Research Article

Development of a technique for assessing the quality of propolis by the content of caffeic acid phenethyl ester by high-performance liquid chromatography with a diode-matrix detector

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ABSTRACT

Propolis is characterized by a wide range of pharmacological properties, such as antioxidant, anti-inflammatory, immunostimulating, hepatoprotective, and antidiabetic, as well as cardioprotective. A number of listed pharmacological properties of propolis is associated primarily with of oxycinnamic acid ester, one of them - caffeic acid phenethyl ester (CAPE) - is considered the most pharmacologically significant. This paper presents the results of the development of an estimation of the quality of propolis with the use of CAPE as a marker component. Using reversed-phase high-performance liquid chromatography with diode-matrix detection, identification was carried out, and the quantitative content of this component in propolis was determined. As an optimal elution mode, a gradient regime was used, with allowed differentiating CAPE from other esters of caffeic acid. In the course of the experiment, it was found that the most suitable extractant for extracting CAPE from propolis is isopropyl alcohol. The optimal color form of propolis, containing the greatest amount of the component to be determined, is green propolis. A quantitative evaluation of the studied component was carried out using the absolute calibration method in the concentration range of the standard sample of CAPE 0.01–0.1%. In the indicated concentration range, the calibration curve was linear; the correlation coefficient was $R^2 = 0.9998$. To assess the comparability and correctness of the methodology, metrological characteristics were calculated. The error of the single determination with the relative error P, 95% was 4.76%. The quantitative content of CAPE in 6 propolis samples taken from different regions of the Russian Federation showed a significant difference in the content of this component and was in the range of 0.106–0.515%. The results of the conducted studies indicate the possibility of assessing the quality of propolis in terms of one of its most significant components - CAPE.

KEY WORDS: Caffeic acid phenethyl ether, Method of absolute graduation, Propolis, Reversed-phase high-performance liquid chromatography

INTRODUCTION

Propolis is a natural resinous substance with a wide range of biological properties, prepared by bees from bud exudates of some plants. As a resinous product, bees are used as a sealant to seal cracks in the walls of the hive, preserve moisture, and primarily to prevent the decomposition of animals and insects killed by bees after intrusion into the hive. Propolis differs in color, depending on the source of its collection and age, and includes mainly several classes of compounds: Waxes, resins, balms, and ether oils. The proportion of these types of substances varies and depends on the place and time of harvesting. These substances get in propolis with resinous secretions of the buds of some trees the bees sip or secret as a result of their metabolism or bring as mechanical impurities. Therefore, propolis contains products of both animal and vegetable origin.

Propolis has an exceptionally wide range of pharmacological properties. First of all, these are antioxidant, anti-inflammatory,[1] immunostimulating,[2] hepatoprotective, and antidiabetic,[3] as well as cardioprotective properties.[4]

However, antitumor properties of propolis are most promising. It was found that a number of its phenolic

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compounds, namely, caffeic acid, its ethers, quercetin, propolines A, B, C, D, E, and F have the property of stopping the growth of tumor cells in the phase of the cell cycle, followed by the development of apoptosis, and also prevent metastasis. In this regard, similar results were obtained both in experiments with inoculation of transplanted syngeneic tumors to animals[5] and on models of chemically induced carcinogenesis.[6] At the same time, a correlation was observed between the antioxidant properties of these compounds and the size of the antitumor effect.[7]

At the same time, there is evidence that this is not the only possible mechanism of antitumor effect of propolis, and it can be realized in part due to the ability to enhance the antitumor cytotoxicity of macrophages *in vivo*.[8]

A number of listed pharmacological properties of propolis is associated primarily with derivatives of oxycinnamic acids. It is known that oxycinnamic acids have a wide range of pharmacological effects, such as antioxidant, antimicrobial, anti-inflammatory, cardioprotective, and antidiabetic.[9] One of them, caffeic acid phenethyl ester (CAPE), first discovered in 1987, is the most important bioactive component of propolis.[10,11] An increased interest in this compound is due to its antitumor, antioxidant, and cytoprotective activity.

It inhibits the growth of tumor cells and is an inhibitor of the transcription factor NF-kB nuclear transcription factor NF-kappa B, known to control the expression of the immune response, apoptosis and cell cycle genes, which is constitutively active in cholangiocarcinoma cells.[12]

This molecule also induces apoptosis by inhibiting NF-kB and activating FasR – antigen of apoptosis 1, the tumor necrosis receptor. As is known, FasR is a receptor of death on the surface of cells, which leads to programmed cell death (apoptosis). Since FasR receptors are found in most mammals in human breast cancer MCF-7 cells, activation with CAPE can lead to antitumor activity in this organ.[13]

In addition, CAPE possesses neuroprotective activity, which is associated with an increase in cholinesterase activity in the blood serum, resulting in the prevention of Alzheimer's disease.[14]

Since the chemical variability of propolis makes it difficult to standardize it; therefore, it is necessary to select reliable markers for assessing the quality of various types of propolis that would correlate with its pharmacological activity.[15]

To analyze CAPE in propolis, a number of analytical techniques have been developed. In particular, the content of caffeic acid and CAPE in propolis was measured using liquid chromatography with an electrochemical detector high-performance liquid chromatography (HPLC/electrochemical detection) in their copresence.[16] There is a technique for determining the derivatives of caffeic acid and flavonoids by HPLC with a diode-array detector (HPLC/DAD).[17]

Using HPLC/ESI/MS in 6 samples of Chilean propolis, a significant difference in the content of CAPE was established.[18]

In addition, gas chromatography-mass spectrometry method was used to determine the content of CAPE and other esters in propolis.[19]

Thus, since CAPE is one of the most pharmacologically significant components of propolis, the objective of this study was to develop a technique for standardizing propolis in terms of this component.

Research Objects and Methods

The objects of this study were 6 pilot propolis samples collected in different regions of Russia. The analyzed objects were used to prepare 80% alcohol extraction by the ethyl maceration method at a ratio of 1:10. The ready-made alcohol extracts were allowed to stand, filtered and further used for analysis. In addition, red, yellow, and green samples of this product were used for comparative quantitative analysis of different color forms of propolis by the content of CAPE. The present research also studied the extracting ability of various solvents to isolate this component from propolis. For this, the samples analyzed were extracted by maceration at a ratio of 1:10 in a range of solvents, namely, methyl, ethyl, isopropyl alcohol, acetone, and ethyl acetate. The obtained extracts were also allowed to stand, filtered, and used for analysis.

The analysis of the ready extracts from propolis was carried out by HPLC.

Chromatography was conducted on Agilent technologies 1200 infinity liquid chromatograph with Agilent 1200 autosampler, a vacuum micro digger, a gradient pump, and a thermostat. The absorption spectra were recorded with Agilent 1200 DAD, with scanning period of 2 nm.

The processing of the spectra and chromatograms was carried out using Agilent chem station software.

The suitability parameters of the chromatographic system were determined by calculating the number of theoretical plates N (column efficiency) (Formula 1):

$$N = 5.545 \times \left(\frac{t_r}{¼_{0.5}}\right)^2 \quad (1)$$

Where *t* - retention time of the analyte, mm;

$\mu_{0.5}$ - width at half height of the peak, mm.

This parameter must be at least 5,000 according to the European Pharmacopoeia.[20]

The efficiency of separation was established by the value of the separation coefficient R_s (Formula 2):

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

Where Δl - the distance between the tops of two adjacent peaks, mm;

$\mu_{0.5(1)}$, $\mu_{0.5(2)}$ - width at half height of the peaks of two components, mm.

The value of this coefficient should be at least 1.5.[8]

The asymmetry coefficient of peak T_f, showing the degree of overload of the stationary phase, was calculated by the formula 3:

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

Where $\mu_{0.5}$ peak width at the height of 5.0% of the baseline, mm;

f - distance between the peak base at the height of 5.0% of the baseline and the perpendicular drawn from its top, mm.

The admissible value of this coefficient is <2.

Mobile phase: 1.0% aqueous solution of formic acid (A) - ethyl alcohol 95% (B); steel column: Ascentis express C_{18} 2.7 μm × 100 mm × 4.6 mm. The flow rate of the mobile phase is 0.5 ml/min; thermostat temperature +35°C; the volume of the injected sample - 1 μl.

For the analysis of CAPE, a gradient elution mode was used, since the presence of related compounds, i.e., other caffeic acid esters having a close polarity, does not allow for efficient determination of this component in the isocratic elution mode.

Table 1: The conditions for the gradient elution of CAPE

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

CAPE: Caffeic acid phenethyl ester

Table 2: Parameters of the suitability of the chromatographic system for the determination of CAPE

t_R	*n*	S, mAU mean	R_s	T_f	W_b
34.636	555896	2998	1.16	0.84	0.1093

t_R - absolute retention time, N - the number of theoretical plates, S, mAU mean - mean area of the chromatographic peak in the chromatogram, R_s - peacks separation coefficient, T_f - asymmetry coefficient, W_b - peak width at a baseline. CAPE: Caffeic acid phenethyl ester

The conditions for the gradient elution of CAPE are given in Table 1.

RESULTS AND DISCUSSION

Identification of CAPE was carried out by coincidence of the retention time and identity of the ultraviolet (UV) profile of the analyzed sample with a standard sample of the test substance of this component [Figure 1].

Figure 1 shows that the retention time of the standard sample of the CAPE is t_R ~ 34.2 min. The UV profile of this component has three absorption maxima: 220, 245, and 329 nm.

Figure 2 shows a chromatogram of alcohol extraction from propolis.

The chromatogram shows that retention time of CAPE is t_R ~ 34.636 min.

To assess the suitability of the chromatographic system used, the fitness parameters shown in Table 2 were calculated.

The calculation of the suitability criteria of the chromatographic system for the determination of CAPE is indicative of compliance with the referenced values. Therefore, this chromatographic system can be recognized as effective for determining the analyte.

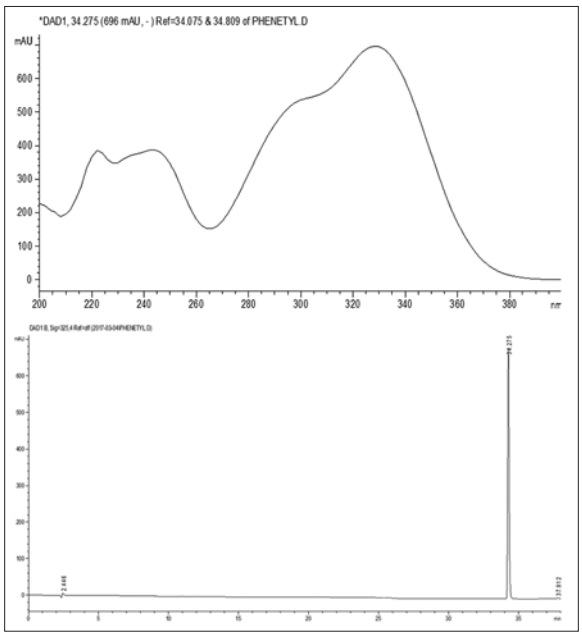

Figure 1: Chromatogram and ultraviolet profile of a standard sample of caffeic acid phenethyl ester

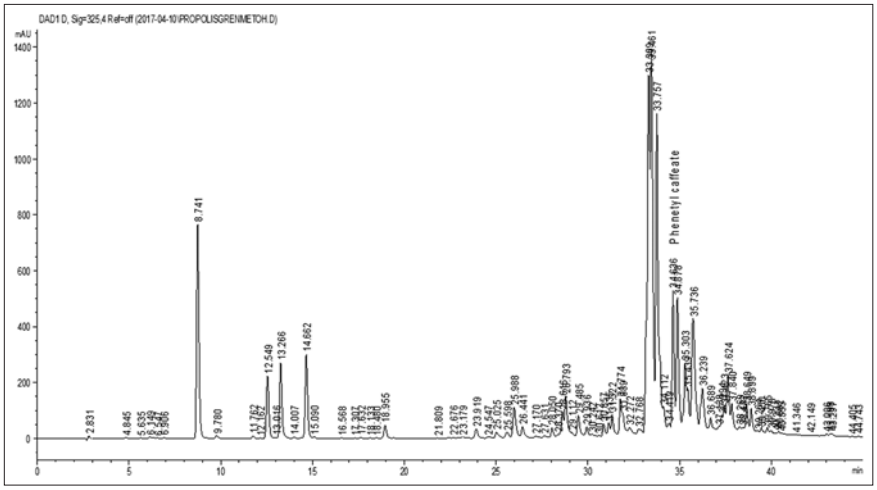

Figure 2: Chromatogram of alcohol extraction from propolis

To determine the efficiency of the extraction capacity of various solvents, to recover CAPE from propolis, a number of extracts from propolis were prepared with the following solvents: Methyl alcohols, ethyl alcohol, isopropyl alcohol, acetone, and ethyl acetate. The obtained extracts were then allowed to stand, filtered, and chromatographed. The extraction efficiency was evaluated by comparing the peak areas of CAPE in the recovered extracts. The results of the comparative analysis are shown in Figure 3.

Thus, it has been found that the most effective extractant for CAPE is isopropyl alcohol.

In addition, we found out which color type of propolis contains the greatest amount of CAPE. To this end, alcoholic extracts from red, yellow, and green propolis were also made, chromatographed, and the obtained peak areas of this component were compared on the obtained chromatograms. The data are shown in Figure 4.

The figure shows that the best color form of propolis containing the largest amount of CAPE is green propolis.

To evaluate the quantitative content of CAPE in propolis, a HPLC analysis method was developed using absolute calibration. For this purpose, a series of 6 calibration solutions were prepared from a standard sample of CAPE including a concentration range of 0.01–0.1% of the analyte. The resulting calibration solutions were chromatographed under the conditions given above, the peak areas were determined and, based on the results, a calibration plot of the peak area of CAPE against the concentration was plotted [Figure 5].

The figure shows that in the indicated concentration range the calibration curve was linear, as evidenced by the value of the correlation coefficient, which was $R^2 = 0.9998$, the regression equation had the form:

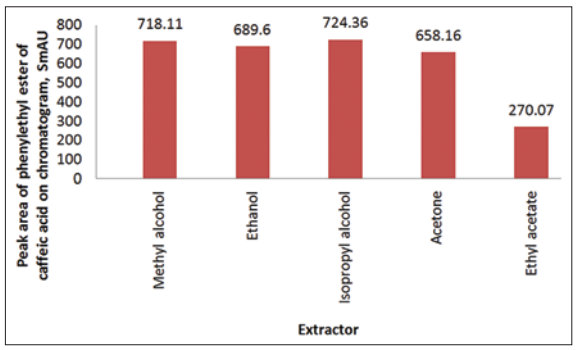

Figure 3: Extraction of phenethyl ether from propolis by different extractants

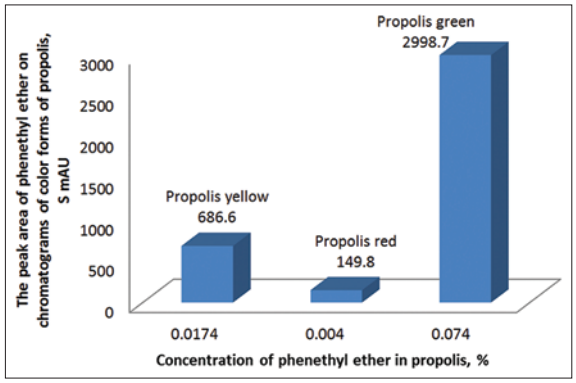

Figure 4: Comparative analysis of the content of caffeic acid phenethyl ester in the color forms of propolis

y=40485x−15.416

Metrological characteristics of the procedure for the quantitative determination of CAPE in 6 alcohol extracts of 1:10 samples of one series of propolis, calculated from the calibration schedule, are presented in Table 3.

According to the data presented in Table 3, the content of CAPE in alcohol extraction of propolis was 0.042 ± 0.002%. The error of the single determination did not exceed 5.0% with a relative error of 95%,

Table 3: Metrological characteristics of the procedure for the quantitative determination of CAPE in propolis by HPLC

f	X	S^2	S	P	t (P, f)	ΔX	ε
1	2	3	4	5	6	7	8
5	0.0422	5.98×10^{-7}	0.00077	95%	2.6	0.00201	4.76%

CAPE: Caffeic acid phenethyl ester, HPLC: High-performance liquid chromatography

Table 4: Quantitative content of CAPE in propolis test samples

Propolis test sample number	1	2	3	4	5	6
CAPE content, %	0.246	0.37	0.14	0.32	0.515	0.106

CAPE: Caffeic acid phenethyl ester

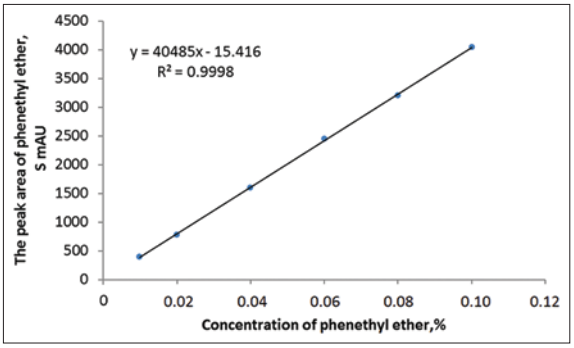

Figure 5: Calibration plot of the peak area of caffeic acid phenethyl ester against the concentration

which is within the range specified by the State Pharmacopoeia.

The developed technique allowed us to analyze 6 prototype propolis samples collected in different regions of Russia in terms of dry matter. The results are shown in Table 4.

As the results in Table 4 show, the content of CAPE in different propolis samples ranged from 0.106 to 0.515%.

CONCLUSION

Using a reversed-phase HPLC with diode-matrix detection made it possible to identify and quantify CAPE in propolis. Using the gradient elution mode, the possibility was demonstrated to separate CAPE from closely related components. In addition, using this technique, it was established that the most effective extractant for CAPE from propolis is isopropyl alcohol, and the optimal color form of propolis containing the greatest amount of the desired component is green propolis. The quantitative content of this component could be characterized using the absolute calibration method. The proposed technique involved linearity in the analyte concentration range of 0.01–0.1% (correlation coefficient $R^2 = 0.9998$) and correctness (single determination error-4.76%). The quantitative content of CAPE in 6 propolis samples taken from different regions of the Russian Federation was calculated, which showed a significant variability and amounted to 0.106–0.515%. The results of the conducted studies indicate the possibility of assessing the quality of propolis by one of its most significant components - CAPE.

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