Phenolic Antioxidants from Betel Leaf (*Piper betel* Linn.) Extract Obtained with Different Solvents and Extraction Time
สารต้านอนุมูลอิสระประเภทฟีนอลิกจากสารสกัดของใบพลู ที่ได้จากตัวทำละลายและเวลาสกัดที่ต่างกัน

Pitchaon Maisuthisakul, Ph.D.
Assistant Professor at the Food Industrial System Department
School of Science, University of the Thai Chamber of Commerce
E-mail: pitchaon__mai@utcc.ac.th, pitchaon@gmail.com

บทคัดย่อ

Piper betel Linn. หรือใบพลู นิยมนำมาบริโภคโดยการเคี้ยวกับหมากและปูนในเอเชียกันมาก ทั้งนี้ข้อมูลเกี่ยวกับสารฟีนอลิกในใบพลูยังมีน้อย งานวิจัยนี้จึงมีความสนใจศึกษากิจกรรมต้านอนุมูลอิสระ ปริมาณฟีนอลิกทั้งหมด สัมประสิทธิ์การแบ่งระหว่างน้ำมัน-น้ำ และปริมาณสารที่สกัดได้ของสารสกัดจากใบพลูที่ได้จากตัวทำละลายและเวลาสกัดที่ต่างกัน โดยทำการสกัดด้วยเมทธานอล เอทธานอล อะซีโตน และเอทธิลอะซีเตทเป็นเวลา 0.5, 1, 3, 4.5, 6, 24 และ 48 ชั่วโมง จากนั้นนำสารสกัดที่ได้มาทำการวิเคราะห์ความสามารถในการกำจัดอนุมูลอิสระของ 2,2-diphenyl-1-picrylhydrazyl (DPPH•) และ 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) cation (ABTS+•) รวมทั้งปริมาณฟีนอลิกทั้งหมดด้วยวิธี Folin-Ciocalteu และความมีขั้วของสารสกัดด้วยวิธีการวัดสัมประสิทธิ์การแบ่งระหว่างน้ำมัน-น้ำด้วยโครมาโตกราฟีเหลวสมรรถนะสูง (HPLC) จากการทดลองพบว่า เอทธิลอะซีเตทซึ่งเป็นสารที่มีขั้วน้อยที่สุดเป็นตัวทำละลายที่เหมาะสมในการสกัดสารฟีนอลิกจากใบพลู ซึ่งสารสกัดที่ได้มีค่ากิจกรรมต้านอนุมูลอิสระ ปริมาณฟีนอลิกทั้งหมดและปริมาณสารที่สกัดได้สูงที่สุด โดยสารฟีนอลิกในใบพลูมีความมีขั้วต่ำเนื่องจากค่าสัมประสิทธิ์การแบ่งระหว่างน้ำมัน-น้ำมีค่าสูง (2.31+0.03) นอกจากนี้สารสกัดที่เวลาสกัดช่วง 4.5 ถึง 6 ชั่วโมง

มีค่ากิจกรรมต้านอนุมูลอิสระสูงเมื่อเปรียบเทียบกับการสกัดที่เวลาอื่น ทั้งนี้ปริมาณสารฟีนอลิกและกิจกรรมต้านอนุมูลอิสระในใบพลูมีค่าสอดคล้องกัน โดยมีความสัมพันธ์ที่ดีระหว่างค่าปริมาณฟีนอลิกทั้งหมดกับค่ากิจกรรมต้านอนุมูลอิสระ DPPH (R = 0.945) และค่าปริมาณฟีนอลิกทั้งหมดกับค่ากิจกรรมต้านอนุมูลอิสระ ABTS (R = 0.979) จากการทดลองนี้ชี้ให้เห็นว่า ตัวทำละลายและเวลาที่ใช้ในการสกัดมีความสำคัญต่อการเตรียมสารสกัดจากใบพลู เพื่อใช้เป็นสารต้านอนุมูลอิสระทางธรรมชาติ

คำสำคัญ: ฟีนอลิก สารต้านอนุมูลอิสระ ใบพลู ตัวทำละลาย เวลาในการสกัด

Abstract

Piper betel Linn., which is commonly known as Betel leaf, is consumed for chewing with Betel nut and lime by some people in Asia. Data on the phenolic compounds of this plant is scarce. This study was aimed at investigating the antioxidant activity, total phenolic content, oil-water partition coefficient and yield of various solvents, and extraction time. Methanol, ethanol, acetone and ethyl acetate extract of Betel leaves extracted for 0.5, 1, 3, 4.5, 6, 24 and 48 hrs were examined for radical scavenging ability against 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) cation (ABTS+•) radicals. Total phenolic content was evaluated according to the Folin-Ciocalteu procedure. The polarity of the plant extract from various solvents was assessed by determining the oil-water partition coefficient by high-performance liquid chromatography (HPLC). Based on the polarity, the ethyl acetate extract, which had less polarity, was suitable for extracting the phenolic compounds from Betel leaves. The extract showed the highest antioxidant activities, total phenolic content and yield. Betel leaf phenolics are found to have less polarity than other phenolic antioxidants due to their high value of oil-water partition coefficient (2.31±0.03). Extraction time from 4.5 to 6 hrs gave the highest antioxidant activity compared with those from other extraction times. Phenolic compounds are in part responsible for antioxidant activities on leaf extracts. Good relationships between phenolic content and radical DPPH scavenging activity (R = 0.945), including phenolics and radical cation ABTS scavenging activity (R = 0.979) of the extracts, were also found. The results indicated that the extraction solvent and time are important for the preparation of the Betel leaf extract for use as a natural antioxidant.

Keywords: Phenolic, Antioxidant, Betel Leaf, Solvent, Extraction Time

Introduction

Piper betel Linn. (Piperaceae) is a tropical Asian vine closely related to the common pepper. The leaves are chewed alone or with other plant materials, including the areca nut (*Areca catechu* Linn.), and lime. Many researches have focused on the red lime betel quid in the past few years. A little information on the Betel leaf was found. The Betel leaf itself has a spicy taste and yields an essential oil widely used as a medicine. Other biological activities described for the essential oil include antifungal, antiseptic and anthelmintic effects (Evans, Bowers, and Funk, 1984). It was evident that Betel leaves contained rich carotenes (80 IU/g fresh wt.), ascorbic acid (1.94 mg/g fresh wt.) and phenolics. Data on the phenolic compounds of this plant is related to chavicol (Amonkar, *et al.,* 1986), chavibetol, chavibetol acetate (Rimando, *et al.,* 1986) and eugenol (Nagabhushan, *et al.,* 1989). The structures of the phenolic compounds are shown in Figure 1.

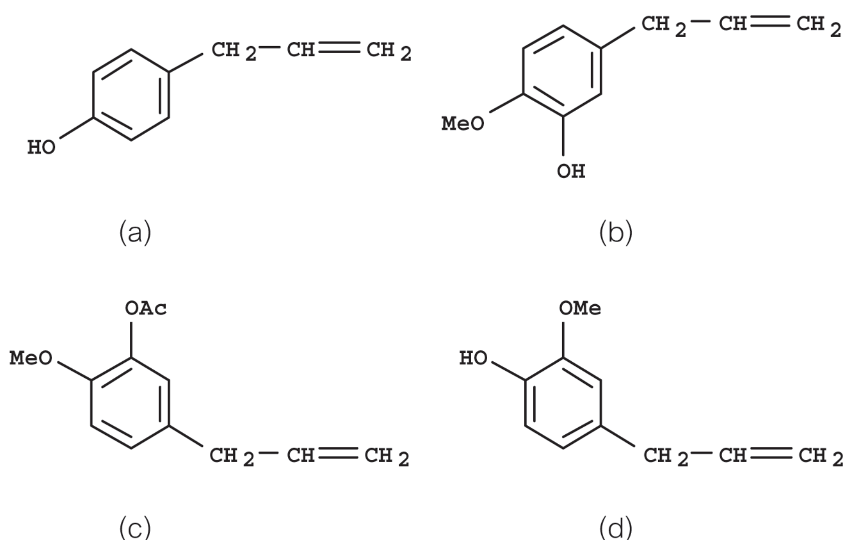

Figure 1 Structures of (a) Chavicol, (b) Chavibetol, (c) Chavibetol Acetate and (d) Eugenol

Several solvent systems have been used to extract antioxidants from Betel leaves. These solvent systems include ethanol, methanol and chloroform (Amonkar, *et al.,* 1986; Nagabhushan, *et al.,* 1989; Rimando, *et al.,* 1986). These different solvent extracts of Betel leaves possess antioxidant, antimicrobial and anti-inflammatory effects. It is noted that a solvent system for extraction is selected according to the purpose of extraction such as preparation for analysis, the nature of interested components, and the physicochemical

properties of the matrix. Consequently, the aim of this research was to compare four solvent systems of 0.5, 1, 3, 4.5, 6, 24 and 48 hrs for the efficacy of the antioxidant extraction activity, total phenolic content, oil-water partition coefficient and yield.

Materials and Methods

1. Materials

One lot of Betel leaves (*Piper betel* Linn.) was purchased from a market place at Prathumthani province during the harvest season in March, 2005. The Betel leaves were cleaned immediately upon return after the purchase, and only sound leaves were selected for the study of the effect of solvent extraction.

Folin-Ciocalteu reagent, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH•), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) cation (ABTS+•) radicals and sodium carbonate 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).

2. Extraction of Betel Leaf Antioxidant

Fresh plant leaves (80 g) were blended for 1 min with solvent (methanol, ethanol, acetone and ethyl acetate) at -20°C and the containers were then flushed with nitrogen and shaken for 4.5 hours in the dark at 30°C. The supernatant, after filtration through cheesecloth and Whatman No. 4 filter paper, was evaporated under vacuum. The sample was freeze-dried and stored in aluminum foil, after flushing with nitrogen, at -20°C until time for analysis to determine their antioxidant activity (Radical DPPH scavenging and radical cation ABTS scavenging activity), total phenolic content, oil-water partition coefficient and yield.

The solvent which gave the highest antioxidant activity, phenolic content and yield was selected to study the effect of extraction time (0.5, 1, 3, 4.5, 6, 24 and 48 hrs) by using the previous procedure. The extracts were kept until the analysis of Radical DPPH scavenging activity, total phenolic content and yield.

3. Determination of Antioxidant Activities

3.1 Radical DPPH Scavenging Activity

Free radical scavenging capacity of Betel leaf extract was evaluated according to the previous reported procedure using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) (Maisuthisakul, Pongsawatmanit, and Gordon, 2007b). The final concentration was 100 μM for DPPH•. The absorbance at 517 nm was measured against a blank of pure methanol at 30 min of reaction used to estimate the remaining radical levels. The DPPH activity was

expressed in terms of EC_{50} (μg.mL^{-1}).

3.2 Radical Cation ABTS Scavenging Activity

The total free radical scavenging capacity of each extract was determined by using the ABTS method. The radical cation ABTS scavenging activity was determined according to Re, *et al.* (1999). Briefly, a mixture of 7 mM ABTS and 2.45 mM potassium persulfate was prepared and allowed to stand at 25°C for 12-16 hours in the dark. The ABTS+• solution was diluted to an absorbance of 0.70 ($\pm$ 0.20) at 734 nm in ethanol before use. The ABTS+• solution (2 mL) was added to 20 µL aliquots of Trolox or sample in water with different concentrations. The activity of each antioxidant was determined within the range of the dose-response curve of Trolox, and the radical-scavenging activity was expressed as the Trolox equivalent antioxidant capacity (TEAC), defined as mmol of Trolox per gram of sample.

4. Determination of Total Phenolic Content

The total phenolic content of the extracts was determined using the Folin-Ciocalteu reagent (Maisuthisakul, Pongsawatmanit, and Gordon, 2007a). Each extract solution (200 µl) was thoroughly mixed with one ml of Folin-Ciocalteu reagent. After mixing for 3 min, 0.8 ml of 7.5% (w/v) sodium carbonate was added and allowed to stand for a further 30 min in the dark. The absorbance of plant extracts and a prepared blank were measured at 765 nm. The total phenolic content is expressed as gallic acid equivalents (GAE) in milligrams per gram dry weight of the plant.

5. Determination of Oil-Water Partition Coefficient

The oil-water partition coefficients of the extract were determined partitioned between soybean oil and water. Soybean oil (2 g) was weighed into a screw-capped centrifuge tube containing a test sample dissolved in HPLC water (2 g) for plant extract. The sample was vortexed three times for 20 sec with a 20 sec interval. The sample was then centrifuged at 6000 rpm for 30 min at 20 °C in a refrigerated centrifuge (Sorvall model super T21, USA). The lower layer was removed from the centrifuge tube with a syringe. The concentrations of the phenolic components in the aqueous phase were determined quantitatively by reversed-phase HPLC. The sample was filtered through a 0.20 µm Millipore filter (type HA) for subsequent analysis by HPLC. The solution (10 µL) was injected into the HPLC and analyzed according to the following conditions: column, Synergi Hydro RP column (150 x 4.6 mm id., 4µm, Phenomenex), fitted with an Allsphere ODS-2 guard column (10 x 4.6 mm id., Alltech). Solvent A was 100% acetonitrile.

Solvent B was 1% formic acid in water. The program was isocratic at 10% A, 90% B for 10 min followed by a linear gradient from 10% to 40% A for 39 min and an isocratic period at 10% A, 90% B for 10 min. The flow rate was 0.5 mL/min^{-1}. Chromatograms were monitored at 320 nm. The concentration of each phenolic compound in the plant extract was quantified using gallic acid as an external standard. The concentration of each phenolic compound in the oil phase (C_{oil}) was calculated as the difference between the total amount of antioxidant in the water before mixing and the amount in the water after mixing with oil (C_{water}). The Partition Coefficient (log P) was calculated as log (C_{oil}/C_{water}).

6. Determination of Yield

The yield of dried extracts based on a dry weight basis was calculated according to Pitchaon Maisuthisakul, Rungnaphar Pongsawatmanit, and Gordon (2007b).

7. Statistical Analysis

Each experiment, from sample preparation to analysis, was repeated in triplicate. Statistics on a completely randomized design were determined using SPSS software program (SPSS Inc., Chicago, IL, USA). The general linear model procedure was applied and Duncan's multiple range test was used to compare the mean values at $P < 0.05$. Mean values and pooled standard error of the mean (SEM) were calculated.

Results and Discussion

1. Effect of Different Solvents on Betel Leaf Extraction

Solvents used for antioxidant extraction had significant effect on the antioxidant activity, total phenolic content, yield and partition coefficient of Betel leaf extract ($P < 0.05$). The antioxidant activity using DPPH radical was expressed as EC_{50} value, defined as the concentration of extract required for 50% scavenging of DPPH radicals in this specified time period. A smaller EC_{50} value corresponds to a stronger antioxidant activity of the plant extract. The antioxidant activity using DPPH and cation ABTS radicals were stronger with less polar solvents. The results showed that the DPPH activity of the extract obtained from ethyl acetate was significantly higher than those obtained from the other solvents. The total phenolic content confirmed this aspect (Table 1). The extracts from solvents which had higher polarity (Table 2) were found to contain rather small amounts of phenolic compound (Table 1). EC_{50} and TEAC value of α-tocopherol was also measured and gave the value equal to 14.95 ± 0.23 μg.mL^{-1} and 2.30 ± 0.03 mmol of Trolox/g sample, respectively. The Betel leaves extracted from ethyl acetate (Table 1) possessed slight antioxidant activity as α- tocopherol. The

yields of plant extracts from various solvents were about 1.34-1.75% (dry weight). These yields varied significantly from different solvent extractions (Table 1).

Table 1 Antioxidant activity, total phenolic content and yield of Betel leaf extracted by different solvents*

Solvent used	DPPH activity (EC_{50}, µg.mL^{-1})	TEAC (mmol Trolox/g sample)	Total phenolic content (mg GAE/g sample)	Yield (%, db)
Methanol	36.65 ± 2.60^{a}	2.01 ± 0.06^{c}	49.89 ± 0.21^{d}	1.34 ± 0.02^{d}
Ethanol	33.85 ± 2.81^{ab}	2.12 ± 0.03^{bc}	50.38 ± 0.08^{c}	1.54 ± 0.01^{c}
Acetone	30.09 ± 1.21^{b}	2.21 ± 0.04^{ab}	53.28 ± 0.19^{b}	1.62 ± 0.00^{b}
Ethyl acetate	17.04 ± 0.51^{c}	2.34 ± 0.06^{a}	55.35 ± 0.14^{a}	1.75 ± 0.00^{a}

Note: * Data followed by different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$. Data were represented as means from three replication measurement.

The polarity of the extract was assessed by determination of the oil-water partition coefficient by HPLC. For the Betel leaf extract, the oil-water partition coefficient was calculated by summing the areas of the three phenolic peaks in the HPLC chromatogram of an aqueous solution of the extract compared with the areas of the three peaks in the aqueous phase after preparation and breaking of an emulsion. The relative areas of the HPLC peaks of the Teaw extract in the aqueous phase before partitioning with oil changed as shown in Figure 2. The oil-water partition coefficient of the Betel leaf extract from ethyl acetate was significantly different from those from the others as shown in Table 2. The HPLC chromatogram of Betel leaves showed that phenolic compounds in plant extracts had less polarity due to the longer retention time of chromatogram (Figure 2). The phenolic compounds of Betel leaves from other literature showed less polarity such as chavicol (log $P = 2.50 \pm 0.21$), chavibetol (log $P = 2.30 \pm 0.23$), chavibetol acetate (log $P = 2.39 \pm 0.24$), and eugenol (log $P = 2.20 \pm 0.23$). Normally, a frequently used descriptor for the estimation of the lipophilicity of phenolic compounds is the partition coefficient. From the results, the partition coefficients of Betel leaf extracts had less polarity due to the value being higher than 0 (Munishwar, *et al.,* 1997) (Table 2). Less polar solvent showed higher extraction efficacy due to the polarity of phenolic compounds of Betel leaf extract. The compounds which have a polarity similar to the solvent, are able to disolve more than those with differing polarities. It can be noted here that less polar solvents gave the higher yield, phenolic content and antioxidant activity from Betel leaves.

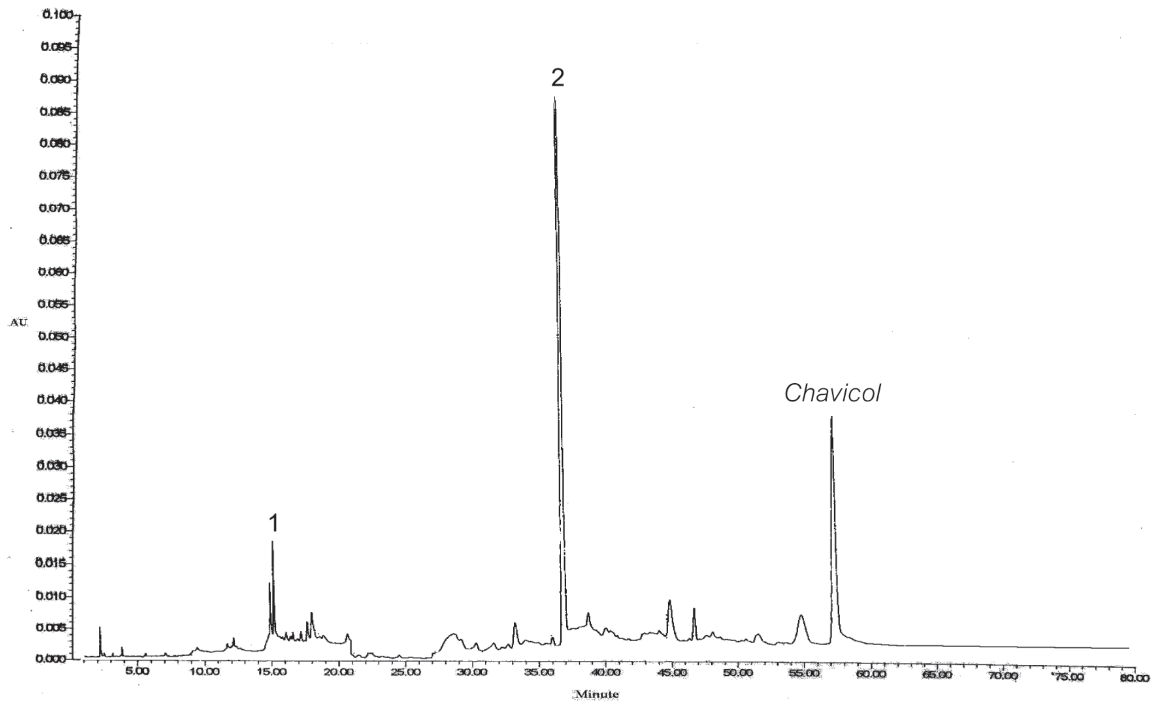

Figure 2 HPLC chromatogram of phenolic compounds in the Betel leaf extract before partitioning into oil; Peak 1 and 2 were unknown phenolic compounds

Table 2 Partition coefficient of solvent and Betel leaf extracts extracted by different solvents*

Solvent used	Partition coefficient of solvent#	Partition coefficient of extract
Methanol	-0.77	2.02±0.01[a]
Ethanol	-0.32	2.09±0.02[ab]
Acetone	-0.24	2.15±0.01[b]
Ethyl acetate	0.66	2.31±0.03[c]

Note: * Data followed by different letters within each column are significantly different according to Duncan's multiple range test at $P < 0.05$. Data represent means from three replication measurements.

Data obtained from literature review.

2. Effect of Different Extraction Times on Betel Leaf Extraction

Extraction time affected yields, total phenolic content and antioxidant activities. For the first stage of extraction, the yield and total phenolic content increased with increased extraction time (Figure 3a and 3b). The yield and total phenolic content remained almost the same after 3 hrs of extraction at 30°C. It was noted that the extraction of small molecules and highly soluble substances would initially be favored due to a high rate of diffusion. After that, large molecules of phenolic compound and less soluble substances would diffuse at a slower rate from the leaves (Gadow, Joubert, and Hansmann, 1997). However, the extraction time at 4.5 gave the lowest EC_{50} value (the highest antioxidant activity) compared with those obtained from the other extraction times (Figure 3c). The EC_{50} increased with increasing extraction time when the extraction time was more than 6 hrs. This may result from the chemical and enzymatic degradation which is likely to be the main mechanism causing the reduction in phenolic content affected to the antioxidant activity (Larrauri, Ruperez, and Saura-Calixto, 1997). From this study, the amount of total phenolic content from 4.5 to 6 hrs did not change (Figure 3b) whereas the antioxidant activity decreased. These suggests that polyphenols can react with other plant components and decompose to form other less active phenolic contents (Guillot, Malnoë, and Stadler, 1996).

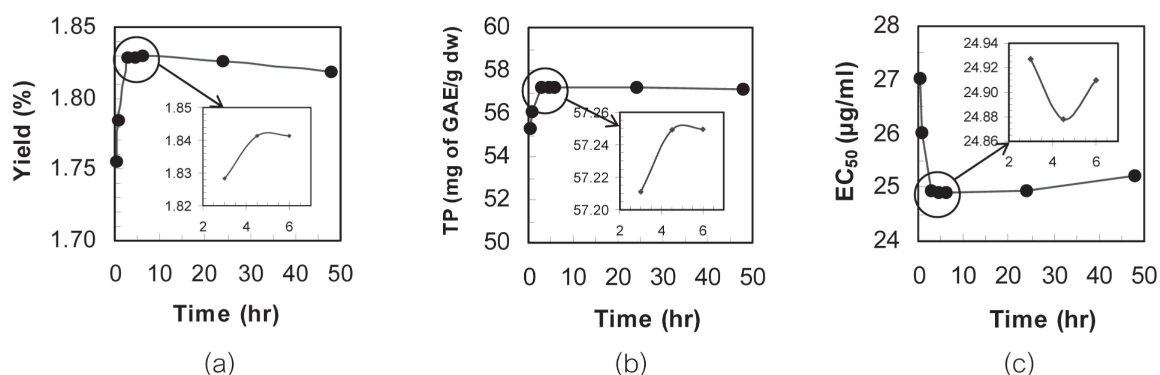

Figure 3 Effect of extraction time on (a) yield of the extract, (b) total phenolic content and (c) antioxidant activity (EC_{50}) of Betel leaf extract

3. Relationships between Yields, Total Phenolic Content and Antioxidant Activities

All data from the experiment were used to find a relationship between yield, total phenolic content, radical DPPH scavenging activity and radical cation ABTS scavenging activity. The results shown in Figure 4 indicate that the relationship of yield and total phenolic content

(R = 0.942) was higher than the relation between yield and radical DPPH scavenging activity (R = 0.918), but less than that of yield and radical cation ABTS scavenging activity (R = 0.986). The results indicated that the levels of yield of Betel leaf extracts are not related only to phenolics, but also antioxidant activities using DPPH and cation ABTS radicals.

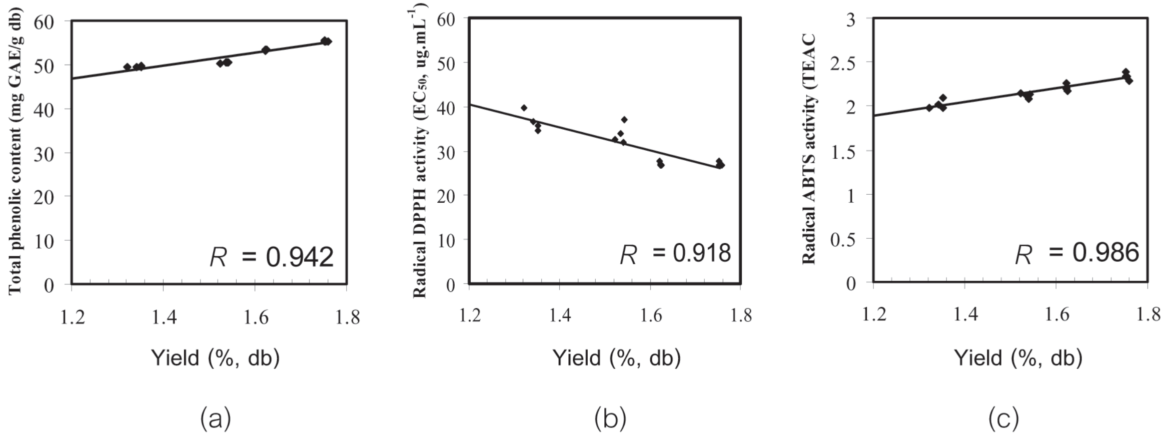

Figure 4 The relationships between (a) yield and total phenolic content, (b) yield and radical DPPH scavenging activity and (c) yield and radical cation ABTS scavenging activity of Betel leaf extract

When we considered the phenolic content and the antioxidant activities of Betel leaves by correlation matrix and regression analysis between the combinations of the independent variables (radical DPPH scavenging activity and radical cation ABTS scavenging activity) and the dependent variable (total phenolic content). The correlation matrix of ABTS activity and the total phenolic content was higher than that of DPPH activity and the total phenolic content (Figure 5). The relationship of the antioxidant activity using DPPH• and ABTS+• was high (R = 0.960) as shown in Figure 6. It is noticed here that most of the phenolic compound of Betel leaves acts as a radical scavenger and the two studied radical scavenging methods gave the same picture of activity.

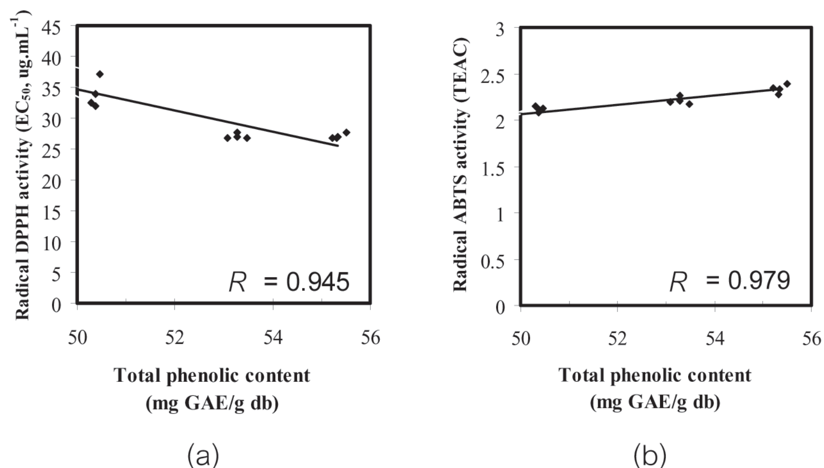

Figure 5 Relationships between (a) total phenolic content and radical DPPH scavenging activity and (b) total phenolic content and radical cation ABTS scavenging activity of Betel leaf extract

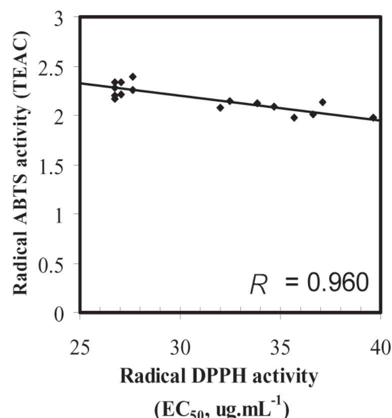

Figure 6 Relationships between radical DPPH scavenging activity and radical cation ABTS scavenging activity of Betel leaf extract

Conclusion

The results of this study indicated that Betel leaf extract is rich in less polarity phenolic compound. Solvents used for extraction also affected the concentration of total phenolic content in extracts. Ethyl acetate was found to be the best solvent for the extraction of antioxidant compounds from Betel leaves due to its nonpolar components. The major bioactive compounds of phenolics in Betel leaves were found to be less polarity. The extraction time at 4.5 hrs is the recommended time to prepare antioxidant extracts from Betel leaf samples. As can also be observed from the present data, a relationship existed between yield, total phenolic content and antioxidant activities for Betel leaf extracts.

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References

Amonkar, A. J., *et al.* 1986. "Hydroxychavicol: a New Phenolic Antimutagen from Betel Leaf." **Food and Chemical Toxicology** 24, 12: 1321-1324.

Evans, P. H., Bowers, W. S., and Funk, E. J. 1984. "Identification of Fungicidal and Nematocidal Components in the Leaves of Piper Betel *(Piperaceae)*." **Journal of Food Agricultural and Chemistry** 32: 1254-1256.

Gadow, A. V., Joubert, E., and Hansmann, C. F. 1997. "Comparison of the Antioxidant Activity of Rooibos Tea *(Aspalathus linearis)* with Green, Oolong and Black Tea." **Food Chemistry** 60, 1: 73-77.

Guillot, F., Malnoë, A., and Stadler, R. H. 1996. "Antioxidant Properties of Novel Tetraoxygenated Phenylindan Isomers Formed during Thermal Decomposition of Caffeic Acid." **Journal of Food Agricultural and Chemistry** 44: 2503-2510.

Larrauri, J. A., Ruperez, P., and Saura-Calixto, F. 1997. "Effect of Drying Temperature on the Stability of Polyphenols and Antioxidant Activity of Red Grape Pomace Peels." **Journal of Food Agricultural and Chemistry** 45: 1390-1393.

Munishwar, N. G., *et al.* 1997. "Polarity Index: the Guiding Solvent Parameter for Enzyme Stability in Aqueous-Organic Cosolvent Mixtures." **Biotechnology Progress** 13: 284-288.

Nagabhushan, M., *et al.* 1989. "Hydroxy-Chavicol: a New Anti-Nitrosating Phenolic Compound from Betel Leaf." **Mutagenesis** 4, 3: 200-204.

Maisuthisakul P., Pongsawatmanit R., and Gordon, M. H. 2007a. "Assessment of Phenolic Content and Free-Radical Scavenging Capacity of Some Thai Indigenous Plants." **Food Chemistry** 100: 1409-1418.

___________. 2007b. "Characterization of the Phytochemicals and Antioxidant Properties of Extracts from Teaw (*Cratoxylum formosum* Dyer)." **Food Chemistry** 100: 1620-1629.

Re, R., *et al.* 1999. "Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay." **Free Radical Biology Medicine** 26: 1231-1237.

Rimando, A. M., *et al.* 1986. "Studies on the Constituents of Philippine Piper Betel Leaves." **Archives of Pharmacal Research** 9, 2: 93-97.

Asst. Prof. Dr. Pitchaon Maisuthisakul received her Doctor of Philosophy in Agro-Industry Product Development from Kasetsart University. She is currently working at the University of the Thai Chamber of Commerce, Bangkok. Her main interest is in food antioxidant, consumer concept development, and sensory analysis.