Original article

Antioxidant activity of ethanolic extracts of propolis by high hydrostatic pressure extraction

Jun Xi[1]* & Zhang Shouqin[2]

1 School of Aerospace, Tsinghua University, Beijing 100084, China
2 College of Biological and Agricultural Engineering, Jilin University, Changchun 130012, China

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Summary The *in vitro* antioxidant activity of the ethanolic extracts of propolis by high hydrostatic pressure extraction (HHPE) was investigated in relationship to its total polyphenol and flavonoid content by β-carotene bleaching and 1,1-diphenyl-2-picrylhydrazyl free radical scavenging assay systems. The results showed that the antioxidant activity of ethanolic extracts of propolis (EEP) samples gradually increased with increasing concentration of the extracts in the concentration range tested. The EEP by HHPE have the same relatively strong antioxidant activities as that by leaching at room temperature (LRT). LRT usually needs 7 days, while HHPE needs only 1 min. From the viewpoints of extraction time, and strong antioxidant activity of its extracts, HHPE was more effective than the conventional extraction methods studied.

Keywords Antioxidant activity, extraction methods, propolis.

Introduction

The degenerative diseases associated with ageing include cancer, cardiovascular disease, immune system decline, brain dysfunction and cataracts (Ames *et al*., 1993). They are also associated with free radicals because oxidative damage to DNA, proteins and other macromolecules accumulates with age and has been postulated to be a major type of endogenous damage leading to ageing (Fraga *et al*., 1990). Superoxide, hydrogen peroxide and hydroxyl radicals, which are mutagens produced by radiation, are also by-products of normal metabolism (Wagner *et al*., 1992). Besides giving rise to mutagenic lipid epoxides, hydroperoxides, alkoxyl and peroxyl radicals, lipid peroxidation is also a major cause of food deterioration, affecting colour, flavour, texture and nutritional value (Halliwell & Gutteridge, 1999).

The consumption of plant foods, such as fruits, vegetables, red wines and juices, provides protection against various diseases, including cancer, cardio and cerebrovascular diseases (Weisburger, 1999). This protection can be explained by the capability of antioxidants in the plant foods to scavenge free radicals, which are responsible for the oxidative damage of lipids, proteins and nucleic acids. Synthetic antioxidants have been used in stabilisation of foods. The most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylated hydroxyquinone (TBHQ), which are applied in fat and oily foods to prevent oxidative deterioration (Loliger, 1991). However, BHA and BHT were found to be anticarcinogenic as well as carcinogenic in experimental animals. Originally, BHA appeared to have tumour-initiating and tumour-promoting action. Recently, it has been established that BHA and BHT can cause formation and promotion of tumour (Botterweck *et al*., 2000). As carcinogenic properties have been reported for some synthetic antioxidants, recent research on the potential applications of natural antioxidants from spices and herbs, for stabilising foods against oxidation, has received much attention (Gu & Weng, 2001).

Propolis, a natural substance collected by honeybees from buds and exudates of certain trees and plants, is thought to be used in the beehive as a protective barrier against enemies. Propolis has been used in folk medicines in many regions of the world (Ghisalberti, 1979) and has been reported to have various biological activities such as antioxidant activity (Kumazawa *et al*., 2004), antibacterial (Kujumgiev *et al*., 1999), antiviral (Amoros *et al*., 1994), antiinflammatory (Wang *et al*., 1993) and anticancer (Kimoto *et al*., 2001) properties. For this reason, propolis is extensively used in food and beverages to improve health and prevent diseases such as inflammation, heart disease, diabetes and cancer (Banskota *et al*., 2001). Propolis usually contains a variety of chemical compounds, such as polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids and amino

*Correspondent: Fax: 861062775033;
e-mail: xijun@mail.tsinghua.edu.cn

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acids. It has been believed that various pharmacological activities of propolis are attributed to phenolics, such as flavonoids and caffeic acids (Kumazawa *et al*., 2004).

High hydrostatic pressure, which means cold isostatic superhigh hydraulic pressure that ranges from 100 to 800 MPa or more (US Food and Drug Administration Center for Food Safety and Applied Nutrition, 2000), is currently considered as an attractive innovative non-thermal process that can effectively inactivate micro-organisms and preserve fresh food products (Knorr, 1993). Exploring the effects of HHP in biotechnology has received increased interest during the last decade (Mozhaev *et al*., 1994). HHP has successfully been applied in the processes of pasteurisation and sterilisation in the food and pharmaceutical industries. Some reports demonstrated some changes in cell morphology and structure, such as cell deformation, cell membrane damage and protein denaturation (Bennett *et al*., 1998; US Food and Drug Administration Center for Food Safety and Applied Nutrition, 2000). According to the mass transfer theory, pressurised cells increased permeability (Yan, 2002). Based on the phase behaviour theory, the solubility is larger while pressure increases (Richard, 1992). The differential pressure between the inner and outer cell membrane is so large that it will lead to instant permeation. Consequently, the concentration between inner and outer cell membranes can reach equilibrium in a short time. Therefore, high hydrostatic pressure extraction (HHPE) has many advantages, such as shorter extraction time, higher extraction yield, etc. (Xi, 2005).

Studies have dealt with the methods of extraction of flavonoids from propolis, such as leaching at room temperature (LRT) (Murad *et al*., 2002) and heat reflux extraction (HRE) (Gu *et al*., 2001). LRT is the most currently applied technique, but it usually needs a few days, even more than 7 days. HRE generally needs a higher temperature (85 °C) during the extraction, which can lead to loss of biological activity in some heat-sensitive ingredients of propolis. HHPE is a novel technique at present, which we had successfully used to extract flavonoids from propolis (Xi, 2005). Propolis obviously possesses antioxidant activity, as reported by many researchers. However, there are no studies on the antioxidant activity of ethanolic extracts of propolis (EEP) by HHPE. Therefore, the purpose of this study was to investigate the antioxidant activity of EEP by HHPE in relationship to its total polyphenol and flavonoid content and evaluate the antioxidant activity of EEP samples by HHPE and conventional extraction methods.

Materials and methods

Materials and instrumentation

Crude propolis that had been collected in Nongan County of Jilin Province (China) was provided by the Jilin Provincial Institute for Drug Control. Galangin, pinocembrin and gallic acid, pharmaceutical grade standard, were purchased from the National Institute for Control of Pharmaceutical and Biological Products (China). Tween 80, ethanol, methanol, chloroform, 2,4-dinitrophenylhydrazine (DNP), sulphuric acid, potassium hydroxide, aluminium chloride and potassium acetate (analytical grade; Beijing Chemical Reagents Company, Beijing, China) were used. TBHQ, β-carotene, linoleic acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich Co. (St Louis, MO, USA). The spectrophotometer (751-GW) was from Shanghai Analytical Instrument Overall Factory (Shanghai, China).

A DL700–0.55 × 1.5 ultra-high pressure isostatic apparatus was purchased from Dalong Machinery Works. (Shanghai, China) (effective volume of vessel: 0.35 L; maximal working pressure: 700 MPa; inner diameter: 55 mm; pressure transmitting media: mixture of transformer oil and kerosene).

Preparation of EEP by HHPE

Crude propolis was frozen at −20 °C and ground in a chilled disintegrator. Then, we exactly weighted 10 g of crude propolis, mixed it with 350 mL of 75% ethanol and placed it in a sterile polyethylene bag. The bag was sealed after eliminating air inside. Then we placed the bag in a hydrostatic pressure vessel in an ultrahigh pressure isostatic apparatus. After processing (high pressure levels: 500 MPa) for 1 min at room temperature, the mixture was filtered through a filter paper. The extracts were centrifuged at 4000 × ***g*** for 10 min, and the supernatants were pooled. The extracts were again centrifuged under the same conditions and the supernatants were pooled. The supernatants that were obtained were combined and concentrated in a rotary evaporator under reduced pressure at 40 °C, and then the supernatant was lyophilised. In this manner, the EEP by HHPE were prepared (Xi, 2005).

Preparation of EEP by conventional extraction methods

The EEP by LRT was obtained as described by Murad *et al*. (2002). In brief, 10 g of propolis was suspended and extracted with 30 mL of 70% ethanol with shaking at room temperature for a week. The mixture was then filtered through a filter paper. The extracts were centrifuged at 4000 × ***g*** for 10 min, and the supernatants were pooled. The extracts were again centrifuged under the same conditions and the supernatants were pooled. The supernatants that were obtained were combined and concentrated in a rotary evaporator under reduced pressure at 40 °C, and then the supernatant was lyophilised. In this manner, the EEP by LRT were prepared.

The EEP by HRE was obtained as described by Gu *et al*. (2001). In brief, propolis ethanol extracts were boiled (10 g of propolis mixed with 40 mL of 95% ethanol in water) at boiling point, at about 85 °C, for 4 h (superboiling of the solution did not occur). The mixture was then filtered through a filter paper. The extracts were centrifuged at 4000 × ***g*** for 10 min, and the supernatants were pooled. The extracts were again centrifuged under the same conditions and the supernatants were pooled. The supernatants that were obtained were combined and concentrated in a rotary evaporator under reduced pressure at 40 °C, and then the supernatant was lyophilised. In this manner, the EEP by HRE were prepared.

Determination of total flavonoid and polyphenol content

Total flavonoid content

Flavone and flavonol content

EEP solution (2 mL) of the test solution, 20 mL methanol and 1 mL 5% aluminium chloride in methanol (w/v) were mixed in a volumetric flask and the volume was made up to 50 mL with methanol. The mixture was left for 30 min and the absorbance at 425 nm was measured. Flavone and flavonol content was calculated as galangin equivalent from a calibration curve (Popova *et al*., 2004). The blank solution was prepared with solvent according to the procedure previously mentioned for the analysis of flavone and flavonol, which contained all the earlier chemicals except galangin equivalent. A calibration curve was constructed with different concentrations of galangin (10–200 μg mL^{-1}) as standard (see Table 1).

Flavanone and dihydroflavonol content

EEP solution (1 mL) of test solution and 2 mL of DNP solution (1 g DNP in 2 mL 96% sulphuric acid, diluted to 100 mL with methanol) were heated at 50 °C for 50 min (water bath). After cooling to room temperature, the mixture was diluted to 10 mL with 10% potassium hydroxide in methanol (w/v). A sample (1 mL) of the resulting solution was added to 10 mL methanol and diluted to 50 mL with methanol (volumetric flask). The absorbance was measured at 486 nm. Calibration was performed using pinocembrin as reference compound (Popova *et al*., 2004). The blank solution was prepared with solvent according to the procedure previously mentioned for the analysis of flavanone and dihydroflavonol, which contained all the earlier chemicals except pinocembrin equivalent. A calibration curve was constructed with different concentrations of pinocembrin (0.1–2.0 mg mL^{-1}) as standard (see Table 1).

Table 1 Calibration equations for the spectrophotometric determination of flavonoids and phenolics in EEP

Compound type	Regression equation	Correlation coefficient
Flavones and flavonols	$C = 727.82A - 1.6724$	0.9995
Flavanones and dihydroflavonols	$C = 7.157A - 0.0035$	0.9998
Total polyphenol	$C = 763.77A - 9.3233$	0.9979

EEP, ethanolic extracts of propolis; *C*, concentration of solution used for colorimetric analysis; *A*, absorbance.

Total polyphenol content

Total polyphenol content was determined by the Folin-Ciocalteau colorimetric method (Kumazawa *et al*., 2002). EEP solution (0.5 mL) was mixed with 0.5 mL of the Folin-Ciocalteau reagent and 0.5 mL of 10% Na_2CO_3, and the absorbance was measured at 760 nm after 1 h incubation at room temperature. Total polyphenol content was calculated as gallic acid equivalent from a calibration curve. The blank solution was prepared with solvent according to the procedure previously mentioned for the analysis of polyphenol, which contained all the earlier chemicals except gallic acid equivalent. A calibration curve was constructed with different concentrations of gallic acid (50–500 μg mL^{-1}) as standard (see Table 1).

Antioxidant activity assays

β-*Carotene bleaching method*

This experiment was carried out by the method of Emmons *et al*. (1999). β-Carotene (3 mg) was dissolved in 30 mL of chloroform, and 3 mL was added to 40 mg of linoleic acid and 400 mg of Tween 80. Chloroform was removed under a stream of nitrogen gas. Distilled water (100 mL) was added and mixed well. Aliquots (3 mL) of the β-carotene/linoleic acid emulsion were mixed with 50 μL of EEP solution and incubated in a water bath at 50 °C. Oxidation of the emulsion was monitored spectrometrically by measuring the absorbance at 470 nm over a 60-min period. The control sample contained 50 μL of solvent in place of the extract. The antioxidant activity is expressed as percentage inhibition relative to the control after a 60-min incubation period using the following equation:

$$AA = \frac{100(DR_C - DR_S)}{DR_C}, \qquad (1)$$

where AA is the antioxidant activity, DR_C is the degradation rate of the control (= $\ln(a/b)/60$), DR_S is the degradation rate in the presence of the sample (= $\ln(a/b)/60$), a is the initial absorbance at time 0 and b is the absorbance at 60 min. EEP samples were evaluated at different concentrations (1–15 μg mL^{-1} ethanol), and TBHQ at 10 μg mL^{-1} was used as the reference sample.

Free radical scavenging activity on DPPH

The scavenging activity of the EEP on DPPH radicals was measured according to the method of Chu *et al.* (2000) with some modifications. An aliquot of 2 mL of 1×10^{-4} mol L^{-1} DPPH radical in ethanol was added to a test tube with 2 mL EEP sample solution with different concentrations (1–15 μg mL^{-1} ethanol). Ethanol was used instead of the EEP sample solution as a control. The reaction mixture was incubated for 1 h at room temperature and the absorbance (Abs) was determined immediately after mixing by measuring at 517 nm with a spectrophotometer. The scavenging activity (%) (SA) on DPPH radicals was calculated by using the following equation. TBHQ (10 μg mL^{-1}) was used as the reference sample.

$$SA = 100(1 - \text{Abs in the presence of sample}/\text{Abs in the absence of sample}) \quad (2)$$

Results and discussion

Extraction yield of total polyphenol and flavonoid

Propolis is collected by honeybees from varies sources. The precise composition of raw propolis varies with the source, thus the content of flavonoids and total polyphenol in propolis varies with the source. The composition of the raw propolis that we used was different from that given in the literature. In order to compare the results of HHPE with other traditional extraction methods, we performed all experiments using raw propolis from the same batch, and the technology of extraction methods (LRT, HRE, HHPE) is exactly similar to that given in the literature (Gu *et al.*, 2001; Murad *et al.*, 2002; Xi, 2005).

Table 2 shows that there was no significant difference ($P < 0.05$) in the extraction yield of flavonoid and total polyphenol among HHPE for 1 min, LRT for 7 days and HRE for 4 h. The extraction time of HRE and LRT was, respectively, about 240 and 10 080 times more than that of HHPE. Thus, HHPE can greatly reduce the extraction time.

Total polyphenol and flavonoid content in EEP

Propolis is commercially available as tinctures or tablets made from ethanol extracts in many countries. The total polyphenol and flavonoid content is reported to be the most abundant and most effective antioxidant in propolis (Scheller *et al.*, 1990). Therefore, we first investigated the total polyphenol and flavonoid content of EEP by HHPE and conventional extraction methods.

Table 3 shows that total polyphenol and flavonoid content of the EEP by HHPE and LRT had no significant difference ($P < 0.05$), and those of EEP by HRE had the lowest values in all EEP samples. The high ethanol concentration [95% (v/v)] used in HRE might have resulted in the extraction of larger amounts of un-polar components and in the decrease of total polyphenol and

Table 3 Total polyphenol and flavonoid content in EEP by HHPE and conventional extraction methods

	Total flavonoid content*		
Methods	Flavone and flavonol	Flavanone and dihydroflavonol	Total polyphenol content*
HHPE	230.4 ± 6.5 a	30.7 ± 4.7 c	290.4 ± 8.7 e
LRT	232.1 ± 3.2 a	31.6 ± 5.2 c	296.0 ± 6.4 e
HRE	167.9 ± 5.5 b	21.8 ± 3.4 d	247.7 ± 7.0 f

Values are means ± standard deviations of triplicate measurement. For different extraction methods, means in every column with different letters were significantly different ($P < 0.05$, Student's *t*-test). The value of flavone and flavonol was expressed as galangin equivalent, flavanone and dihydroflavonol as pinocembrin equivalent, and total polyphenols as gallic acid equivalent. HHPE, high hydrostatic pressure extraction; LRT, leaching at room temperature; HRE, heat reflux extraction.
*Expressed as mg g^{-1} of ethanolic extracts of propolis.

Table 2 Extraction yield of total polyphenol and flavonoid by HHPE and conventional extraction methods

			Extraction yield of total flavonoid*	
Methods	Time	Extraction yield of total polyphenol*	Flavone and flavonol	Flavanone and dihydroflavonol
HHPE	1 min	6.43 ± 0.34 a	5.10 ± 0.34 b	0.68 ± 0.21 c
LRT	7 days	6.35 ± 0.44 a	4.70 ± 0.41 b	0.64 ± 0.34 c
HRE	4 h	6.51 ± 0.41 a	4.66 ± 0.39 b	0.71 ± 0.28 c

HHPE: 500 MPa HHPE pressure, HHPE for 1 min, 75% ethanol concentration, 1:3.5 (g mL^{-1}) solid/liquid ratio. LRT: 70% ethanol concentration, 1:3.5 (g mL^{-1}) solid/liquid ratio, at room temperature. HRE: 95% ethanol concentration, 1:4 (g mL^{-1}) solid/liquid ratio, at boiling point about 85 °C. Values are means ± standard deviations of triplicate measurement. For different extraction methods, means in every column with different letters were significantly different ($P < 0.05$, Student's *t*-test). The value of flavone and flavonol was expressed as galangin equivalent, flavanone and dihydroflavonol as pinocembrin equivalent, and total polyphenols as gallic acid equivalent. HHPE, high hydrostatic pressure extraction; LRT, leaching at room temperature; HRE, heat reflux extraction.
*Expressed as percentage, g/g of crude propolis.

Table 4 Antioxidant activity (%) of EEP by HHPE and conventional extraction methods at different concentrations by β-carotene-linoleic acid system

	Sample concentration (μg mL^{-1})					
Sample	**1**	**4**	**6**	**8**	**10**	**15**
EEP by HHPE	39.2 ± 3.2 a	49.1 ± 2.5 c	54.6 ± 2.7 e	61.3 ± 1.8 g	70.1 ± 4.3 i	75.5 ± 3.3 k
EEP by LRT	40.4 ± 4.1 a	48.7 ± 4.2 c	55.4 ± 4.1 e	62.2 ± 4.2 g	68.6 ± 3.8 i	74.6 ± 2.8 k
EEP by HRE	33.4 ± 3.6 b	40.5 ± 3.6 d	47.8 ± 3.6 f	53.6 ± 3.7 h	61.2 ± 2.6 j	67.2 ± 4.1 m
TBHQ	–	–	–	–	85.5 ± 4.3 n	–

Values are means ± standard deviations of triplicate measurement. For different extraction methods, means in every column with different letters were significantly different ($P < 0.05$, Student's *t*-test).
EEP, ethanolic extracts of propolis; HHPE, high hydrostatic pressure extraction; LRT, leaching at room temperature; HRE, heat reflux extraction; TBHQ, tert-butylated hydroxyquinone.

flavonoid content. Therefore, the total polyphenol and flavonoid content in EEP by HRE was low.

Antioxidant activity

β-Carotene bleaching method

Table 4 shows the antioxidant activity of EEP by HHPE and conventional extraction methods at different concentrations by β-carotene-linoleic acid system. The antioxidant assay, using the discoloration of β-carotene, is widely used, because β-carotene is extremely susceptible to free radical-mediated oxidation. β-Carotene is discoloured easily by the oxidation of linoleic acid, as its double bonds are sensitive to oxidation (Singh *et al.*, 2002). EEP samples were evaluated at the different concentrations (1–15 μg mL^{-1}) for the assay, and TBHQ was compared at 10 μg mL^{-1} under the same conditions.

As shown in Table 4, EEP samples by HHPE and LRT had stronger antioxidant activity than that by HRE, and the antioxidant activity of EEP by HHPE and LRT had no significant difference ($P < 0.05$) at the given concentrations. The antioxidant activity of EEP samples varied significantly with different concentrations ($P < 0.05$). The antioxidant activities of EEP by HHPE and conventional extraction methods gradually increased with increasing concentration of the extracts. The EEP by HHPE and conventional extraction methods showed positive correlation between their antioxidant activity and concentration. The EEP by HHPE at 15 μg mL^{-1} showed the highest scavenging activity (75.5%), but the value was much lower than that of TBHQ at 10 μg mL^{-1} (85.5%).

The antioxidant activity shown in Table 4 seemed to correlate with total polyphenol and flavonoid of EEP (Table 3). Positive correlations were found between total polyphenol and flavonoid content in the EEP and their antioxidant activities. Kumazawa *et al.* (2004) investigated the antioxidant activity of propolis samples from various geographical origins and reported that the correlation between total polyphenol and flavonoid content and antioxidant activity was significant.

Scavenging activity of DPPH radical

Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. The method of scavenging DPPH free radicals can be used to evaluate the antioxidant activity of specific compounds or extracts in a short time.

Table 5 shows the scavenging activity of EEP by HHPE and conventional extraction methods at different concentrations. The scavenging activity of EEP samples varied significantly with different concentrations ($P < 0.05$). The scavenging activity of EEP by HHPE

Table 5 Scavenging activity (%) of EEP by HHPE and conventional extraction methods at different concentrations

	Sample concentration (μg mL^{-1})					
Sample	**1**	**4**	**6**	**8**	**10**	**15**
EEP by HHPE	59.8 ± 4.1 a	64.7 ± 3.1 c	70.4 ± 3.9 e	76.3 ± 2.3 g	82.3 ± 2.7 i	86.8 ± 4.3 k
EEP by LRT	57.6 ± 3.6 a	62.2 ± 3.8 c	69.6 ± 2.8 e	77.1 ± 3.2 g	81.5 ± 3.6 i	87.1 ± 3.4 k
EEP by HRE	50.3 ± 4.4 b	56.4 ± 4.5 d	60.8 ± 3.4 f	69.4 ± 4.7 h	73.6 ± 3.4 j	80.6 ± 3.8 m
TBHQ	–	–	–	–	87.6 ± 4.2 n	–

Values are means ± standard deviations of triplicate measurement. For different extraction methods, means in every column with different letters were significantly different ($P < 0.05$, Student's *t*-test).
EEP, ethanolic extracts of propolis; HHPE, high hydrostatic pressure extraction; LRT, leaching at room temperature; HRE, heat reflux extraction; TBHQ, tert-butylated hydroxyquinone.

and conventional extraction methods gradually increased with increasing concentration of the extracts. The EEP samples showed positive correlation between their scavenging activity and concentration. Strong DPPH radical scavenging activity was found in the EEP possessing high total polyphenol and flavonoid content (Tables 3 and 5). We also found that the DPPH free radical scavenging activity shown in Table 5 seemed to correlate with the antioxidant activity shown in Table 4. EEP sample by HRE, which had weak antioxidant activities in the assay system using the discoloration of β-carotene (Table 5), exhibited weak DPPH free radical scavenging activity.

The scavenging activity of EEP by HHPE and LRT was significantly ($P < 0.05$) higher than that of EEP by HRE, and the scavenging activity of EEP by HHPE and LRT had no significant difference ($P < 0.05$) in the concentration range tested. The EEP by LRT at 15 μg mL^{-1} exhibited 87.1% scavenging activity which was comparable to that of the TBHQ standard at 10 μg mL^{-1} (87.6%).

Though other antioxidants were probably present in these EEP samples, total polyphenol and flavonoid content could make a significant contribution to the antioxidant activities in these extracts. Having established the antioxidant activities in these EEP samples, the chemical characteristics of the antioxidative components in these extracts will be further investigated.

Conclusions

In this study, the *in vitro* antioxidant activity of EEP samples by HHPE and the conventional extraction was investigated. The results showed that the antioxidant activity of EEP samples by HHPE and the conventional extraction methods gradually increased with increasing concentration of the extracts in the concentration range tested. The EEP by HHPE and LRT had relatively strong antioxidant activities, which might correlate with the total polyphenol and flavonoid content in their EEP samples. Antioxidant activity of EEP by HHPE was the same as that of EEP by LRT. LRT usually needs a few days, even more than 7 days, while HHPE needs only 1 min. These findings further illustrate that HHPE has a bright prospect for extracting flavonoid from propolis.

High hydrostatic pressure extraction is suitable for fast extraction of flavonoids from propolis, as its extracts have strong antioxidant activity and it is more rapid, safer, and eco-friendly than conventional extraction methods. Food and medicinal industries will benefit from this emerging technology.

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