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OPTIMIZATION OF TEMPERATURE CONDITION IN PRESSURE LIQUID EXTRACTION OF PROPOLIS SAMPLES

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Abstract

Propolis is a natural resinous substance collected by honey bees from buds and exudates of trees. Bees use it as a glue, general-purpose sealer, and draft excluder for beehives. Known in folk medicine since ancient times, propolis has attracted much attention in recent years as a useful ingredient applied in medicine, domestic products, and food products since it possesses various biological properties, including antimicrobial, antioxidative, and anti-ulcer properties. In this study, pressure liquid extraction (PLE) was used to extract the different chemicals from bee propolis samples. The temperature condition in PLE was optimized based on the amount of materials extracted as determined by UV-Vis absorbance. The extracts were also analyzed for phenolic content using Folin-Ciocalteu assay and correlated with the antioxidant activity obtained using DPPH and ABTS assays. The optimized temperature was found to be 60°C. It was observed that above 60°C, a technical problem was encountered that led to the instrument's failure to extract the propolis samples.

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Introduction

Propolis is a resinous substance harvested by bees from the buds and bark of some plants.[1-2] Known as bee glue,[3] it acts as the adhesive and cement of beehives, and in most cases, it is used to seal cracks and cavities as well as to smooth and seal the interior of the beehive.[2-4] Honeybees also use it as a powerful chemical defense against various predators.

Propolis is currently well known as an effective healing and nutraceutical material widely used in folk medicine traditions worldwide to treat several syndromes. Over the past few decades, evidence has accumulated for the beneficial properties of propolis in terms of potent antibacterial, antiviral, analgesic, anti-inflammatory, antioxidant, immunomodulatory, antitumor, hepatoprotective, and photoprotective effects.[5-6]

The extraction method is the most important and key step in removing the bioactive constituents from propolis.[7] There are several conventional extraction methods that have been used, such as hydrodistillation and organic solvent extraction such as Soxhlet and maceration. The use of these so-called traditional methods are limited because of high energy costs, increased solvent use, high temperatures that are detrimental to thermally labile substances, and solvent residues from dissolved substances that reduce the quality of the extracted materials.[8] Maceration is commonly used for propolis samples and is accompanied by soaking in an appropriate solvent. Although this method is low cost, it is still deemed time-consuming and requires 1-10 days.[9]

Modern methods such as ultrasonic extraction (ultrasound extraction), microwave extraction, and supercritical liquid extraction have been used.[7, 10-12] These modern techniques are considered greener and quicker in extracting the active chemicals from bee propolis. This is because they use considerably less solvent when compared to standard methods. Sonication uses sonic energy to disrupt cell membranes, disrupt cell wall structures, and accelerate solvent diffusion across the membrane. On the other hand, microwave extraction uses microwaves that can easily penetrate the pores of the sample to uniformly and rapidly heat the solvent trapped in the pores. Supercritical extraction uses CO_2 in a supercritical state. These newer methods have higher extraction yields and shorter extraction times than traditional methods. In the case of microwave-assisted extraction and supercritical fluid, less solvent is utilized.[13]

In this study, pressure liquid extraction (PLE) or accelerated solvent extraction (ASE) was used to extract bee propolis. Only a few studies have reported using this method to extract bioactive materials from propolis.[9, 14-15] It is an automated extraction technology that takes advantage of high temperature and pressure and it is a rapid method just like the other modern techniques mentioned above but is more environmentally friendly because it makes use of much less solvent volume.[16]

The optimized temperature was determined by looking at the absorbance of the extracts. In addition to this, the phenolic content using Folin-Ciocalteu was also determined and correlated with the antioxidant activity using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays.

Materials and Methods

Reagents and Chemicals

Folin-Ciocalteu's phenol reagent, anhydrous sodium carbonate, 2,2-diphenyl-1-bicrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate and methanol were all purchased from Sigma Aldrich (St. Louis, MO). The alcohol solvent made up of 90% ethanol with 5% methanol and 5% isopropyl alcohol were obtained from Fisher Chemical (Waltham, MA) while gallic acid was purchased from Acros Organics (Geel, Belgium).

Propolis Preparation and Extraction

The propolis samples used were obtained from University of

the Philippines Los Baños (UPLB) bee program based in College, Laguna, Philippines. Approximately 0.50 grams of propolis was ground and placed in a 10 mL extraction cell of a Dionex ASE 350 unit. For the PLE, most conditions for the method were kept constant with the exception of the temperature. The static time, which was the number of minutes to contain the cell contents at the set temperature of was at one minute with 5 static cycles (wash 5x) after it was heated for 5 minutes at the set temperature. The solvent used in extraction was 3:1 ratio of alcohol solution and distilled water and a pressure equal to 1500 psi.

Absorbance

A JASCO v-570 spectrophotometer (Easton, MD) was used to obtain the absorbance of each extract. Pure ethanol was also used as a blank and its absorbance was recorded as well. The absorbance of the sample was obtained by using 20 uL of the extract added with 1 mL of the solvent (3:1 alcohol:distilled water mixture). This was done at least three times and all readings were averaged for comparison purposes.

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Phenolic Content and Antioxidant Activity

The phenolic content was determined using the Folin-Ciocalteu assay. This was carried out by mixing 20 uL of each sample or standard (gallic acid) with 100 uL of Folin-Ciocalteu reagent (10%) for 4 minutes before adding 100 uL of 1.0 M Na_2CO_3. After 2 hours of incubation, the absorbance at 650 nm was obtained. A blank containing the solvent used in extraction was also utilized.

The DPPH and ABTS tests were used to assess the antioxidant activity of each extract. Both experiments were carried out in a microplate reader with various extract concentrations employed. A methanolic solution of 0.2 mM DPPH was prepared fresh while ABTS was prepared by mixing ABTS with potassium persulfate in a 1:1 ratio 16 hours before use. Extracts of different volumes (20 uL, 10, 5 uL, 2 uL and 1 uL) was mixed with 200 uL of the reagent solution (DPPH and ABTS). The absorbance reading was obtained at 519 nm (for DPPH) and 650 nm (for ABTS) after incubation of mixtures for 30 minutes (DPPH) and 5 minutes (ABTS) in the dark. Alcohol solution served as a negative control, and gallic acid served as a positive control.

The Biotek Cytation 5 Image Reader was used to get all absorbance readings for phenolic content and antioxidant activity. Antioxidant activity was expressed as an IC_{50} (ug/mL), the half-maximal effective concentration and calculated using the formula below. The IC_{50} value was generated by plotting with %scavenging activity (formula given below) of each sample against their specific concentrations. This plot generates a sigmoidal curve, for which the point of 50% effectiveness can be determined from the logarithmic equation.

$$\%Scavenging\ Activity = \frac{\text{blank absorbance} - \text{sample absorbance}}{\text{blank absorbance}} x\ 100$$

Blank absorbance = just DPPH or ABTS solution
Sample absorbance = DPPH or ABTS added with propolis sample

Statistical Analysis

Statistical analysis using student's t-test ($p < 0.05$) was performed on the experimental data (absorbance in specific wavelengths and assays) to compare if data obtained at different temperatures are significant. Correlation analysis of the phenolic content and IC_{50} from DPPH and ABTS assays at different temperatures was also determined using Microsoft Excel.

Results and Discussion

Several factors are usually taken into consideration when optimizing conditions. For PLE application and extraction in general, the order of importance of parameter is typically: (1) solvent; (2) temperature; (3) time; (4) repetitions; (5) pressure.[17] In this study, the focus is on the temperature while the other parameters are kept constant. Although ethanol is the most popular solvent the production of propolis extracts,[18] a 70% ethanol solution is widely employed as solvent of choice for extracting biologically active components of propolis in most commercial products.[10] Another study reported 70 and 80% ethanolic extracts had the greatest antioxidant activity.[19] Because of this, a 3:1 ethanol:distilled water mixture was utilized as the solvent system in the study.

The temperature condition in extracting chemicals using PLE was optimized in this study. Although the temperature conditions used ranges from 40°C to 80°C, technical problem was observed for those in 70°C and 80°C. The sample was not extracted at these temperatures as the pressure exceeded the set maximum pressure limit forcing the extraction run to be discontinued. No extract was collected and upon opening the cell, some solvent was observed mixed with the solidified propolis samples thus clogging the ASE system that results in pressure higher than the one that was set. The extract collected at 60°C is darker compared to the other extracts from different temperature (Figure 1). Although the same extracting volume was set (~10 mL), more volume was collected at 60°C (~14 mL). An earlier study[9] showed this same problem with another propolis sample where it solidifies at 100°C. It is possible that both the pressure and high temperature force the sample to clump together thereby clogging the ASE system resulting to pressure build up and stopping the run.

Figure 2 shows the absorbance from the collected extracts at different temperature. As temperature increases from 40°C to 60°C, there is also a significant increase in the absorbance. As expected, the absorbance profile are the same since the propolis materials used are from the same sample. Distinct peak regions

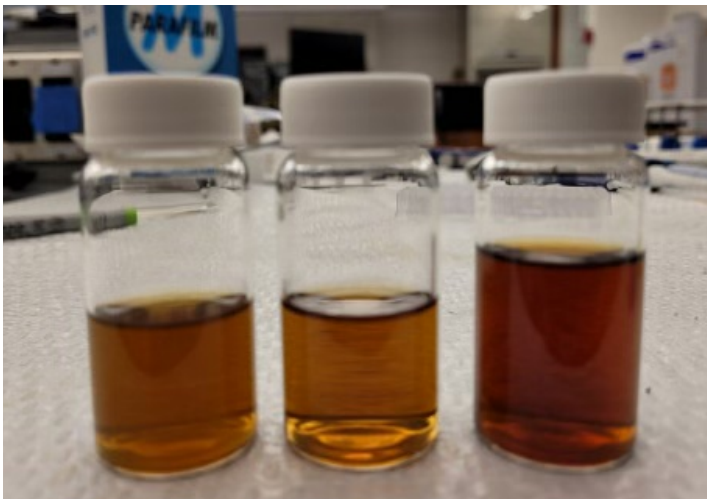

Figure 1. Propolis extract obtained using Dionex ASE at different temperature conditions. From left to right: 40°C, 50°C, 60°C.

can be observed: 240, 290, and 330 nm. However, a higher peak at around 240 nm was observed at 60°C with respect to 220 nm which is higher in lower temperature (40°C and 50°C). These peak regions have been observed in absorbance from other propolis samples and indicative of the presence of phenolic compounds such as flavonoids which have been reported to be found in the 290-400 nm regions.[20]

Results of Folin-Ciocalteu assay (Table 1) showed more phenolic compounds are extracted at higher temperature. The values obtained are within those reported in different studies.[9,21-22] Among these propolis samples within the range of the phenolic content obtained in the sample is a Brazilian green and brown propolis reported to have 31.88-204.30 mg GAE/g[21] sample, another Brazilian brown propolis with 57.9-1614.8 mg GA/g sample and a 151 mg GAE/g propolis sample from Portugal.[22] The reported amount in this study is lower than the amount of phenolic compounds extracted from another propolis sample from the Philippines using ASE at 100°C.[9]

There is also an increase of antioxidant activity (as shown by decreasing IC_{50} values) with increase in temperature. Results from both antioxidant assays expressed as IC_{50} value showed significant difference at all temperatures except between 40°C and 50°C for DPPH. The sample extracted with 60 °C had the lowest IC_{50} values for both the DPPH and ABTS assays (Table 1). At all temperatures, the IC_{50} values for DPPH are significantly lower than the ABTS values (Figure 3).

Results from the two assays are very similar to one study where most of the Indian propolis samples analyzed in antioxidant activity using the two assays (8 out of 10) has lower IC_{50} for the DPPH assay than ABTS assay.[23] Results from the two assays are usually different due to the following reasons.[24-25] First, the ABTS assay is known to be less selective than the DPPH assay in reacting with donors of hydrogen atoms because it is reduced by OH-aromatic groups (unlike DPPPH) that do not contribute significantly to antioxidant activity. Second, phenolic compounds react differently with free radicals in the water phase (ABTS assay) and organic phase (DPPH assay), Third, small molecules may be able to better access the active center of ABTS radicals and latsly, ABTS radicals are more reactive than DPPH radicals.[26] The antioxidant activity obtained by DPPH is strongly positively correlated to that of the ABTS assay with a correlation coefficient of 0.965. In terms of the relationship of the antioxidant activity with phenolic content, both are negatively correlated with DPPH having a higher correlation coefficient (-0.978) in comparison to ABTS (correlation coefficient = -0.888).

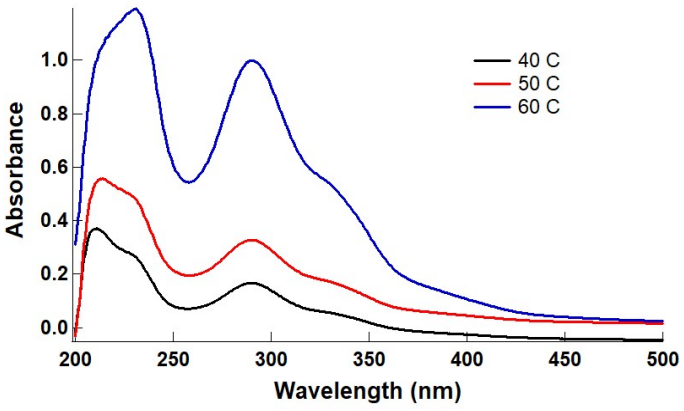

Figure 2. Absorbance of propolis extracts collected by PLE at different temperature.

Table 1. Phenolic content and antioxidant activity (DPPH and ABTS) of propolis extracts obtained by PLE at different temperature.

	40°C	50°C	60°C
Phenolic Content (mg GA/g)*	70.46 ± 4.40	84.37 ± 5.03	175.96 ± 18.43
DPPH (IC_{50} mg/mL)**	1.81 ± 0.31	1.42 ± 0.41	0.69 ± 0.10
ABTS (IC_{50} mg/mL)*	2.37 ± 0.17	1.74 ± 0.08	1.25 ± 0.01

*significantly different with respect to temperature
**significantly different with respect to temperature except between 40°C and 50°C

Conclusion

The optimized temperature for the pressure liquid extraction of propolis samples was determined taking in consideration the absorbance, phenolic content and antioxidant activity of the collected extracts. As the temperature increases from 40° to 60°C, more phenolic compounds were extracted giving higher absorbance and stronger antioxidant activity. A higher phenol content is significant as it means more source of chemicals with biological activities including antioxidant activity. However, further increase in temperature above 60°C cause solidification of propolis samples resulting in technical problem associated with pressure build-up exceeding the limit set for maximum pressure.

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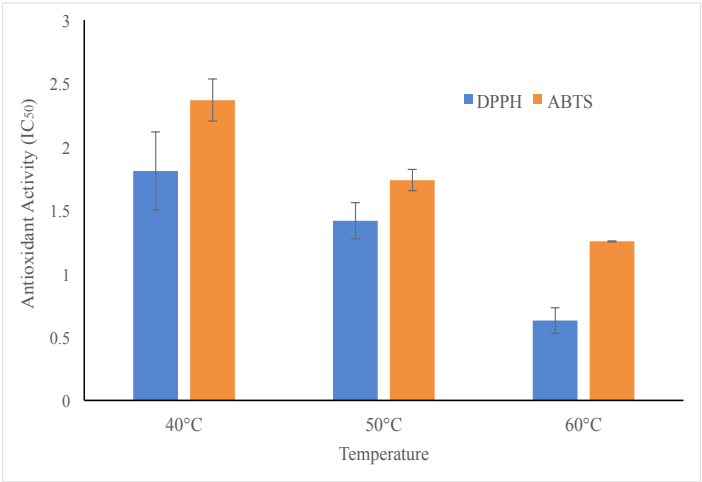

Figure 3. Comparison of antioxidant activities (IC_{50}) using ABTS and DPPH assays at different temperature. The IC_{50} between ABTS and DPPH are significantly different ($p < 0.05$) at all temperatures.

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