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Research Article

Development of an anti-inflammatory extract from leaves and immature fruits of walnuts

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ABSTRACT
The article presents the results of studying the composition of walnut leaves (Juglansregia L.) growing in the Republic of Uzbekistan. Methods of obtaining dry extract extraction from raw materials and standardization of tannins contained in walnut leaves. Pharmacological study of toxicity and anti-inflammatory effect of walnut leaf extract.

Keywords: Juglansregia L., walnut, dry extract, biologically active substances.

INTRODUCTION

Walnuts (Juglansregia L., fam. Juglandaceae) are of great interest for science and practice. Leaf preparations (decoction and infusion) are characterized by their tart and bitter taste and binding properties due to the tannins they contain, which is why they are often used as an anti-diarrhea binder. In folk medicine, infusions and decoctions of walnut leaves are used to improve metabolism and heart disease, with chronic eczema, dermatomycosis, exudative diathesis, scrofulaise and rickets, purulent wounds, scrofula, boils, carbuncle (sometimes for this purpose prepare ointments in sunflower oil), with lung oil, skin and other forms of tuberculosis, diseases of the mucous membrane of the mouth and throat as an appetizing agent and in gastric diseases, for the treatment of diabetes mellitus, gout, anemia and avitaminosis, pain, dysmenorrhoea and venereal diseases, frostbite, and for strengthening and stimulating hair growth.

OBJECTIVE OF THE STUDY

To develop a method for obtaining a dry extract from the leaves and immature fruits of walnut, allowing for the optimal yield of biologically active compounds, by selecting the technological operations that ensured the simplification of the technology, reducing the duration of the process, increasing the yield of extractive substances and expanding the spectrum of specific activity of the target product. Leaves and immature walnut fruits collected during May-July in Tashkent and Andijan regions were used for the study. The leaves are complex, successive and the edges are whole. Before drying, the leaves were sorted. Leaves decomposed in one layer were naturally dried at room temperature in an air-conditioned room with periodic turning over. As a result of the natural drying method, the whole dried leaves of the plant were obtained. In the dried raw materials the tannins content, residual moisture and the quantity of heavy metals and trace elements were determined.

Residual moisture: Residual moisture content was determined first of all in dried raw materials. Residual moisture content of samples (leaves) was determined on the Model-"SF-1" moisture analyzer. The working temperature of the moisture meter is 105°C. The duration of the measurement is 10 minutes until the sample reaches a constant mass. To determine the humidity, an exact weight of at least 5 g is taken and the test result is displayed on the screen of the device. The quantitative content of residual moisture for the studied samples was 4.2% respectively.

Heavy metals are detected on an optical emission spectrometer with inductively coupled argon plasma Optima-2400 DV (USA). Heavy metals should not exceed 0.01% in the preparation. Table 1 presents the results of the study of quantitative determination of micro- and macroelements, according to which it can be said that the content of harmful substances did not exceed permissible norms and the presence of mercury, lead and arsenic was not detected [1].

Table 1: Quantity of heavy metals and trace elementswalnut leaf

No	Indicators	Leaves	
		mg/kg	%
1.	Phosphorus	9385,15	0,93
2.	Lead	n/a	n/a
3.	Cadmium	0,0109	0,000109
4.	Mercury	n/a	n/a
5.	Copper	8,765	0,0087
6.	Zinc	12,384	0,0012
7.	Arsenic	n/a	n/a
8.	Natrium	20528,41	2
9.	Potassium	32435,437	3,24
10.	Iron	460,28	0,046

*Note. Preparation of lead acetate solution: place 15 g of ammonium acetate in a 100 ml flask and dissolve in water, add 0.5 ml of ice acetic acid and dilute with water to the mark [1].

Determination of tannins. When extracting tannins from raw materials, most often use the method of extraction of hot water, so the extraction of air-dry raw materials for qualitative analysis was carried out at a total ratio of raw materials: water (hydromodule) 1:10 according to the methodology set out in the State Pharmacopoeia XI edition. About 0.5 g of dry extract is placed in a conical flask with a capacity of 100 ml, pour 20 ml of distilled water and heated in a water bath with a refrigerator back at a temperature of 40-500 C for 1 hour. Filter the resulting extract. Place 5 ml of filtrate in a tube and add 0.2 ml 10% lead acetate solution in acetic acid solution (10%) (deposition of hydrolysable tannins), then filter the sludge and add 0.5 g of iron ammonium alum to the resulting solution. The solution acquires a black and green colouring. Precipitation reactions include interaction of tannins with the following reagents - with 1% gelatin solution, prepared on 10% sodium chloride solution, with alkaloid salts, 5% potassium bihromate solution, with 10% acetic lead solution, with bromine water and with a mixture of 40% formaldehyde solution and concentrated hydrochloric acid, and to color reactions - with iron ammonium alum. When tannins interact with 1% gelatin solution prepared in 10% sodium chloride solution, sludge is formed or solution turbidity occurs. If excess gelatin is added, the turbidity disappears. Deposition of tannins with alkaloid salts (quinine sulfate) forms white sludge. Tannins in interaction with 5% solution of potassium bihromate form brown sludge or turbidity. At interaction with 10 % solution of acetic lead tannins of hydrolyzed group form flaky sediment. The tannins of the condensed group form flake sediment in reaction with bromine water when heated. The 10% lead acetate solution in the acetic solution (10%) precipitates the hydrolysisable tannins, while the condensed tannins remain in the solution, which with iron ammonium alum give a black and green colouring. The mixture of 40% formaldehyde solution and concentrated hydrochloric acid precipitates the condensed tannins, and the hydrolysable tannins remain in the solution [2, 3]. The results of qualitative research are presented in summary table №2.

Table 2: Results of qualitative reactions to tannins extraction from walnut leaves

No	Reagents	Results
1.	1% gelatinsolution	Blurring
2.	Quininesulphate	whitesludge
3.	5% potassiumbichromatesolution	brownmud
4.	10% aceticleadsolution	flakesludge
5.	Brominewater	flakesludge
6.	1) 10% lead acetate solution in acetic acid solution (10%) 2) ironammoniumalum	sludge black-and-greencolouring
7.	Mixture of 40% formaldehyde solution and concentrated hydrochloric acid	sludge

For quantitative determination of tanning substances such methods as: gravimetric (based on quantitative deposition of tanning substances with gelatin, salts of heavy metals, etc.) are used.), titrimetric (based on oxidative reactions, primarily with potassium permanganate), photoelectric colorimetric (based on the ability of tannins to form stable colored reaction products with salts of oxide iron, phosphorus tungsten acid, etc.), spectrophotometric (based on comparative analysis of the optical density of standard and studied samples) and others [2, 3]. But as it follows from the literature data, the method of permanganatometry is more acceptable due to its efficiency and accuracy. This method is based on light oxidation of tannins with potassium manganese oxide in an acidic medium in the presence of indigosulfonic acid. At the end point of titration, the color of the solution varies from blue to golden-yellow [1]. According to the method of the State Pharmacopoeia XI determination of tannins by permanganatometry was carried out in the following way. About 2 g (precise hinge) of crushed raw materials, sifted through a sieve with a hole diameter of 3 mm, poured 250 ml of boiling water and heated in a water bath for 30 minutes with frequent mixing. Then for 30 minutes the extraction was set at room temperature and filtered through a paper folded filter into a 100 ml flask and brought to the mark with water. 25 ml of extraction was placed in a 1-liter conical flask, 750 ml of water and 25 ml of indigosulphonic acid solution were added and titration was done at constant stirring with 0.02 mol/l potassium permanganate until golden yellow colouring. A control experiment was conducted simultaneously.

1 ml solution of potassium permanganate (0.02 mol/l) corresponds to 0.004157 g of tannins in terms of tannin.

The quantitative content was calculated by formula:

$$X = \frac{(V - V1) \times 0{,}004157 \times 250 \times 100 \times 100}{m \times 25 \times (100 - W)}$$

where,

V - volume of permanganate potassium solution (0.02 mol/l) used for titration of extraction, in ml;

V1 - volume of permanganate potassium solution (0.02 mol/l), spent for titration in control experiment, in ml;

m - raw material hinge, d;

0.004157 - quantity of tannins corresponding to 1 ml of potassium permanganate solution (0.02 mole/l) (in terms of tannin), in g;

W - loss in mass during drying of raw materials, in % [4].

The result of the quantitative content of tannins.

$$\% = \frac{(2{,}6 - 0{,}5) \times 0{,}004157 \times 250 \times 100 \times 100}{1{,}0118 \times 25 \times (100 - 3{,}9)} = 8{,}98$$

As can be seen from the obtained data, the tannins content is 8.98%.

Choice of extraction method. The following methods of extraction were considered as possible methods of alcohol extraction from walnut leaves: maceration, fractional maceration, percolation and recolation. All the methods used are widely used in industry to obtain alcoholic beverages from plant raw materials. After completion of each stage or extraction as a whole, the obtained extractions were merged by gravity. The remaining meal was squeezed out after extraction and the resulting plums were combined with the corresponding extracts. Combined extractions were defended at a temperature no higher than +10°C for 2 days, after which they were filtered. Dry leaves of the plant were used in the experiment, which were shredded to the required size. The obtained product was dried in the rotary evaporator at temperature from 40-500C. The experimental part of the work was done according to the traditional scheme, starting with the establishment of technological parameters of medicinal plant raw materials (DLR) and ending with the development of quality standards for the finished product.

Technological properties of herbal medicinal raw materials include the degree of grinding, fractional composition, bulk weight, extractant absorption coefficient and mass flowability, duration of extraction and the number of phase contacts of raw materials and extractant (multiplicity). The choice of the optimum degree of grinding was determined by the quantitative content of tannins in the extractions. The extractions obtained during the extraction experiment were analyzed. The quality of extracted extracts was assessed by the dry residue content. Thus, the optimal method was chosen for reperkolation (Table 3).

Table 3: Selection of extraction method

Methodsofextraction	Maceration	Fractionalmaceration	Repertoire	Percolation
Dryresidue	3,35	3,48	7,51	6,48

The resulting liquid extract is dark brown with a greenish tint and a specific odour. Further this liquid extract was evaporated under vacuum on the rotary evaporator and dried under vacuum to obtain a dry extract with residual moisture not exceeding 5%. The received dry extract represents a hygroscopic powder of brown colour with a specific smell.

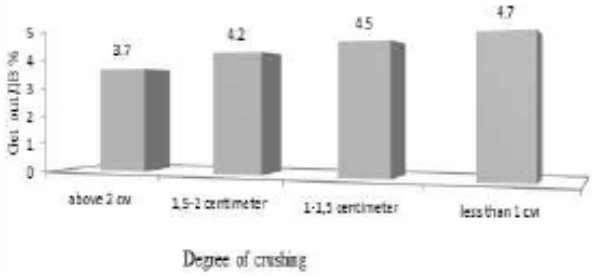

Fig.1: Results of study of the degree of raw material crushing

Selection of the optimal extractant. The experiment was conducted on three samples, selected on the basis of literature data. We chose 40%, 70% and 96% ethyl alcohol solution as the samples. Extraction of walnut leaves was carried out from the canopies of the same sample of raw materials, under the same conditions (at room temperature, for 3 hours), the ratio of raw materials - extractant 1:10. The extractions were a dark, muddy liquid, and when sedimentation was formed sediment. As a result of the experiment, the optimal extractant was chosen as 40% ethyl alcohol, as the highest yield of tannins was extracted by this extractant (Table 4).

Table 4: Selection results of the optimal extractant

No	Estragent	Extractantyield,%
1.	40% ethylalcohol	21,15
2.	70% ethylalcohol	18,37
3.	96% ethylalcohol	12,5

Influence of the degree of crushing of raw materials on the yield of tannins from walnut leaves. The completeness of the target substance extraction depends to a great extent on the degree of the plant raw materials shredding. When searching for the optimum value of the degree of raw material shredding, we used leaves which were shredded to different degrees: coarser than 2 cm, 1.5-2 cm, 1-1.5 cm, shallower than 1 cm. As expected (Fig. 1), with finer grinding of the raw material, the yield of tannins increased. Thus, the yield of tannins when using raw materials larger than 2 cm will reach 1.2%, when grinding 1.5-2 cm - 1.9%, when grinding 1-1.5 cm - 2.1% and when less than 1 cm - 2.2%. However, taking into account the fact that excessively fine grinding of the raw material considerably complicates the filtration of the obtained extractant and, moreover, the yield of tannins from leaves crushed less than 1 cm varies insignificantly, our choice was stopped at 1-1,5 cm. Selection of optimum temperature. An increase in temperature has a significant effect on the extraction of plant substances, as the process of diffusion accelerates as the temperature increases. Therefore, we studied the effect of the temperature factor on the extraction process. The experiments were conducted with the raw materials under the same conditions. The study of temperature influence on tannins extraction process was carried out at room temperature (18-200C), at 30-400C and at 40-500C. From the received data (Fig.2) it is visible, that influence of temperature on tanning substances output has special influence.

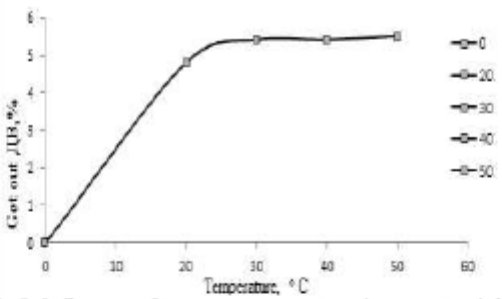

Fig.2: Influence of temperature on tanning agents yield

Study of extraction dynamics. In order to prevent unreasonable expenses during extraction of plant raw materials, it is necessary to study the duration of the extraction process. Therefore, we studied the dynamics of the extraction process in order to determine it and establish the phase equilibrium moment. In the experiments, we established the time required for the most complete depletion of raw materials at four phase contacts. In the literature we have data on the study of the dynamics of the process at different methods of extraction. However, it depends first of all on the type of raw materials used [5]. Experiments to determine the phase equilibrium moment were carried out using the technique described in this paper. In the process of extraction after a specified time, a sample was taken from the extractor and analyzed for the amount of extracted tannins. The study of the extraction dynamics showed that the equilibrium concentration of tannins at the first phase contact is achieved in - 8 h, at the second - 6 h, at the third - 4 h and at the fourth - 2 h (Table 5). The total yield of tannins was 8.3% and 5.1%, with the highest yield at the first extraction being 4.2% and 2.1%, at the second - 2.3% and 1.5%, at the third - 1.2% and 1.0% and the fourth - 0.6% and 0.5%, respectively. The fourth extraction is not expedient due to low yield of substances, but the resulting extraction may be used in the extraction of a new portion of raw materials.

Table 5: Results of the extraction dynamics study

phasecontact	Extractiontime, ч	Yield, FS from content in raw materials %
I	1,0	1,4
	2,0	1,7
	4,0	1,9
	6,0	2,1
	8,0	2,2
II	1,0	0,9
	2,0	1,3
	4,0	1,5
	6,0	1,6
III	1,0	0,6
	2,0	0,9
	4,0	1,1
IV	1,0	0,4
	2,0	0,6

Based on the data of Table 5, we have prepared a picture. 3, on which the curves are given. On which we can observe that with the depletion of raw materials the relative extraction rate decreases, as evidenced by the curves.

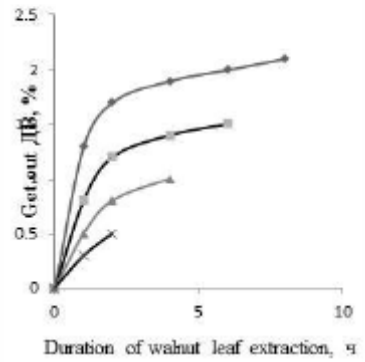

Fig. 3: Effect of extraction duration on DV output

Pharmacological study of toxicity of dry walnut leaf extract. Acute toxicity of dry walnut leaf extract was studied using a common method described in the literature, a single administration of drugs with the definition of LD50 and toxicity class. For the experiment we used white non-breeding mice of males and females in the amount of 18 heads, body weight 19-21 g, aged under quarantine for 14 days. Before and during the experiments the mice were in vivarium at air temperature + 20-22°C, humidity - no more than 50%, volume of air exchange (extractor hood: inflow) - 8:10, in light mode - day - night. Mice were placed in standard plastic cells and kept on a standard diet [6, 7].

Carrying out the experiment: for the experiment to study the acute toxicity of dry extract from walnut leaves, mice were divided into 3 groups, the dry extract was injected as follows:

1 group (6 mice) - per os in dose 10000 mg/kg (0.4 ml);

Group 2 (6 mice) - per os in a dose of 15000 mg/kg (0.6 ml);

Group 3 (6 mice) - per os in a dose of 20000 mg/kg (0.8 ml).

Then mice of all groups were observed hourly during the first day of the experiment in the laboratory conditions, and survival rate during the experiment, general condition, possible seizures and death were used as indicators of the animals' functional state. From the second day the observations were made daily, for 2 weeks in the conditions of the vivarium, while observing the general state and activity, peculiarities of behavior, reaction to tactile, pain, sound and light stimuli, frequency and depth of respiratory movements, heart rate, the state of hair and skin, the position of the tail, the amount and consistency of faecal masses, the frequency of urination, changes in body weight, and other indicators. All experimental animals were kept in the same conditions and on a common diet with free access to water and food. After the experiment, LD50 and toxicity class of the compared preparations were determined [8]. Microcirculation disturbance and edema formation are the main signs of living tissue inflammation. Numerous mediators and modulators of inflammation take part in formation of acute inflammatory reaction. When studying the acute toxicity of the preparation of a dry extract from walnut leaves, the following data were obtained

Group 1 (dose 10000 mg/kg): after administration, mice remained active during the day, no changes in behavior or functional state were observed. The condition of wool and skin was usual without changes, food and water were not refused and no death of mice was observed. On the second day and in the following period no pathological changes in behavior and physiological indices of mice were observed. Water and feed consumption was normal, and growth and development retardation were not observed. There were no deaths of mice for 14 days. Group 2 (dose 15000 mg/kg): no visible changes were observed after administration of the drug during the day of mice, no changes in behavior and functional state were observed. The condition of wool and skin was usual without changes, food and water were not refused and no death of mice was observed. On the second day and in the following period no pathological changes in behavior and physiological indices of mice were observed. Water and feed consumption was normal, and growth and development retardation were not observed. There were no deaths of mice during 14 days (Table 8).

Group 3 (dose 20000 mg/kg) after administration mice showed short-term lethargy and lack of mobility, which was 30-40 minutes later. After 1 hour the mice returned to their

previous state, the behaviour was active and physical indicators did not deviate from normal.
On the second day and during the whole period of observation for 14 days no changes were observed in mice' behavior and other physical indices, mice willingly consumed food and water, reactions to light and sound stimuli remained normal, coat and skin were clean, urination and calavage were normal, weight and growth of mice did not lag behind in development. No mice were killed.

Table 6: Determination of acute toxicity of dry walnut leaf extract

№ gr.alive-x	dosage of		route of administration introductions	Result
	mg/kg	millilitre		
1	10000	0,4	Per os	0/6
2	15000	0,6	Per os	0/6
3	20000	0,8	Per os	0/6
LD50	>20000			

Injection of a larger dose was not possible because according to HF the maximum amount of injected liquid at intragastric administration in white mice, weight 19-22g is 0.8 ml, as well as due to the density of the resulting suspension. In this connection, a dose of > 20 000 mg/kg is proposed for LD50 [1, 8]. According to the classification of toxicity of substances, the preparation belongs to Class VI - relatively harmless. Pharmacological study of the anti-inflammatory effect of walnut leaf extract. The anti-inflammatory effect of dry walnut leaf extract was studied using the method "carrageeninoedema of the paw in rats" on 12 white rats, body weight 180 - 200 g of both sexes. In rats, the volume of the leg was measured three times beforehand in a normal way. The average of three measurements was calculated for the initial volume. Acute inflammatory reaction (oedema) was reproduced under the sole injection of 0.1 ml 1% carrageenin solution. Expression of inflammatory reaction was estimated 3 hours after the induction of inflammation. The change of paw volume was measured with the help of pletizmometer - water chamber with 24 mm diameter and curved outlet tube [8].
The anti-inflammatory effect (ABI) was calculated by formula:
By HPE = 1- (Po :Pk) x 100,
where:
Po - an increase in foot volume in an experimental group,
Pk is an increase in foot volume in the control group.The drug was administered intragastrically for 7 days. For the experiment the rats were divided into 2 groups of 6 heads each. The medications were injected as follows:
group - control - intragastric water purified + 0.1 ml 1% carrageenin solution; group - experimental - intragastric 200 mg/kg suspension of dry walnut leaf extract + 0.1 ml 1% carrageenin solution. The results obtained in the study of anti-inflammatory activity of dry walnut leaf extract showed that the preparation has a reliable anti-inflammatory activity in the studied dose (Table 7). Dry extract from walnut leaves with intragastric administration in a dose of 200 mg/kg after 3 hours significantly reduced swelling of the inflamed foot by 15.5% compared to control.

Table 7: Anti-inflammatory activity of dry walnut leaf extract

Animal weight (r)	Drug dosage	Volume of healthy foot, ml	Foot volume after carrageenin injection,	Foot gain, ml	%
	мг/кг		3 ч	3 ч	
Reference Group					
187		1,0	1,5	0,5	
190		0,9	1,4	0,5	
195		1,0	1,5	0,5	
199	-	1,1	1,5	0,4	-
200		1,0	1,6	0,6	
185		0,9	1.4	0,5	
193±6,3		0,98 ± 0,07	1,48 ± 0,07	0,5± 0,06	
Dry extract of walnut leaves					

193		1,0	1,2	0,2	
200		1,1	1,3	0,2	
197		1,0	1,3	0,3	
189		0,9	1,1	0,2	
200	200	1,1	1,3	0,2	15,5
185		1,0	1,3	0,3	
194 ± 6,1		1,0 ± 0,07 P>0,05	1,25 ± 0,08 P<0,05	0,23±0,05 P<0,05	

CONCLUSION

The qualitative reactions to tannins showed a large quantity (8.98%) of tannins in walnut leaves. According to the content of dry residue the most optimal method of extraction was chosen - reperkolation, and the most optimal extractant - 40% ethyl alcohol. According to the substances' toxicity classification, the preparation belongs to Class VI - relatively harmless. Dry extract from walnut leaves with intragastric administration in a dose of 200 mg/kg has significantly reduced swelling of the inflamed foot in rats, showing that it has an anti-inflammatory effect.

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