiments on the extraction of

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furostanol saponins from *Tribulus terrestris* established the following parameters: extractant – 70% ethanol; degree of shredding of raw materials – 2–6 mm; process temperature – 20–30 °C.

Tribulus terrestris contains steroidal glycosides (A – trillin, B – ruskogenin monoside, C – dioscin, D – dioscinin, and longipetalosides A–C) [15–17], furastanol saponins (chloromaloside, terrestoneiside, trillin, dioscine, protodioscin, terfyranosides, and others) [13, 18–23] flavonoids (rutin, kaempferol-3-rutinoside, rhamnetin-3-rutinoside, isorhamnetin-3-rutinoside, tamaraxetin-3-rutinoside, azaleatin-3-rutinoside) [24, 25], alkaloids, vitamin C, resinous substances, fatty oil, coumarins, and dyes [26]. Therefore, *Tribulus terrestris* contains various classes of compounds. When extracted with 70% ethanol, accompanying substances pass into the extract along with furostanol saponins, which requires a specific sequence for cleaning the extract.

The aim of this study was to develop a method for purifying extracts obtained from *Tribulus terrestris* with 70% ethanol and to select the optimal drying conditions for purified furostanol saponins.

Experimental part

For holding experiments, raw materials were procured from *Tribulus terrestris* collected between August 20 and September 10 in the Tashkent region of the Republic of Uzbekistan. In the raw materials used for experiments, the content of the sum of furostanol saponins based on protodioscin was 2.5%.

Determination of the dry mass of solutions and extracts.

Five millilitres of an analytical sample (extract or solution) was placed in a weighted dish, evaporated in a water bath, dried for 3 hours at 102.5±2.5 °C, cooled in a desiccator for 30 minutes and weighed.

The mass fraction of the dry residue (in%) was calculated by Formula (1):

$$W = \frac{m_2 - m_1}{m} \times 100 \quad (1)$$

where m – sample mass, g; m_1 – *the mass of the cup, g;* and m_2 – the mass of the cup with residue after drying, g.

Quantification of furostanol saponins.

Approximately 0.05 g (accurately weighed) of extract was added to a 50 ml volumetric flask, 30 ml of 70% ethanol was added, the solid was dissolved, and the solution volume was brought to the mark with 70% ethanol. To 2.0 ml of the filtrate were added 3.0 ml of methanol and 5.0 ml of reagent A; the mixture was stirred and heated at 58±2 °C in a water bath for 2 hours and cooled.

The optical density of the test solution and the standard solution of the dry extract of *Tribulus terrestris* (with the content of furostanol saponins of at least 45% based on protodioscin) was measured relative to the control solution on a spectrophotometer at a wavelength of 515 nm in a 10 mm cuvette.

In experiments, a working standard sample (WSS) was used to determine the quantitative content, which value (P) is 48.68%, respectively standardized relative to the standard sample of protodioscin (P=98.23%).

The percentage of furostanol saponins content in terms of protodioscin was calculated using Formula (2):

$$\% = \frac{D_1 \times m_0 \times 50 \times P}{D_0 \times 50 \times m_1} = \frac{D_1 \times m_0 \times P}{D_0 \times m_1} \quad (2)$$

where D_1 is the optical density of the test solution; D_0 is the optical density of the standard solution; m_0 is the weight of a standard sample in mg; m_1 is the weight of raw materials in mg; and P is the content of a standard sample of protodioscin in %.

Preparation of reagent A. One gram of p-dimethylaminobenzaldehyde was dissolved in a 66:34 mixture of methanol and hydrochloric acid (35 to 39%) in a 100 ml volumetric flask, and the volume of the solution was brought to the mark with the same mixed solvent.

Preparation of a standard solution. Approximately 50.0 mg (accurately weighed) of the working standard sample (WSS) was placed in a 50 ml volumetric flask, dissolved in 70% alcohol, and filtered through filter paper; the first portions of the filtrate were discarded.

To 2.0 ml of the filtrate were added 3.0 ml of methanol and 5.0 ml of reagent A; the mixture was stirred and heated at 58±2 °C in a water bath for 2 hours and cooled.

Preparation of the control solution. Methanol (5.0 ml) and reagent A (5.0 ml) were stirred and heated at 58±2 °C in a water bath for 2 hours and cooled.

Residual amounts of organic solvents.

Approximately 0.2 g (accurately weighed) of the substance was placed in a 10 ml volumetric flask and dissolved in 5 ml of propyl alcohol. The volume of the solution was brought to the mark and mixed.

One microlitre of the resulting solution and a solution containing standard samples of organic solvents were alternately chromatographed on a gas chromatograph with a flame ionization detector, with at least 5 chromatograms obtained under the following conditions: glass column with a size of 0.3 × 250 cm; sorbent – chromaton N-AW-DMCS with a particle size of 0.16 mm; stationary phase – SE52; various column temperatures ranging from 50 to 110 °C according to the programme; evaporator and detector temperature – 230 °C; and carrier gas velocity (nitrogen) = 20 ml/min.

The % content of organic solvent (X) in the substance was calculated by Formula (3):

$$X = \frac{B_1 \times m_0 \times 100}{B_0 \times m_1} \tag{3}$$

where B_1 – the average value of the ratios of chromatographic peak areas for the organic solvent and the test solution; B_0 – the average value of the ratios of chromatographic peak areas for the organic solvent and the standard sample in the organic solvent; m_1 – the weight of the sample, g; and m_0 – the mass of the sample for a standard sample of the organic solvent.

Preparation of standard samples of organic solvents (ethanol, chloroform, dichloromethane, n-butanol). Approximately 1.16 g (accurately weighed) of an organic solvent (ethanol 96%, chloroform, dichloromethane, *n*-butanol) was placed in a 100 ml volumetric flask and the volume was brought up to the mark with propyl alcohol and mixed. From each standard solution, 2 ml was removed, placed in a gas-liquid chromatography vial, stirred, and filtered through a Millipore filter.

Purification of the extract from Tribulus terrestris. A total of 2.5 kg of air-dried raw material was extracted six times with 70% ethanol at room temperature and infused for 8 hours. The combined extract was evaporated to 1.0 litres, and 2.0 litres of water were added. The remaining alcohol was removed from the extract, and its concentration was reduced to 2.0 l. As a result, an extract was obtained with a dry matter content of 12.5% and a furostanol saponin content of 2.8%. The extract was divided into 4 parts, 0.5 litres each. Then, to remove lipophilic substances from the extract, the aqueous solutions were washed five times with 0.5 litres of organic solvents immiscible with water, such as chloroform, dichloromethane, extraction gasoline, and ethyl acetate. The obtained extracts were analysed to determine the yields of accompanying substances and furostanol saponins.

Selection of a solvent for the extraction of furostanol saponins from a purified aqueous solution. Two kilograms of raw material was extracted, concentrated, and diluted with water, as in the previous experiment. To remove the remaining alcohol, the mixture was concentrated to 2.0 litres and treated 3 times with 2 l of chloroform and then 2 times with 2 litres of ethyl acetate. The purified aqueous solution of saponins was divided into four equal portions. From the first solution, saponins were extracted with a chloroform – 2-propanol (1 : 1) mixture, from the second with a chloroform – butanol (1 : 1) mixture, from the third with a chloroform-methanol (1 : 1) mixture, and from the fourth with 500 ml butanol; extractions were performed six times under the same conditions. The obtained extracts were dehydrated with sodium sulfate, the solvent was distilled off under vacuum, and the product was dried and analysed.

The effect of temperature in drying aqueous solutions of furostanol saponins. A total of 250 kg of air-dried *Tribulus terrestris* was extracted, concentrated, diluted with water, and treated with chloroform and ethyl acetate under the conditions described in the previous two experiments. From the purified aqueous solution, furostanol saponins were extracted four times with butanol. The butanol extract was concentrated and dissolved in water until approximately 7% of the dry residue in the solution was formed. To remove the residual butanol, the aqueous solution of saponins was concentrated to a content of 10% dry residue. The resulting solution was divided into six portions, which were dried at different temperatures with a solution feed rate of 70 l/h and a spray head rotation speed of 7500 rpm.

Optimal concentration for drying aqueous solutions of furostanol saponin. Solutions of furostanol saponins containing 5, 10, 15, 20 and 25% dry residues were dried in a spray dryer under the conditions described for the previous experiment. The resulting dry extracts were analysed and, on the basis of the results, the effect of residue concentration on the quality of the dry extract was evaluated.

Results and discussion

The State Unitary Enterprise “State Center for Expertise and Standardization of Medicines, Medical Products, and Medical Equipment” of the Ministry of Health of the Republic of Uzbekistan approved temporary pharmacopeia article 42 Uz-3282–2018 “Herb *Tribulus terrestris”* for raw materials and temporary pharmacopeia article 42 Uz-3283–2018 for the substance “dry extract Tribulus”.

In terms of organoleptic and physicochemical indicators, “Dry extract Tribulus” must meet the requirements given in Table 1.

Studies on the selection of the optimal solvent for removal of accompanying hydrophobic substances by liquid-liquid extraction have established that when the extract is treated with ethyl acetate, the yield of lipophilic substances is greatest, but ethyl acetate also extracts furostanol saponins along with accompanying substances. When processing the extract with benzene, fewer furostanol saponins are lost; however, the ability to extract associated substances is limited. Good results were obtained when the extract was treated with chloroform and dichloromethane, since they extracted the accompanying substances almost equally. However, the boiling point of dichloromethane (39.6 °C) is much lower than that of chloroform (61.2 °C). Considering that the loss of dichloromethane is three times greater than that of chloroform, the use of the latter is beneficial from an economic point of view at this stage (Table 2).

In some experiments there were transfers of significant amounts of basic substances from solution to organic solvent, which was due to increased solubility in the presence of significant amounts of hydrophobic substances. To reduce the loss of basic substances and achieve maximum purity of the treated solution, a scheme is proposed for sequential treatment of an aqueous solution with nonpolar solvents and then with more polar solvents. In particular, processing of the bottom residue of the extract *Tribulus terrestris* with chloroform removes the maximum number of lipid-like compounds with minimal losses of furostanol saponins. Ethyl acetate recovered few polar substances from prepurified solutions, and mainly removed the dark colouring. Such sequential purification made it possible to reduce the loss of furostanol saponins by up to a factor of three upon treatment with ethyl acetate.

Experiments on the dynamics of purification of the extract from lipophilic substances showed that for nearly exhaustive removal of accompanying substances from the bottom residue, at least three extractions with chloroform and then two extractions with ethyl acetate were required. In this case, 3.4% (from the mass of raw materials) of accompanying hydrophobic substances were extracted.

Research on the extraction of furostanol saponins from a purified aqueous solution showed that the extraction capacities of mixtures comprising chloroform – 2-propanol (1 : 1), chloroform – butanol (1 : 1), and chloroform – methanol (1 : 1) were not sufficient. For exhaustive extraction of furostanol saponins from an aqueous solution, butanol is the optimal solvent (Table 3).

Table 3 also shows that a fourth butanol extraction does not meet the requirements of temporary pharmacopeia article 42 Uz-3283-2018 with respect to the content of furostanol saponins. However, preliminary experiments showed that the combined first four fractions of butanol extracts contained 46% furostanol saponins after drying. Based on this, fourfold extraction with butanol was proposed to extract furostanol saponins from a purified aqueous solution.

The selection of the drying conditions for the purified aqueous solution of saponins from *Tribulus terrestris* was carried out in a ZPG 150 spray dryer (China), and the solution to be dried was fed into the chamber from top to bottom using a spray head.

Experiments on the optimum temperatures of the coolant at the inlet and outlet showed that when drying an aqueous solution of saponins in a spray dryer, the temperature of the coolant should be 165–170 °C at the inlet and 85–90 °C at the outlet (Table 4).

Based on the results given in Table 5, it was established that the dry residue content in an aqueous solution of saponins should be at least 10% and not more than 15%.

In all samples of dry extract obtained from *Tribulus terrestris* under the above conditions, the residual amount of butanol did not meet the requirements of temporary pharmacopeia article 42 Uz-3283-2018, and residual amounts of other organic solvents were present. Such problems are often observed when organic solvents remain in the bottoms. To eliminate this problem, we propose the following preparation stage for the solution to be dried: at the end of concentrating the butanol extract, it is necessary to supply purified water in portions to establish a ratio of at least 1 kg of dry mass in the solution to 30 litres of supplied water while continuing concentration to give a dry residue content of 10% (Table 6).

Table 1. Specifications of the substance “Dry extract Tribulus”

№	Indicators	Norms
1	Description	Amorphous powder with light brown to brown colour and a specific odour
2	Solubility	Soluble in water, slightly soluble in methanol, very slightly soluble in 50% ethyl alcohol
3	Authenticity	Absorption maximum at 515±2 nm (furostanol saponins)
4	Outside impurities	On the chromatogram, the main spot of the test solution of the drug corresponded to the size of the main spot of the comparison solution. The appearance of a second main spot (protodioscin) is allowed
5	pH	3.0–5.0
6	Weight loss upon drying	Not more than 5.0%
7	Sulfated ash	Not more than 10%
8	Heavy metals	Not more than 0.001%
9	Residual organic solvents:	
	– ethanol	Not more than 0.5% (5000 ppm)
	– chloroform	No more than 0.06% (600 ppm)
	– *n*-butanol	Not more than 0.5% (5000 ppm)
10	Amount of furostanol saponins	Not less than 45.0%

Table 2. Effect of hydrophobic extractants on purification of *Tribulus terrestris* extract from lipophilic impurities

Extractant	The output of furostanol saponins, as a % of the content in the raw material	The output of related substances, as a % of the mass of raw materials
Chloroform	3.5	3.2
Dichloromethane	3.7	3.5
Benzine	0.02	1.8
Ethylacetate	17.7	5.9

Table 3. Yields of furostanol saponins depend on the nature of the solvent and the dynamics of their extraction from an aqueous solution

Number plums	The yield of dry extract, in g to the mass of raw materials	Content of furostanol saponins in dry extract, %
Chloroform – propanol-2 (1 : 1)		
1	2.76	20.8
2	1.72	18.3
3	0.92	15.7
4	0.55	12.6
5	0.26	9.6
6	0.09	7.4
Chloroform – butanol (1 : 1)		
1	3.52	24.3
2	2.14	22.6
3	1.86	19.8
4	0.72	17.6
5	0.29	12.5
6	0.12	9.4
Chloroform – methanol (1 : 1)		
1	4.88	40.6
2	3.00	35.2
3	1.76	29.7
4	0.94	25.6
5	0.35	22.3
6	0.17	20.1
Butanol		
1	6.64	48.2
2	5.20	46.1
3	3.78	45.6
4	2.11	43.6
5	1.25	40.0
6	0.85	35.1

Table 4. Change in yield and quality of dry extract depending on the temperature of the heat carrier during drying in a spray dryer

Heat carrier temperature, °C		Dry extract moisture, %	Dry extract yield, kg
at the entrance	at the exit		
150	70	8.0	1.19
160	75	5.4	1.29
165	85	4.0	1.44
170	90	2.6	1.40
175	95	1.4	1.32

Table 5. Change in yield and quality of dry extract depending on the concentration of the supplied solution to the spray dryer

Solution concentration, % dry residue	Dry extract moisture, %	Dry extract yield, kg to raw material weight	Dry extract colour
5	6.8	1.27	Light brown
10	4.2	1.45	Light brown
15	2.5	1.44	Brown
20	2.2	1.38	Dark brown
25	1.6	1.32	Dark brown

Table 6. Influence of the amount of water supplied during the concentration of the butanol extract on the residual amount of organic solvents in the dry extract obtained from *Tribulus terrestris*

Ratio of dry mass and supplied water, kg : l	Residual amount of organic solvents, ppm		
	Ethanol	Chloroform	*n*-butanol
1 : 20	600.0	250.0	6000.0
1 : 30	400.0	175.0	3000.0
1 : 35	300.0	120.0	2000.0
1 : 40	150.0	90.0	900.0

Based on the results obtained, we propose the following technology for the production of substances from *Tribulus terrestris*: 50 kg of raw materials were loaded into an extractor, which was then poured into 215 litres of 70% ethanol and extracted at room temperature for 8–10 hours. The first ethanol extract (145 litres) was filtered into a collection tank. A total of 145 litres of 70% ethanol was poured into the extractor, and a second extraction was carried out. Then, the third – sixth extractions were carried out in the same way as the second. The resulting 870 litres of extract was concentrated to 25 litres in an evaporator. The concentrate was poured into the reactor, 12 litres of purified water and 25 litres of chloroform were fed in, mixed thoroughly, and left for 30 minutes to separate the phases. The aqueous layer was separated and washed 2 more times with the same solvent. To prepare a purified aqueous solution of furostanol saponins, ethyl acetate was fed into the reactor. The mass was stirred for 10 minutes and allowed to settle for 10–20 minutes. After settling, the ethyl acetate layer was separated. Then, a new portion of ethyl acetate was fed into the reactor, and a second extraction was carried out in a manner similar to the first. Butanol was added to a reactor with a purified aqueous solution of furostanol saponins and stirred, and the butanol extract was separated after settling. The extraction process was repeated 3 more times. The butanol extracts were combined and concentrated. At the end of the process, 45 litres of water were fed into the reactor and concentrated to 15 litres. An aqueous solution of furostanol saponins was dried in the spray dryer with heat carrier temperatures of 170 °C at the inlet and 90 °C at the outlet, a solution feed rate of 80 l/h, a spray head rotation speed of 8000 rpm and a heat carrier velocity of 2000 kg/h.

One kilogram of “dry extract Tribulus” was obtained with a furostanol saponin content of 48%. The yield of the substance was 3.2% by weight of the raw material, and the yield of furostanol saponins was 61.5% based on the content in the raw material.

Conclusion

This technology was developed at the Scientific and Technological Center for GMP Requirements of the Institute of Chemistry of Plant Substances; 400 kg of “Dry extract Tribulus” with 45% furostanol saponin content was produced and, in accordance with the contract, was implemented by FE “Nobel Pharmsanoat”. On the basis of this substance, FE “Nobel Pharmsanoat” produces 250 mg of the drug “Tales Energo”, coated tablet No. 30.

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