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Phenolic compounds content in *Vitex agnus-castus* L. and *V. cannabifolia* Sieb. growing in Ukraine

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ABSTRACT

There are many studies dedicated to phytochemistry of *Vitex agnus-castus* and *V. cannabifolia* growing in their endemic conditions, but there is no available information about constituents of ones growing in temperate climates. Also the data of phytochemical composition of stems and inflorescences are insufficient. The TLC and HPLC methods were used for identification and quantification, while UV-spectrophotometry was used for estimation of sums of flavonoids, polyphenols and cinnamic acids in stems, inflorescences and leaves. The content of caffeic acid was much lower that it was reported for raw material collected in Turkey. The highest total polyphenols content was found in inflorescences and leaves of each plant (up to 6.65%). The highest total flavonoids content was in *V. agni-casti* inflorescences (7.59%). In the rest of raw material types it was no higher than 2.23%. The total cinnamic acids content is higher in *V. cannabifoliae* raw material (up to 6.10%).

Keywords: *V. agnus-castus*, *V. cannabifolia*, orientin, casticin, caffeic acid.

1. Introduction

Phytochemical investigation of medicinal herbs growing in Ukraine is one of the main aims of modern Ukrainian pharmaceutical science. Plants recently introduced into our flora as well as endemic ones are promising for pharmacognostic investigation and further development of new plant drugs.

Chaste-tree (*Vitex agnus-castus* L.) and variant of Chinese chaste-tree called *Vitex cannabifolia* Sieb. (Lamiaceae) are interesting species for phytochemical study. *V. agni-casti* fruits are well-known remedy for treatment of female reproductive system diseases^[1, 2, 3] while roots, leaves and fruits of *V. cannabifolia* have been used in folk medicine as anti-inflammatory, analgesic, sedative and antipyretic medicine since ancient times. Also decoction from bark of *V. cannabifolia* has been used for treatment of disenteria, enteritis, malaria, and digestive disorders^[2, 4, 5]. Flavonoids vitexin, isovitexin, casticin, apigenin, luteolin^[6, 7, 8, 9], iridoids agnuside, aucubin^[9, 10, 11], terpenes, lignanes and other compounds^[5, 12, 13, 14, 15, 16, 17, 18] were isolated from leaves and fruits of *V. agnus-castus* and *V. cannabifolia*, collected in places of origin. Although these plants are endemic in areas with subtropical climate such as India, China, Malaysia and Nepal, they are successfully cultivated in temperate zones. Both species are introduced into Ukrainian flora by National Botanical Garden named after M.M. Hrysko in Kyiv. We supposed that phytochemical constituents of *V. agnus-castus* and *V. cannabifolia* growing in conditions of temperate climate, grey and black soils and moderate moisture (as in Ukraine) may differ in quality and quantity from those were reported for subtropical regions, but there are no available publications with certain data.

Another problem is only leaves and fruits of *V. agnus-castus* and *V. cannabifolia* are relatively well-studied, while the data of phytochemical composition of stems and inflorescences are insufficient. So the aim of our study was determination of flavonoids, cinnamic acid derivatives and polyphenols in leaves, stems and inflorescences of *V. cannabifolia* and *V. agnus-castus* as a part of ongoing phytochemical research of these plants growing in Ukraine.

2. Materials and methods

2.1 Standards and reagents

All reagents and solvents were of analytical grade. Authentic standards for TLC and HPLC analysis (vitexin, orientin and caffeic acid) were obtained from Sigma-Aldrich (St. Louis, MI, USA), while casticin was acquired from Ausmausco Pharma (Shanghai, China) and

ferulic acid was purchased from Xian Plant Bio-engineering (Xian, China). The following solvents: trifluoroacetic acid, acetonitrile, formic acid and acetic acid were got from Sigma-Aldrich, and ethyl acetate was acquired from TD Ukrkhimekspro (ТД Укрхимекспро, Kyiv, Ukraine).

2.2 Sampling

The leaves, inflorescences and stems of *V. cannabifolia* and *V. agnus-castus* were collected in National Botanical Garden named after M.M. Hryshko in August 2012 from garden's collection of New Cultures Department. The person responsible for *Vitex* species introduction and cultivation was Olga Korablova, PhD in agriculture, the senior engineer in New Cultures Department.

2.3 Phenolic compounds extraction and derivatization

The extraction was performed by the common method for polar compounds [19]. Briefly, 2 g of the dried specimens were extracted with 20 ml of 70% ethanol for 30 min at 90 °C. The extraction procedure was repeated five times and the resulting extracts were pooled and rised volume into 50 ml by same extragent.

2.4 TLC-analysis

Thin-layer chromatography analyses were performed using "Silufol UV-254" plates in further solvent system: ethyl acetate, formic acid, acetic acid and water (100:11:11:26). Detection was performed by NP/PEG reagent: the plate was dipped in 1% methanolic solution of diphenylborinic acid aminoethylester, dried in the stream of cold air and then dipped in 5% ethanolic solution of PEG 4000. Examination was observed under UV 365 nm.

2.5 UV/VIS spectrophotometry

A Hewlett Packard UV/VIS 8452A spectrophotometer and 1 cm pathlength disposable cells were used for spectral measurements.

2.6 Flavonoids

The 3 ml of 5% aluminium chloride in 70% ethanol was added to 1 ml of extract in 25 ml volumetric flask, after 10 min total solution volume was brought with 5% acetic acid in 70% ethanol to the mark. After 40 min the absorbance was measured at 400 nm wavelength. The blank solution was mixture of 3 ml of 5% aluminium chloride in 70% ethanol and 5% acetic acid in 70% ethanol brought to volume of 25 ml. The total flavonoid content (x_1) expressed as orientin was calculated using following equation: $x_1 = A \times c \times 1250 / A_0$, where A was the absorbance of test solution at 400 nm; A_0 — the absorbance of orientin standard solution; c — the concentration of orientin standard solution.

2.7 Cinnamic acid derivatives

10 g of sodium nitrite and 10 g of sodium molybdate were placed in 25 ml volumetric flask. Added 15 ml of water, mixed, and brought total solution volume with water to the marc. Filtered (solution A).

1 ml of extract, 2 ml of 0,5 M hydrochloric acid, 2 ml of solution and 2 ml of sodium hydroxide diluted were placed in 25 ml volumetric flask. The total solution volume was brought to 25 ml with water (test solution). The compensation liquid was prepared using all reagents except extract that were placed

in 25 ml volumetric flask and diluted with water till the mark. Measured the absorbance at 505 nm. Calculated the percentage content of cinnamic acid derivatives (x_2), expressed as caffeic acid, using the following expresion: $x_2 = A \times 1250 / 332$, where A was the absorbance at 505 nm; 332 - the specific absorbance of caffeic acid.

2.8 Polyphenols

The pharmacopaeian determination of tannins in herbal drugs was carried out.

2.9 HPLC-analysis

High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-20 module system (Shimadzu Corporation, Kyoto, Japan) using diode array detection (DAD). Separations were accomplished using a Luna C18 (2) 100A 250×4,60 mm 5 micron column (Phenomenex, Torrance, USA) at 35 °C. The flow rate was 1 ml/min; injection volume was 5 µl. Prior to injection all samples were diluted 10 times and filtered through a 0.45 mm Millipore membrane filter. Chromatograms were examined at 350 nm UV-light.

Table 1: The program of gradient elution

Time, min	Solvent A ^a	Solvent B ^b
5	95	5
35	75	25
40	75	25
60	50	50
62	20	80
65	20	80
65,01	95	5
80	95	5

^a 0.1% trifluoroacetic acid in water; ^b0.1% trifluoroacetic acid in acetonitrile; All measurements were made in triplicate.

3. Results and discussion

3.1 TLC-method

The two cinnamic acid derivatives (chlorogenic and caffeic acids) along with two flavonoids (casticin and orientin) were identified by the TLC-method in leaves, stems and inflorescences of investigated plants. While orientin (water-soluble flavon C-glucoside) is the widespread constituent among Lamiaceae, as well as chlorogenic and caffeic acids, casticin (lipophilic aglicone with four methoxy groups, another name — vitexicarpin) is reported to be a marker compound for *V. agni-casti* raw material. But spots with the same colour and Rf-values as casticin spots, were also found on all of the *V. cannabifoliae* chromatograms.

Also there were found two unidentified blue spots (perhaps inherent to cinnamic acid derivatives or coumarins) on chromatograms, and five yellow and orange spots, which probably correspond to flavonoids.

Analyzed extracts of *V. agnus-castus* and *V. cannabifolia* which were collected in Ukraine did not show the presence of vitexin, while vitexin is reported to be one of the prevailing flavon in *Vitex* spp. growing in Asia [20, 21].

The values of Rf of different flavonoids and cinnamic acid derivatives are shown in the Table 2.

Table 2: TLC-profile of *V. agnus-castus* and *V. cannabifolia* flavonoids and cinnamic acid derivatives

Compound assignment	Presense of spot in certain raw material						Rf	Colour of spot
	Inflorescences of VAC ^a	Leaves of VAC	Stems of VAC	Inflorescences of VC ^b	Leaves of VC	Stems of VC		
casticin	+	+	+	+	+	+	0,97	dark-blue
caffeic acid	+	+	+	+	+	+	0,81	blue
NI ^c	++	+	+	++	++	+	0,75	yellow
NI	+	-	-	+	+	+	0,73	blue
NI	+	+	+	+	+	+	0,69	orange
orientin	+++	++	+	+++	+++	++	0,63	orange
NI	+	+	+	++	++	+	0,58	yellow
chlorogenic acid	+	+	+	+	+	+	0,48	blue
NI	+	+	+	+	-	+	0,33	orange
NI	+	+	+	+	+	+	0,29	orange
NI	+	+	+	+	+	+	0,13	blue

^aV. agnus-castus; ^bV. Cannabifolia; ^cNI — not identified compound.

3.2 UV-spectrophotometry

The results of total phenolic compounds content estimation in different types of raw material are shown in the Table 3.

The highest total polyphenols content was found in inflorescences and leaves of each plant (40 - 199% more than in stems). Comparing two these plants, polyphenolic content in *V. cannabifolia* was 44% higher in inflorescences, 15% - in leaves, 166% - in stems. Talking about low-molecular tannins, which could be adsorbed by hide powder, the differences in their content in these two plants were not so big. Their content in *V. agni-casti* inflorescences was 1.85% (calculating into dry mass), while in *V. cannabifolia* it was 2.21% (19% higher), leaves of *V. agnus-castus* contained 1.90% of low-molecular tannins versus *V. cannabifolia* — 1.44% (31% lower). And only stems of *V. cannabifolia* contained 260% higher amount of polyphenols adsorbed by hide powder than *V. agni-casti* stems.

The highest total flavonoids content expressed as orientin was found in *V. agni-casti* inflorescences (7.59% calculating by dry mass). In the rest of raw material types it was no higher than 2.23% (3.4 times lower). This data is higher than was earlier reported for *V. agnus-castus* growing in Asia, according to which there are 2.7% of flavonoids in leaves and no more

than 1.5% in other parts of plant. Also we compared our results with early reported from Croatia [22]: raw material, collected in Ukraine, contained up to 3 times more flavonoids, but significantly less (more than 2 times) tannins. The sum of flavonoids in different parts of *V. cannabifolia* was discovered for first time and showed the same difference in accumulation. In general, total flavonoids content was higher in stems and inflorescences of both species, while in stems its content was up to ten times lower. That is the reason we recommend leaves and inflorescences of these plants for further studies (especially inflorescences of *V. agnus-castus*) as a possible source of flavonoids. These types of raw material are valuable for further study as they are the only nowadays discovered sources of casticin in Ukraine.

The total cinnamic acid derivatives content was higher in *V. cannabifoliae* raw material (37.2%, 23.5% and 77.36% higher in inflorescences, leaves and stems respectively). As for tannins and flavonoids, inflorescences and leaves of these plants contained 1.5 - 5 times more cinnamic acid derivatives than stems, and leaves of *V. cannabifolia* could be recommended as a source of cinnamic acid derivatives according to their bigger mass rate.

Table 3: Total polyphenols, flavonoids and cinnamic acid content in *V. agnus-castus* and *V. Cannabifolia*

Type of raw material	Total content in air-dry raw material, %			
	Polyphenols adsorbed by hide powder	Total polyphenols	Total flavonoid content expressed as orientin	Total cinnamic acid derivatives content expressed as caffeic acid
Inflorescences of VAC	1.85±0.04	4.61±0.08	7.59±0.05	3.84±0.08
Leaves of VAC	1.90±0.03	4.98±0.06	2.23±0.02	3.92±0.02
Stems of VAC	0.40±0.02	1.54±0.05	0.22±0.01	0.78±0.07
Inflorescences of VC	2.21±0.08	6.65±0.07	2.19±0.03	6.10±0.05
Leaves of VC	1.44±0.06	5.75±0.04	1.98±0.01	5.12±0.03
Stems of VC	1.04±0.03	4.10±0.06	0.71±0.01	3.46±0.07

3.3 HPLC-method

The HPLC-profile of leaves, inflorescences and stems of *V. agnus-castus* and *V. cannabifolia*, which is shown in the Table 4, reveals orientin was the major flavonoid in all discovered types of raw material. Its content was the highest in inflorescences of *V. cannabifolia* (1.8% calculating into dry mass) and in the leaves of both species (2.09% and 1.85% in *V. agnus-castus* and *V. cannabifolia* respectively). The

accumulation of orientin in stems was visibly lower (up to eight times lower than in leaves). These data did not match with that was recently reported in literature for raw material collected in Asia, according to which casticin, vitexin and kempferole are prevailed flavonoids in *Vitex* spp. Peaks of casticin were much smaller than peaks of prevailing compounds on all the chromatograms (see Fig. 1) and its content did not exceed 0, 08%. We suppose it was the result of shorter vegetating period

of investigated plants.

Ferulic acid was mostly cumulated in inflorescences and leaves of *V. cannabifolia* and *V. agnus-castus*. For caffeic acid the difference in accumulation in different parts of the plants was not so big, its content in stems was not more than 38% lower than in leaves. In general caffeic acid was mostly

cumulated in *V. cannabifolia* while ferulic acid slightly prevailed in *V. agnus-castus*. These cinnamic acid derivatives were also found in *V. agnus-castus* growing in Turkey [21, 23], but their content in Ukrainian raw material was much lower: not more than 0.06% of caffeic acid in *V. agnus-castus* growing in Ukraine versus almost 0.30% in Turkish one.

Table 4: The content of identified compounds in *V. agnus-castus* and *V. cannabifolia* (mean values)

Compound	Rt	Content in air-dry raw material, %					
		Inflorescences of VAC	Leaves of VAC	Stems of VAC	Inflorescences of VC	Leaves of VC	Stems of VC
Caffeic acid	20.36	0.038	0.037	0.023	0.057	0.058	0.043
Orientin	27.26	1.000	2.090	0.215	1.802	1.852	0.664
Ferulic acid	29.56	0.253	0.318	0.045	0.206	0.211	0.060
Casticin	61.31	0.054	0.077	0.006	0.004	0.004	0.0003

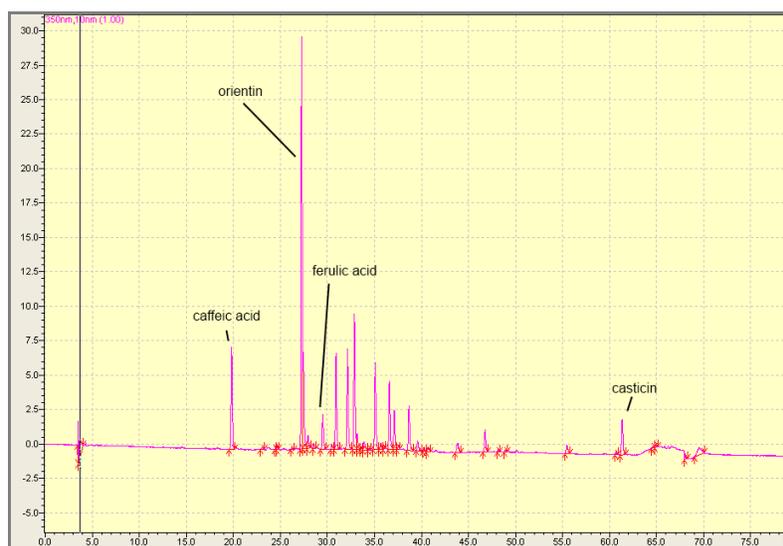


Fig 1: HPLC-DAD profile of *V. agnus-castus* leaves extract.

4. Conclusions

This study showed the difference in qualitative and quantitative content of some phenolic compounds in *V. agnus-castus* and *V. cannabifolia* growing in areas with different climate and soil types. Talking about raw material collected in Ukraine, no vitexin was found and content of casticin was much lower than expected while the major flavonoid in investigated plants was orientin, perhaps because of shortness of vegetating period. The caffeic acid content in Ukrainian species was also approximately five times lower than in Turkish ones. But the total content of flavonoids was even higher than it was founded earlier.

Also in this study we compared the total content of flavonoids, cinnamic acids and polyphenols in leaves, stems and inflorescences of *V. agnus-castus* and *V. cannabifolia* at first time. According to results leaves of *V. cannabifolia* growing in Ukraine could be recommended as a source of cinnamic acids and leaves of both leaves – as a source of orientin.

Because of difference in phytochemical content the further study of pharmacological activities for *V. agnus-castus* and *V. cannabifolia* growing in Ukraine are needed.

5. Acknowledgement

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