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High hydrostatic pressure extraction of flavonoids from propolis

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Abstract: A high hydrostatic pressure extraction (HHPE) method is presented for the extraction of flavonoids from propolis. Various experimental conditions of the HHPE process, such as solvents, ethanol concentration (35–95%, v/v), HHPE pressure (100–600 MPa), HHPE time (1–10 min) and solid/liquid ratio (1:5–1:45 g cm^{-3}), were investigated to optimize the extraction process. The extraction yield with HHPE for 1 min was higher than those using extraction at room temperature for 7 days and heat reflux extraction for 4 h respectively. From the viewpoints of extraction time, the extraction efficiency and the extraction yield of flavonoids, HHPE was more effective than the conventional extraction methods studied.

Keywords: propolis; flavonoids; high hydrostatic pressure extraction; extraction

1 INTRODUCTION

Propolis is a strongly adhesive resinous substance collected, transformed and used by bees to seal holes in their honeycombs, smooth out the internal walls and protect the entrance against intruders.[1] Propolis has a long history of being used in traditional medicine dating back to at least 300 BC[2] and has been reported to have a broad spectrum of biological activities, such as anticancer, antioxidant, anti-inflammatory, antibiotic, and antifungal activities, etc.[1,3,4] At least 200 compounds have been identified in different propolis samples, including fatty and phenolic acids and esters, substituted phenolic esters, flavonoids, terpenes, β-steroids, aromatic aldehydes and alcohols, sesquiterpenes, naphthalene and stilbene derivatives.[5–7] In most reports, the biological or pharmacological activity was associated with phenolic compounds, mainly with flavonoids and aromatic acids and esters.[1,8] Flavonoids are potent antioxidants, free radical scavengers and metal chelators: they inhibit lipid peroxidation and exhibit various physiological activities, including antihypertensive and anti-arthritic activities.[9,10] Flavonoids are frequently used as the main index for product evaluation of propolis.[11] Propolis has recently become popular as a health drink and is used extensively in food and beverages in various parts of the world, where it is claimed to improve health and prevent diseases such as inflammation, heart disease, diabetes, cancer, etc. These facts mean that there is renewed interest in the extraction of flavonoids from propolis.

High hydrostatic pressure extraction (HHPE) is a novel technique that at present is used for the high pressure processing of food[12] in the extraction of active ingredients from natural biomaterial. It is not high pressure homogenization technology or supercritical fluid extraction. High hydrostatic pressure means cold isostatic superhigh hydraulic pressure that ranges from 100MPa to 800MPa or more. High hydrostatic pressure can cause some structural changes in structurally-fragile foods, such as cell deformation, cell membrane damage, protein denaturation[12–14] and so on. Based on the phase behavior theory, the solubility is greater as the pressure increases.[15,16] According to the mass transfer theory, the rate of mass transfer = pressure/resistance, ie pressurized cells show increased permeability.[17] The higher the hydrostatic pressure is, the more solvent can enter into the cell and the more compounds can permeate the cell membrane. Under the process of HHPE, the differential pressure between the cell interior and the exterior of cell membranes is so large that it will lead to rapid permeation. Consequently, the concentration between the cell interior and the exterior of cell membranes can reach equilibrium in a short time.

Herbs contain many compounds, of varying polarity. The extraction solvents used in HHPE vary with the compounds present. Water, and hydrophilic and lipophilic organic solvents of different concentration can be used. Thus, HHPE can be applied to the extraction of strongly polar, weakly polar and nonpolar compounds using different solvents, examples of these

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compounds are glucides, coumarins, lignans, quinines, flavonoids, terpenes, tannins, triterpenoids, cardiac heterosides, glycosides, aglycones, alkaloids, etc.

HHPE is operated at room temperature without any heating process, except for the rise in temperature resulting from compression. HHPE has several principal procedures. Firstly, the raw herb is mixed with solvent. Secondly, the mixture is treated with high hydrostatic pressure. Lastly, the mixture, after processing, is filtered to remove the solid particles. Thus the extraction solution of HHPE can be prepared which contains the active ingredients that we need. The extractions of herbs can replace chemicals and be used as drugs as well as additives (flavor, color, etc) in the food and cosmetic industries, which has attracted many people's attention such as biologists, chemists, pharmaceutists, doctors, nutritionists, etc.

There have some reports about extraction of flavonoids from propolis,[11–22] such as extraction at room temperature, heat reflux extraction, etc. However, there no reports have been on the use of HHPE for the extraction of flavonoids from propolis. So, the purpose of this study was to develop an HHPE method and evaluate HHPE and conventional extraction methods for the extraction of flavonoids from propolis.

2 MATERIALS AND METHODS

2.1 Materials and instrumentation

Crude propolis that had been collected in Nongan County of Jilin Province (China) was provided by Jilin Provincial Institute for Drug Control. Rutin, pharmaceutical grade standard, was purchased from National Institute for Control of Pharmaceutical and Biological Products (China). Ethanol, methanol, sodium bicarbonate, aluminum chloride and potassium acetate (Beijing Chemical Reagents Company; analytical grade) were used. Superhigh pressure isostatic equipment (DL700-0.55 × 1.5) was purchased from Shanghai Dalong Superhigh Pressure Machine Co, Ltd. The spectrophotometer (751-GW) was from Shanghai Analytical Instrument Overall Factory.

2.2 High hydrostatic pressure extraction

Crude propolis was frozen at −20 °C and ground in a chilled disintegrator. Then, we weighed exactly 10g of propolis and mixed it with a quantity of an appropriate solvent. After being processed with superhigh pressure equipment for several minutes, the mixture was filtered through filter paper. Propolis extraction solution was finished.

2.3 Conventional extraction methods

Traditional extraction methods are reported in the literature, such as extraction at room temperature[21,22] and heat reflux extraction.[11]

2.3.1 Extraction at room temperature[22]

Propolis ethanol extracts were prepared (30g of propolis, making up the volume to 100 cm^3 with 70% ethanol) in the absence of bright light, with moderate shaking, at room temperature. After a week, the extracts were filtered, and finally concentrations were calculated.

2.3.2 Heat reflux extraction[11]

Propolis ethanol extracts were boiled (10g of propolis, mixed with 40 cm^3 of 95% ethanol in water) at boiling point, about 85 °C, for 4 h (Superboiling of the solution did not occur). Then, the extracts were filtered through filter paper. Finally, concentrations were calculated.

2.4 Estimation of total flavonoids in propolis

The content of total flavonoids was with measured the aluminum chloride method of colorimetry.[23] We selected rutin as the standard sample, and then determined the content of total flavonoids by colorimetry. All experiments and analysis were performed in triplicate.

2.4.1 Making of the standard curve

Absorbed accurately 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 cm^3 of 200 $\mu g\,cm^{-3}$ standard rutin solution, put them into seven 25 cm^3 volumetric flasks respectively, joined 3 cm^3 aluminium chloride (0.1 $mol\,dm^{-3}$) and then 5 cm^3 potassium acetate (1.0 $mol\,dm^{-3}$) into volumetric flasks, diluted with 75% ethanol solution in water, shook evenly, placed for 40 min. With blank solution as reference solution, the absorbance (A) at Vis 415 nm was determined with a 1 cm quartz cell. Thus the standard curve can be drawn be:

$$A = 0.0296C + 0.0032, \text{ with } R^2 = 0.9999$$

where, A, absorbance at Vis 415 nm; C, concentration of solution used for colorimetric analysis, $\mu g\,cm^{-3}$.

2.4.2 Determining of the samples

An exactly weighed (L(g)) amount of propolis was mixed with V (cm^3) of an appropriate solvent. After processing with superhigh pressure equipment for several minutes, the mixture was filtered through filter paper. Then, we absorbed 5 cm^3 filtrates, put them into a 50 cm^3 volumetric flask, and reached the scale accurately with 75% ethanol concentration in water. Then, we absorbed two 5 cm^3 dilute solutions, and put them into 25 cm^3 volumetric flasks. Next, we added 3 cm^3 aluminium chlorine (0.1 $mol\,dm^{-3}$) and 5 cm^3 potassium acetate (1.0 $mol\,dm^{-3}$) into volumetric flasks, diluted with 75% ethanol concentration in water, shook them evenly, and left them for 40 minutes. With blank solution as the reference solution, the absorbance (A) at Vis 415 nm was determined with a 1 cm quartz cell. Then, the concentration of solution used for colorimetric analysis was determined according to the standard curve. Finally, the extraction yield of flavonoids was calculated according to eqn (1), and

the errors were controlled to less than 0.5% through duplicated experiments and analysis.

$$\text{The extraction yield of flavonoids (\%, w/w)} = \frac{C \times V \times 25 \times 10}{5 \times L \times 10^6} \times 100\% \quad (1)$$

where, C, concentration of solution used for colorimetric analysis according to the standard curve, $\mu g\,cm^3$; V, total volume of extraction solvent, cm^3; L, mass of propolis sample, g.

2.5 Statistical analysis

The data were expressed as mean ± SE. Statistical analysis was performed using analysis of variance following the Duncan's multiple range test for specific comparisons. A value of $P < 0.05$ was considered statically significant.

3 RESULTS AND DISCUSSION

3.1 The effect of different solvents on the extraction yield of flavonoids

Figure 1 shows that methanol can be used to obtain higher extraction yields of flavonoids than using ethanol, sodium bicarbonate or distilled water. Ethanol can give a higher extraction yield of flavonoids than using sodium bicarbonate or water. The Flavonoids of propolis are easily soluble in organic solvents, such as methanol, ethanol, aether, acetone, etc, and poorly soluble in water.[24] The polarities of high-concentration methanol and ethanol are weak, while that of water is very strong. Furthermore, under the process of high pressure, the solubility is greater as the pressure increases.[15,16] Thus the extraction yields of flavonoids in methanol and ethanol are higher. Even though the extraction yield of flavonoids in methanol is slightly higher than in ethanol, the fact that ethanol is non-toxic, and easy to recycle and mix with water in different ratios means that it was chosen to extract flavonoids from propolis.

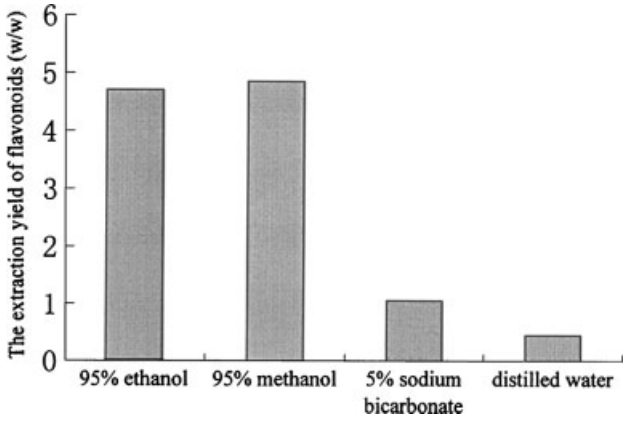

Figure 1. The effect of different solvents on the extraction yield of flavonoids. Solvent: 200 cm^3, Propolis: 10 g, HHPE pressure: 500 MPa, HHPE time: 5min, solid/liquid ratio: 1:20 g cm^{-3}.

3.2 The effect of ethanol concentration on the extraction yield of flavonoids

Figure 2 shows that the extraction yield of flavonoids from propolis was greatly influenced by the ethanol concentration in water. When the volume of ethanol in the solvent was lower than 75% (v/v), the extraction yield was increased with the increase of ethanol concentration. The reason is that the solubility of flavonoids in ethanol solution increases with the increasing concentration of ethanol. When the volume of ethanol in the solvent was higher than 75% (v/v), the extraction yield decreased slowly with the increase in ethanol concentration. The higher concentration of ethanol may affect the composition, or the quality of flavonoids under the process of higher pressure, this will be investigated in further research. Therefore, 75% (v/v) ethanol concentration in water was used in the following experiments.

3.3 The effect of HHPE pressure on the extraction yield of flavonoids

Figure 3 shows that the extraction yield of flavonoids was influenced by HHPE pressure. When the HHPE pressure was increased from 100 to 600MPa, the extraction yield of flavonoids was increased from 4.19 to 4.73%. It is obvious that HHPE pressure is useful for improving the extraction yield of flavonoids. Based on the phase behavior theory, the solubility is greater as the pressure increases.[15,16] According to the mass transfer theory, pressurized cells show increased permeability.[17] The higher the hydrostatic pressure is, the more solvent can enter into the cell and the more compounds can permeate cell membrane. So increasing the HHPE pressure could increase the extraction yield of flavonoids. However, the higher the HHPE pressure, the more expensive the equipment, the more energy would be consumed and the safety

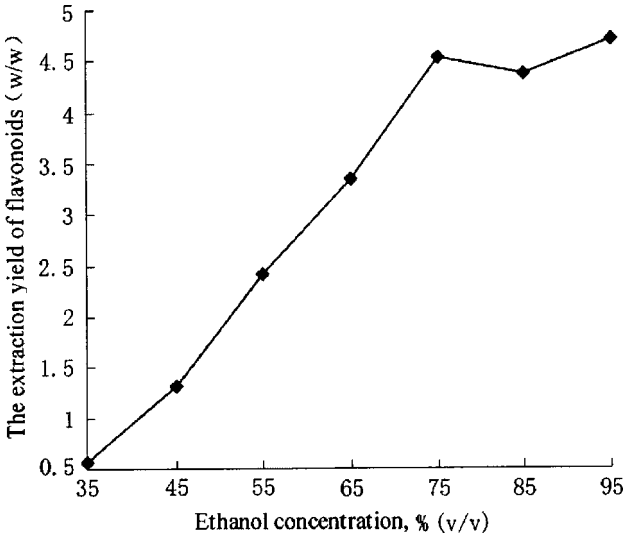

Figure 2. The effect of ethanol concentration in water on the extraction yield of flavonoids. Solvent: ethanol/water, propolis: 10g, HHPE pressure: 500MPa, HHPE time: 5 min, solid/liquid ratio: 1:20 g cm^{-3}.

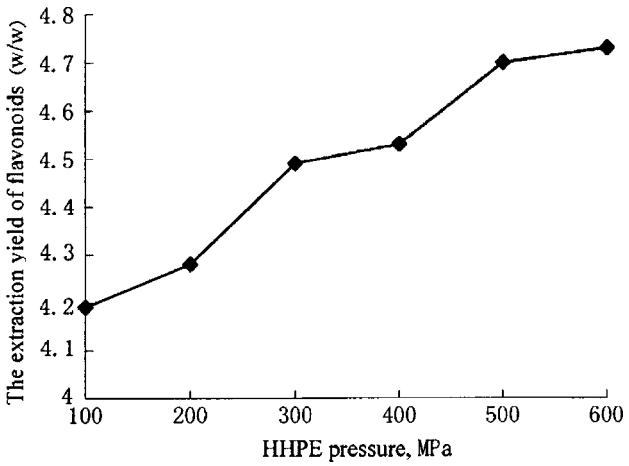

Figure 3. The effect of HHPE pressure on the extraction yield of flavonoids. Solvent: 75% ethanol concentration, propolis: 10 g, HHPE time: 5 min, solid/liquid ratio: 1:20 g cm^{-3}.

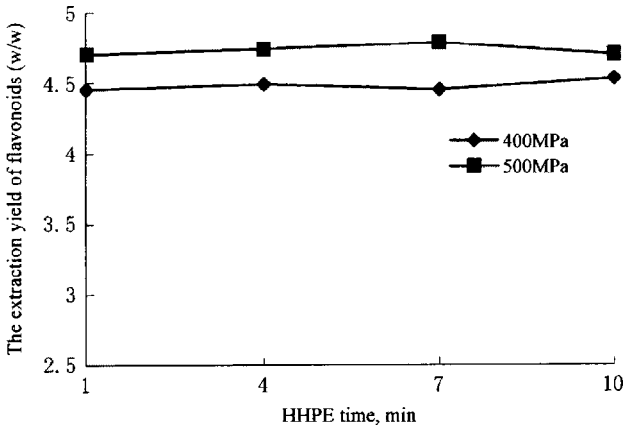

Figure 4. The effect of HHPE time on the extraction yield of flavonoids. Solvent: 75% ethanol concentration, propolis: 10 g, solid/liquid ratio: 1:20 g cm^{-3}.

factor would decrease. So 500MPa HHPE pressure was chosen to extract flavonoids from propolis.

3.4 The effect of HHPE time on the extraction yield of flavonoids

Figure 4 shows the effect of HHPE time on the extraction yield of flavonoids. The results indicate that the extraction yield of flavonoids does not vary with the increase in the duration of the HHPE, which means the duration of HHPE has no close relationship to the increase in the extraction yield of flavonoids. It may be that the equilibrium of pressure between the inside and outside of the cell could be achieved in a short time. So an HHPE time of 1 min was used in the following experiments.

3.5 The effect of solid/liquid ratio on the extraction yield of flavonoids

Figure 5 shows that the extraction yield of flavonoids was increased with the increase in the solid/liquid ratio. When the solid/liquid ratio was increased from 1:5 to 1:45 (g cm^{-3}), the extraction yield of flavonoids was increased from 4.19 to 5.25%. It is obvious that the

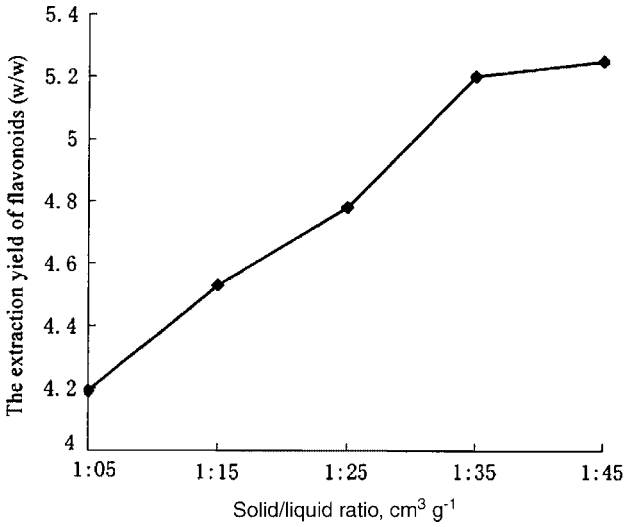

Figure 5. The effect of solid/liquid ratio on the extraction yield of flavonoids. Solvent: 75% ethanol concentration, propolis: 10g, HHPE pressure: 500 MPa, HHPE time: 1 min.

solid/liquid ratio is useful for improving the extraction yield of flavonoids. But if the extraction was carried out under a higher solid/liquid ratio, the concentration of flavonoids in the extraction solution was low. The solid/liquid ratio of 1:35 (g cm^{-3}) was sufficient to reach the high extraction yield, and it was used in further experiments.

3.6 Comparison of HHPE and conventional extraction methods

Propolis is collected by honeybees from varies sources. The precise composition of raw propolis varies with the source,[25] thus the content of flavonoids in propolis varies with the source.[24] The composition of the raw propolis that we used must be different from that given in the literature. In order to compare the results of HHPE with other traditional extraction methods,[11,22] we performed all experiments using raw propolis from the same batch, and the technology of extraction methods (extraction at room temperature,[22] heat reflux extraction[11]) is exactly the same as that given in the literature.

Table 1 shows that the HHPE for 1min gave higher extraction yield of flavonoids than the extraction at room temperature for 7 days and heat reflux extraction for 4h respectively. The results show that the durations of heat reflux extraction and extraction at room temperature were respectively about 240 and 10 080 times more than that of HHPE. Thus, HHPE can greatly reduce the extraction time among all extraction methods.

4 CONCLUSION

Conditions for HHPE of flavonoids from propolis have been studied. HHPE has been shown to be an efficient method for extraction of flavonoids from propolis. Compared with the conventional extraction methods, the HHPE procedure provided higher extraction yield,

Table 1. Comparison of the results of the extraction yield with HHPE and conventional extraction methods

Extraction method	Extraction time	The extraction yield of flavonoids (%, w/w)
Extraction at room temperature	7 days	4.70 ± 0.21
Heat reflux extraction	4 h	4.56 ± 0.11
High hydrostatic pressure extraction	1 min	5.10 ± 0.14

High hydrostatic pressure extraction: 500 MPa HHPE pressure, HHPE for 1 min, 75% ethanol concentration, 1:35 (g cm^{-3}) solid/liquid ratio, at room temperature. Heat reflux extraction: 95% ethanol concentration, 1:4.0 (g cm^{-3}) solid/liquid ratio, at boiling point about 85 °C. Extraction at room temperature: 70% ethanol concentration, 1:3.5 (g cm^{-3}) solid/liquid ratio, at room temperature.

higher extraction selectivity, required a shorter time, and was less labor intensive.

HHPE is suitable for fast extraction of flavonoids from propolis. Food and medicinal industries will benefit from this emerging technology, for it is more rapid, safer, and eco-friendly than conventional extraction methods.

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