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NATURAL DRUG

PHENOLIC ACIDS IN THE FLOWERS OF *Althaea rosea var. nigra*

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Abstract: Distribution of phenolic acids in the flowers of *Althaea rosea var. nigra* has been studied by 2D-TLC and HPLC methods. The phenolic acids occurring in these fractions have been identified as ferulic, vanillic, syringic, *p*-coumaric, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic and caffeic acids. By means of the HPLC methods the contents of major phenolic acids were estimated. From among the phenolic acids analyzed the syringic, *p*-hydroxybenzoic and *p*-coumaric acids are dominant. Total content of phenolic acids was determined by the Arnov's method.

Keywords: *Althaea rosea* (L.) *Cav. var. nigra, Malvaceae,* phenolic acids, 2D-TLC, HPLC, Arnov's method

Althaea rosea (L.) is a popular garden plant, and its dark-violet flower variety (*Althaea rosea* (L.) *Cav. var. nigra*) is used in traditional medicine. Hollyhock flowers (*Malvae arboreae flos sine or cum calycibus*) are used as mucilage for prophylaxis and therapy of diseases and discomforts of the respiratory and the gastrointestinal tracts, for urinary complaints and externally for ulcers, inflammations and as a capillary protectant too (1). In folk medicine, flowers of hollyhock are regarded as an emmenagogue. The mechanism of this effect is unknown, but some estrogenic or non-estrogenic substances present in the plant are supposed to be involved. According to Commission E monographs, the effectiveness of the claimed application has not been documented, so a therapeutic administration cannot be recommended, except for the use of hollyhock flowers as a brightening agent in herbal tea mixtures.

According to literature data, the infusion and methanolic extract tested on rats influenced their hormonal activity and the morphology of their sexual organs (2-4).

As follows from earlier chemical studies of hollyhock flowers, they contain large quantities of polysaccharides (5), flavonoids, mainly derivatives of kaempferol, quercetin, luteolin and myricetin (6), anthocyanidins, chiefly derivatives of delphinidin (7) and an unknown component with estrogenic activity.

Numerous compounds showing estrogenic properties of different chemical structure have been found in different plants. The best known are isoflavonoids, but their presence in the hollyhock flowers has not been confirmed. It has been established that derivatives of cinnamic acids, mainly *p*-coumaric and ferulic acids, also show estrogenic activity and could influence the reproductive activity (8). The estrogenic effect of *p*-hydroxybenzoic acid was reported by Lemini (9). It is possible that the estrogenic effect is a result of interaction of different components of hollyhock. The lack of data on the phenolic acids in *Althaea rosea var. nigra*, which could be responsible for the estrogenic properties, prompted us to examine this plant for their content.

EXPERIMENTAL

Plant material

Flowers of *Althaea rosea var. nigra* (commercial material from Kawon Hurt, Gostyń) was investigated.

Extraction procedure

The dried whole flowers, petals and calyxes of *Althaea rosea var. nigra* (5 g x 250 mL) were extracted with boiling methanol and with a mixture of methanol-water (1:1) on a water bath under reflux condenser. The extracts were combined, then concen-

trated under vacuum to the syrupy consistence and hot water (50 mL) was poured into them. After 24 h, the ballast substances were filtered off and the filtrates were extracted with ether (10 x 50 mL). The combined ethereal extracts were dried with anhydrous sodium sulfate followed by evaporation under reduced pressure. As a result, ethereal fractions were obtained: F (from the methanolic extract from whole flowers), F' (from the methanolic-aqueous extract from whole flowers), P (from the methanolic extract from petals), P' (from the methanolic-aqueous extract from petals), C (from the methanolic extract from calyxes), C' (from the methanolic-aqueous extract from calyxes) (Figure 1). The fractions were used to check for presence and content of phenolic acids.

Qualitative analysis

TLC-2D analysis (two-dimensional thin layer chromatography)

The fractions studied F, F', P, P', C, C' and the standards were dissolved in methanol, the solutions were spotted on cellulose plates 100 x 100 x 0.1 mm (DC–Alufolien, Merck, Darmstadt) and analyzed by TLC technique using mobile phases: toluene – glacial acetic acid –water (6:8:2 v/v/v) – the first direction and glacial acetic acid – water (15:85 v/v) – the second direction. Chromatograms were examined in UV light (λ = 366 nm) and in daylight after visualization with a mixture of diazotized sulfanilic acid and 20% sodium carbonate solution (1:1). The results of TLC analysis are presented in Table 1.

Quantitative analysis

Arnov's method

The total amount of phenolic acids in whole flowers, their parts (petals and calyxes) and in the infusion from whole flowers was determined by Arnov's method (Specol–11, λ = 490 nm, Arnov's reagent) (10, 11).

HPLC analysis

The content of individual phenolic acid was determined by HPLC method. The HPLC analysis was carried out on a Merck Hitachi apparatus model LaChrom D–7000 connected to a computer analytical program HSM and consisted of a pump model L–7100, DAD detector model L–7445, autosampler model L–7200 or injection valve (Rheodyne) with 20 µL sample loop.

The fractions studied F, F', P, P', C, C' and the standards were dissolved in methanol, the solutions was filtered through 0.25 µm filter (Chromafile type O45/25, Macherey-Nagel and 20 µL of the filtrate were analyzed by HPLC under the condition described below.

Chromatographic conditions: precolumn: LiChrospher 100 RP 18e (4 x 4 mm); column: LiChrospher 100 RP 18e (250 x 4.6 mm); mobile phase: aqueous phosphoric acid buffer (pH = 2.2) : methanol, flow rate: 0.8 mL/min; detection: UV (220–330 nm). Peaks were considered to originate purely from the analyte when the agreement between the spectra was better than 99%.

Table 1. The presence of phenolic acids in the flowers, petals and calyxes of *Althaea rosea var. nigra* examined by 2D-TLC method.

Phenolic acids	R_f values of standard solution		Studying fractions					
	I direct.	II direct.	**F**	**F'**	**P**	**P'**	**C**	**C'**
ferulic *trans*	0.81	0.46	+	+	+	+	+/-	+
ferulic *cis*	0.80	0.75	-	+/-	+	+	-	+/-
syringic	0.78	0.50	+++	+	+++	-	+/-	+
p-hydroxybenzoic	0.40	0.74	++	++	++	++	+	+
vanillic	0.80	0.72	+	++	+	+	-	-
p-coumaric *trans*	0.46	0.62	++	+++	+++	+++	+	+
p-coumaric *cis*	0.45	0.79	++	+++	++	++	+	+
caffeic *trans*	0.17	0.46	-	+	-	+	+/-	+/-
caffeic *cis*	0.16	0.62	-	+	-	+	+/-	+/-
p-hydroxyphenylacetic	0.40	0.86	+	-	+	-	-	-

Explanations: + present (+ + +, + +, + according to color intensity), +/- only detected in UV light (low intensity of the fluorescence), – absent

Fractions from the methanol extract from: **F –** whole flowers, **P –** petals, **C –** calyxes,

Fractions from the methanol-water extract from: **F' –** whole flowers, **P' –** petals, **C' –** calyxes

The mixture of each phenolic acid from Table 2 was used to prepare a calibration curve. Each standard substance (Merck) was dissolved in methanol to obtain six different concentrations (1.0–5.0 mg/100 mL). The correlation coefficients were better than 0.999. For the repeatability, the obtained coefficients of variation were between 1.6 and 2.0 (n = 6). The retention times (t_R) for the standards are presented in Table 2.

The results of HPLC quantitative analysis are given in Table 3.

An exemplary chromatogram of HPLC of phenolic acids from the ethereal fraction from the methanolic-aqueous extract from the petals (P') is presented in Figure 2.

RESULTS

The study was undertaken to confirm the presence and determine the amount of phenolic acids in the methanolic and methanolic-aqueous extracts from whole flowers, petals and calyxes of *Althaea rosea* (L.) *Cav. var. nigra*. The flowers, petals and calyxes of hollyhock flowers were extracted separately with methanol and methanol-water mixtures. The presence of phenolic acids in the extracts was examined by 2D-TLC method (Table 1). By comparison with standards the derivatives of cinnamic (ferulic, *p*-coumaric, caffeic), benzoic (*p*-hydroxybenzoic, vanillic, syringic) acids and *p*-hydroxyphenylacetic acid were identified. *p*-Coumaric, syringic and *p*-hydroxybenzoic acids were detected almost in all fractions. In the petals almost all of detected phenolic acids were found (except caffeic acid in methanolic extract, syringic and *p*-hydroxyphenylacetic acids in methanolic-aqueous extract). In the calyxes the vanilic and *p*-hydroxyphenylacetic acids were not found, whereas the presence

Table 2. HPLC retention times of standard phenolic acids.

Standard phenolic acids	Retention time [t_R]
ferulic	13.30
izoferulic	14.50
syringic	9.20
p-hydroxybenzoic	8.10
m-hydroxybenzoic	10.30
p-coumaric	12.70
chlorogenic	6.50
caffeic	8.70

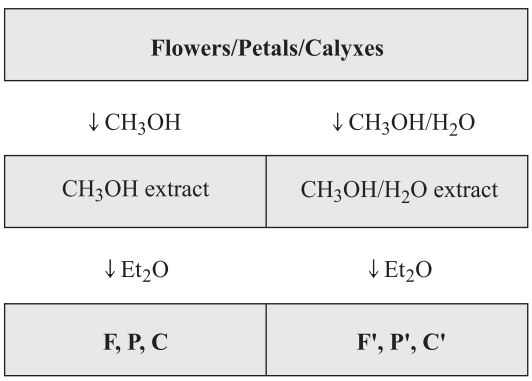

Figure 1. The scheme of extraction procedure of the flowers of *Althaea rosea var. nigra*.

Table 3. The contents of phenolic acids in the flowers, petals and calyxes of *Althaea rosea var. nigra* determined by HPLC method.

Phenolic acids	Studied fractions/content [mg%]*					
	F	**F'**	**P**	**P'**	**C**	**C'**
ferulic	1.18	2.35	2.28	4.23	0.87	1.78
izoferulic	–	0.33	1.53	0.78	–	0.20
syringic	27.45	8.37	47.10	–	0.26	1.57
p-hydroxybenzoic	9.22	7.16	18.27	11.68	4.29	1.38
m-hydroxybenzoic	14.98	7.73	–	–	–	4.66
p-coumaric	6.98	15.15	14.06	28.09	2.14	2.89
chlorogenic	–	0.08	0.12	–	0.09	0.03
caffeic	–	5.82	–	13.17	1.34	1.70
the total content of phenolic acid	59.81	46.99	83.36	57.95	8.98	14.21

*Fractions from the methanol extract from: **F –** whole flowers, **P –** petals, **C –** calyxes
Fractions from the methanol-water extract from: **F' –** whole flowers, **P' –** petals, **C' –** calyxes

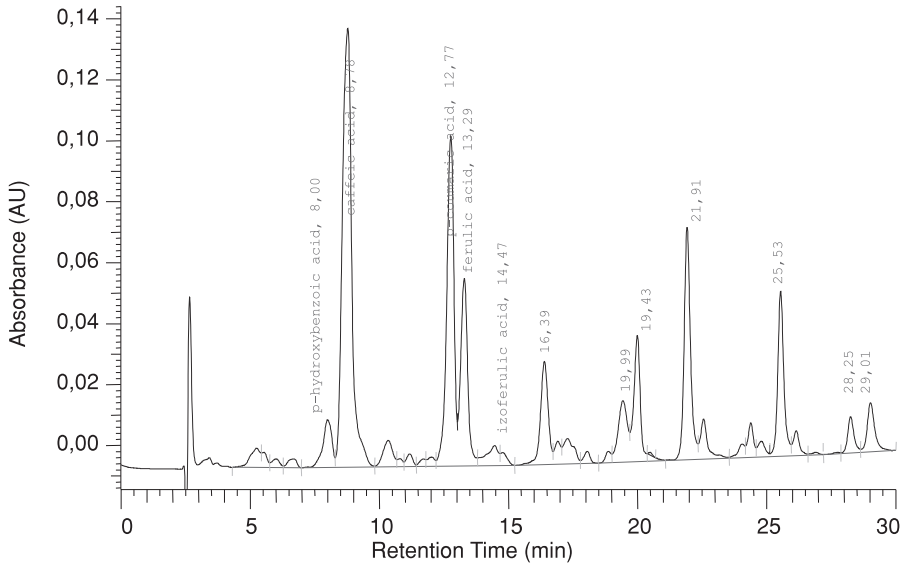

Figure 2. The HPLC chromatogram of phenolic acids in the ethereal fraction from the methanolic-aqueous extract from the petals (P') of *Althaea rosea var. nigra*.

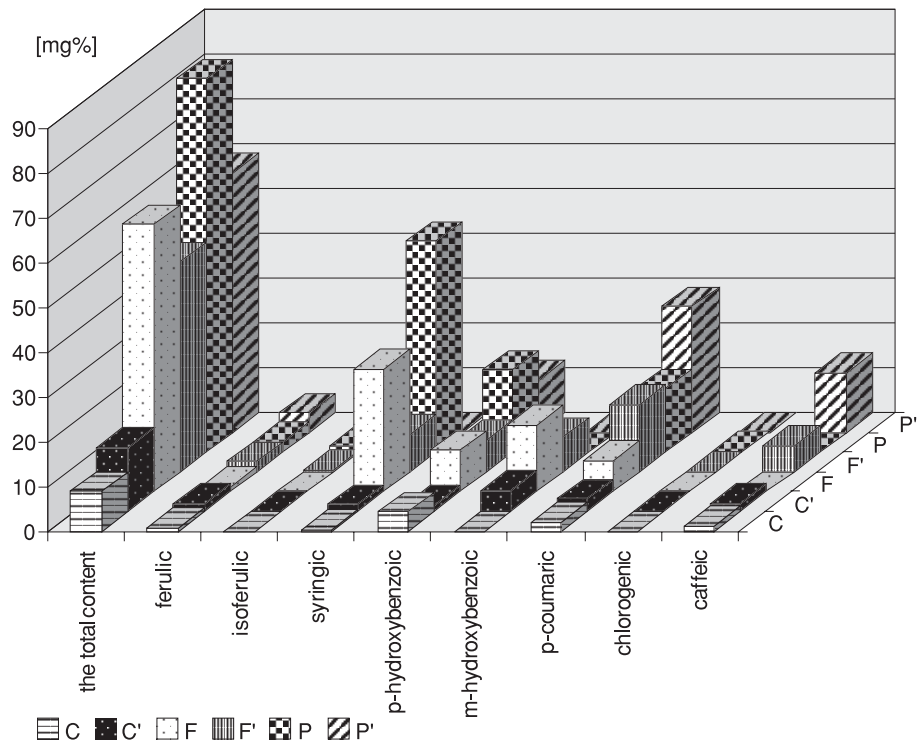

Figure 3. The contents of phenolic acids in the flowers, petals and calyxes of *Althaea rosea var. nigra*.

of the ferulic and caffeic acids was confirmed only in UV light. The total amount of phenolic acids calculated as caffeic acid in the ethereal fractions from the isopropanol extracts from the whole flowers and they parts was determined by Arnov's method. The total content of phenolic acids in whole flowers amounts to 60 mg%, in petals – 120 mg% and in calyxes – 30 mg%.

The content of several phenolic acids was determined by HPLC method (Table 3, Fig. 3). The contents of vanillic and *p*-hydroxyphenylacetic acids could not be determined by the HPLC method

because of the lack of reference standards of appropriate purity, however, additionally the contents of *m*-hydroxybenzoic and chlorogenic acids were found. These acids occurred in very low concentrations in the extracts and that is why their presence was not detected by the TLC method. In all examined extracts the syringic, *p*-hydroxybenzoic and *p*-coumaric acids were dominant.

The methanolic extract from the petals contained the following acids in the greatest contents: syringic (47.10 mg%), *p*-hydroxybenzoic (18.27 mg%), *p*-coumaric (14.06 mg%) and ferulic acids (2.35 mg%), whereas the methanolic-aqueous extract contained mainly *p*-coumaric (28.09 mg%), caffeic (13.17 mg%), *p*-hydroxybenzoic (11.68 mg%) and ferulic acids (4.23 mg%).

In the methanolic extract from the calyxes the acids: *p*-hydroxybenzoic (4.29 mg %) and *p*-coumaric (2.14 mg %) and in the methanolic-aqueous extract mainly the *p*-hydroxybenzoic (4.66 mg%) and *p*-coumaric (2.89 mg %) were found, but their contents were lower than in the petals.
A comparative analysis has shown that the type of the extrahent used has significant influence on the quantitative and qualitative composition of the fractions studied. Methanol was proved to be a better extrahent than a methanol-water mixture only for *p*-hydroxybenzoic acid. For the acids: ferulic, *p*-coumaric and caffeic acids the best solvent was the methanol-water mixture.

DISCUSSIONS AND CONCLUSION

In traditional folk medicine the hollyhock flowers were regarded as an emmenagogue. Literature data have proved that the infusion and methanolic extract influence the hormonal activity and the morphology of the sexual organs of the rats (2-4). The exact component of this plant responsible for the estrogenic activity has not been identified yet. The content of phenolic acids in this plant was not investigated, although according to literature data, this compounds can influence the estrogenic activity. The *in vivo* test proved that *p*-hydroxybenzoic acid was estrogenic. Subcutaneous administration of this compound produced an estrogen-like effect in the mice inducing vaginal cornification and uterotrofic activity in both immature female mice and ovariectomized animals. These effects were dose-dependent (9). In another study it was confirmed that the derivative of cinnamic acids (mainly ferulic and *p*-coumaric acids) could regulate the reproductive effort in a small herbivorous rodent *Microtus montanus*. The addition of the derivative of cinnamic acids to the diet inhibits development of the uterus and decreased breeding performance in this animal (8).

The phenolic acids: *p*-hydroxybenzoic, *p*-coumaric, ferulic, syringic dominated in the both examined extracts (methanolic and methanolic-aqueous). They may contribute to the estrogenic activity of this plant.

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