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Research Article

HPLC-DAD assay of phenols profile in *Antennaria dioica* (L.) Gaertn

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Abstract

Anternnaria dioica (*Asteraceae* family) – is a perennial herb, commonly found in dry grasslands and sandy or stony places from Eurasian areas. It is known in traditional medicine as antioxidant, diuretic, choleretic and anti-inflammatory remedy. This species should be reconsidered as possible sources of phenols, mainly flavonoids and hydroxycinnamic acids. Thus, the aim of this study was to validate a chromatographic method for detection of phenols and their identification in *A. dioica* herb. HPLC-DAD method was evaluated in terms of linearity, precision, repeatability, accuracy, LOD and LOQ. The calibration curves of thirteen reference substances were linear ($R^2 > 0.99$) over the range of 5–400 μg/mL, the LODs and the LOQs were in the range of 0.1–0.3 μg/mL and 0.2–1.0 μg/mL, respectively. During HPLC-DAD assay two flavones – luteolin, apigenin; flavonol – quercetin and three its glycosides – rutin, hyperoside and isoquercitrin; coumarins: coumarin and umbelliferone; five hydroxycinnamic acids – chlorogenic, caffeic, *p*-coumaric, *trans*-ferulic and rosmarinic were identified in *A. dioica* herb. This phytochemical study of *A. dioica* confirms that this plant material is a rich source of phenolic compounds.

Keywords

Antennaria dioica Gaertn, phenolic compounds, flavonoids, hydroxycinnamic acids, HPLC-DAD

Introduction

Asteraceae family, which includes more than 1600 genera, with over 23,000 species, widespread in different types of regions all over the world and is the largest family of flowering plants (Babotă et al. 2018; Rolnik and Olas 2021). Several classes of compounds from *Asteraceae* species were studied and tested for different bio-activities and were reported as having medicinal potential (Dudova 2018; Rosche et al. 2018). Among these compounds, a special attention has been given to phenols, and especially to flavonoids and hydroxycinnamic acids, which provide for these species important uses in the pharmaceutical, cosmetics and food industry, that are due to their important medicinal properties as the antioxidant, anti-inflammatory, antifungal or antibacterial ones (Bumrungpert et al. 2018; Dabeek and Marra 2019; Savych 2021; Savych and Mazur 2021; Savych and Milian 2021). In this context and taking into consideration the fact that in the last decades these compounds have shown a significant importance in the field of medicinal compounds (Savych and Marchyshyn 2021a, 2021b; Savych et al. 2021f, 2021d), the *Asteraceae* species should be reconsidered as possible sources of phenols.

Antennaria dioica (L.) Gaertn. (*A. dioica*, Stoloniferous Pussytoes) is the plant belonging to the same tribe (*Gnaphalieae*) of the *Asteraceae* family. It is a perennial herb, commonly found in dry grasslands and sandy

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or stony places from Eurasian areas. It is known in the traditional medicine for its use in cases of biliary and respiratory tract diseases (Dudova 2018). The folk medicine of different countries cites common uses for preparations obtained from *A. dioica* herb, exploited for their antioxidant, diuretic, choleretic and anti-inflammatory properties (Babotă et al. 2018; Rolnik and Olas 2021). Today, there is not enough information about the phytochemical composition of this species, so the study of the profile of biologically active substances is relevant.

In addition, it is important for medicine and pharmacy to study new promising plant species, as they can be a source of new drugs that can have a numerous of advantages over synthetic agents, namely, they are low-toxic (Savych and Mala 2021), have a mild pharmacological effect and possibility to be used for long periods of time without significant side effects, have a complex activity through a numerous of biologically active compounds (Savych and Nakonechna 2021; Savych et al. 2021b, 2021c, 2021i, 2022).

Thus, the aim of this study was to validate the chromatographic method for detection of phenols and their identification in *Antennaria dioica* herb.

Materials and methods (experimental part)

Plant materials

Aerial parts of the Antennaria dioica Gaertn. were harvested in Western Ukraine, region (48°13'23.2"N, 25°11'42.0"E), during a mass flowering period in 2019. The raw materials were then dried, crushed and stored according to the general Good Agricultural and Collection Practice (GACP) requirements (WHO 2003). Plants were authenticated by prof. Svitlana Marchyshyn, Department of Pharmacognosy with Medical Botany, Ivan Horbachevsky Ternopil National Medical University, Ternopil, Ukraine. A voucher specimen No. 189 is kept in departmental herbarium for future record.

Chemicals and standards

Chemical reference substances (CRS) of chlorogenic acid, caffeic acid, *p*-coumaric acid, *trans*-ferulic acid, rosmarinic acid, apigenin, luteolin, coumarin, 7-hydroxycoumarin, quercetin, quercetin-3-galactoside, quercetin-3-rutinoside, quercetin-3-glucoside were of primary reference standard grade (≥ 95% purity HPLC) and were purchased from Sigma-Aldrich Chemical Company (Germany). Methanol (≥ 99.9% purity HPLC), trichloroacetic acid (TCA) (> 99% purity HPLC), acetonitrile (HPLC grade) was purchased from ThermoFisher Scientific (USA). Water used in the studies was produced by MilliQ Gradient water deionizaton system (USA).

Extraction procedure

The sample of herbal raw materials was ground into a powder by laboratory mill, then about 500 mg (accurately weighed) was selected and placed into flask with 5–10 mL of 60% methanol (v/v). The extraction was carried out in an ultrasonic water bath at 80 °C for 4 hours. The resulting extract was centrifuged at 3000 rpm and filtered through disposable membrane filters with pores of 0.22 μm (Sumere et al. 2018).

Instrumentation and chromatographic conditions

Content of phenols in the herbal raw material was studied by high performance liquid chromatography coupled with diode array detector (HPLC-DAD) (Savych et al. 2021e, 2021g) using 3D LC System from Agilent Technologies 1200 (USA) equipped with a G1313A autosampler, a G1311A quaternary pump, a G1316A thermostat and a G1315B diode array detector (Savych et al. 2021). The separation was performed on a Zorbax SB-Aq chromatographic column (4.6 mm ± 150 mm, 3.5 μm) (Agilent Technologies, USA).

Table 1. Chromatographic conditions.

Flow rate	0.7 mL/min
Eluent supply pressure	10000–12000 kPa
Column temperature	25 °C
Injection volume	20 μL
Detection	255 nm, 320 nm, 330 nm
Scan time	0.6 sec
Range of absorbance spectra	200–400 nm.

Mobile phase A – 0.1% TCA, mobile phase B – acetonitrile. Samples were chromatographed in gradient mode (Table 2).

Table 2. Gradient mode.

Chromatography time, min	Mobile phase A, %	Mobile phase B, %
	Flavonoids	
0:00	88	12
30:00	75	25
33:00	75	25
38:00	70	30
40:00	60	40
41:00	20	80
	Hydroxycinnamic acids	
0:00	95	5
8:00	92	8
15:00	90	10
30:00	80	20
40:00	60	40
41:00	25	75

Method validation

Validation of HPLC-DAD method to quantify of phenols was evaluated in terms of linearity, precision, repeatability,

accuracy, limit of detection (LOD) and limit of quantification (LOQ) according to the International Conference on Harmonization (ICH) guidelines.

Stock solutions

1 mg of each standard was dissolved in 1 mL of methanol. The solutions were filtered through disposable membrane filters with pores of 0.22 μm. All filtered standards were kept at -18 °C.

Standard calibration solution

The stock solutions of each CRS were dissolved in methanol and diluted together to give concentrations in range 5–400 μg/mL for evaluation of the calibration range.

Linearity

Linearity was assessed by using six concentration levels of each standard calibration solution with three injections. Using the peak areas on the chromatogram, a calibration curve was plotted against the known concentrations of the standard solutions. Linear least-squares regression was used to analyze the standard curves of each CRS and the correlation coefficient (R^2) of the regression formula were used to validate the linearity.

LOD and LOQ

The values for LOD and LOQ were calculated based on the data obtained during linearity testing in the low concentration range of the test solution, using the following formulas: LOD = 3.3 * s / Slope; LOQ = 10 * s / Slope.

Precision

To validate precision, three different concentrations of sample extracts were used for intra-day (repeatability) analysis, and only one concentration for the inter-day (intermediate precision) analysis in triplicate. Intra and inter-day precision were examined by calculating the percent of relative standard deviation (% RSD) of standards on the same day and on three different days, respectively.

Accuracy

The accuracy of each sample was tested by recovery method. Three different levels of standard solutions (25, 50, and 100 μg/mL) were spiked into the extract. The spiked and un-spiked samples were evaluated under the same condition in triplicate, then percent recoveries were calculated by comparing the measured amount of those standards with the amount added.

Results and discussion

The chromatographic method was validated by evaluating linearity range, precision, repeatability, accuracy, LOD and LOQ. The linearity of the method was evaluated by studying its ability to obtain an analyte response linearly proportional to its concentration in a given range. To determine that parameter, calibration curves were generated by injection in triplicate of standard solutions at six concentration levels and their correlation coefficients were calculated. As can be seen in Table 3, the linearity of HPLC-DAD method was good, since R^2 were in the range of 0.997–0.999.

Table 3. Validation parameters for HPLC-DAD method.

Compound	Linear range, μg/mL	R^2	Precision, % RSD	Repeatability, % RSD	Accuracy, %	LOD, μg/mL	LOQ, μg/mL
coumarin	5–300	0.998	1.89	1.24	101.14	0.1	0.3
quercetin	5–300	0.999	0.64	0.42	100.06	0.1	0.2
luteolin	5–400	0.999	1.73	1.23	100.36	0.2	0.5
quercetin-3-galactoside	5–300	0.999	2.19	1.40	97.12	0.1	0.3
quercetin-3-rutinoside	5–300	0.998	1.91	1.33	101.10	0.2	0.7
quercetin-3-glucoside	5–400	0.999	1.41	0.89	101.25	0.1	0.3
apigenin	5–300	0.997	2.36	1.24	99.22	0.3	1.0
chlorogenic acid	5–300	0.997	2.79	1.92	106.10	0.1	0.3
caffeic acid	5–400	0.999	1.40	0.95	99.67	0.2	0.5
p-coumaric acid	5–400	0.999	0.38	0.21	100.36	0.2	0.5
trans-ferulic acid	5–300	0.999	1.54	1.28	99.66	0.1	0.2
rosmarinic acid	5–400	0.998	0.48	0.39	100.09	0.1	0.4
7-hydroxycoumarin	5–300	0.999	0.89	0.94	100.12	0.1	0.2

The precision of the method was evaluated by injecting three times the same sample spiked with three levels of concentration (covering the specific range for each compound) during three consequent days. Repeatability was calculated by analysing three times the same sample. Both parameters were evaluated by RSDs that were in the range of 0.38% – 2.79% for inter-day precision and were from 0.39% to 1.92 for intra-day precision (Table 3).

The accuracy of HPLC-DAD method was evaluated by the recovery test. In this way, three samples, previously analyzed, were spiked at three concentration levels of the target compounds and were injected by triplicate. The recoveries of all compounds ranged between 97.12% and 106.10% (Table 3).

HPLC-DAD method allowed the detection of phenols in the range of 0.1–0.3 μg/mL; the quantification in the range of 0.2–1.0 μg/mL, as it is shown in Table 3.

The results of identification and quantification of phenols in *A. dioica* herb are represented in Table 4. During HPLC-DAD assay two flavones – luteolin, apigenin; a flavonol – quercetin and three its glycosides – rutin, hyperoside and isoquercitrin; coumarin and umbelliferone – hydroxycoumarin; five hydroxycinnamic acids – chlorogenic, caffeic, *p*-coumaric, *trans*-ferulic and rosmarinic acid were identified (Figs 1–3).

The quantitative determination showed that the main hydroxycinnamic acids were rosmarinic (944.1±0.22 μg/g) and chlorogenic (793.5±0.19 μg/g), regarding the flavonoids, isoquercitrin (164.5±0.17 μg/g) and luteolin (126.4±0.18 μg/g) are prevailed in *A. dioica* herb (Table 4).

Flavonoids that were detected during HPLC-DAD analysis have powerful antioxidant activities, which are manifested due to their chemical structure, which provides the cleavage of hydrogen atoms (Shashank and Abhay et al. 2013; Panche et al. 2016). In addition, they increase the production of glutathione (GSH) and antioxidant enzymes – superoxide dismutase (SOD) and catalase (CAT), as well as inhibit xanthine oxidase, which is involved in the generation of ROS (Enogieru et al. 2018; Xu et al. 2019; Savych and Polonets 2021; Savych and Sinichenko 2021).

Table 4. Results of HPLC-DAD analysis of phenols in *Antennaria dioica* Gaertn. herb.

Identified substance	UV-spectrum λ max, nm	t_R, min (SD±0.02)	Content in the herbal raw materials, μg/g
coumarin	255	14.95	27.6±0.15
quercetin	255	15.77	12.8±0.14
luteolin	255	16.42	126.4±0.18
quercetin-3-galactoside	255	18.10	38.3±0.11
quercetin-3-rutinoside	255	19.40	53.2±0.11
quercetin-3-glucoside	255	19.98	164.5±0.17
apigenin	330	35.66	33.6±0.12
chlorogenic acid	330	22.53	793.5±0.19
caffeic acid	320	30.99	62.3±0.16
p-coumaric acid	320	33.80	31.1±0.14
trans-ferulic acid	320	39.04	72.4±0.13
rosmarinic acid	330	40.44	944.1±0.22
7-hydroxycoumarin	330	42.47	35.5±0.14

Note: Values are expressed as mean ± SD (n = 4).

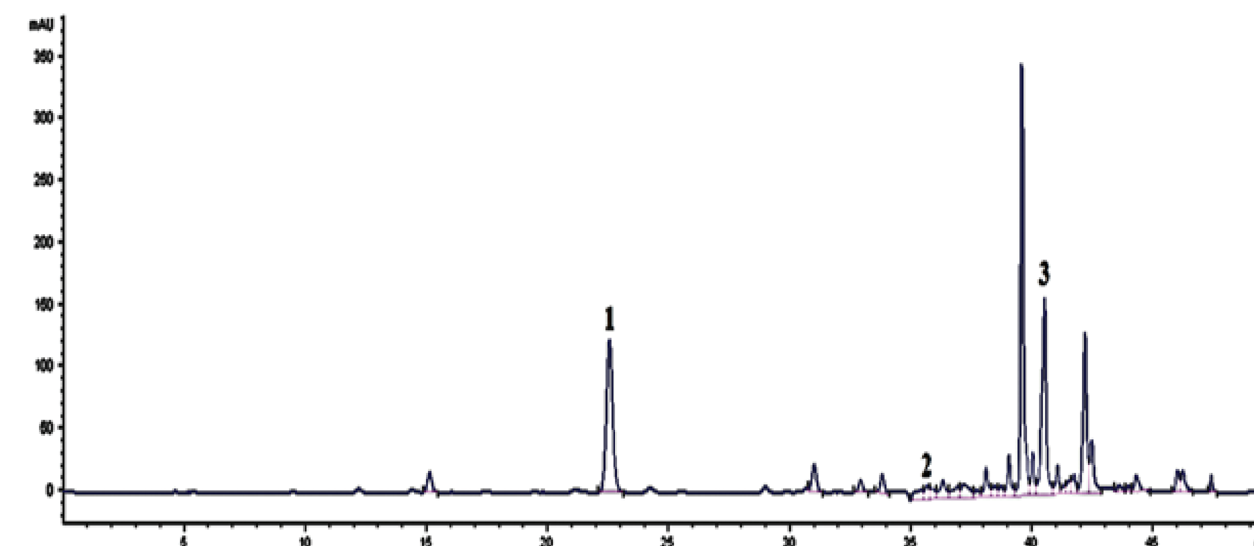

Figure 1. HPLC-DAD chromatogram of phenols identified in *A. dioica* herb, λ = 320 nm: **1** – chlorogenic acid; **2** – apigenin; **3** – rosmarinic acid.

Flavonoids exhibit a numerous of pharmacological effects, such as antioxidant, antihyperglycemic, antidiabetic, anti-inflammatory, cardiovascular, neuroprotective, hepatoprotective, antiallergic, antiosteoporotic, anticancer, antiplatelet and vasodilatory properties (Kawser et al. 2016; Savych et al. 2021a).

Phenylpropanoic acids have potential antioxidant properties, which are realized by cleavage of hydrogen atoms, which reduces the number of free radicals, lipid peroxidation products and inhibits an oxidative stress (Alam et al. 2016; Adisakwattana 2017; Stähli et al. 2019). In addition, they can regulate lipid metabolism by lowering triglycerides, low-density lipoproteins and cholesterol that is signified to prevent the development of cardiovascular diseases (Salazar-Lopez et al. 2017).

Phenols exhibit anti-inflammatory properties, which is manifested by a decrease in edema, effective suppression of proinflammatory cytokines and reduced neutrophil infiltration (Tsang et al. 2016). Phenolic substances are very important agents for prevention and treatment of diabetes mullites because they have a hypoglycemic effect, which is manifested by various mechanisms – inhibition of α-glucosidase, stimulation of insulin secretion,

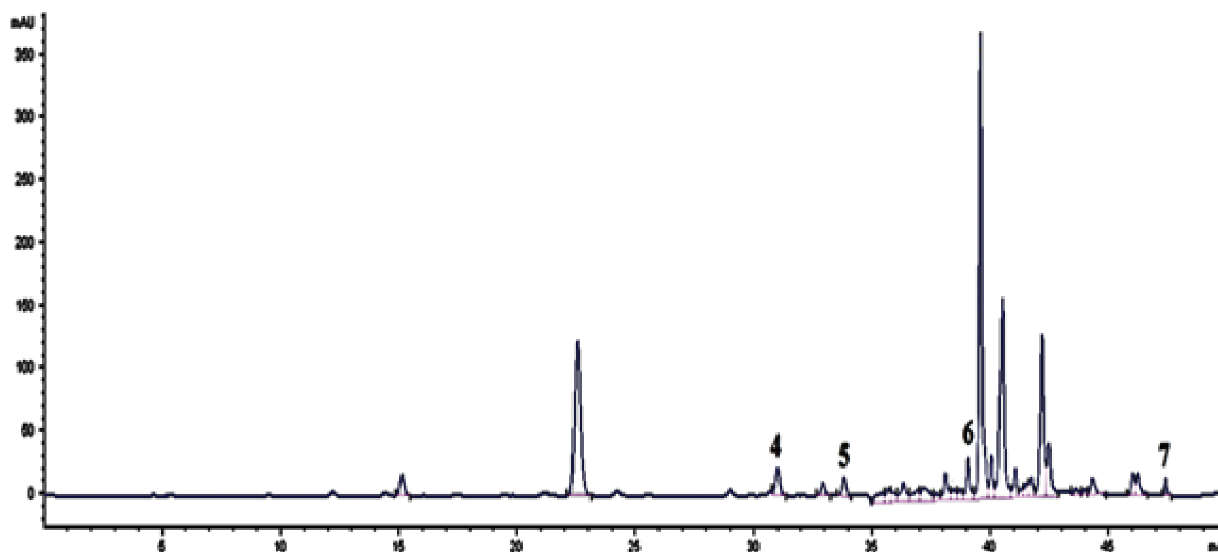

Figure 2. HPLC-DAD chromatogram of phenols identified in *A. dioica* herb, λ = 330 nm: **4** – caffeic acid; **5** – *p*-coumaric acid; **6** – *trans*-ferulic acid; **7** – 7-hydroxycoumarin (umbelliferone).

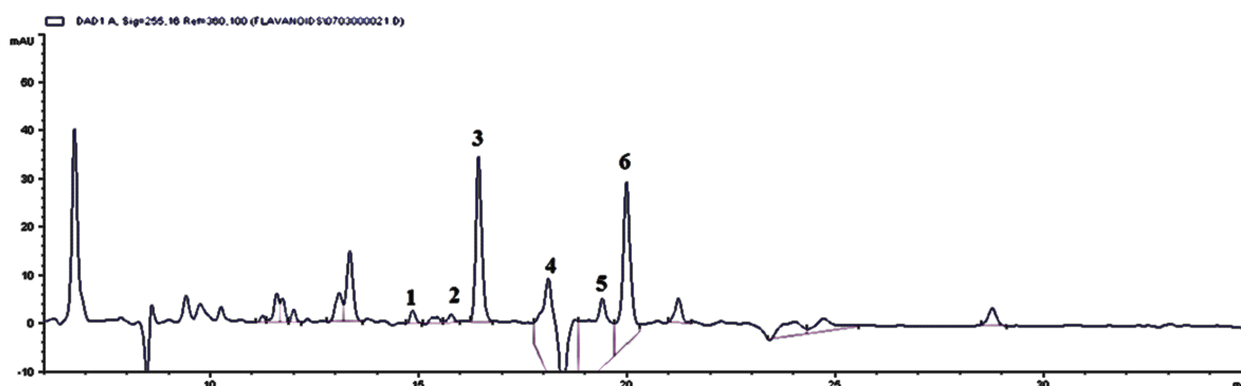

Figure 3. HPLC-DAD chromatogram of phenols identified in *A. dioica* herb, λ = 255 nm: **1** – coumarin; **2** – quercetin; **3** – luteolin; **4** – quercetin-**3**-galactoside (hyperoside); **5** – quercetin-**3**-rutinoside (rutin); **6** – quercetin-**3**-glucoside (isoquercitrin).

improving the functioning of pancreas β-cells, increased glucose utilization; antioxidant effect due to its ability to prevent protein glycation and lipid peroxidation of the membrane, neutralizing the formed free radicals (Santana-Galvez et al. 2017; Kang et al. 2019; Savych and Basaraba 2021; Savych et al. 2021h).

This phytochemical study of *A. dioica* confirms that this plant material is a rich source of phenolic compounds.

Conclusions

The method was validated in terms of linearity, precision, repeatability, accuracy, LOD and LOQ. HPLC-DAD assay of phenols revealed that *A. dioica* represent important sources of bioactive compounds with a wide range of pharmacological activities. It was identified two flavones – luteolin, apigenin; flavonol – quercetin and three its glycosides – rutin, hyperoside and isoquercitrin; coumarin and umbelliferone – hydroxycoumarin; five hydroxycinnamic acids – chlorogenic, caffeic, *p*-coumaric, *trans*-ferulic and rosmarinic acid in *A. dioica* herb. The quantitative detection showed that the main hydroxycinnamic acids were rosmarinic and chlorogenic acids, their contents were 944.1±0.22 μg/g and 793.5±0.19 μg/g, respectively. Regarding flavonoids, the largest amounts were of isoquercitrin (164.5±0.17 μg/g) and luteolin (126.4±0.18 μg/g).

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