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Water-ethanol extraction of hydroxycinnamonic acid from brown knapweed

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Abstract
Five hydroxycinnamonic acids were found and the presence of chlorogenic and caffeic acids was confirmed. The dominating compound is caffeic acid. The following optimal conditions of water-ethanol extraction of the total hydroxycinnamonic acids from brown knapweed are experimentally selected: volume ethanol fraction – 80%; extraction time – 6 hours; extraction temperature – 80ºC; extraction multiplicity- three times; the ratio of brown knapweed and 80% ethanol – 1:50; the degree of brown knapweed grinding – 500 μM (microns). The nature of the extraction of the total hydroxycinnamonic acids is determined by the dominating compound – caffeic acid. Total content of hydroxycinnamonic acids in brown knapweed taking into account the selected optimal extraction conditions makes, on the average, 2.6%. Volume ethanol fraction; time, temperature and multiplicity of extraction statistically significantly affect the concentration of hydroxycinnamonic acids in the extractions obtained. The most critical factor of water-ethanol extraction is extraction time.

Keywords: Brown knapweed, hydroxycinnamonic acids, optimal extraction conditions

Introduction
Brown knapweed (*Centaurea jacea* L.) is a perennial herbaceous plant of the sunflower family (*Asteraceae* Dumort.) widely growing on the territory of the Republic of Belarus and Europe. It grows on meadows, forest glades, forest borders, in lighted forests, in cleared spaces, on roadsides, sodded fills, dry slopes, garbage areas and among shrubs. It is found in the Republic of Belarus, the European part of Russia, Siberia, the Far East, the Ukraine, Europe (from Scandinavia to Italy, from the Baltic states to Great Britain) and Asia. It is a stranger in North America. The homeland of the plant is considered the Mediterranean [1].
Flavonoids are found in brown knapweed: apigenin; 5,7,4´-trihydroxy-3,6´-dimethoxyflavone and its 7-glucoside; centaurein, centaureidin, yacein, yaceosidine [2]. It is established that brown knapweed extractions and individual phenolic compounds isolated from it: flavonoids – cirsiliol, apigenin, hispidulin, eupatorin, isocampferide, axillarin, centaureidin, 6-methoxycampferol, 3-methyl ether, quercetin and hydroxycinnamonic acids (HCA) chlorogenic, caffeic and ferulic acids, exert a cytotoxic effect on HeLa, MCF-7, and A431 tumor cells [3, 4]. The content of flavonoids in brown knapweed makes 3.36% to 4.44%, HCA – from 1.36% to 2.23% and depends on the concentration of ethanol used to obtain the extractions. Using cathodic voltammetry, it is shown that ethyl acetate fraction (in comparison with chloroform and butanol fractions) of 70% ethanol extraction (in comparison with extraction of 40% and 96% of ethanol) has the greatest antioxidant activity [5].
In traditional medicine brown knapweed is used for dropsy, jaundice, heart diseases, headaches and stomachaches, for uterus diseases, amenorrhea and as a diuretic. For external use it's administered for rinsing in tonsillitis, in the form of compresses – for diathesis and eczema in children, dermatoses, in the form of baths and steaming – for joint pain, rheumatism, muscle and tendon stretching. Swollen areas are powdered by the plant pulvis. Thus, plants of *Centaurea* species are used in traditional medicine for diuretic, choleretic and anti-inflammatory effects [6–10]. HCA are poorly studied compounds of brown knapweed. This group of biologically active substances (BAS) exhibits various pharmacological properties. Besides, the presence of antioxidant and anti-inflammatory properties in brown knapweed extractions can be explained due to the presence of this BAS group. Caffeic acid effects the cells of the immune system exhibiting at the same time an immunotropic and anti-inflammatory effect [11–14]. Taking into account that the content of HCA in brown knapweed extractions and their antioxidant properties depend on the volume ethanol fraction used for their preparation,

it is reasonable to conduct the study for selecting optimal conditions of HCA water-ethanol extraction from brown knapweed which provide the greatest yield of these compounds from raw material to the extracting solvent Brown knapweed is included in the Specialized National Inventory of Plants, Mushrooms and Animals of the Republic of Belarus which contains information on the chemical composition for using in the national economics of Belarus. Essential oil of brown knapweed exhibits bactericidal and fungicidal effect. The extractions from brown knapweed leaves inhibit trypsin, chymotrypsin, and subtilisin [15].

The aim of the work is to choose optimal conditions for HCA water-ethanol extraction from brown knapweed (volume fraction of ethanol; time, temperature and extraction multiplicity; ratio of raw material and extracting solvent; the degree of grinding raw material).

Materials and Methods

Brown knapweed was harvested in the places of natural growth in the vicinity of Vitebsk (Ulanovichi village) and Borisov district (Kastritsa village) of the Republic of Belarus during mass flowering in the middle of July in 2016–2017. The herb presented an aboveground part including flowers, leaves and stems cut at a distance of about 30 cm from the apex. Harvested raw material was dried by the air-shadow method. As extracting solvents 96% ethanol (chemically pure), purified water and their mixtures with a volume ethanol fraction were used: 20, 40, 60, and 80%.

The selection of optimal conditions of water-ethanol extraction has been performed by us earlier on the example of another medicinal plant raw material [16].

Identification and assay of HCA was carried out by high performance liquid chromatography (HPLC) on the liquid chromatograph Agilent 1260 coming with the four solvent feeding and degassing system G5611A, the diode array detector G1315D, the column heating oven G1316C and the autoinjector (autosampler) G5667A. Data collection, processing of chromatograms and absorption spectra was carried out by the program Agilent OpenLAB.

For carrying out the studies the chromatographic column Zorbax SB with the length of 0.25 m and ID 4.6 mm filled with octylsilyl silica gel for chromatography with the particle size of 5 μm was used, the column temperature: 30° C.

A mixture of acetonitrile and 0.01 M potassium dihydrogen phosphate solution adjusted with phosphoric acid to pH 3.0 ± 0.2, in a volume ratio 15:85 was used as the mobile phase. The elution mode is isocratic. The eluent flow rate is 1.0 ml / min. The time of the chromatographic analysis made 60 minutes. Injected sample volume: 20.0 μl.

Detection wavelength – 280 nm. Absorption spectra are recorded in the wavelength range of 190 – 400 nm with the step of 1 nm for BAS in the tested extractions.

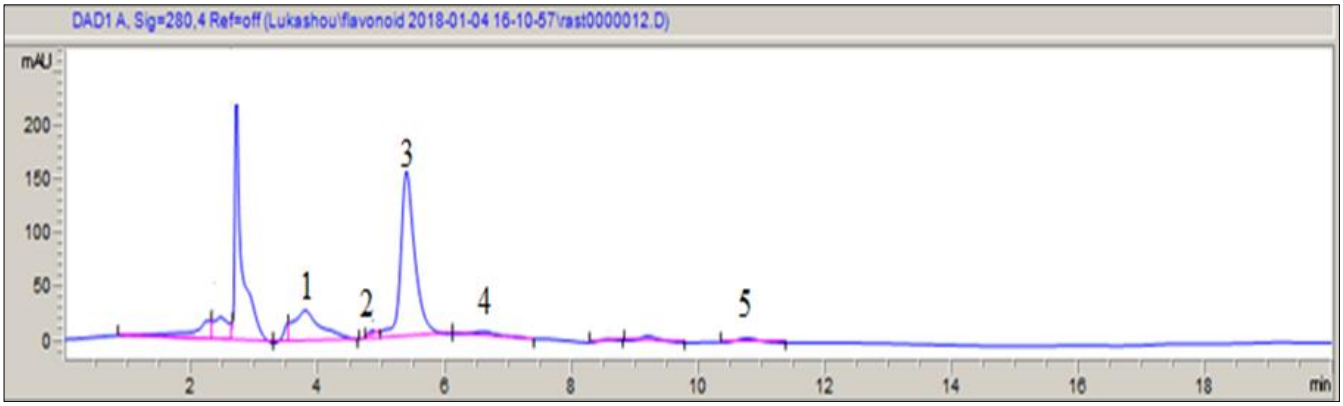
Identification of HCA was carried out by comparing the retention factors and absorption spectra of the substances in the tested extractions with the standard HCA samples and the base including spectral and chromatographic characteristics of 15 phenolic acids [17].

0.5 g / L solutions of chlorogenic and caffeic acids standard samples were prepared on 70% ethanol for injecting into the chromatograph. Relative abundance of HCA was calculated by the internal normalization method. Total concentration of HCA was calculated by the external standardization technique expressed as caffeic acid which was the dominating BAS among HCA.

Each test was made three times (P = 95%; n = 3). The results were presented as $\bar{X}\pm\Delta_{\bar{x}}$, where $\bar{X}$ –average value; $\Delta_{\bar{x}}$ – half-width of the medium sized confidence interval. Comparison of two groups of values was performed using Student's t-test. Variance analysis was performed to identify statistically significant effect of the factors on HCA extraction. The values were statistically significantly different at p<0.05.

Results and discussion

Figure 1 shows that five HCA are found in the test extractions from brown knapweed with retention coefficients of 1.7 – 5.0. Chlorogenic and caffeic acids are identified. The dominating HCA was caffeic acid which relative content depending on the extraction conditions ranged from 49% to 98%.



1 – Chlorogenic acid (1,7); 3 –caffeic acid (2,5); 2, 4 and 5 – unidentified hydroxycinnamonic acids with retention factors 2,3; 4,1 and 5,0 correspondingly

Fig 1: Extraction chromatogram obtained at the extraction with 60% ethanol from drown knapweed

Maxima and arms in the absorption spectra of brown knapweed HCA were in the following ranges: 218; 240; 298 and 324. Figures 2–7 show the relation of HCA concentrations (μg / ml) in the extractions obtained from brown knapweed to the volume ethanol fraction; time, temperature and extraction multiplicity; the ratio of raw materials and extracting solvent; the degree of raw material grinding respectively.

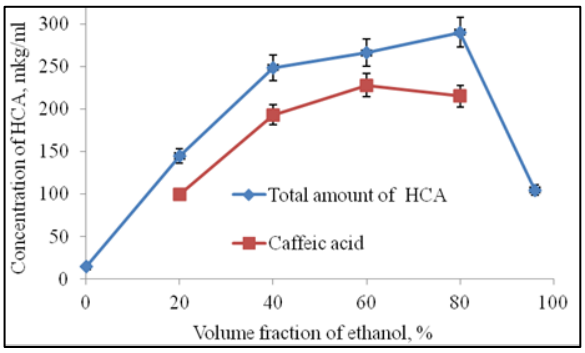


Fig 2: Relation of HCA concentration in the extractions to the volume ethanol fraction

Figure 2 shows that maximum concentration of the total HCA is determined in the extraction obtained by the extraction with 80% ethanol which is statistically significant more ($p = 4.8 \times 10\text{-}6$) by 9.1% (rel.) than with the extraction of 60% ethanol. Steady increase in the content of HCA was observed in the range of volume ethanol fractions from 0% to 80%; fall off (2.8 times) in the concentration of the total HCA was observed in passing from the extraction with 80% ethanol to the extraction with 96% ethanol.

The concentration of caffeic acid is 5.9% (rel.) more in the extraction obtained by the extraction with 60% ethanol in comparison with the extraction with 80% ethanol. Maximum extraction with 80% ethanol was explained by the increased extraction (2.5 times) of chlorogenic acid compared to 60% ethanol. Thus it is possible to use different volume ethanol fractions for separate extraction of caffeic acid (extraction with 60% ethanol) and other HCA (in particular chlorogenic acid (extraction with 80% ethanol)).

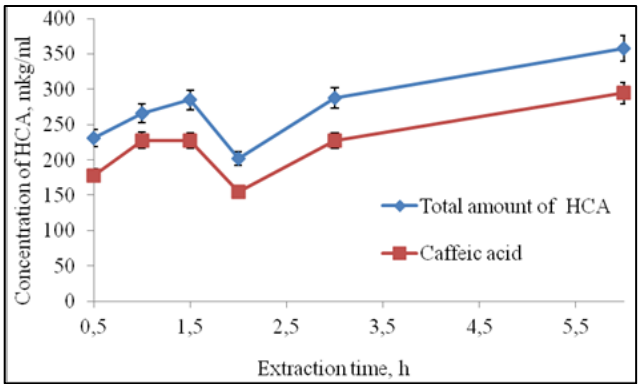


Fig 3: Relation of HCA concentration of in the extractions to the time of extraction

Figure 3 shows that maximum concentration of the total HCA is characteristic of the extraction obtained during extraction for 6 h which is statistically significant by ($p = 1.0\ 10^{-4}$) 1.3 times than at the time of extraction for 3 h. Extraction lasting from 0.5 h to 1.5 h and from 3 h to 6 h led to steady increase in the content of the total HCA (including caffeic acid), at the time of extraction for 2 h minimum extraction was observed which is statistically significant ($p = 8.7\ 10^{-7}$) 1.4 times less than the content at the time of extraction for 1.5 hours. Increase in the concentration of the total HCA during extraction for 0.5 h to 1.5 h is associated with the release of HCA from the cytoplasm through the cell walls taking into account internal diffusion, and with extraction from 3 h to 6 h – with the release of HCA from cell organelles (endoplasmic reticulum, vacuoles, plastids others) and other supramolecular structures. Minimum extraction for 2 h is associated with the mass release of active forms of enzymes splitting HCA into the liquid medium which were inactivated during long-term heating (more than 3 h). Thus a six-hour extraction is reasonable for carrying out the most complete extraction of the total HCA from brown knapweed.

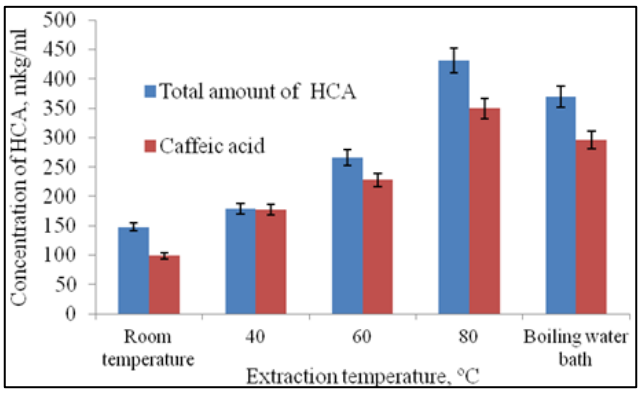


Fig 4: Dependence of HCA concentration in the extractions on the temperature of extraction

Figure 4 shows that maximum total HCA concentration is marked during the extraction at the temperature of 80 ° C which is statistically significant ($p = 7{,}2\ 10^{-5}$) 17% more (rel.) than during the extraction in boiling water bath which is explained by the decrease in the volume of extracting solvent involved in the extraction process in boiling water bath owing to its evaporation ($t_{eth\ boil}$ = 78 ° C) and almost complete inactivation of the enzymes breaking down HCA at 80° C. In the range from room temperature to heating at the temperature of 80° C the rise of the total HCA concentration (including caffeic acid) was observed. During extraction in boiling water bath compared to the extraction at the temperature of 80° C the concentrations of caffeic acid and other HCA decreased on the average by 17–22% (rel.).

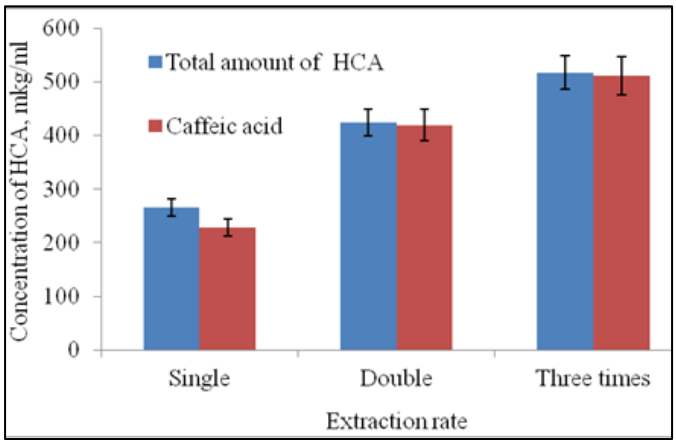


Fig 5: Dependence of HCA concentration in the extractions on the extraction multiplicity (raw material depletion)

Figure 5 shows that maximum HCA concentration (including caffeic acid) was extracted at triple extraction which indicated insufficient depletion of raw material during single and double extraction. With triple extraction the concentration of the total HCA is statistically significant ($p = 1.8\ 10^{-9}$) 22% more (rel.) than with double extraction. With the increase of the number of extractions HCA concentration increased. While conducting double and triple extraction addition of a new portion of the extracting solvent facilitated the behavior of convective and molecular diffusion owing to the increase of the concentration gradient.

At triple extraction with equal volumes of the extracting solvent for one hour the concentration of the total HCA, statistically significant ($p = 1.1\ 10^{-3}$) 1.4 times as much, exceeded the same parameter for the single extraction with the equal volume of the extracting solvent for 6 hours which indicated the achievement of dynamic equilibrium between the concentrations of HCA in the raw material (solid phase) and liquid phase at single extraction.

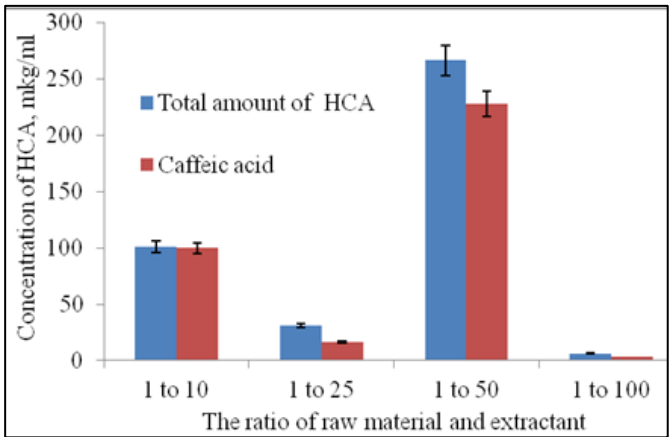


Fig 6: Dependence of HCA concentration in the extractions on the ratio of raw material and extracting solvent

Figure 6 shows that maximum concentration of HCA (including caffeic acid) was achieved when the ratio of raw material and extracting solvent was 1:50 which is statistically significant ($p = 6.7\ 10^{-16}$) 2.3 times as much than with the ratio of raw material and extracting solvent 1:10. The necessity of using sufficiently big volume of the extracting solvent for carrying out the extraction can be associated with its significant consumption for the dilation of ground herb particles of brown knapweed and an increase in the concentration gradient.

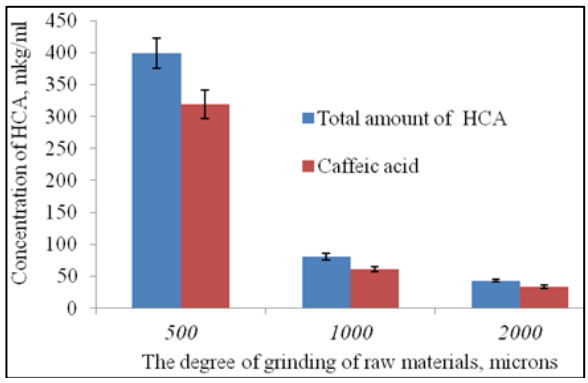


Fig 7: Dependence of HCA concentration in the extractions on the degree of raw material grinding

Figure 7 shows that maximum content of HCA was extracted from brown knapweed with the grinding degree of *500* μm. With the increase in particle size of raw material total concentration of HCA (including caffeic acid) decreased in the obtained extractions. Figures 2–7 show that the nature of the extraction of the total HCA was determined by dominating BAS — caffeic acid. At the same time relative content of the other HCA depending on the time, temperature and multiplicity of extraction; the ratio of raw materials and the extracting solvent; the grinding degree of raw material changed equally and repeated the dependences for caffeic acid concentrations.

Total content of HCA in brown knapweed, expressed as caffeic acid calculated using selected conditions of water-ethanol extraction and determined by HPLC, made 2.6 ± 0.13% which is 16% (rel.) as much as the data given in the origin [5] and obtained using non-selective direct spectrophotometry. The degree of effect of water-ethanol extraction conditions on the yield of the total HCA and caffeic acid from brown knapweed was studied using variance analysis (table).

Table 1: The results of a variance analysis of the effect of factors on water-ethanol extraction of HCA from brown knapweed

The factor Studied Name of HCA	Significance level (p)					
	Volume ethanol fraction	Extraction time	Extraction temperature	Multiplicity of extraction	Ratio of raw material and extracting solvent	The degree of raw material grinding
Caffeic acid	$1{,}8 \cdot 10^{-3}$	$7{,}0 \cdot 10^{-7}$	$1{,}7 \cdot 10^{-2}$	$1{,}0 \cdot 10^{-2}$	0,49	$9{,}5 \cdot 10^{-2}$
Total	$2{,}0 \cdot 10^{-2}$	$2{,}3 \cdot 10^{-7}$	$14{,}5 \cdot 10^{-3}$	$5{,}4 \cdot 10^{-3}$	0,41	$8{,}4 \cdot 10^{-2}$

The table shows that volume ethanol fraction; time, temperature, and multiplicity of extraction statistically significantly ($p < 0.05$) effected the concentration of the total HCA and caffeic acid in water-ethanol extractions obtained from brown knapweed. Besides, the time of extraction effected water-ethanol extraction most of all since the lowest values of significance levels were obtained for this factor.

Thus, the time of extraction can be considered as the most critical factor of HCA water-ethanol extraction from brown knapweed. Therefore, close adherence of extraction time mode is required, which most significantly effects the content of HCA in the extractions obtained. Selected conditions of HCA water-ethanol extraction from brown knapweed can be used to develop the procedure of caffeic acid assay and the amount of the total HCA in brown knapweed and also technologies for producing galenical medicines based on the given plant raw material.

Conclusion

Three HCA were additionally determined in water-ethanol extractions from brown knapweed in addition to the known chlorogenic and caffeic acids. Dominating HCA is caffeic acid. Optimal conditions for water-ethanol extraction of HCA from brown knapweed are selected: volume ethanol fraction – 80%; extraction time – 6 h; extraction temperature – 80ºC; extraction multiplicity- triple; the ratio of brown knapweed and 80% ethanol – 1:50; the grinding degree of brown knapweed – *500* microns. Maximum concentration for caffeic acid was observed at the extraction with 60% ethanol.

Total content of HCA determined using HPLC method based on the above mentioned optimal extraction conditions makes 2.6 ± 0.13%.

Volume ethanol fraction; time, temperature and multiplicity of extraction statistically significantly ($p < 0.05$) effect the total HCA content in water-ethanol extractions from brown knapweed. The most critical factor is the time of extraction.

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