ORIGINAL ARTICLE

Chemical study of the composition and quantitative analysis of hydroxycinnamic acids in experimental samples of propolis

Elena T. Zhilyakova[1], Oleg O. Novikov[1], Marina Y. Novikova[2], Dmitriy I. Pisarev[1], Tatyana V. Lupina[1]

[1]Department of Pharmaceutical Chemistry and Pharmacognosy, Belgorod State University, Belgorod 308015, Russia, [2]Department of Pharmaceutical Technology, Belgorod State University, Belgorod 308015, Russia

Abstract

Background and Objective: This article presents the results of the determination of hydroxycinnamic acids in 6 experimental propolis samples. Using reversed-phase high-performance liquid chromatography in a gradient elution mode is able to identify hydroxyl and cinnamic acids by comparing retention times of analytes, the standard samples and the corresponding acids according to the results of diode-matrix detection. **Method:** Six prototype propolis samples from different regions of Russia were taken as research objects. From the experiment, samples were prepared extracts by 80% ethanol extraction, according to the traditional production scheme of tinctures in a ratio of 1:10. It was found that the composition of propolis has a stable set hydroxycinnamic acids comprising about 6 components: Trans-p-coumaric, ferulic, isoferulic, caffeic, 3,4-dimethyl caffeic and cinnamic acids. The internal normalization method calculates the specific weight of each of the components within the specified group. **Results:** It has been established that about 90% of all oxycinnamic acids are trans-para-coumaric, ferulic, and caffeic acids with a predominance of the first. The quantitative content of the dominant hydroxycinnamic acids in propolis prototypes was determined by the absolute calibration method. The content of trans-para-coumaric acid was in the range 1.43-2.1%; ferulic acid - 0.93-1.6%; and caffeic acid - 0.193-0.44%. **Conclusion:** The results of the conducted experiments make it possible to recommend an assessment of the quality of propolis by any of the dominant acids.

Key words: Absolute calibration method, hydroxycinnamic acids, propolis, reversed-phase high-performance liquid chromatography

INTRODUCTION

Propolis is a product of the livelihoods of bees, which is a sticky resinous mass with a characteristic aromatic odor and a bitter-burning taste. Propolis differs in color and can have a red, green, or yellow color depending on the source and harvest season.[1,2]

The component composition of propolis includes polyphenolic compounds, resins, balsams, essential oils, and wax. Polyphenolic compounds are represented by flavonoids, tannins, oxycoumarins, and hydroxycinnamic acids. Resins consist mainly of organic acids. Balsams - a mixture of essential oils that cause the aroma and taste of propolis, tannins, and aromatic aldehydes. Propolis also contains microelements, fatty acids, and amino acids. However, the chemical composition of propolis can vary widely, depending on the region of origin and plants from whose buds the product was made. Moreover, propolis samples taken from even one hive do not always have the same chemical composition.[1,3]

One of the most important groups of biologically active propolis compounds, which determine the nature of its pharmacological action, are hydroxycinnamic acids.

Hydroxycinnamic acids are substances, the source of which are almost all higher plants. The wide spectrum of diverse

Address for correspondence:
Elena T. Zhilyakova,
Department of Pharmaceutical Technology,
Belgorod State University 85, Pobedy Street,
Belgorod 308015, Russia.
E-mail: Novikov@bsu.edu.ru

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pharmacological effects of these compounds with mild or moderate side effects reveals their ability as therapeutic agents.

At present, a number of pharmacological effects have been established for *in vitro* and *in vivo* experiments for hydroxycinnamic acids, namely, antioxidant, antimicrobial, anti-inflammatory, cardioprotective, and antidiabetic.[4]

Antioxidant activity *in vitro* of such acids as coffee, chloroorganine, synapic, ferulic, and p-coumaric is manifested in the protection of low-density lipoproteins from peroxidation.

The antimicrobial activity of hydroxycinnamic acids is caused by the appearance of irreversible changes in the property of the cell membranes of microbes due to a change in the hydrophobicity, a decrease in the negative surface charge, and the appearance of local rupture or pore formation in cell membranes, followed by the leakage of significant intracellular components.

The anti-inflammatory activity primarily of caffeic acid derivatives is attributed to the inhibition of the excessive production of pro-inflammatory mediators, such as nitric oxide (NO), PGE2, and the expression of the protein iNOS and COX-2, as well as pro-inflammatory cytokines (interleukin-1b and tumor necrosis factor-a).

The antidiabetic activity of hydroxycinnamic acids has a number of complex mechanisms. P-coumaric acid lowers blood glucose levels, improves blood lipid profile and significantly increases plasma insulin levels. Ferulic acid causes an increase in the release of insulin, affects the level of glycogenolysis of the liver, caffeic acid mainly contributes to reduce the hepatic glycogenolysis.

Ferulic acid competitively exhibited inhibitory activity on 3-hydroxy-3-methylglutaryl coenzyme A reductase and activate glucokinase, which help reduce hypercholesterolemia and hyperglycemia, respectively.

Neuroprotective activity of hydroxycinnamic acids is the ability to inhibit the deposition of amyloid β-peptide leading to Alzheimer's disease in the brain parenchyma. For example, phenethyl ester of caffeic acid, one of the components of propolis, is able to improve the activity of cholinesterase in the blood serum.

Cardioprotective activity of hydroxycinnamic acids is primarily associated with their antioxidant effect.

The aforementioned series of effects is associated with the structural features of hydroxycinnamic acids. The presence of an unsaturated bond in the side chain of these molecules is most important for their activity. In addition to the structural features that are important for antioxidant activity, are:

- Molecules with an ortho- or para-dihydroxyphenyl group or 4-hydroxy-3-methoxy groups have higher antioxidant activity.[5]
- Esterification to glycosides maintains or enhances antioxidant activity.
- Esterification in the primary hydroxyl group of the glycoside improves the acceptor activity against radicals.[6]
- The presence of the orthodihydroxyphenyl group (catechin fragment) is important for antioxidant activity, as well as an increase in the activity level in groups of up to three not higher than this activity.[7]
- Substitution in the main fragment of the molecule of oxycinnamic acids leads to various pharmacological effects. For example, m-hydroxy or p-methoxy moieties in the cinnamic acid structure serve as important functional groups as effective insulin liberators, while 3,4-dihydroxycinnamic acid (coffee) exhibits hepatoprotective activity.[8]

Since the composition of hydroxycinnamic acids of propolis varies from source literature, and the question remains on which group of substances to standardize this object, the objective of this study was an objective comparative assessment of the composition of hydroxycinnamic acids in various propolis samples to establish the possibility of its adequate standardization for this group of substances.

METHODS

Six prototype propolis samples from different regions of Russia were taken as research objects. From the experimental samples were prepared extracts by 80% ethanol extraction, according to the traditional production scheme of tinctures in a ratio of 1:10. The recoveries obtained were filtered and used direct for analysis.

Chromatographic separation of alcohol extracts of propolis was carried out on a liquid chromatograph "Agilent Technologies 1200 Infinity" with automatic sampler Agilent 1200, vacuum microdegger, gradient pump, and thermostat. The absorption spectra were recorded with a diode-array detector of the series Agilent 1200, scanning period - 2 nm.

The spectra and chromatograms were processed using software "Agilent Chem Station."

The efficiency of the column was established by calculating the number of theoretical plates N according to the formula 1.

$$N = 5.545 \times \left(\frac{t_r}{\mu_{0.5}}\right)^2 \quad (1)$$

Where t - is the retention time of the substance to be determined mm;
$\mu_{0.5}$ - is the width at half peak height, mm.

The optimum criterion of column efficiency is not <5000.[9]

The efficiency of the separation of adjacent peaks is determined by calculating the separation factor R_s by the formula 2, the value of which according to the European Pharmacopoeia should not be <1.5:

$$R_s = \frac{\Delta l}{\mu_{0.5(1)} + \mu_{0.5(2)}} \quad (2)$$

Where Δl - is the distance between the peaks of two adjacent peaks mm;
$\mu_{0.5(1)}$, $\mu_{0.5(2)}$ - is the widths at half the height of the peaks of the two components mm.

The shape of the chromatographic peak was determined by calculating the peak asymmetry (T_f) according to the formula 3, the optimal value of which is <2.

$$T_f = \frac{\mu_{0.05}}{2 \times f} \quad (3)$$

Where $\mu_{0.05}$ - is the peak width at a height of 5.0% of the baseline (mm);
f - is the distance from the beginning of the peak at 5.0% of the baseline to the perpendicular drawn from its apex (mm).

Mobile phase: 1.0% aqueous solution of formic acid (A) - ethyl alcohol 95% (B);

Steel column Ascentis express C_{18} 2.7μm × 100 mm × 4.6 mm.

The flow rate of the mobile phase is 0.5 ml/min;

Temperature of throstat is +35°C;

Sample volume is 1 μl.

A gradient elution regime was used to separate the hydroxycinnamic acids since the presence of similar structural fragments in their molecules that cause a similar polarity does not allow them to be adequately separated in the isocratic mode of elution.

The conditions for the gradient elution of polyphenols, including hydroxycinnamic acids, have been developed previously[10] and have also been used to study propolis and are given in Table 1.

Table 1: Conditions for the gradient elution of hydroxycinnamic acids of propolis

Time, min	A,%	B,%
0	90	10
10	80	20
20	70	30
30	50	50
40	10	90

RESULTS

The identification of hydroxycinnamic acids was carried out by the identity of the retention time of the test components, with the witness substances registered under similar experimental conditions and following the results of diode-matrix detection. Under the above chromatographic conditions, the SS of the caffeic acid has a retention time of t_r ~7.4 min, ferulic acid t_r ~ 11.8 min, isoferulic ~ 13.28, trans-para-coumaric acid ~ 11.3 min, 3,4-dimethyl-caffeic acid ~ 17.6 min, and cinnamic acid ~ 25.18 min.

To solve the problem of choosing the detection wavelength, the profiles of ultraviolet (UV) spectra of the sought hydroxycinnamic acids were analyzed. It is shown in Figure 1.

Figure 1 shows that trans-para-coumaric acid is characterized by the presence of two absorption maxima at wavelengths of 230 and 310 nm, with a maximum at 310 nm having a high intensity and specificity. Caffeic acid and its derivatives have almost the same profiles of UV spectra, the maxima are observed at wavelengths of 237 and 325 nm, the latter being more intense. Cinnamic acid absorbs at wavelengths of 223 and 278 nm. Based on the obtained data, 310 nm for trans-para-coumaric acid, 325 nm for caffeic acid derivatives, and 280 nm for cinnamic acid were used as analytical wavelengths.

The relative content of individual components was calculated by the method of internal normalization, by the ratio of the area of the chromatographic peak of the individual component to the sum of the areas of all peaks of registered oxycinnamic acids according to formula 4:

$$X_i = \frac{S_i \times 100}{\sum S} \quad (4)$$

Where Si - is the average value of the peak area of the component on the chromatograms of the sum;
∑S - is the average value of the sum of all areas of peaks on chromatograms.

The chromatogram of the separation of alcohol extraction from propolis is shown in Figure 2.

As can be seen in the Figure 2, all the components present on the chromatogram are separated along the baseline, which indicates good selectivity of the chromatographic conditions used. The results of calculating the fitness parameters of the applied chromatographic system are shown in Table 2.

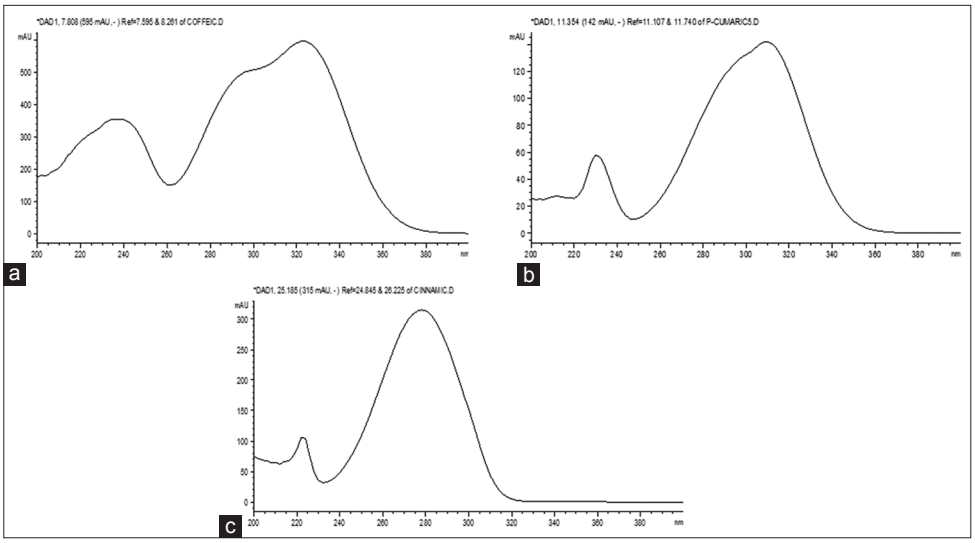

Figure 1: Ultraviolet spectra of standard samples of some hydroxycinnamic acids, (a) caffeic acid, (b) trans-para-coumaric acid, (c) cinnamic acid

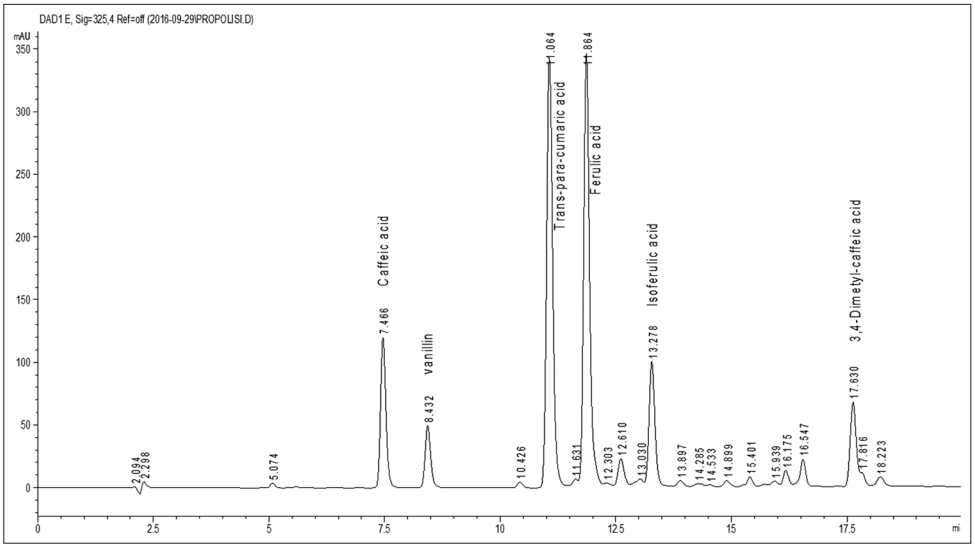

Figure 2: Chromatogram of 80% alcohol extraction from propolis (detector diode-matrix λ_{max} - 325 nm)

Table 2: Indicators of the suitability of the chromatographic system for the determination of oxycinnamic acids in propolis

t_R	N	S, mAU average	R_s	T_f	W_b	Identified component
7.466	20506	867	12.32	0.8	0.1227	Caffeic acid
11.064	37928	5732	3.05	0.84	0.1393	Trans-para-coumaric acid
11.864	44773	3317	1.22	0.79	0.1320	Ferulic acid
13.278	54958	412	1.19	0.84	0.1333	Isoferulic acid
17.630	96895	400	1.84	1.01	0.1333	3,4-dimethyl caffeic acid
24.634	135041	224	2.18	1.1	0.1578	Cinnamic acid

t_R: Absolute retention time, N: The number of theoretical plates, S, mAU average: The average area of the chromatographic peak in the chromatogram, R_s: The peak separation coefficient, T_f: The asymmetry coefficient, W_b: The peak width on the baseline

The results of calculating fitness criteria presented in Table 2 (N>5000, R_s>1.5, T_f<2) generally correspond to the referenced values. Therefore, it can be argued that the applied chromatographic system can be found suitable for the determination of oxycinnamic acids in propolis.

A number of regularities can be noted in the chromatographic behavior of hydroxycinnamic acids. Since the hydroxycinnamic acids, the compounds are sufficiently polar, the grafted non-polar stationary phases have high mobility in comparison with other phenolic compounds due to limited penetration into the hydrophobic phase of the medium. However, various substituents have a significant effect on their mobility. Thus, the most mobile is caffeic acid containing two polar hydroxyl groups. In trans-para-coumaric acid, one hydroxyl group is less, which reduces its polarity, thus inhibiting mobility. Methoxylation significantly reduces mobility since the polarity of the molecule decreases. Therefore, ferulic, isoferulic, and especially 3,4-dimethyl caffeic acid have the lowest mobility.

The investigated propolis prototypes showed identical composition of oxycinnamic acids. In all samples, trans-para-coumaric, ferulic, isoferulic, caffeic, 3,4-dimethyl-caffeic, and cinnamic acids were present in different respects.

Using the peak areas of the hydroxycinnamic acid components obtained in the course of chromatography [Table 2], the internal normalization method was used to calculate the specific gravity of each of the components within the specified group.

The percentage distribution of hydroxycinnamic acids within the group is shown in Figure 3.

The data presented in Figure 3 show that in propolis from the group of hydroxycinnamic acids the greatest content is accounted for by trans-para-coumaric, ferulic, and caffeic acids.

For the quantitative determination of oxycinnamic acids in propolis, the method of absolute calibration was used. At the same time, the content of the dominant oxycinnamic acids was estimated: Trans-para-coumaric, ferulic, and caffeic. To this end, pre-formed calibration curves prepared from calibration solutions of standard samples of caffeic, ferulic, and trans-para-coumaric acids.

1. Preparation of solutions of standard samples of ferulic, caffeic, and trans-para- coumaric acids.

For the construction of calibration charts of 0.025 g (analytical sample) of trans-para-coumaric acid (CAS No. 501-98-4, 99.7%; Sigma-Aldrich), caffeic acid (CAS No. 331-39-5, 99.0%; Dr. Ehrenstorfer GmbH), and ferulic acid (CAS1135-24-6, acros 99.0%) was transferred to 25 ml volumetric flasks, 20 ml of ethyl alcohol 95% was added, shaken until completely dissolved, and brought to the mark with the indicated solvent (solution A).

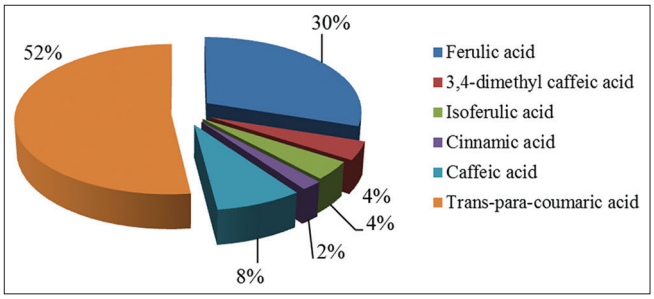

Figure 3: Percentage distribution of hydroxycinnamic acids in propolis within the group

A series of calibration solutions consisting of 6 samples was then prepared from solution A obtained. For this purpose, in each of 6 measuring flasks with a capacity of 25 ml with a pipette, solution A was transferred in volumes: 0.5; 1.0; 2.0; 3.0; and 4.0 ml, the contents of the flasks were thoroughly mixed and adjusted with ethanol 95% to the label (solutions B).

Then, 1 µl of prepared calibration solutions were injected into the chromatograph, including solution A and their peak areas were noted. The results were translated into a graphical form by plotting a calibration curve for the area of the peak (S) versus the amount of the injected substance (C%). Thus, the range of concentrations of calibration solutions was 0.002-0.1%.

In the indicated concentration range, the calibration curves in all three cases had a rectilinear dependence, the regression equations had the form: Trans-para-coumaric acid: y = 81807×-34.936 (R^2 = 1); caffeic acid: y = 58224× (R^2 = 1); ferulic acid: y = 91745×-1320 (R^2 = 0.993), where x - the concentration of the corresponding acid in %, y - chromatographic peak area, R^2 - correlation coefficient.

The results of the calculation of the content of hydroxycinnamic acids in propolis of different experimental samples are presented in Table 3.

The data presented in Table 3 indicate that the content of trans-para-coumaric acid in the propolis test samples was in the range 1.43-2.1%; ferulic acid - 0.93-1.6%; caffeic acid - 0.193-0.44%.

CONCLUSION

Thus, during the conducted studies, it was established that the composition of the propolis samples studied is characterized by a fairly stable, uniform type of hydroxycinnamic acid, including para-trans-coumaric, ferulic, caffeic, isoferulic, 3,4-dimethyl-caffeic and cinnamic acids. All components are well separated under conditions of gradient chromatography. The dominant components of propolis are para-trans-coumaric, ferulic, and caffeic acids, occupying an average of about 90% of the total amount of hydroxycinnamic acids. By the method of absolute calibration, it was possible to estimate the quantitative content of each of the main components, and it was found that trans-para-coumaric acid is the largest. The reproducible composition of hydroxycinnamic acids in propolis, their good chromatographic behavior under

Table 3: The content of hydroxycinnamic acids in laboratory samples of propolis

Number of propolis prototype	1	2	3	4	5	6
Content, % of trans-para-coumaric acid	1.60	1.43	1.546	2.1	1.81	1.76
Content, % of ferulic acid	1.16	0.93	1.08	1.60	1.30	1.30
Content, % of caffeic acid	0.352	0.193	0.40	0.44	0.364	0.41

reversed-phase high-performance liquid chromatography conditions, allow us to recommend the standardization of propolis by any of the dominant components of this group of compounds.

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