Original Article

Intensification of the Extraction Process of Flavonoids and Hydroxycinnamic Acids from *Tanacetum vulgare* L. Flowers

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Abstract

Background and Purpose: It has been shown that taking into account the polyvalence of action, tansy (*Tanacetum vulgare* L.) is a promising raw material for obtaining substances based on it as active pharmaceutical ingredients for the development of new potential herbal medicinal products. **Materials and Methods:** Ultrasound-assisted extraction (UAE) has been considered as one of the promising methods to optimize the technology of extracting biologically active substances (BAS) from *T. vulgare* flowers. **Results:** The advantages of the method, the mechanism of ultrasound action on plant cells, and the main factors affecting this process are indicated. **Conclusion:** The optimal technological parameters that allow the extraction of flavonoids calculated with reference to luteolin and hydroxycinnamic acids calculated with reference to chlorogenic acid in the maximum amount, namely, the raw material and extractant ratio, time, and the extraction frequency, have been determined. The high efficiency of UAE has been proven.

Keywords: *Complex of biologically active substances, pharmaceutical substance, Tanacetum vulgare, ultrasound-assisted extraction*

Introduction

There has been a steady demand for medicinal plant raw material in recent decades. The share of herbal medicinal products has already reached 30–50% of the total volume of the pharmaceutical market, and the trends in recent years indicate a further constantly growing demand both in Ukraine and worldwide.[1,2] All of this demonstrate the significant potential of herbal medicine as natural products combine high efficacy, relative safety, and the therapeutic window.[3-5] This has become possible due to the development of more advanced methods of isolation of target active substances, modern approaches to the intensification of the extraction process, and methods for standardization of the medicinal raw material, substances, and herbal medicinal products.[6,7]

Sources of medicinal plants are both wild and cultivated plants. However, only those medicinal plants that can be cultivated and are scientifically and practically significant can be promising for medical use, and their harvesting is economically profitable.[8,9] Such a plant is tansy (*Tanacetum vulgare* L.), which contains essential oil, flavonoids, tannins, and bitter substances, carotenoids, ascorbic acid, alkaloids, and organic acids in its composition.[10,11] Due to its biologically active substances (BAS), tansy has a wide range of pharmacological activities such as membrane-stabilizing, antioxidant, hepatoprotective, choleretic, anti-inflammatory, antispasmodic, capillary-strengthening, antimicrobial, antisclerotic, anthelmintic, phytoncidal, etc.[11-19]

At the pharmaceutical market of Ukraine, there are only two drugs based on *T. vulgare* flowers: "Tansy flowers" medicinal plant raw material in packs and filter bags and "Phytohepatol" phytospecies produced by PJSC "Liktravy."[20] Taking into account the polyvalence of action of the raw material, the development of new herbal medicinal products based on it is of high practical interests for modern medicine.

Traditional extraction methods (maceration, percolation) used in manufacturing herbal medicinal products are time-consuming and inefficient for modern industrial production.[21,22] This substantiates a considerable interest of manufacturers in modern extraction methods and their intensification (pulse, vacuum pulse and electric pulse treatment, microwave extraction, extraction using ultrasound or supercritical

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How to cite this article: Herbina N, Ruban O, Andryushayev O, Hohlova L. Intensification of the extraction process of flavonoids and hydroxycinnamic acids from *Tanacetum vulgare* L. flowers. J Rep Pharma Sci 2022;11:125-31.

Received: 16 Sep 2021
Accepted: 08 May 2022
Published: 29 Jun 2022

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Access this article online
Website: www.jrpsjournal.com
DOI:10.4103/jrptps.JRPTPS_133_21
Quick Response Code:

fluids); it allows us to significantly increase the yield of extractives.[23,24]

Of the aforementioned methods, one of the promising methods of intensive extraction is ultrasound-assisted extraction (UAE), which uses the action of intense high-frequency sound waves.[25] Under the ultrasonic action, solid and liquid particles vibrate and accelerate; due to it substances from plant cells quickly diffuse from the solid phase into the extractant. An increase in the intensity of ultrasound in the liquid leads to the fact that intramolecular forces are not able to hold the molecular structure, so it is destroyed, and bubbles are formed, i.e., the cavitation process occurs. The collapse of bubbles can cause physical, chemical, and mechanical effects that lead to the destruction of biological membranes and the unhindered penetration of the extractant into the plant cell.[26]

The use of ultrasound has significant advantages over traditional raw material processing technologies. In particular, it provides a deeper penetration of the solvent into the material with a cellular structure, reduces the processing time, provides a higher product yield and reproducibility, reduces the solvent consumption, increases the speed of the process, and allows the extraction of thermolabile substances.[22] The equipment does not require large maintenance costs; less energy is used for processing. Thus, the process becomes more environmentally friendly and economically justified and it is of crucial importance for modern industrial production.[27]

Therefore, the aim of our work was to determine the optimal conditions of UAE for obtaining flavonoids and hydroxycinnamic acids from *T. vulgare* flowers.

Materials and Methods

Plant material

T. vulgare flowers were harvested in the Kharkiv region (Ukraine) in the phase of mass flowering (August). The plant raw material was dried using the air-shadow method at a temperature not exceeding 40°C, avoiding exposure to direct sunlight. The dried raw material was subjected to the quality control in accordance with the monograph of the State Pharmacopoeia of Ukraine (SPhU), 2nd ed., Supp. 2 "Tansy flowers." The raw material was ground to a particle size of 2–3 mm.[28]

The research was conducted using the equipment of the Educational and Scientific Institute of Applied Pharmacy of the National University of Pharmacy (Kharkiv, Ukraine).

Ultrasound-assisted extraction

The selected method for obtaining extracts was remaceration using ultrasound. It was found in previous studies that the optimal extractant for the plant tested was ethyl alcohol at a concentration of 70%.[29]

The extraction was performed in a PEX 1 ultrasonic extraction reactor (R.E.U.S., Contes, France) hermetically sealed; the soaking time of the raw material was 15 ± 1 min, and the ultrasound frequency was 35 kHz. The resulting extracts were kept at a temperature of 8 ± 2°C for 2 days and filtered through an ash-free paper filter under vacuum.

A dry residue of the extracts obtained was determined by the method specified in Article 2.8.16 of the SPhU.[30] An aliquot of 2 mL of the extract was placed in a flat-bottomed cup with a diameter of 50 mm and a height of about 30 mm, evaporated to dryness on a water bath and dried in an oven at 100–105°C for 3 h, then cooled in a desiccator and weighed.

The quantitative determination of the total amount of flavonoids calculated with reference to luteolin and the total amount of hydroxycinnamic acids calculated with reference to chlorogenic acid was performed by a unified spectrophotometric method described in the monograph of the SPhU, 2nd ed., Supp. 2 "Tansy flowers" using a Specord 200 two-beam spectrophotometer (Analytik Jena, Germany).[28]

Quantitative determination of the total amount of flavonoids

Stock solution 1

Five milliliters of the extract was placed in a 100 mL flask and diluted to the volume with the same solvent (70% ethanol).

Then the study was conducted according to the method.

Test solution 1

Five milliliters of *Stock solution* 1 was placed in a round-bottomed flask and evaporated to dryness under reduced pressure. The resulting residue was transferred to a 25 mL volumetric flask using 8 mL of the mixture of methanol RS–anhydrous acetic acid RS (10:100). A round-bottomed flask was rinsed with 3 mL of the mixture of methanol RS–anhydrous acetic acid RS (10:100), and the washing liquid was placed in the same 25 mL volumetric flask. Then 10.0 mL of the solution containing 25.0 g/L of boric acid RS and 20.0 g/L of oxalic acid RS in anhydrous formic acid RS was added to the solution obtained, and the solution was diluted to 25.0 mL with anhydrous acetic acid RS.

Compensation solution 1

Five milliliters of *stock solution* were placed in a round-bottomed flask and evaporated to dryness under reduced pressure. The resulting residue was transferred to a 25 mL volumetric flask using 8 mL of the mixture of methanol RS–anhydrous acetic acid RS (10:100). A round-bottomed flask was rinsed with 3 mL of the mixture of methanol RS–anhydrous acetic acid RS (10:100), and the washing liquid was placed in the same 25 mL volumetric flask. Then 10.0 mL of anhydrous formic acid RS was added to the solution obtained, and the solution was diluted to 25.0 mL with anhydrous acetic acid RS.

Stock solution 2

Approximately 0.010 g (accurate weight) of luteolin RS (SPhU) was placed in a 100 mL volumetric flask, dissolved in 70 mL

of methanol RS, the solution was diluted to the volume with the same solvent, and mixed.

Reference solution

One milliliter of Stock solution 2 was transferred to a 25 mL volumetric flask. Then 10.0 mL of the solution containing 25.0 g/L of boric acid RS and 20.0 g/L of oxalic acid RS in anhydrous formic acid RS was added, and the solution was diluted to 25.0 mL with anhydrous acetic acid RS.

Compensation solution 2

One milliliter of Stock solution 2 was transferred to a 25 ml volumetric flask, 10.0 mL of anhydrous formic acid RS was added, and the solution was diluted to 25.0 mL with anhydrous acetic acid RS.

The optical density of the test solution was measured 30 min after the preparation of Compensation solution 1 at a wavelength of 410 nm. In parallel, the optical density of the *Reference solution* in relation to *Compensation solution* 2 was measured.

The content of the total amount of flavonoids expressed as luteolin in the extract was calculated by the following formula:

$$X(\%) = \frac{A_1 \times m_0 \times 20 \times P \times 100\%}{A_0 \times m \times (100w) \times 100},$$

where A_1 is the optical density of *Test solution* at a wavelength of 410 nm;

A_0 is the optical density of *Reference solution* at a wavelength of 410 nm;

m_0 is the sample weight of *luteolin RS* (SPhU), g;

m is the sample weight of the raw material tested, g;

P is the luteolin content in *luteolin RS* (SPhU), %;

w is the loss of the raw material on drying, %.

Quantitative determination of the total amount of hydroxycinnamic acids

Stock solution.

Five milliliters of the extract was placed in a 100 mL volumetric flask and dilute d to the volume with 70% ethanol.

Test solution.

One milliliter of Stock solution 2 was placed in a 10 mL volumetric flask; 2 mL of 0.5 M hydrochloric acid solution, 2 mL of a freshly prepared solution of 10 g of sodium nitrite RS, 10 g of sodium molybdate in 100 mL of water RS, and 2 mL of dilute sodium hydroxide solution RS were successively added stirring after each addition, diluted to the volume with water RS and mixed.

Compensation solution.

One milliliter of Stock solution 2 was placed in a 10 mL volumetric flask; 2 mL of 0.5 M hydrochloric acid solution and 2 mL of dilute sodium hydroxide solution RS were successively added stirring after each addition, diluted to the volume with water RS and mixed.

The optical density of the test solution was measured immediately at a wavelength of 525 nm in a cuvette with a layer thickness of 10 mm using compensation solution as a reference solution.

The content of the total amount of hydroxycinnamic acids expressed as chlorogenic acid (%) in the extract was calculated by the following formula:

$$X = \frac{A \times 1000 \times 100}{188 \times m \times 100},$$

where A is the optical density of the *test solution* at a wavelength of 525 nm;

m is the sample weight of the raw material tested, g.
For the calculation, the specific absorption index of chlorogenic acid equal to 188 was used.

Statistical analysis

Statistical analysis was carried out with the Microsoft Excel 2016 software. All experimental determinations were done in triplicate, and results were presented as a mean value ± confidence interval (CI). Significant levels were determined at $P < 0.05$.

Results

An important processing method that contributed to the effective implementation of the ultrasonic extraction process is to provide continuous and intensive mixing of the extractant and the crushed plant raw material in the working chamber; it improves the access of the extractant to each particle. Therefore, the raw material–extractant ratio must be strictly determined.

To scientifically substantiate the optimal ratio of the plant raw material mass to the volume of the extractant, the extracts with the ratios of 1:5, 1:10, and 1:15 were obtained. The efficiency of BAS extraction was evaluated by the dry residue index expressed as 1.0 g of the raw material. The extraction was performed using an ultrasound at a temperature of 30°C for 60 min. The experimental data are shown in Figure 1.

As shown in Figure 1, the yield of the dry residue increases with increasing the raw material–extractant ratio. However, at a ratio of 1:15, the dry residue increases insignificantly, therefore, for economic reasons, the optimal ratio is 1:10.

To evaluate the effect of ultrasound on the extraction process, comparative extractions with and without ultrasound under the same conditions were performed. The experiment duration was 60 min. The yield of the dry residue and the concentration of flavonoids and hydroxycinnamic acids in the extracts obtained were compared. In this case and in further studies, the

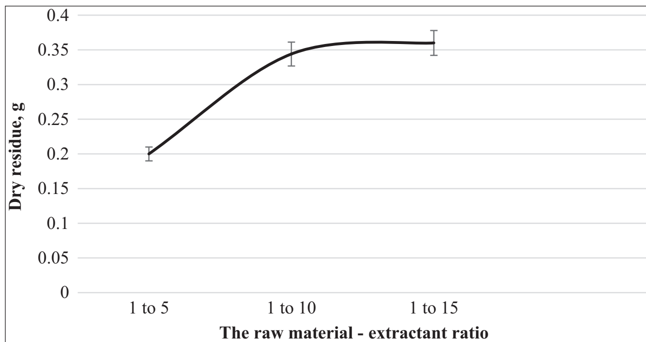

Figure 1: The dependence of the dry residue yield on the ratio of the plant raw material mass to the volume of the extractant
***Note*: *n* = 3, *P* < .05**

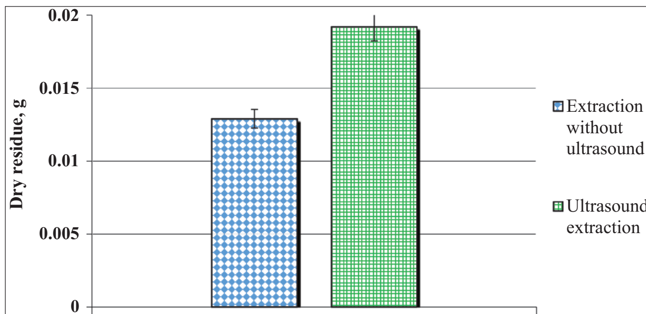

Figure 2: The ultrasound effect on the amount of extractives
***Note:* *n* = 3, *P* < 0.05**

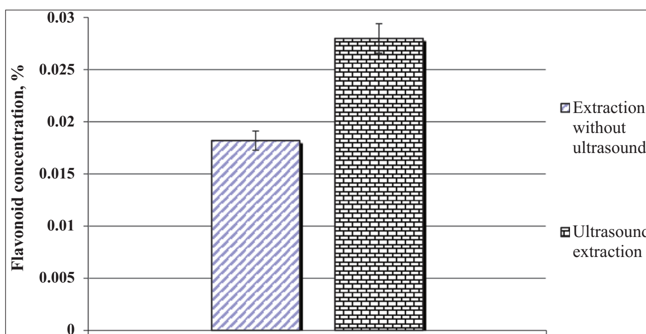

Figure 3: The ultrasound effect on the amount of flavonoids
***Note*: *n* = 3, *P* < 0.05**

effectiveness of BAS extraction was evaluated and calculated with reference to 1 mL of the extract.

The results showed that the yield of the dry residue from tansy flowers using UAE was higher by 33% compared with the extraction without ultrasound [Figure 2].

The results of the quantitative determination of the content of phenolic compounds in the extracts calculated with reference to luteolin and the total amount of hydroxycinnamic acids calculated with reference to chlorogenic acid also showed a higher yield of 35% and 13%, respectively, compared with the extraction without ultrasound [Figures 3 and 4]. This confirms the effectiveness of ultrasound used for obtaining extracts from *T. vulgare* flowers.

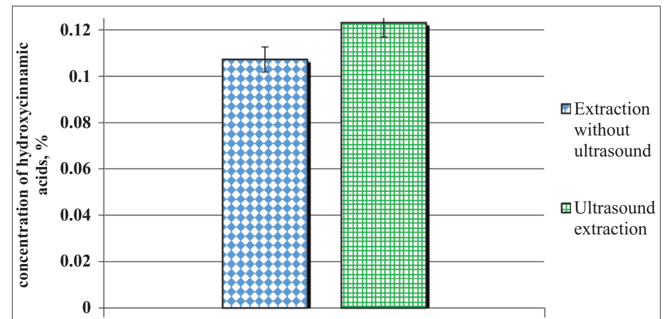

Figure 4: The ultrasound effect on the amount of hydroxycinnamic acids
***Note*: *n* = 3, *P* < 0.05**

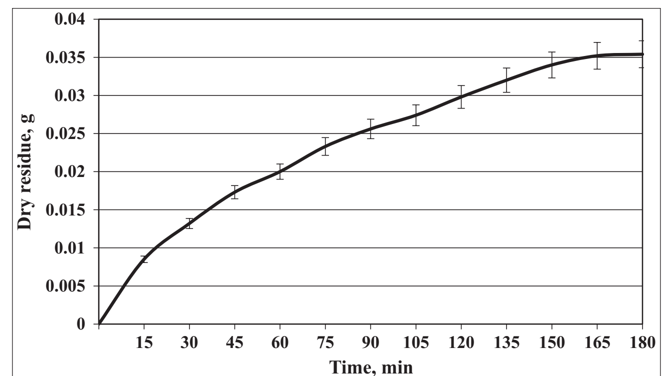

Figure 5: Determination of the optimal time of the first extraction by the dry residue
***Note*: *n* = 3, *P* < 0.05**

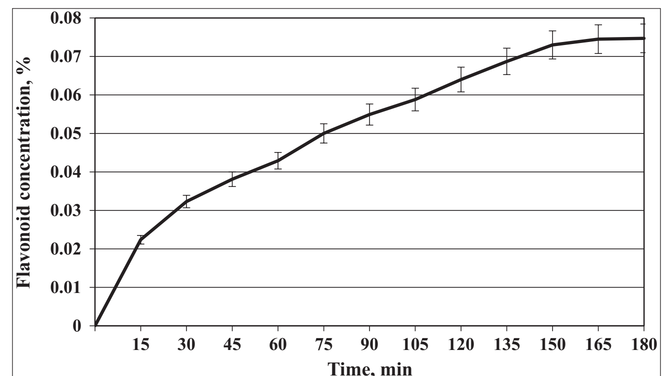

Figure 6: Determination of the optimal time of the first extraction by the amount of flavonoids
***Note*: *n* = 3, *P* < 0.05**

The next step was to determine the optimal duration of the first stage of extraction, which allowed extracting the maximum amount of BAS from the raw material of *T. vulgare* flowers. Extract samples were tested every 15 min. The research results are shown in Figures 5–7.

As the process time increased, the dry residue yield increased proportionally. However, this increase occurred within the first 150 min of the extraction with a maximum amount of the dry residue of 0.0340. Increasing time to 180 min, only an insignificant increase of extractives was observed, indicating

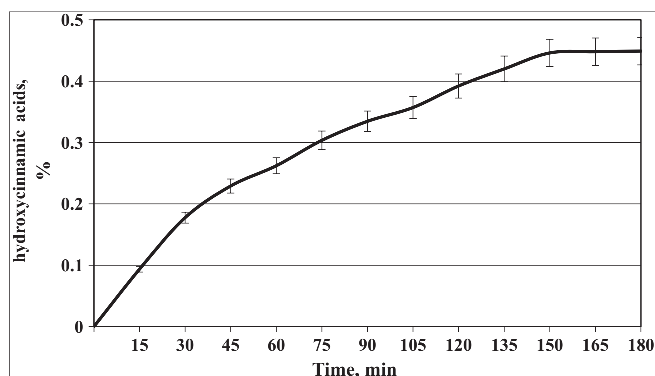

Figure 7: Determination of the optimal time of the first extraction by the amount of hydroxycinnamic acids
***Note*: *n* = 3, *P* < 0.05**

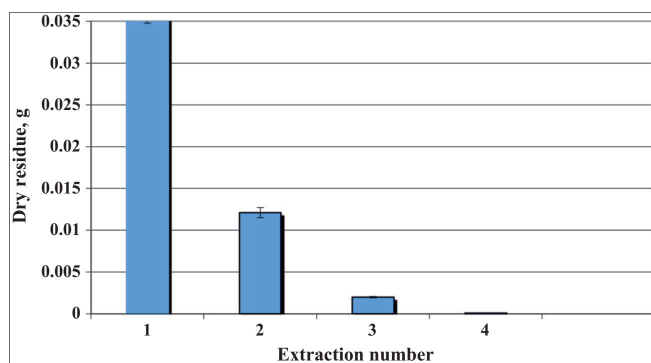

Figure 8: Determination of the optimal extraction frequency
***Note*: *n* = 3, *P* < 0.05**

the achievement of an equilibrium concentration between the raw material and the extractant [Figure 5].

The results of the experimental studies [Figures 6 and 7] revealed that the concentration of flavonoids and hydroxycinnamic acids ~~has been~~ increased to 150 min and amounted to 0.073% and 0.446%, respectively. In the next few minutes, there was a significant slowdown in the concentration growth, indicating the achievement of the extraction process equilibrium.

When determining the optimal extraction conditions, the dry residue yield depending on the extraction frequency was also taken into account. The extractant renewal allowed maintaining the difference in concentrations in the raw material and the extractant, thereby increasing the degree of depletion of the raw material. Thus, it was necessary to determine the feasibility of the extractant renewal; for this purpose, the quantitative content of extractives in subsequent extractions was found. The extraction was performed in four stages at a ratio of the raw material to the extractant of 1:10 for 150 min at 30°C. The first, second, third, and fourth extractions were performed under the same conditions with draining the extract and adding the fresh portion of 70% ethyl alcohol after each extraction. The research results are shown in Figure 8.

The results of the studies have shown that for the complete depletion of the raw material, one renewal of the extractant is required, i.e., the two-stage extraction. Further third and fourth extractions were irrational as the yield of dry residues (0.002 and 0.0001, respectively) was insignificant.

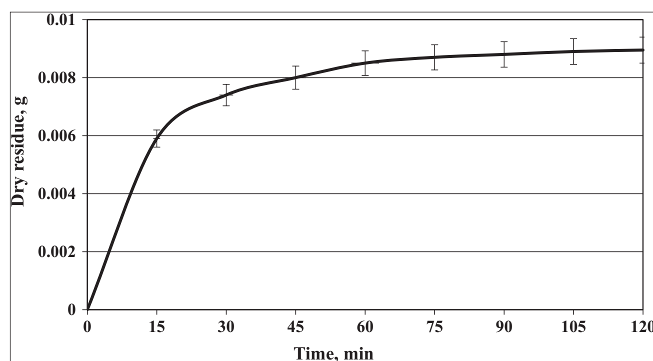

Figure 9: Determination of the optimal time of the second extraction by the dry residue
***Note*: *n* = 3, *P* < 0.05**

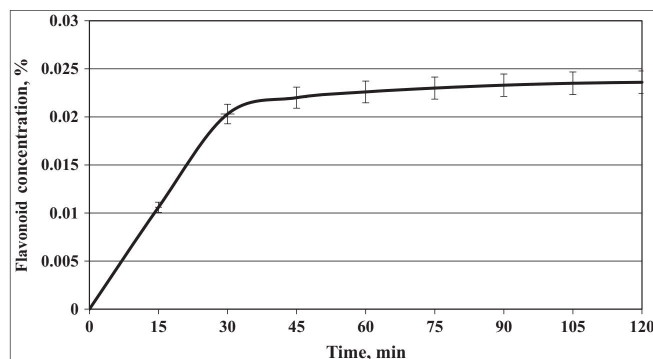

Figure 10: Determination of the optimal time of the second extraction by the amount of flavonoids
***Note*: *n* = 3, *P* < 0.05**

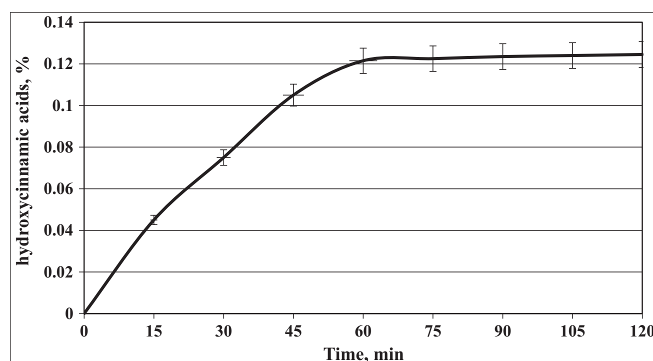

Figure 11: Determination of the optimal time of the second extraction by the amount of hydroxycinnamic acids
***Note*: *n* = 3, *P* < 0.05**

At the next stage of the study, it was necessary to determine the optimal extraction time with the second portion of the extractant. The results of the study are shown in Figures 9–11.

The experimental data presented in Figure 9 showed that during the second extraction, the yield of the dry residue was much lower; its increase was observed up to 60 min of the process and amounted to 0.0085. Further extraction was impractical

as a linear relationship indicating the maximum degree of the raw material depletion was detected.

The maximum extraction of the amount of flavonoids during the second extraction occurred for up to 30 min and amounted to 0.0226%. Then there was a slight increase in their yield, indicating the complete depletion of the raw material [Figure 10].

In contrast to flavonoids, the time required to extract the maximum concentration of (0.1215%) during the second extraction was 60 min; a further increase in time did not significantly increase their yield [Figure 11].

Thus, based on the results obtained, the optimal time of the first extraction of *T. vulgare* flowers was 150 min, whereas the second extraction was 60 min. It confirms a high efficiency of the technology for obtaining extracts from *T. vulgare* flowers through UAE in a short period of time.

Conclusions

Taking into account the polyvalence of the pharmacological action of *T. vulgare* flowers and a limited number of drugs based on it, the raw material has proven to be promising for the development of new herbal medicinal products.

The advantages of using ultrasound as an intensified method to optimize the technology of extracting BAS from *T. vulgare* flowers have been theoretically substantiated and experimentally proven. It has been found that the yield of the total amount of flavonoids and hydroxycinnamic acids increased by 35% and 13% compared with dynamic maceration, indicating the efficiency and cost-effectiveness of this method.

The optimal technological parameters which allowed extracting the maximum amount of BAS from the plant raw material studied using ultrasound have been determined. They are the ratio of the raw material to the extractant—1:10, the optimal frequency of extractions—2, the time of the first extraction—150 min, the time of the second extraction—60 min.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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