RESEARCH ARTICLE

Development of a Method for Producing Purified Spinach Extract with High Content of 20-Hydroxyecdysone and Polyphenols

Zorin S. N., Petrov N. A., Perova I. B., Malinkin A. D., Bokov D. O.,* Bessonov V. V.,

Federal Research Centre of Nutrition, Biotechnology and Food Safety, Moscow, 109240, Russian Federation

Received: 22th October, 2020; Revised: 20th June, 2021; Accepted: 14nd September, 2021; Available Online: 25th September, 2021

ABSTRACT

Adaptogens are biologically active substances providing state unspecific increased resistance in stressful situations. Spinach (*Spinacia oleracea* L.) is one of the most promising food plants containing adaptogens (ecdysteroids and polyphenols complexes). This research aims to obtain the final purified spinach extract (FPSE) with a high content of 20-hydroxyecdysone (20E), flavonoids, and oxalic acid-free from food raw materials – spinach leaves. This work is necessary to further use spinach as part of functional food ingredients in specialized food products. Total polyphenols were determined by the Folin-Ciocalteu method, oxalic acid content – by permanganate titration. The 20E content was determined by HPLC-MS using an Agilent 1100 chromatograph with an Agilent 6410 mass detector. Individual flavonoid content and profile were determined using an ultimate 3000 liquid chromatography syste'm with a diode array detector (DAD) and a TSQ Endura triple quadrupole mass spectrometric detector (MSD). FPSE was obtained by water extraction of freeze-dried powdered spinach leaves (FDPSL), ultrafiltration, and sorption on a C18 column. In the FPSE, the 20E content was 12,17 ± 1,24 mg/g; total polyphenols – 19.3 ± 1.6%; flavonoids – 41.9 ± 4.1%, oxalic acid was absent. Flavonoid profile included patuletin-3-glucosyl-(1→6)-apiosyl-(1→2)-glucoside, patuletin-3-glucosyl-(1→6)-glucoside, patuletin-3-(2''feruloylglucosyl)-(1→6)-apiosyl-(1→2)-glucoside, patuletin-3-(2''feruloylglucosyl)-(1→6)-glucoside, axilyarin-4'-glucuronide (spinatoside), 5,3',4'-trihydroxy-3-methoxy-6:7-methylenedioxyflavone-4'-glucuronide, 5,4′-dihydroxy-3-methoxy-6:7-methylenedioxyflavone-4′-β-D-glucuronide, 5,4'-dihydroxy-3,3'-dimethoxy-6:7-methylenedioxyflavone-4'-glucuronide. As a result of the study, a laboratory method of producing functional food ingredient (adaptogen spinach extract) perspective for further industrial scaling and producing biologically active food supplements has been developed.

Keywords: 20-hydroxyecdysone, Flavonoids, Spinach, *Spinacia oleracea* L.

International Journal of Pharmaceutical Quality Assurance (2021); DOI: 10.25258/ijpqa.12.3.23

How to cite this article: Zorin SN, Petrov NA, Perova IB, Malinkin AD, Bokov DO, Bessonov VV. Development of a Method for Producing Purified Spinach Extract with High Content of 20-Hydroxyecdysone and Polyphenols. International Journal of Pharmaceutical Quality Assurance. 2021;12(3):276-281.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Adaptogen is a substance that modifies the body state and provides unspecific increased resistance (SUIR), reducing stressful effects. In modern society, the increasing demand for adaptogens is due to factors such as susceptibility to psycho-emotional stress, chronic fatigue syndrome, physical and mental stress. There is a growing trend in the production of synthetic adaptogenic drugs. However, plant adaptogens consumption as an alimentary factor is one of the most promising approaches to increase the SUIR. Plant adaptogens devoid of many disadvantages of chemotherapeutic agents: addiction, toxicity, and the development of adverse reactions. The use of plant extracts containing phytoadaptogens as components of specialized food products (SFP) meets the challenges of rational nature management of plant resources.[1-3]

Notably, plant adaptogens, such as phenolic compounds, are structurally similar to catecholamines. Also, phytoecdysteroids are close in structure to cortisol. 20-hydroxyecdysone is the most well-studied compound from the phytoecdysteroids group. The prospects of using 20-hydroxyecdysone (20E) in food dietary supplements (FDS) and SFP for athletes' nutrition were discussed very detailed.[4] 20E has an anabolic effect that the influence of this compound can explain (as such or its metabolites) on the activity of the protein kinase B-(RKV)/Akt signal macromolecule; it is key in the regulation of cellular activity.[5]

Attempts to develop complex adaptogenic herbal preparations have already been made. For example, the

*Author for Correspondence: *fmmsu@mail.ru*

substance "Serpisten", containing 20-hydroxyecdysone and 25S-inocosterone, exhibits pronounced adaptogenic properties. The combination of phytoecdysteroids and polyphenols leads to a synergism of adaptogenic effects and other pharmacological properties.[6-9] The crude extract from a wild plant Crowned Serpenta (*Serratula coronata* L.) has similar properties. The consumption of *S. coronata* extract contributes to the adaptation to new conditions and accelerates the learning and memory processes.[10-11]

Individual ecdysteroids (mainly of synthetic origin) or ecdysteroid-containing extracts of wild medicinal plants (*Rhaponticum carthamoides* (Willd.) Iljin, *S. coronata, Ajuga turkestanica* (Regel) Briq., *Pfaffia paniculata* (Mart.) Kuntze, *Cyanotis* D. Don) are the main components in adaptogenic drugs and SFP. The production of new functional food ingredients (FPI) from plant raw materials, containing phytoecdysteroids and polyphenols complexes, with adaptogenic properties is a very upcoming research area. The development of FPI of plant origin is necessary for the production of various types of SFP.[4,5,12]

The spinach (*S. oleracea* L.) is one of the promising food plants containing ecdysteroids and polyphenols (flavonoids) complexes.[13-15] In this regard, this research aims to obtain purified spinach extract with a high content of 20-hydroxyecdysone and flavonoids for the preparation of FFI from spinach leaves for further SFP production.

MATERIALS AND METHODS

Plant Material

The spinach (*S. oleracea* L.) leaves were purchased in the grocery network of the Moscow and immediately frozen. The moisture content in the leaves of raw spinach was about 95%.

Extracts Preparation

Spinach samples were lyophilized by a LS 500 machine (manufactured by PROINTECH, Pushchino, Russian Federation (RF)) and ground using a laboratory blender (Fimar FRI, Italy) to a powder state. The optimal ratios of powdered spinach leaves/extraction solvent and the incubation time and ethanol content in the extraction solvent were determined in preliminary experiments. Extraction was carried out at room temperature for 60 minutes at a mass ratio of spinach leaf powder/extraction solvent 1/39 with stirring and 20 and 40% ethanol. The resulting suspension was clarified by centrifugation for 30 minutes at 4000 rpm (centrifuge "Beckman J-6B"). The supernatant was taken and ultrafiltered through a membrane with a pore diameter of 10 kD (laboratory setup for micro-and ultrafiltration based on the ASF-018 filter holder. Vladisart, Russian Federation) with the collection of a low molecular weight fraction and its subsequent concentration on a reverse osmosis unit with a roll membrane filter "URF-1812" (production of Vladisart, RF). Residual amounts of ethanol (in extraction with ethanol) were removed on a rotary evaporator (60°C). Oxalic acid was removed from the obtained 20E-rich extract by preparative low-pressure liquid chromatography on a C18 column (4.0 × 9.0 cm). The extract was passed through a column then washed successively with distilled water and 70% ethanol. Ethanol was removed on a rotary evaporator (60°C). The resulting products were freeze-dried. The scheme for obtaining final purified spinach extract (FPSE) enriched 20E is shown in Figure 1.

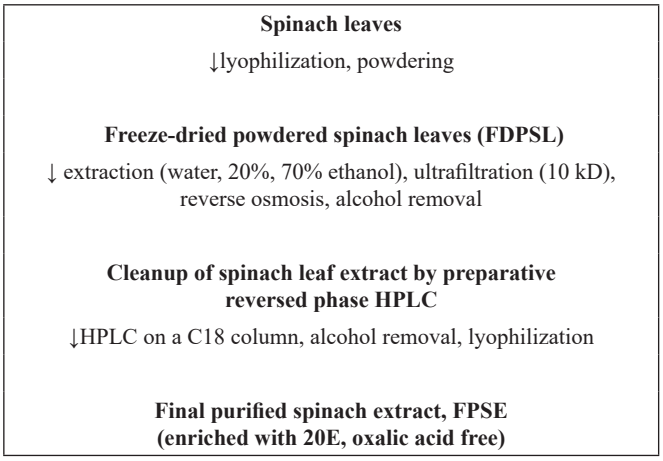

Figure 1: Scheme for obtaining the final purified spinach extract (FPSE).

Oxalic Acid

The determination of oxalic acid in spinach extracts was carried out by the method of permanganate titration as described in work (sensitivity of the method 10 μg/mL).[16] The oxalic acid was precipitated by the addition of 12% ammonia solution to 50 mL of a pre-filtered extract till a slightly alkaline reaction appeared (pH = 8); 10 mL of calcium chloride buffer solution was added (pH = 4.6). The final mixture was incubated for 48 hours at a temperature of 3–7°C. The precipitated calcium oxalate was separated from the supernatant by centrifugation for 15 minutes at 3000 rpm, washed with hot (70–90°C) water until a negative reaction to chlorine. The precipitate was dissolved in 15 mL of hot 10% sulfuric acid, the amount of residual oxalic acid in the solution was determined by titration with 0.1 N permanganate solution.

20-hydroxyecdysone

The 20E content was determined by HPLC using an Agilent Technologies 1100 chromatograph with an Agilent Technologies 6410 mass detector. 0.1% solution of formic acid in water (mobile phase A) and acetonitrile (mobile phase B) were used for the following gradient elution program on Poroshell 120 EC-C18 column (3.0 × 50 mm, 2.7 μm particle size): 0 minute 5% B, 5 minutes 27% B, 5.5 minutes 90% B, 8.5 minutes 90% B, 9.5 minutes 5% B, 13.5 minutes 5% B. Flow rate – 0.4 mL/min. The 20-hydroxyecdysone (98% Cat No.: 234697, CAS 5289-74-7; J&K Scientific GmbH, Germany) was used as a standard sample.

The parameters of the mass detector were as follows. Ionization source parameters: atomizing gas pressure – 2.8 bar; drying gas temperature – 350°C; drying gas flow rate – 10 L/min; voltage on the fragmented – 98 V. Recorded mass transitions in MS/MS mode: from 481.3 to 445.4 with a collision energy of

8 eV (used for quantitative analysis), from 481.3 to 371.4 with a collision energy of 12 eV (used for qualitative confirmation); the polarity of the studied ions – positive; the voltage on the capillary – 4000 V. 980 µL of a 50% methanol solution of in water was added to 20 µL of the extract before analysis then vortexed and centrifuged with a relative centrifugation force of 18,407 g for 10 minutes, respectively.

Total Polyphenols

Total polyphenols (TP), expressed as gallic acid equivalents (GAE), were determined by the Folin-Ciocalteu method.[17]

Flavonoids

The study was performed using an Ultimate 3000 liquid chromatography system with a diode array detector (DAD) and a TSQ Endura triple quadrupole mass spectrometric detector (MSD). HPLC-DAD conditions: Phenomenex Luna C18 column (150×4.6 mm, 5 µm particle size); mobile phase A – 0.1% solution of formic acid in water, B – 0.1% solution of formic acid in acetonitrile; gradient elution: 0-10 minutes 15–30% B, 10–20 minutes 30–45% B, 20–30 minutes 45–60% B, 30–35 minutes 60–70% B, 35–38 minutes 70% B, 38–40 minutes 70-15% B, 40-50 minutes 15% B; eluent flow rate – 0.5 mL/min; column temperature – 25ºC; autosampler temperature – 20ºC; sample volume – 5 µL; DAD at λ = 350 nm and λ = 338 nm; spectra were recorded in the wavelength range of 200–400 nm. MS conditions: ionization by a heated electrospray with the registration of positive HESI-MS^+ and negative ions HESI-MS^-; voltage in the positive mode 3500 V, in the negative – 2500 V; the temperature of the heated capillary 350ºC, the evaporator – 400ºC; mass scanning in the range of 150–1500 m/z. Data processing was performed using Thermo Xcalibur 3.0.63 software. Available flavonoid standards have been purchased from Sigma-Aldrich (Merck) for quantitative analysis.

The contents of 20E, TP, and flavonoids profile were determined in the freeze-dried powdered spinach leaves, intermediate extracts, and FPSE, respectively.

RESULTS AND DISCUSSION

The results of 20E determination in freeze-dried powdered spinach leaves (FDPSL), in extracts and final purified spinach extract (FPSE) are presented in Table 1.

HPLC-DAD-MS data of spinach extracts flavonoids is presented in Table 2 and in Figure 2. The main flavonoid in all products obtained with a different type of extraction solvent is 5,3',4'-trihydroxy-3-methoxy-6:7-methylendioxyflavone-4'-glucuronide (more than 50% of total flavonoids content).

The extraction effectiveness (the transition of a substance from the FDPSL directly to the extraction phase) of polyphenols and 20E practically did not depend significantly on the extractant's composition. It is only slightly increased with increasing ethanol concentration by up to 40%.

The most enriched FPSE was obtained by water extraction of FDPSL, ultrafiltration, and sorption on a C18 column. The content of 20E in the FPSE increased by almost 30.4 times compared with dried spinach leaves. The content of 20E in the

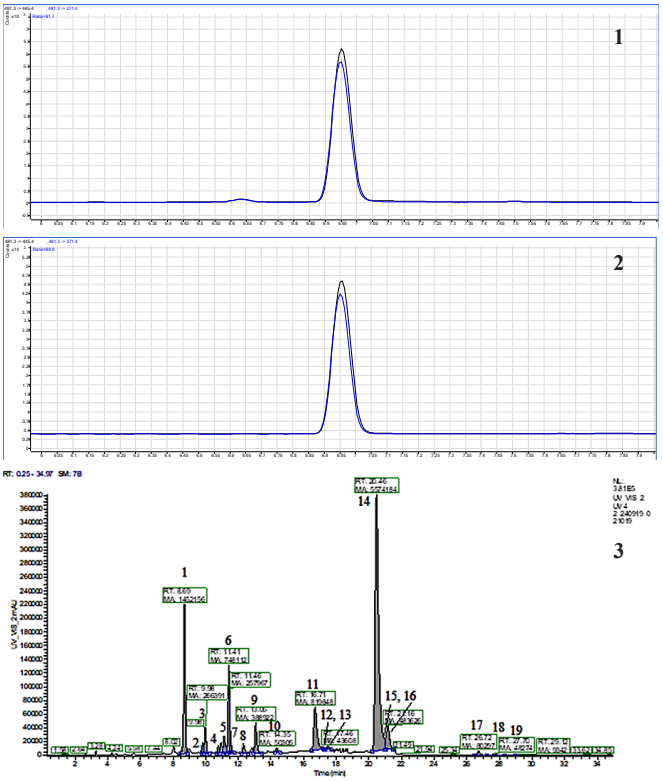

Figure 2: Typical HPLC-MS/MS chromatogram of FPSE (1) and a standard sample (2) with a peak of 20E; chromatogram of spinach extract 2 with 20% ethanol, λ=350 nm, numbering according to Table 2 (3).

Table 1: The content of 20E and flavonoids in FDPSL and extracts

		Flavonoids			*20E*		
Solvent	*Sample*	*Weight. %*	*% yield*	*The concentration ratio*	*mg/g*	*% yield*	*The concentration ratio*
Water	FDPSL*	1.21 ± 0.12	28.7%	34.7	0.40 ± 0.04	54.6	30.43
	FPSE**	41.9 ± 4.1			12.17 ± 1.24		
20% ethanol	FDPSL	1.26 ± 0.12	25.4%	31.8	0.35 ± 0.04	57.7	26.74
	FPSE	40.1 ± 3.9			9.36 ± 0.91		
40% ethanol	FDPSL	1.38 ± 0.13	28.6%	29.5	0.35 ± 0.04	60.3	28.63
	FPSE	40.3 ± 0.39			10.02 ± 0.99		

*Freeze-dried powdered spinach leaves (FDPSL)
**Final purified spinach extract (FPSE)

Table 2: Peak numbers, names, retention times (Rt), UV-absorption maximum wavelengths, mass spectral data, and content of spinach extracts flavonoids

No	Flavonoid	Rt, min (± 0.2 min)	λ_{max}, nm (± 2 nm)	HESI-MS$^+$	HESI-MS$^-$	Content, %		
						1 (water)	2 (20% ethanol)	3 (40% ethanol)
1	Patuletin-3-glucosyl-(1→6)-apiosyl-(1→2)-glucoside	8.7	258, 270 shield, 350	827.06 $[M+K]^+$, 811.21 $[M+Na]^+$, 789.24 $[M+H]^+$, 657.17 [M-apiose*+H]$^+$, 495.37 [M–apiose–glucose+H]$^+$, 333.06 [M–glucosyl-(1→6)-apiosyl-(1→2)-glucose+H]$^+$	787.30 $[M-H]^-$, 655.40 [M–apiose–H]$^-$	4.64	5.52	5.47
2	Spinacetin-3-glucosyl-(1→6)-apiosyl-(1→2)-glucoside	9.9	255, 270 shield, 350	841.32 $[M+K]^+$, 825.08 $[M+Na]^+$, 803.37 $[M+H]^+$, 671.53 [M-apiose+H]$^+$, 509.51 [M–apiose–glucose+H]$^+$, 347.05 [M-glucosyl-(1→6)-apiosyl-(1→2)-glucose+H]$^+$	801.27 $[M-H]^-$	0.40	0.41	0.26
3	Patuletin-3-glucosyl-(1→6)-glucoside	10.0	260, 270 shield, 350	685.95 $[M+K]^+$, 679.12 $[M+Na]^+$, 657.22 $[M+H]^+$, 495.25 [M–glucose+H]$^+$, 333.00 [M-glucosyl-(1→6)-glucose+H]$^+$	655.20 $[M-H]^-$	0.66	1.01	0.99
4	Patuletin-3-(2''coumaroyl-glucosyl)-(1→6)-apiosyl-(1→2)-glucoside	10.8	258 shield, 275, 310 shield, 350	957.28 $[M+Na]^+$, 935.04 $[M+H]^+$, 803.01 [M-apiose+H]$^+$, 332.99 [M-(2''coumaroyl-glucosyl)-(1→6)-apiosyl-(1→2)-glucose+H]$^+$	933.40 $[M-H]^-$, 801.45 [M–apiose-H]$^-$	0.25	0.26	0.25
5	Patuletin-3-(2'' coumaroyl-glucosyl)-(1→6)-apiosyl-(1→2)-glucoside isomer	11.1	258 shield, 275, 310 shield, 350	957.28 $[M+Na]^+$, 935.04 $[M+H]^+$, 803.01 [M-apiose+H]$^+$, 333.20 [M-(2''coumaroyl-glucosyl)-(1→6)-apiosyl-(1→2)-glucose+H]$^+$	933.34 $[M-H]^-$	0.70	0.81	0.74
6	Patuletin-3-(2''feruloyl-glucosyl)-(1→6)-apiosyl-(1→2)-glucoside	11.4	250, 275, 337	987.22 $[M+Na]^+$, 965.20 $[M+H]^+$, 833.31 [M-apiose+H]$^+$, 671.09 [M-apiose-glucose+H]$^+$, 333.06 [M-(2''feruloylglucosyl)-(1→6)-apiosyl-(1→2)-glucose+H]$^+$	963.41 $[M-H]^-$	2.68	2.84	2.94
7	Spinacetin-3-glucosyl-(1→6)-glucoside	11.5	258, 266, 350	709.26 $[M+K]^+$, 693.25 $[M+Na]^+$, 671.41 $[M+H]^+$, 508.98 [M-glucose+H]$^+$ 347.03 [M–gentiobiose+H]$^+$	669.27 $[M-H]^-$, 345.26 [M–gentiobiose–H]$^-$	0.67	0.97	0.46
8	Spinacetin-3-(2''feruloyl-glucosyl)-(1→6)-apiosyl-(1→2)-glucoside	12.3	250 shield, 270, 340	1001 $[M+Na]^+$, 979.03 $[M+H]^+$, 847.45 [M-apiose+H]$^+$, 685.26 [M-apiose-glucose+H]$^+$, 347 [M-(2''feruloylglucosyl)-(1→6)-apiosyl-(1→2)-glucoside+H]$^+$	977.41 [M - H]$^-$	0.44	0.44	0.35
9	Patuletin-3-(2''feruloyl-glucosyl)-(1→6)-glucoside	13.0	250 shield, 270, 335	855.18 $[M+Na]^+$, 833.03 $[M+H]^+$, 333.27 [M-(2''feruloylglucosyl)-(1→6)-glucoside+H]$^+$	831.25 [M - H]$^-$	1.06	1.47	1.53
10	Spinacetin-3-(2''feruloylglucosyl)-(1→6-glucoside	14.3	258, 270, 335	869.38 $[M+Na]^+$, 847.11 $[M+H]^+$, 347 [M-(2''feruloylglucosyl)-(1→6)-glucoside+H]$^+$	845.34 [M - H]$^-$	0.18	0.18	0.19
11	Axillarin-4'-glucuronide (Spinatoside)	16.7	250 shield, 270, 340	523.13 $[M+H]^+$, 347.01 [M–glucuronic acid+H]$^+$	521.30 [M - H]$^-$, 345.00 [M–glucuronic acid -H]$^-$	3.28	3.12	3.64
12	Unidentified flavonoid glucuronide	17.2	275, 335	507.18 $[M+H]^+$, 331.21 [M– glucuronic acid +H]$^+$	505.13 [M – H]$^-$ 329.31 [M–glucuronic acid -H]$^-$	0.06	traces	0.07

No	Flavonoid	Rt, min (± 0.2 min)	λ_{max}, nm (± 2 nm)	HESI-MS$^+$	HESI-MS$^-$	Content, % 1 (water)	2 (20% ethanol)	3 (40% ethanol)
13	Jaceidin-4'-glucuronide	17.5	254, 270, 350	537.13 $[M+H]^+$, 360.91 [M– glucuronic acid +H]$^+$	535.27 [M - H]$^-$, 359.02 [M– glucuronic acid -H]$^-$	0.21	0.16	0.24
14	5,3',4'-trihydroxy-3-methoxy-6:7-methylendioxy-flavone-4' -glucuronide	20.5	250 shield, 278, 340	521.14 $[M+H]^+$, 344.98 [M– glucuronic acid +H]$^+$	519.03 [M - H]$^-$, 343.21 [M – glucuronic acid - H]$^-$	23.75	21.23	21.08
15	5,4′-dihydroxy-3-methoxy-6:7-methylendioxy-flavone-4′-β-D-glucuronide	21.0	278, 332	505.09 $[M+H]^+$, 329.33 [M– glucuronic acid +H]$^+$	503.29 [M - H]$^-$, 327. [M – glucuronic acid - H]$^-$	1.75	0.94	1.17
16	5,4'- dihydroxy-3,3'-dimethoxy-6:7-methylendioxyflavone-4'-glucuronide	21.2	250 shield, 278, 340	535.04 $[M+H]^+$, 359.32 [M– glucuronic acid +H]$^+$	533.50 [M - H]$^-$, 357.40 [M – glucuronic acid - H]$^-$	1.17	0.46	0.55
17	5,3',4'- trihydroxy-3-methoxy-6:7-methylendioxy flavone	26.7	258, 276, 350	345.04 $[M+H]^+$	343.07 [M – H]$^-$ 328.06 [M – O]$^-$	0.01	0.07	0.02
18	Unidentified flavonoid glycoside	27.7	280, 330	697.13 $[M+H]^+$	695.11 [M – H]$^-$	0.03	0.18	0.33
19	Unidentified flavonoid glycoside	29.1	280, 330	681.14 $[M+H]^+$	679.12 [M – H]$^-$	-	0.03	0.11
	Total flavonoids					41.93	40.10	40.39

*Hereinafter: dehydrated sugars (-H_2O), water is lost upon forming a glycosidic bond

FPSE was 12.17 ± 1.24 mg/g in FPSE No 1 (water extraction). Given the moisture content in relation to raw spinach, the content of 20E in the FPSE increased by more than 110 times. The maximum flavonoid content was 41.9 ± 2.9 % in the FPSE No 1. TP content in the FPSE (No 1-3) varied 19.3-23.1% (No 1 – 19.3 ± 1.6%, 22.9 ± 1.9%, 23.1 ± 2.0%) that shows similar results in the content of polyphenols.

Oxalic acid should not be in the FPSE; it is contraindicated for people with increased stomach acidity and kidney diseases. Since its use leads to an exacerbation of these diseases and causes the formation of calcium oxalates, which are deposited in the knee and elbow joints and the cervical vertebrae. Thus, a high 20E and polyphenols content is accompanied by the absence of oxalic acid significant amounts in the FPSE.

CONCLUSION

A laboratory method for obtaining FFI from spinach leaves has been developed. This method allows producing final purified spinach extract with a high content of 20-hydroxyecdysone and polyphenols from food raw materials – spinach leaves. FPI from spinach is a valuable substance for producing adaptogenic functional food products, dietary supplements, and biologically active food supplements.

ACKNOWLEDGMENT

The study was financially supported by the Russian Science Foundation, grant No. 19-16-00107, "New functional food ingredients of adaptogenic action for the enhancement of working capability and cognitive potential of human organism".

Authors would like to thank Eller K.I., Mazo V.K., Sidorova Yu.S. for useful discussions and constant feedback.

REFERENCES

1. Jaremenko KV. Lazarev's theory of state unspecific increased resistance (SUIR) and adaptogens as a basis of preventive medicine. Psychopharmacology and Biological Narcology. 2005; 5(4): 1086-1092. Available from: https://clinpharm-journal.ru/articles/
2. Zabrodin ON. Conception of N.V. Lazarev about adaptogens in aspect of teaching about of nervous trophism. Psychopharmacology and Biological Narcology. 2005; 5(4): 1108-1112. Available from: https://clinpharm-journal.ru/articles/
3. Molinos DÁ. Effects of adaptogen supplementation on sport performance. A recent review of published studies. Journal of Human Sport and Exercise. 2013; 8(4): 1054-1066. Available from: doi.org/10.4100/jhse.2013.84.15
4. Volodin VV, Sidorova YuS, Mazo VK. 20-Hydroxyecdysone – plant adaptogen: an anabolic effect, possible use in sports nutrition. Voprosy Pitaniia. 2013; 82(6): 24-30. Available from: https://www.voprosy-pitaniya.ru/en/jarticles_diet/233.html
5. Sidorova YuS, Selyaskin KE, Zorin SN, Vasilevskaya LS, Volodin VV, Mazo VK. In-vivo study of *Serratula coronata* L. extract on biomarkers of general adaptation syndrome. Traditional medicine 2014; 1 (36): 57-62. Available from: http://www.tradmed.ru/n36_9.shtml
6. Volodin VV, Pchelenko LD, Volodina SO, Kudryashova AG, Shevchenko OG, Zagorskaya NP. Pharmacological estimate of

new containing ecdysteroid substance "Serpisten". Rastitel'nye resursy. 2006; 42(3): 113-130. Available from: https://www.elibrary.ru/item.asp?id=9275187

7. Kudyasheva AG, Andreeva LI, Volodin VV, Volodina SO. Biochemical parallels of cellular adaptive reactions in chronic low-intensity irradiation and the action of the phytoecdysteroid drug Serpisten. Radiation biology. Radioecology. 2015;55(1):43-50.
8. Shirshova TI, Politova NK, Beshlei IV, Volodin VV, Burtseva SA. Antimicrobial activity of natural ecdysteroids from *Serratula coronata* L. and their acyl derivatives. Pharmaceutical Chemistry Journal. 2006; 40(5): 268-271. Available from:
9. Selyaskin KE, Sidorova YS, Zorin SN, Volodin VV, Mazo VK. Effect of *Serratula coronata* extract on apoptosis activity in rats. Pharmaceutical Chemistry Journal. 2016; 50 (5): 315-319. Available from: doi.org/10.1007/s11094-006-0106-7
10. Odinokov VN, Galyautdinov IV, Nedopekin DV, Khalilov LM, Shashkov AS. Phytoecdysteroids from the juice of *Serratula coronata* L . (*Asteraceae*). Insect Biochemistry and Molecular Biology. 2002; 32(2): 161-165. Available from: 10.1016/S0965-1748(01)00106-0
11. Zharikov Ya A, Volodina SO, Volodin VV, Kaneva LA. Effect of infusion of *Serratula coronata* on the metabolism and growth young sheep. Russian Agricultural Sciences. 2019; 3: 51-53. Available from: doi.org/10.31857/S2500-26272019351-53
12. Bezmaternykh KV, Volodin VV, Volodina SO, Smirnova GV, Oktyabrsky ON. Study of antioxidant activity and adaptogenic activity of plant extracts containing ecdysteroids and polyphenols. In the book: Phenolic compounds: fundamental and applied aspects. Proceedings of the IX International Siposium. 2015; 499-502.
13. Bakrim A, Maria A, Sayah F, Lafont R, Takvorian N. Ecdysteroids in spinach (*Spinacia oleracea* L.): biosynthesis, transport and regulation of levels. Plant Physiology and Biochemistry. 2008; 46(10): 844-854. Available from: doi.org/10.1016/j.plaphy.2008.06.002
14. Edenharder R, Keller G, Platt K L, Unger K K. Isolation and characterization of structurally novel antimutagenic flavonoids from spinach (*Spinacia oleracea*). Journal of Agricultural and Food Chemistry. 2001; 49(6): 2767-2773. Available from: doi.org/10.1021/jf0013712
15. Koh E, Charoenprasert S, Mitchell AE. Effect of organic and conventional cropping systems on ascorbic acid, vitamin C, flavonoids, nitrate, and oxalate in 27 varieties of spinach (*Spinacia oleracea* L.). Journal of Agricultural and Food Chemistry. 2012; 60(12): 3144-3150. Available from: doi.org/10.1021/jf300051f
16. Shirley Navis M, Subila S. Study on the presence of oxalate ions in guava and sapota fruits. International Journal of Advanced Science and Research. 2017; 2(1): 15-17. Available from: http://www.allsciencejournal.com/archives/2017/vol2/issue1/1-12-17
17. Silgleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology. 1999; 299:152-178. Available from: doi.org/10.1016/S0076-6879(99)99017-1