SUPPLEMENTARY MATERIAL

Application of Quality by Design Approach to the Pharmaceutical Development of Anticancer Crude Extracts of *Crocus sativus* Perianth

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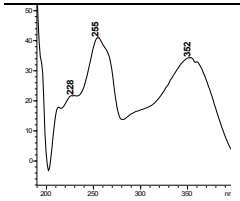
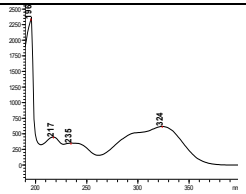
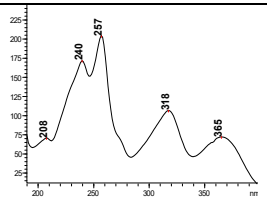
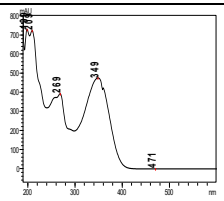
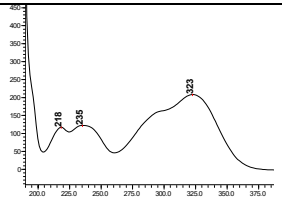
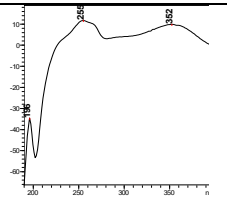
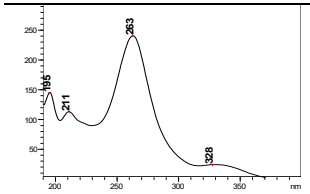
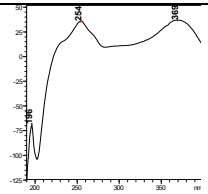
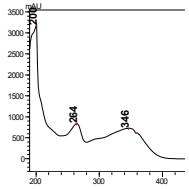
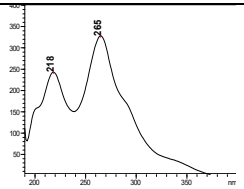
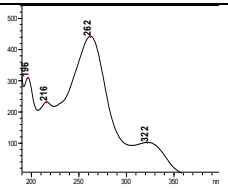
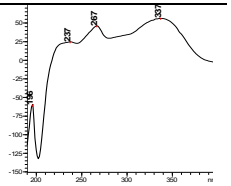
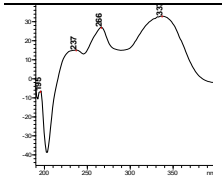
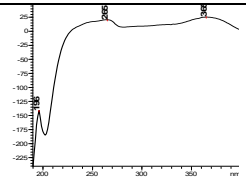
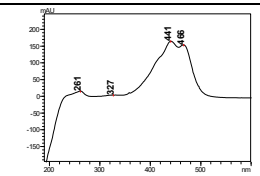
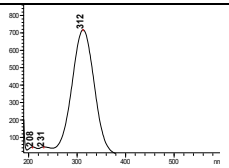
Table S1. Precision and stability of the quantified compounds.

	Compound	Concentrate (μg/mL)	Precision				Repeatability	
			Intra-Day (n=3)		Inter-Day (n=3)		Recovery (%)	RSD (%)
			RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)		
1	Mangiferin	9.06	0.33	100.46	0.29	100.41	100.29	0.25
		36.25	0.24	99.66	0.32	100.45	100.03	0.39
		145	0.22	100.32	1.10	98.45	99.58	0.99
2	Isoorientin	0.73	0.37	101.15	0.72	101.97	101.17	0.88
		11.6	0.91	100.78	0.58	10.62	99.98	0.55
		46.42	0.45	100.15	0.67	99.92	100.03	0.27
3	Ferulic acid	7.06	0.68	100.22	0.90	98.29	99.11	0.69
		28.25	0.93	98.20	0.29	99.31	99.60	0.57
		56.5	1.22	100.24	0.46	98.28	100.12	0.49
4	Rutin	2.53	1.26	100.35	0.62	100.15	100.18	0.55
		10.12	1.29	101.12	0.80	99.21	100.65	0.92
		20.24	0.76	99.56	1.14	100.94	99.78	0.31
5	Isoquercitrin	5.57	0.86	100.26	0.41	100.23	100.13	0.69
		22.28	1.12	101.27	0.98	99.24	99.64	0.90
		44.56	0.80	99.58	0.91	100.92	97.79	0.49
6	Crocin	1.23	0.87	102.5	0.80	101.36	101.07	0.85
		18.4	1.25	99.19	0.91	100.37	98.99	1.06
		73.6	1.22	98.97	0.81	100.27	100.29	0.98
7	Tectoridin	16.25	1.35	101.93	1.57	102.24	101.39	0.98
		65	1.13	101.92	0.72	101.03	100.98	0.95
		260	0.30	99.57	0.03	99.96	99.84	0.23
8	Astragalin	0.37	0.47	98.86	0.95	101.79	99.42	0.93
		5.96	0.88	101.51	0.81	99.26	100.12	1.06
		23.85	0.82	99.86	1.07	100.07	100.45	0.19
9	Apigenin 7-*O*-glucoside	4	0.46	100.75	0.90	98.76	100.38	0.53
		16	0.45	100.89	0.64	98.62	100.45	0.63
		32	0.62	100.70	0.20	99.03	102.35	0.85
10	Quercetin	2.51	1.07	100.71	0.33	98.81	99.86	0.80
		10.4	0.81	99.30	0.44	102.23	99.15	0.61
		20.08	0.64	100.27	0.57	100.22	100.14	1.07
11	Kaempferol	2.29	0.48	100.69	0.44	99.81	100.35	0.49
		9.16	0.77	100.97	1.01	99.54	100.49	0.68
		18.32	1.14	99.66	1.12	100.84	99.83	0.34
12	Iristectorigenin B	7.5	1.23	101.76	1.64	102.35	101.36	0.97
		30	1.01	102.88	1.23	101.76	101.54	1.01
		120	0.07	99.90	0.33	99.53	99.81	0.25
13	Nigricin	11.31	1.19	101.70	1.21	101.73	101.14	0.98
		45.25	0.37	99.47	1.19	101.70	100.39	0.96
		181	0.57	99.19	0.48	99.33	99.50	0.43
14	Safranal	1.33	0.57	101.53	0.67	101.07	100.27	0.65
		10.64	0.78	98.68	0.53	99.14	99.58	0.77
		42.56	1.02	100.47	0.86	100.12	99.14	1.04
15	Caffeic acid	11.49	1.05	102.02	0.52	98.49	100.01	0.46
		45.96	1.08	98.78	0.67	99.73	99.39	0.99
		91.92	0.64	100.35	0.95	98.17	100.17	0.37
16	Apigenin	4	0.74	100.75	0.57	98.76	100.56	0.79
		16	0.89	100.89	0.29	98.62	98.96	0.71
		32	0.88	100.70	0.70	98.03	99.80	1.02

Table S2. The specificity of the quantified compounds.

Isoquercitrin	Caffeic acid	Mangiferin	Isoorientin	Ferulic acid	Rutin
350 nm[b] and 24.83 min[c]	310 mn and 14.18 min	270 mn and 14.18 min	310 mn and 17.66 min	310 mn and 21.64 min	310 nm and 22.48 min
					
Tectoridin	**Quercetin**	**Astragalin**	**Iristectorigenin B**	**Nigricin**	**Apigenin**
269 nm and 29.89 min	310 nm and 43.71 min	260 nm and 31.66 min	269 nm and 49.15 min	269 nm and 49.50 min	340 nm and 47.90 min
					
Apigenin 7-D-glucoside	**Kaempferol**	***trans*-Crocetin bis(*β*-D-gentiobiosyl) ester**	**Safranal**		
340 nm and 33.49 min	370 nm and 48.99 min	28.37 min	51.23 min		
					

Note: [a]Compound; [b]Detected at wavelength, λ, nm, c – Retention time, min

Table S3. Rank for Risk Quantification of S, P, and D of Parameter

The severity of excursion (S):
5 – Highest - a slight change in the parameter can lead to a significant change in the quality of the plant extract
4 - High - primary changes in the parameter lead to an almost imperceptible change in the quality of the plant extract
3 - Moderate - a parameter, only if it fluctuates in a wide range, can cause a significant impact on the quality of the extract
2 - Low impact - always negligible
1 - No impact
The probability of occurrence of the excursion (P)
5 - Highest probability that the parameter can appear
4 - May appear under certain conditions
3 - Sometimes may be present
2 - Almost never happens
1 - not typical
The detection of excursion (D)
5 - Danger or failure will not be detected
4 - Probably it will not be possible to establish the presence of a failure
3 - Sometimes not detected
2 - Always detected
1 - the danger is visible at the very beginning of the production process

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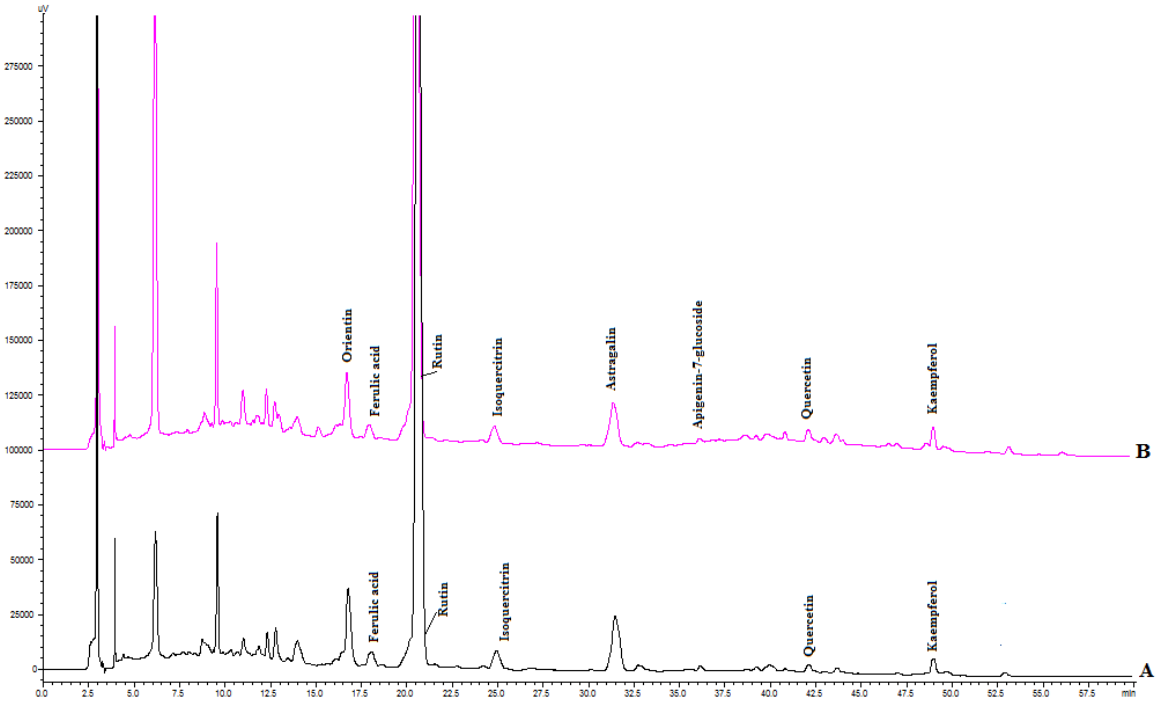

Figure S1. HPLC chromatograms of *C. sativus* perianth water dry (B) and ethanolic dry (A) extracts at 310 nm.

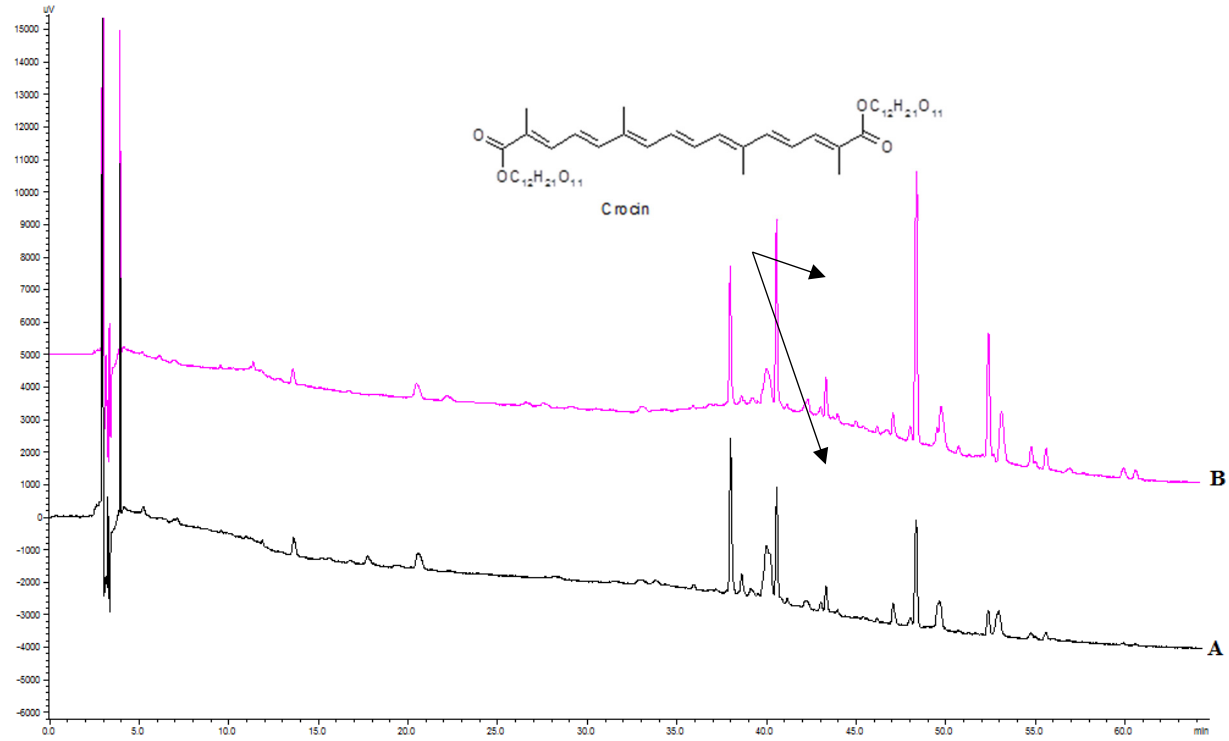

Figure S2. HPLC chromatograms of *C. sativus* perianth water dry (B) and ethanolic dry (A) extracts at 440 nm.

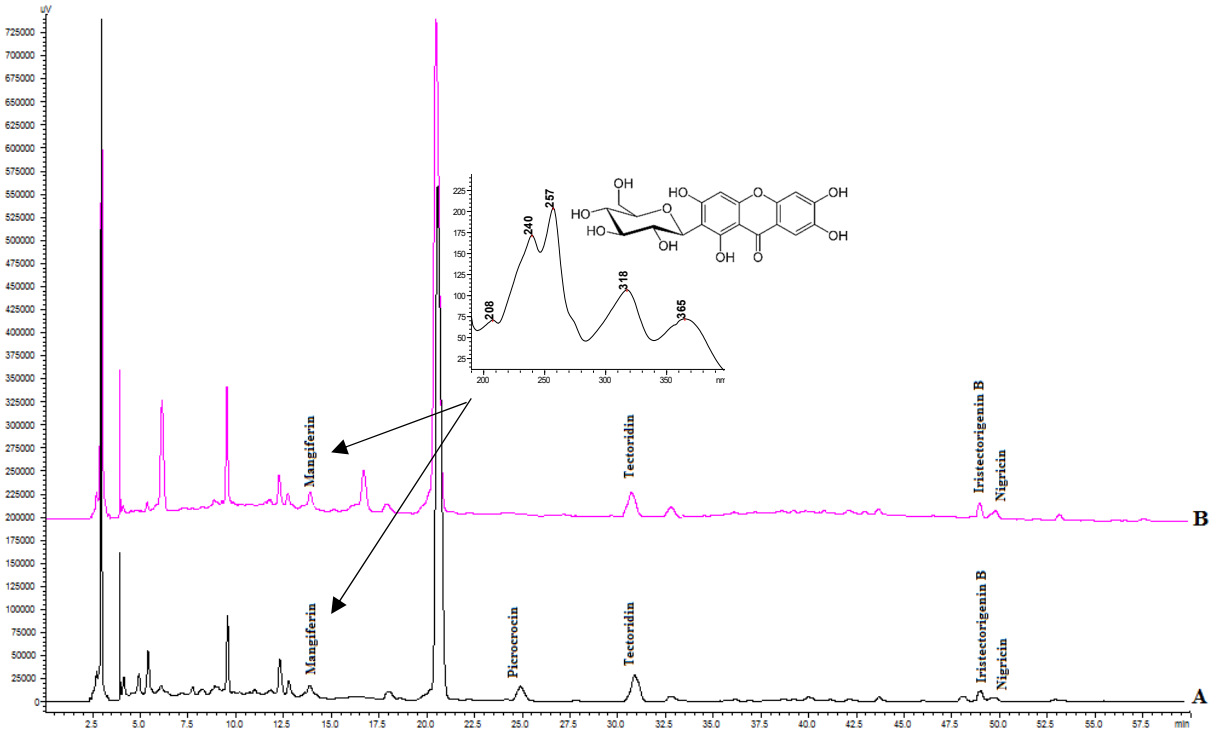

Figure S3. HPLC chromatograms of *C. sativus* perianth water dry (B) and ethanolic dry (A) extracts at 250 nm.

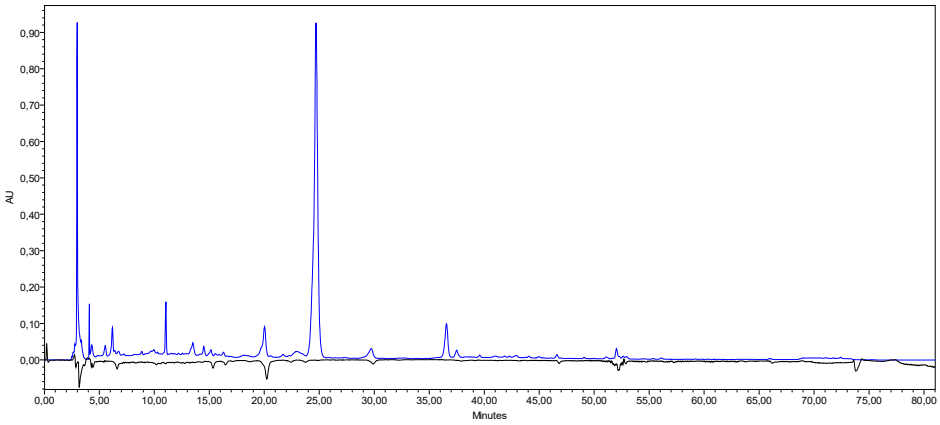

Figure S4. HPLC-ABTS chromatograms of *C. sativus* perianth water extract at 256 nm/650 nm

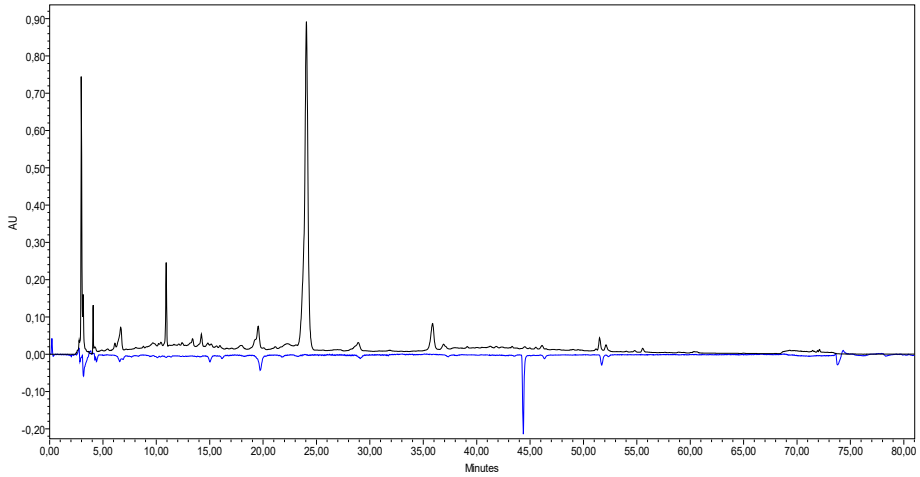

Figure S5. HPLC-ABTS chromatograms of *C. sativus* perianth ethanol extract at 264 nm/650 nm.

Details of procedures from Section 2 of main text are described below, including HPLC methodology and bioactivity assays.

HPLC-PDA and UPLC-MS conditions, HPLC post-column assay

Waters Alliance 2695 (Waters, Milford, USA) separation module system equipped with Waters 2487 UV/VIS and Waters 996 PDA diode-array detector (DAD) were used. HPLC grade water was obtained from a water purifying system (Millipore, Bedford, MA, USA). 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, purity ≥ 98%), potassium persulphate (purity ≥ 99 %) were purchased from Sigma (St. Louis, MO, USA), and Trolox (purity ≥ 98%) from Fluka Chemika (Buchs, Switzerland). The compounds separation was carried out utilizing an ACE C18 column (250 mm × 4.6 mm, 5.0 μm, Pennsylvania, USA) with a YMC guard cartridge ODS-A (3.0 μm, 10 mm × 4.0 mm). The binary solvent system of the mobile phase comprised solvent A (0.1% acetic acid in water) and solvent B (acetonitrile). A following linear gradient program was applied: 0–8 min, 5–15% B; 8–30 min, 15–20% B; 30–48 min, 20–40% B; 48–58 min, 40–50% B; 58–65 min, 50%; 65–66 min, 50–95% B. The injection volume of the sample solution was 20 μL. After PDA detection, the ABTS solution was mixed with the mobile phase carrying the analytes in the reaction coil. Empower Software Chromatographic Manager System (Waters Corporation, Milford, USA) was used to analyze the data. The ABTS post-column chromatograms were detected at the wavelength of 650 nm using Waters 2487 UV/VIS detector (Waters Corporation). The standard antioxidant Trolox (0.3995 μmol/g) was used for the preparation of the calibration curves. Trolox equivalent antioxidant capacity (TEAC) was used to express antioxidant activity. The value was calculated as μmol Trolox equivalent (TE) for 1 g of dry mass (DM) of the plant material using the following formula: $TEAC = c \times V/m$ (μmol/g) where 'c' is the Trolox concentration in μM established from the calibration curve, 'V' is the plant material extract volume in L, and 'm' is the weight (precise) in g.

In vitro assessment of cytotoxic activity

Cell culture

Human melanoma cancer cell line IGR39 and human triple-negative breast cancer cell line MDA-MB-231 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM Glutamax medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum and 1% antibiotic mixture (10,000 U/mL penicillin and 10 mg/mL streptomycin; Gibco). All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability assay

IGR39 and MDA-MB-231 cells were seeded (3,000 and 5,000 cells/well, respectively) in 96-well plate and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were affected by various concentrations of the tested extracts (from 1 mg/mL to 31.25 μg/mL). As a background control, the only medium without cells was used and the medium with 0.4% ethanol served as the negative control. After 72 hours of incubation with the extracts, 20 μL of MTT (5 mg/ml) was added into each well and incubated for 4 hours under the same conditions. The supernatant was removed and 100 μL DMSO was added. The absorbance was measured at 570 nm and 630 nm, and EC_{50} (half-maximal effective concentration of a drug at which 50% of its maximum response is observed) values were calculated.

Instruments. Separation of the compounds was achieved using a Nexera X2 LC-30AD HPLC system (Shimadzu, Kyoto, Japan). The system comprises an on-line degasser, a quaternary pump, SIL-30AC autosampler (Shimadzu), CTO-20AC thermostat (Shimadzu), a column temperature controller and a SPD-M20A diode array detector (DAD). Other instruments used in the investigation were an Ultrasonic Cleaner Set (Wise Clean WUC-A06H, Witeg Labortechnik GmbH, (Wertheim Germany), Libra UniBloc AUW120D (Shimadzu Analytical Scale, Kyoto, Japan); pH-meter—Knick Electronic Battery-operated pH Meter 911 PH (Portamess, Berlin, Germany), and class A analytical vial that meet requirements of the State Pharmacopoeia of Ukraine (SPhU, 2015). For the experiment used drying cabinet SNOL (Ukraine), muffle furnace SNOL (Ukraine), kitchen mixer (Moulinex, Italy), laboratory scales Electric «Certus Balance» (Ukraine), analytical electronic scales «Axis ANG 100C» (Ukraine), rotary evaporator «Heidolph 2 WB eco, Laborata400 efficient» (Germany).