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Effect of Sample Preparation Methods and Extraction Time on Yield and Antioxidant Activity from Kradonbok (*Careya sphaerica* Roxb.) Leaves

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ABSTRACT

Kradonbok (*Careya sphaerica* Roxb.), one of Thai indigenous vegetables, was selected for studying the effect of sample preparation method and extraction time on antioxidant activity. The results showed that sample preparation methods gave significant effect on total phenolic content and antioxidant activity but had no marked effects on yield of the extract. Freezing, especially fast freezing, for keeping the leaves until the analysis time gave a higher yield of the extract, a higher total phenolic content and a greater antioxidant activity compared with those obtained from other drying methods. Kradonbok leaves kept by fast freezing were extracted with ethanol for 0.25, 0.5, 1, 3, 4.5, 6, 24, 48, 96 and 168 h. The results showed that yield of the extract and total phenolic content were almost constant after 3 h of extraction time at room temperature. However, extraction time from 4.5 to 6 h gave the lowest EC_{50} value, comparing with those from other extraction times. These results suggested that fast freezing was the appropriate preparation method to keep the plant leaf for further investigating the antioxidant activity and extraction time to obtain the highest yield of the extract and antioxidant activity should be carried out for 4.5 to 6 h.

Key words: Kradonbok, antioxidant activity, sample preparation methods, extraction time

INTRODUCTION

Currently, there is an increasing demand to evaluate the antioxidant properties from plants which contain a variety of substances called "phytochemicals" (Pratt, 1992). The term "phytochemicals" refers to naturally occurring chemical substances present in plants especially those with biological activity (Caragay, 1992). Most phytochemicals include phenolic compounds (flavonoids, phytoestrogens), and glucosinolates (Johnson, 2001). Many plant polyphenolics with antioxidant properties have been studied and proposed for protection against the oxidation (Pokorny, 2001). The most important mechanism is their reaction with free radicals and forming inactive products.

In general, processing conditions and drying methods affect yield and the retention of antioxidant activity (Moure *et al*., 2001). The effect of temperature has been studied and showed that drying with air at 100°C or higher gave a significant reduction in extracted polyphenols (Larrauri *et al*., 1997). In addition, the antioxidant activity of samples dried with air at 100°C was reduced by 28% comparing with that obtained from drying at 60°C but did not significantly affect the extracted polyphenols with respect to freeze-drying. From

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previous reports, there were several preparation processes before extraction such as drying plants with hot air oven at 40°C and keeping at room temperature (Miean and Mohamed, 2001; Bocco *et al*., 1998), or drying samples with air at 25°C and keeping at room temperature (Siddhuraju *et al*., 2002), or keeping fresh plants in a still air freezer between -25 to -30°C (Amakura *et al*., 2000; Velioglu *et al*., 1998). Extraction time affects the antioxidant activity differently. A significant increment of extracted polyphenols from strawberry and white grape was found when they were extracted between 2 min and 4 h (Wang *et al*., 1996).

In Thailand, many indigenous plants belonging to various families are utilized as food and medicine. Kradonbok is one of Thai indigenous vegetables. It is favorably consumed fresh and mostly found in the Northeast of Thailand. Its name is different in different local areas, for instance, Kradon, Phak-Kradon, Kradonbok and Kradonkhok (Northeast). The shoots, young leaves and young flowers of the plant are traditionally eaten. It tastes a little sourness and astringency due to their phenolic phytochemicals. Kradonbok has some health benefits such as using leaves for healing a wound (Vuttithammawech, 1997). Therefore, it was of interest to investigate the total yield and antioxidant activity of Kradonbok (*Careya sphaerica* Roxb.) leaves from various sample preparation methods for storage before the extraction analysis due to the seasonal harvest. In addition, the sample preparation method that provides the highest extraction yield and total antioxidant activity will be selected to study the appropriate extraction time.

MATERIALS AND METHODS

Plant and chemicals

One lot of Kradonbok leaves (*Careya sphaerica* Roxb.) was purchased from cultivated place at Amphur Trakarnpeaudphol in Bureerum province during harvest season in April 2002. Immediately upon arrival after harvesting, Kradonbok was cleaned and selected only sound leaves for studying the sample preparation method for further extraction.

Folin-Ciocalteu reagent, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, hexamethyltetramine, aluminium chloride, rutin were purchased from Sigma Chemical Co. (St.Louise, USA). Gallic acid was purchased from Acros Organics (New Jersey, USA). The other chemicals and solvents used in this experiment were reagent - grade quality and purchased from Sigma - Aldrich (Milwaukee, USA).

Determination of the appropriate sample preparation methods

The fresh Kradonbok leaves containing about 75.3% moisture content were used. Five different sample preparation methods for sample storage were investigated. These sample preparing methods were (1) hot air drying by tray dryer at 40° C for 18 h (Bocco *et al*., 1998) with air velocity about 0.5 m/s; (2) vacuum drying by vacuum dryer at 40°C, 100 mmHg (EYELA, model VOS-300SD, Japan) for 10 h; (3) air drying at room temperature 25°C for 12 h with air velocity about 3.2 m/s; (4) slowing freezing by freezing fresh leaves in freezer at -30°C after packing in HDPE bag; (5) fast freezing by contacting with dry ice and further freezing at -30°C. All dried samples were stored at room temperature in PE bag while frozen samples were stored in the freezer at -30°C until extraction for the yield of the extract and antioxidant activity determination.

All samples were sampled out every 10 days. The samples prepared from drying methods were analyzed for their water activity using a thermoconstanter (Novasina, Zurich, Switzerland) at 25°C (Pongsawatmanit *et al*., 2002) during storage period. The Kradonbok extracts was obtained by extracting the ground leaves with 95% ethanol for 30 min at room temperature with

shaking to ensure the complete extraction (modified from Velioglu *et al*., 1998), then filtrating through Whatman No. 1 filter paper. Samples were centrifuged (15 min, 1500g). The supernatant was evaporated under reduced pressure (50°C, 50 mmHg), and kept in airtight amber bottles after flushing with nitrogen gas for 30 s (Azizah *et al*., 1999). The dried extracts were stored in freezer at -30°C until analysis time by determining their total phenolic compounds and antioxidant activity (EC_{50}). The fresh sample leaves were also investigated for the comparison.

Determination of the appropriate extraction time

The sample preparation methods which gave the highest yield and the highest antioxidant activity were selected to study the effect of extraction time (0.25, 0.5, 1, 3, 4.5, 6, 24, 48, 96 and 168 h) by using the previous procedure. The extracts were evaluated with respect to their yield of the extract, total phenolic content and antioxidant activity.

Determination of yield

The dried extracts were weighed, and percentage of yield for each sample was calculated using following equation:

$$\text{Yield (\%, dry basis)} = (W_1 \times 100)/W_2 \quad (1)$$

Where W_1 was the weight of extract after evaporation and W_2 was the dry solid weight of stored sample.

Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu reagent (Kähkonen *et al*., 1999). Extracted samples (200 µl) were mixed with one ml of Folin-Ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%w/v) for 30 min in the dark, and then centrifuged at 3300g for 5 min. Absorption was measured at 765 nm (UV-VIS 1601 spectrophotometer, Shimadzu). Total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry matter.

Determination of antioxidant activity using DPPH radical

The antioxidant activity of samples was evaluated according to Masuda *et al*. (1999). An appropriate amount of extract sample was added into 4.9 ml methanol to give a serial dilution of the extracts in methanol at 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, $1:10^6$. Then 100 µl of 5 mM DPPH in methanol solution was added into each diluted sample solution. After the solutions were left to stand at 37°C for 30 min in the dark, absorbance of sample solution without DPPH, sample solutions containing DPPH and DPPH solution without sample extract were recorded at 517 nm (UV-VIS 1601 spectrophotometer, Shimadzu). DPPH radical scavenging activity was calculated modifying from Tachibana *et al*., (2001) using the equation

$$\text{DPPH radical scavenging activity (\%)} = [A_0 - (A_1 - A_S)]/A_0 \times 100 \quad (2)$$

Where A_o was the absorbance of the DPPH solution without sample extract, A_1 was the absorbance of the DPPH solution in the presence of the plant extract, and A_s was the absorbance of the sample extract solution without DPPH. Antioxidant activity as EC_{50} was determined from the relationship of DPPH radical scavenging activity and concentration of extract. The EC_{50} is defined as an amount of antioxidant required for causing a 50% reduction in the absorbance of DPPH.

Statistical analysis

The data are mean values of at least two replicate experiments. Analysis of variance was carried out and significant difference among treatments was determined by Duncan's multiple

range test ($p<0.05$).

RESULTS AND DISCUSSION

Comparison of sample preparation methods on yields and antioxidant activities

The yields of Kradonbok leaf extraction prepared from various methods were about 2% (dry weight) while yields of other leaf extraction methods reported in literature ranged between 2 to 15% (Demo *et al.*, 1998). These yields were not significantly different during storage for each preparation method (Figure 1). However, freezing methods seem to get the highest yield compared with those obtained from the other drying methods.

Sample preparation for storage before analysis is necessary even the total phenolic content showed a lower value compared with the fresh leave (24.41±0.03 mg of GAE/g). The total phenolic content of dried extracts was significantly different ($p<0.05$) and reduced by about 3, 9, 12, 20 and 25% for fast freezing, slow freezing, air drying at 25°C, vacuum drying at 40°C and hot air drying at 40°C, respectively. Total phenolic content and antioxidant activity (lower EC_{50}) of extracts prepared from freezing showed the higher values than those prepared from other drying methods (Figure 2a and 2b). The total phenolic content obtained from slow freezing method was lower than that obtained from fast freezing because larger ice crystals grew during freezing which would damage plant cells and cause a loss of antioxidant activity. The enzymes from plant cell such as lipoxygenase can oxidize polyphenols (Akoh and Min, 1997). The total phenolic content obtained from air drying was higher than those obtained from vacuum drying and hot air drying (Figure 2a) because some phenolic content may be degraded by higher temperature (Moure *et al.*, 2001). This result also found in fresh Mulberry leaves that the amount of flavonoid was higher in air-dried samples than that in oven-dried samples, probably due to decomposition after storage (Zhishen *et al.*, 1999). The amount of total phenolic content in dried samples decreased during the storage due to phenolic decomposition which may result from higher water activity (a_w) of the dried samples (Figure 3) ranging from 0.3 to 0.6. Water activity correlates with the stability of foods in terms of chemical reactions and microbial growths. Usually water activity value of higher than 0.4 enhances the deterioration of oxidation ability (Akoh and Min, 1997). The water activity increased because the moisture content could penetrate through PE plastic bag (Kadoya, 1990) during storage.

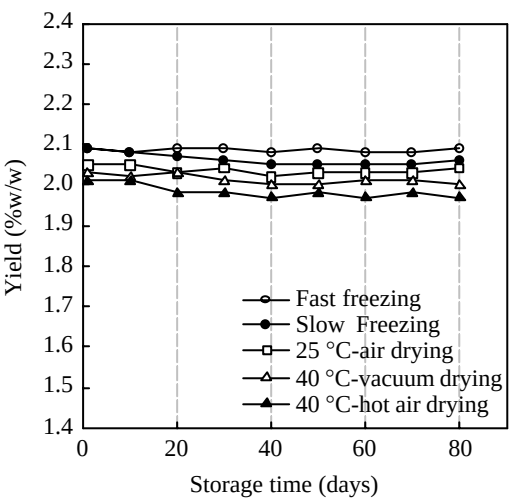

Figure 1 Yields of crude extract from Kradonbok leaves obtained from various sample preparation methods.

Comparison of extraction times on yields, total phenolic content and antioxidant activities

Extraction time affected yields, total phenolic content and antioxidant activities. For the first stage of extraction, the yield and total phenolic content increased with increasing the extraction time (Figure 4a and 4b). The yield and total phenolic content remained almost the same after 3 h of extraction at room temperature. However, the extraction time from 4.5 to 6 h gave the lowest EC_{50} value (highest antioxidant activity),

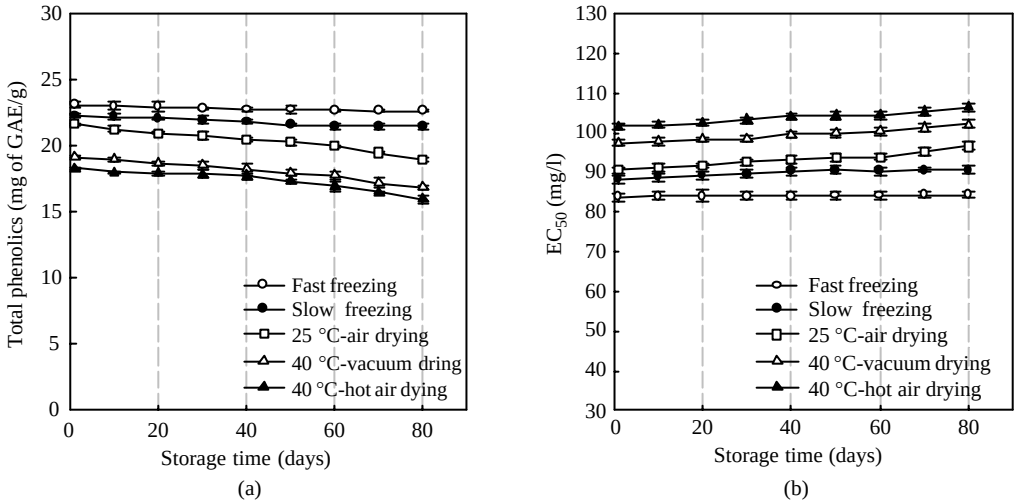

Figure 2 Total phenolics (a) and EC_{50} (b) of the dried extracts from Kradonbok leaves obtained from various sample preparation methods.

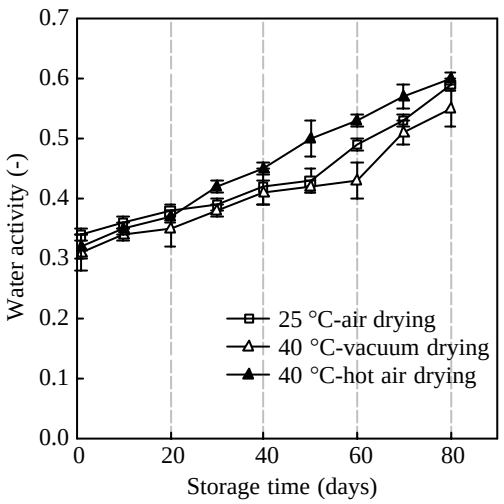

Figure 3 Water activity of the dried leaves from Kradonbok obtained from various drying preparation methods.

compared with those obtained from extraction time of less than 4.5 h or longer than 6 h. The EC_{50} increased with increasing extraction time (Figure 4c) when the extraction time was more than 6 h. This may result from chemical and enzymatic degradation which is likely to be the main mechanism causing the reduction in polyphenol content affected to antioxidant activity (Larrauri *et al*., 1997). From this study, the amount of total phenolic content (about 6%) did not change whereas the antioxidant activity decreased. These suggested that polyphenols can react with other plant components and decompose to form other less active phenolic contents (Guillot *et al*., 1996).

CONCLUSIONS

Freezing, especially fast freezing, for keeping the Kradonbok leaves until the time of analysis, gave the higher yield, total phenolic content and antioxidant activity (lower EC_{50}) compared with those obtained from other drying preparation methods. Extraction time for 4.5 to 6 h with ethanol at room temperature of Kradonbok leaves prepared from fast freezing gave the lowest EC_{50} value which indicated the highest antioxidant activity.

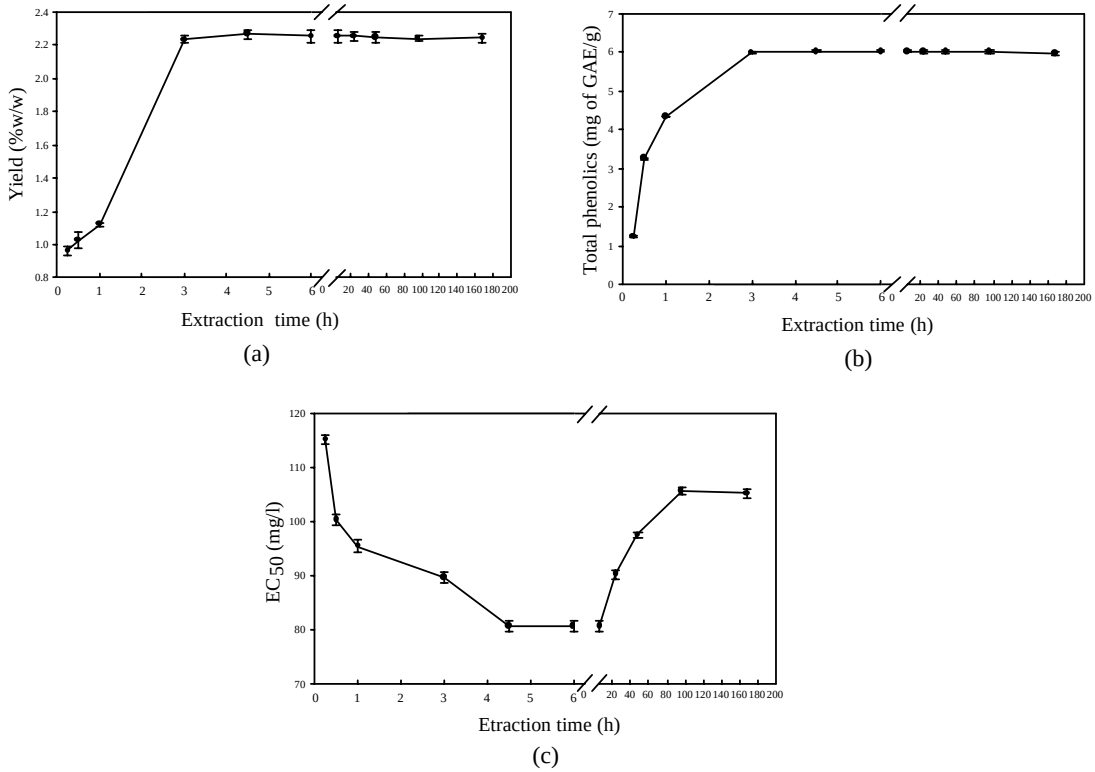

Figure 4 Yield of the extract (a), total phenolics (b) and EC_{50} (c) as a function of extraction time of Kradonbok leaves kept in the frozen state before analysis.

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