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ภาคผนวก ก

Standard curve ของ Trolox

วิธีเตรียม Standard curve ของ Trolox ทำดังต่อไปนี้

1. ชั่ง ABTS (2,2-azino-bis (3-ethylbenzene-thiazoline-6-sulfonic acid), MW = 548.68) มาละลายใน ethanol ปรับปริมาตรให้ได้ความเข้มข้น 7 mM
2. ชั่ง $K_2S_2O_8$ (Potassium persulfate, MW = 270.32) มาละลายในน้ำปรับปริมาตรให้ได้ความเข้มข้น 2.45 mM
3. ผสมสารละลาย ABTS และสารละลาย Potassium persulfate อัตราส่วน 8:12 (โดยปริมาตร) เก็บในที่มืดและเย็น เป็นเวลา 16-18 ชั่วโมง
4. เจือจางด้วย ethanol ในสัดส่วนสารผสมต่อ ethanol เท่ากับ 5:100 โดยปริมาตร แล้วนำไปวัดค่าการดูดกลืนแสง ปรับด้วย ethanol เพื่อให้ได้ค่าการดูดกลืนแสงเท่ากับ 0.7 ± 0.2
5. ชั่ง สารมาตรฐาน Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, MW = 250.29) มาละลายใน ethanol ปรับปริมาตรให้ได้ความเข้มข้น อยู่ในช่วง 50 – 300 µM
6. นำสารละลายที่ได้ ไปวัดค่าการดูดกลืนแสงของสารละลายผสมภายหลังตั้งทิ้งไว้นาน 30 นาที ที่ความยาวคลื่น 750 nm ได้ผลดังตารางต่อไปนี้

หมายเหตุ การเติม Reagent ก่อนวัดการดูดกลืนแสง มีรายละเอียด ดังต่อไปนี้

- Trolox = Trolox 20 µL + $ABTS^{+}$ 180 µL
- Blank = Trolox 20 µL + EtOH 180 µL
- Positive = EtOH 20 µL + $ABTS^{+}$ 180 µL
- Negative = EtOH 200 µL

7. % Inhibition สามารถคำนวณได้ดังสมการต่อไปนี้

$$\% \text{ Inhibition} = \frac{[A_{control}] - [A_{Trolox}]}{[A_{control}]} \times 100$$

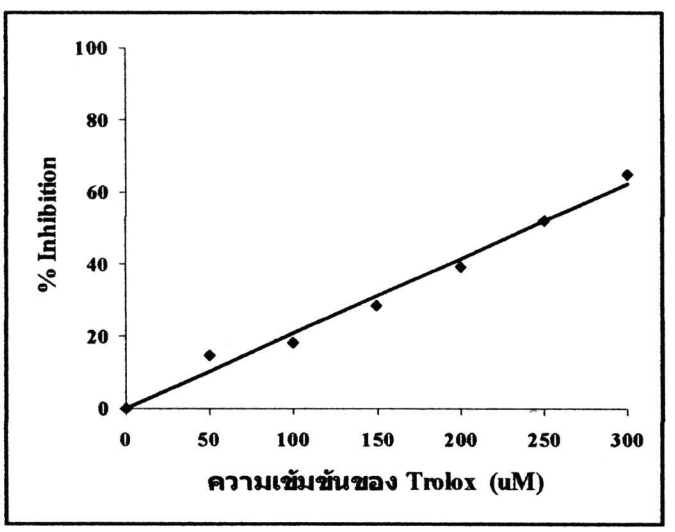
เมื่อ $[A_{control}] = [A_{positive}] - [A_{negative}]$

$[A_{Trolox}] = [A_{Trolox}] - [A_{Blank}]$

ผลการวัดค่าการดูดกลืนแสงและการคำนวณ แสดงในตารางต่อไปนี้ และสามารถเขียนเป็น standard curve ของ Trolox ได้รูปดังต่อไปนี้ พบว่าเส้นกราฟมาตรฐานเป็นเส้นตรงภายใต้สมการเส้นตรง คือ $y = 0.2128x$ และมีค่า $R^2 = 0.9754$

ตารางแสดง % Inhibition ของ Trolox ที่ความเข้มข้นต่าง ๆ

Trolox concentration (μM)	% inhibition
0	0.00
50	14.79
100	18.17
150	28.56
200	38.92
250	51.98
300	69.29



รูปแสดง Standard curve ของ ABTS

ภาคผนวก ข

Standard curve ของ $FeSO_4$

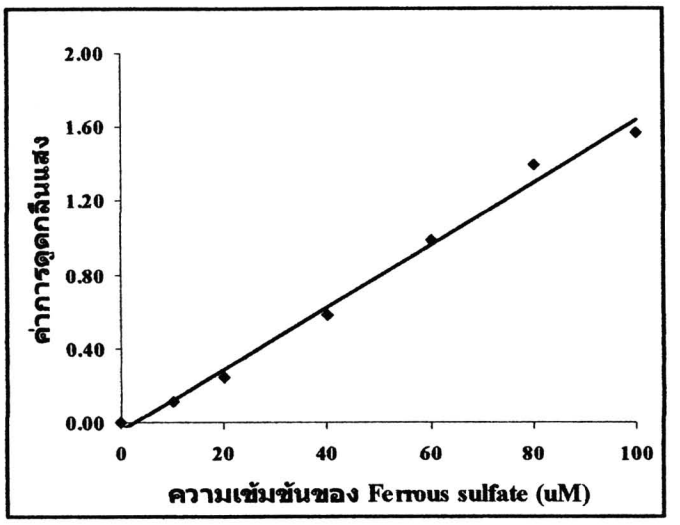
วิธีเตรียม Standard curve ของ $FeSO_4$ ทำดังต่อไปนี้

1. เตรียมสารละลาย $FeSO_4$ ในน้ำ ให้ได้ความเข้มข้น 1.0 mM
2. นำสารละลายที่ได้ในข้อ 1 มาเจือจางด้วยน้ำจนครบ 1.0 มิลลิลิตร ตามตารางต่อไปนี้ เพื่อให้ได้ความเข้มข้นของ $FeSO_4$ ตามตารางต่อไปนี้
3. นำสารละลายที่ได้ในข้อ 2 มาเจือจางต่อด้วยน้ำ เพื่อให้ได้ความเข้มข้นของ $FeSO_4$ เท่ากับ 0 – 100 µM
4. นำสารละลายที่ได้ไปวัดค่าการดูดกลืนแสงที่ 595 นาโนเมตร

ผลการวัดค่าการดูดกลืนแสงของ $FeSO_4$ ความเข้มข้นต่าง ๆ แสดงในตารางต่อไปนี้ และสามารถเขียนเป็น standard curve ของ $FeSO_4$ ได้รูปดังต่อไปนี้ พบว่าเส้นกราฟมาตรฐานเป็นเส้นตรงภายใต้สมการเส้นตรง คือ $y = 0.0172x - 0.0771$ และมีค่า $R^2 = 0.9907$

ตารางแสดงค่าการดูดกลืนแสงของ $FeSO_4$ ที่ความเข้มข้นต่าง ๆ

Final concentration (µM)	ค่าการดูดกลืนแสง
0	0.00
10	0.11
20	0.24
40	0.58
60	0.99
80	1.39
100	1.57



รูปแสดง Standard curve ของ $FeSO_4$

ภาคผนวก ค

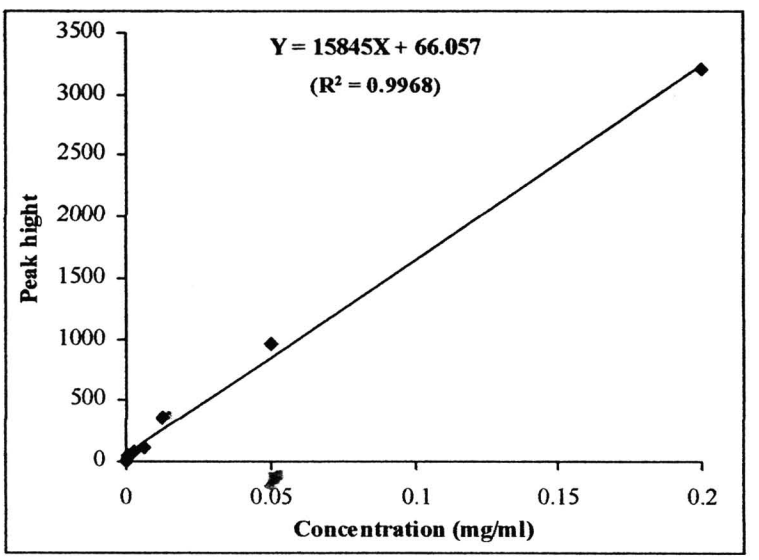
Standard curve ของ Gallic Acid

วิธีเตรียม Standard curve ของ Gallic Acid ทำดังต่อไปนี้

1. เตรียมสารละลาย Standard Gallic acid 20 mg% w/v ในน้ำ
2. เจือจางสารละลายที่เตรียมได้ด้วยน้ำ เพื่อให้ได้สารละลายที่มีความเข้มข้นต่าง ๆ ดังนี้ 0.5, 1.0, 2.5, 6.25, 12.5, 50.0, และ 200.0 µg/ml นำไปฉีดเข้าเครื่อง HPLC แล้วหาค่าความสูงของ peak โดย UV detector ที่ 280 nm ได้ผลดังแสดงในตารางต่อไปนี้

ความเข้นข้นของ Gallic acid (µg/ml)	ค่าความสูงของ Peak ที่ 280 nm
0.00000	0.00
0.00050	30.75
0.00100	40.76
0.00250	73.54
0.00625	121.18
0.01250	363.24
0.05000	954.95
0.20000	3207.89

3. นำค่าที่ได้มา plot แสดงความสัมพันธ์ระหว่างความเข้มข้นของ Gallic acid และค่าการดูดกลืนแสง พบว่าเส้นกราฟมาตรฐานเป็นเส้นตรงภายใต้สมการเส้นตรง คือ y = 15845x + 66.057 และมีค่า R^2 = 0.9968



รูปแสดง Standard curve ของ Gallic acid

ภาคผนวก - ง

Reprint

เรื่อง

Antioxidant active principles isolated from

Psidium guajava grown in Thailand

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Antioxidant Active Principles Isolated from *Psidium guajava* Grown in Thailand

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Abstract

Antioxidant active compounds were isolated from methanol crude extracts of the leaves of Guava (*Psidium guajava* L.) grown in Thailand. The isolated compounds were screened for their *in vitro* antioxidant activity by a DPPH free radical scavenging assay. Results indicate that three isolated compounds contribute importantly to the antioxidant activity of guava leaves, providing a scientific basis for the use of this plant in traditional medicine. Their structures were determined on the basis of spectroscopic and chemical methods. The most active compound was found to be quercetin along with two flavonoid compounds, quercetin-3-*O*-glucopyranoside and morin. The isolated quercetin, quercetin-3-*O*-glucopyranoside and morin showed significant scavenging activity with IC_{50} of 1.20±0.02, 3.58±0.05 and 5.41±0.20 µg/ml, respectively.

Keywords

Guava, *Psidium guajava*, Flavonoid, Antioxidant activity

Introduction

Free radicals are produced in normal and/or pathological cell metabolism. Oxidation is essential to many living organisms for the production of energy to fuel biological processes. However, the uncontrolled production of oxygen derived free radicals is involved in the onset of many diseases such as cancer, rheumatoid arthritis, cirrhosis and arteriosclerosis as well as in degenerative processes associated with aging. Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and tissue damage [1]. In recent years, there has been increasing evidence that reactive oxygen species (ROS) are associated with pathological conditions such as atherosclerosis [2] and carcinogenesis [3], as well as with aging [4]. Thus, a lot of attention has focused on dietary antioxidants which may have a potential for therapeutic use and prevention of these diseases. Investigations into the health maintaining properties of plants have resulted in the identification of a wide array of bioactive compounds in plants that include flavonoids, phenolics, limonoids, carotenoids, coumarins, phytosterols, etc. Based on recent research, several compounds from fruits and vegetables were found to possess anticarcinogenic and antioxidant activities [5]. Furthermore, flavonoids and

carotenoids have also been shown to inhibit cancer cell proliferation in vitro [6]. Antioxidant activity by scavenging of reactive oxygen species is important in preventing potential damage to cellular components such as DNA, proteins, and lipids. In the course of screening for antioxidants in Thai medicinal plants, the methanol extract of the leaves of Guava showed a scavenging activity toward ABTS free radical decolorization assay and Ferric reducing power (FRAP) assay [7]. Guava (*Psidium guajava* L., Myrtaceae) leaves have been used in folk medicine of Thailand as an antidiarrheal [8] and antidysenteric; extemally, they have been used as a deodorant of mouth odor [9]

The aim of this work was to determine the active principles from leaves of guava grown in Thailand, and to assess their antioxidant properties.

Experimental

Materials and physicochemical study

Melting points were determined on a Yanako melting point apparatus. IR spectra were recorded with a JASCO FT/IR-230 spectrophotometer. NMR spectra were recorded on a JEOL JNM-α400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). Chemical shifts are shown as δ values, using tetramethylsilane (TMS) as an internal reference. Column chromatography was carried out on Cosmosil 75μm C_{18}-OPN (Nacalai Tesque), Sephadex LH-20 (GE Healthcare Biosciences AB), Silica gel 60 (230-400 mesh) (Merck), Toyopearl-HW40C (Tosoh Co.) and MCI-gel CHP-20P (Mitsubishi Co.). TLC was performed on pre-coated RP-18 F_{254} (0.25 mm)

(Merck), and spots were detected by UV (254 nm) and by 10% H_2SO_4 spraying reagent followed by heating. Water was deionized and purified using a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals used were of the highest commercial grade available, purchased from Wako Pure Chemical Industries.

Plant material

The dried leaves of *P. guajava* were collected at Chiang Mai Province, Thailand. A voucher specimen is deposited in the herbarium, Faculty of Pharmacy, Chiang Mai University, Thailand.

Extraction and isolation

The dried leaves of *P. guajava* (800 g) were partitioned by successive extraction with *n*-hexane, EtOAc, *n*-butanol and MeOH to give *n*-hexane fraction (15.0 g), EtOAc fraction (13.9 g), *n*-butanol fraction (11.9 g) and MeOH fraction (151.5 g), respectively.

The antioxidant activity of the resulting fractions was determined by the use of the DPPH method. The highly active methanolic fraction (20 g) was subjected to silica gel column chromatography (Cosmosil C_{18}-OPN, 75 µm, 6.0 cm i.d. x 40 cm) and eluted with MeOH of increasing polarities with water (5 to 100% MeOH). The volume of each collected fraction was 100 ml to give 6 fractions (A, B, C, D, E, and F). High antioxidant activity was found in fraction C, D and E. The highly active fraction C (1.0 g) was re-chromatographed over a Sephadex LH-20 column (2.5 cm i.d. x 100

cm) with MeOH, acetone:MeOH (1:1) and acetone. The most active fraction obtained (48.8mg) was then subjected to silica gel column chromatography (Silica gel 60, 230-400 mesh, 1.0 cm i.d. x 20 cm) and eluted with *n*-hexane:EtOAc, EtOAc, EtOAc:acetone, acetone and MeOH to yield compound **1** (14.9 mg) from *n*-hexane:EtOAc (3:7) fraction.

Fraction D (1.804 g) was chromatographed over Toyopearl HW-40C (1.0 i.d.x70 cm) with aqueous MeOH (5, 10, 20, 30, 50, 70, and 100%) and 70% aqueous acetone. The 10% MeOH eluate (61.90 mg) was separated to Cosmosil 75μm C18-OPN (1.0 i.d.x20 cm) and eluted with 5, 15, 25, 50, and 100% MeOH to yield compound **2** (4.0 mg).

Fraction E (1.133 g) was chromatographed over Cosmosil 75μm C18-OPN (2.3 i.d.x13 cm) and eluted with 20, 30, 40, 50, and 100% ethanol. The 30% ethanol eluate (691.30 mg) was re-chromatographed over Cosmosil 75 μm C18-OPN (2.0 i.d.x12 cm) and eluted with 25, 50, 75, and 100% ethanol. The 50% ethanol eluate (567.50 mg) was subjected to column chromatography over MCI-gel (1.0 i.d x 53 cm) with 30, 40, 50, 60, and 100% MeOH to give four factions. The second fraction (231 mg) was re-chromatographed over Toyopearl HW-40C and eluted with 50, 53, 55, 57, 60, and 100% MeOH and 70% acetone to yield compound **3** (33.7 mg).

Free Radical-Scavenging Activity Assay

DPPH radical scavenging is considered a good in vitro model and is widely used to conveniently assess antioxidant efficacy. In its radical form, DPPH free radical has

an absorbance at 515 nm which disappears when DPPH is reduced by an antioxidant compound or a radical species to become a stable diamagnetic molecule. As a result, the color changes from purple to yellow [9]. This color change is taken as an indication of the hydrogen donating ability of the tested compounds.

The DPPH radical scavenging activity of the samples was estimated according to the methods of Brand-Williams et al., 1995 [9] and Gamez et al., 1998 [10] with some modification. Samples in MeOH (100 µl) were added to a solution of DPPH radical in MeOH (200 µM, 100 µl), and the reaction mixture was left to stand for 30 min at room temperature in the dark. The scavenging activity of samples was estimated by measuring the absorption of the mixture at 515 nm, which reflects the amount of DPPH radical remaining in the solution. The scavenging activity was expressed as the IC_{50}, the concentration of samples required for scavenging 50% of DPPH radical in the solution.

Results and Discussion

Phenolic compounds are the major group that contributes to the antioxidant activity of vegetables, fruits, cereals and other plant based materials. In our previous work, different solvents were used for the extraction of phenolic compounds from guava leaves and we have reported [7] that a methanol extract gave high antioxidant activities from 24 samples of plant species commonly found in Thailand. Therefore, in the present study, the bioactive compounds from guava leaves were isolated and the chemical structures were identified.

The dried leaves of *P. guajava* were extracted sequentially with *n*-hexane, EtOAc, *n*-butanol and MeOH in order to identify the fraction with the highest antioxidant activity. As reported previously, the methanolic fraction gave the highest scavenging activity. The methanolic fraction was then fractionated and purified according to the antioxidant test. The active purified principles were analyzed for their chemical structures by IR, ^{13}C-NMR, ^{1}H-NMR, MS analyses and in comparison with the data of authentic quercetin [10-12], morin [13] and quercetin-3-*O*-glucopyranoside [14-15]. Results revealed the three active principles as compound **1**, compound **2**, and compound **3** as follows.

Compound **1** was obtained as a pale yellow powder of melting point 300°C (decomposed) and EI-MS *m/z*: 302 $[M]^+$. IR absorption band at 3293.82, 1616.06, 1511.92 and 1166.72 were consistent with the presence of hydroxyl, carbonyl, aromatic ring and ether groups respectively. The proton and carbon NMR of this compound were shown in Fig 1 and 2, respectively. The ^{1}H-NMR (400MHz, CD_3OD): δ 6.17 (1H, s, H-6), 6.37 (1H, s, H-8), 6.86 (1H, d, J=8.4, H-5'), 7.62 (1H, d, J=8.6, H-6'), 7.72 (1H, s, H-2'). The ^{13}C-NMR (100MHz, CD_3OD): δ 94.58 (C-8), 99.41 (C-6), 104.69 (C-10), 116.18 (C-2'), 116.39 (C-5'), 121.85 (C-6'), 124.32 (C-1'), 137.37 (C-3), 146.38 (C-3'), 148.19 (C-4'), 148.93 (C-2), 158.41 (C-9), 162.67 (C-5), 165.72 (C-7), 177.50 (C-4). From these results, compound **1** was considered quercetin and its chemical structure is shown in Fig 3.

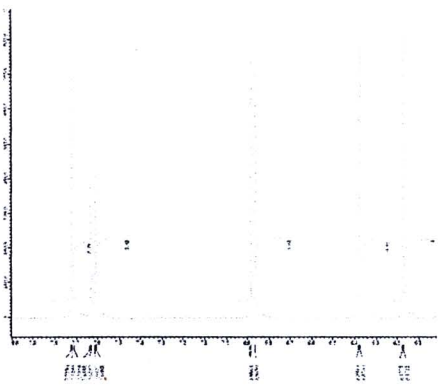

Fig. 1 ^{1}H NMR spectrum of compound **1**

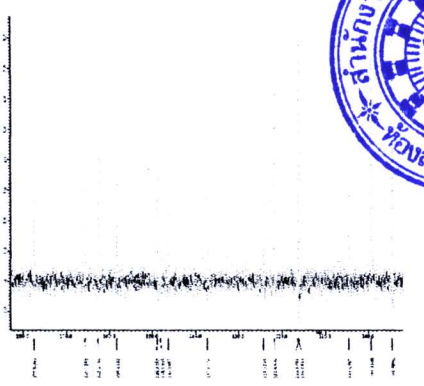

Fig. 2 ^{13}C NMR spectrum of compound **1**

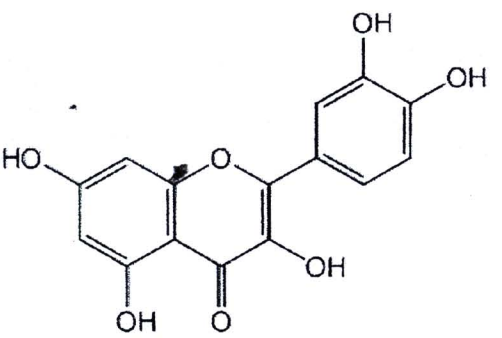

Fig. 3 Chemical structure of compound **1**

Compound **2** was obtained as a yellow powder of melting point 300°C (decomposed) and EI-MS *m/z*: 302 $[M]^{+}$. IR absorption bands at 3484.74, 1604.48, 15263.15, 1052.94.were consistent with the presence of hydroxyl, carbonyl, aromatic ring and ether groups respectively. The proton and carbon NMR of this compound were shown in Fig 4 and 5, respectively. The ^{1}H-NMR (400MHz, CD_3OD): δ 6.19 (1H, s, H-6), 6.39 (1H, s, H-8), 6.87 (1H, d, H-5'), 7.63 (1H, d, H-6'), 7.68 (1H, s, H-3'). The

^{13}C-NMR (100MHz, CD_3OD): δ 93.41 (C-8), 98.58 (C-6), 104.46 (C-10), 114.90 (C-3'), 115.84 (C-5'), 121.78 (C-6'), 121.94 (C-1'), 132.10 (C-3), 144.73 (C-2), 148.73 (C-4'), 157.02 (C-9), 158.02 (C-2'),161.80 (C-5), 164.71 (C-7), 177.96 (C-4). From these results, compound 2 was considered morin and its chemical structure is shown in Fig 6.

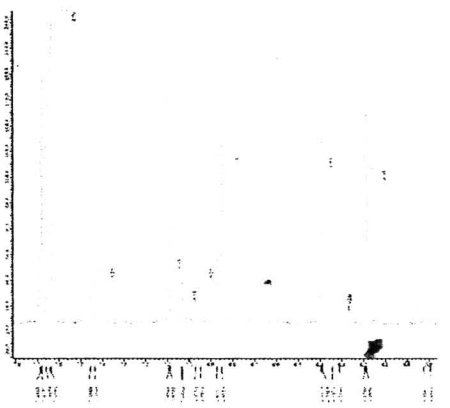

Fig. 4 ^{1}H NMR spectrum of compound **2**

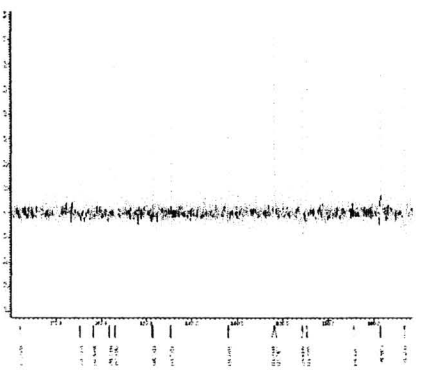

Fig. 5 ^{13}C NMR spectrum of compound **2**

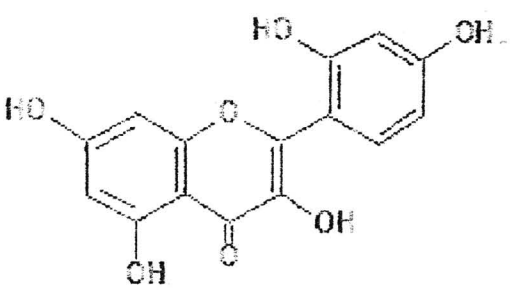

Fig. 6 Chemical structure of compound **2**

Compound **3** was obtained as a yellow powder. It showed melting point at 220-225 °C and EI-MS *m/z*: 464 $[M]^+$. IR absorption bands at 3739.30, 1648.84, 1562.06, 1492.63, 1295.93, 1054.87, 622.89 were consistent with the presence of hydroxyl, carbonyl, aromatic ring and ether groups respectively. The proton and carbon NMR of this compound were shown in Fig 7 and 8, respectively. The ^{1}H-NMR (600MHz, CD_3OD): δ 3.42 (1H, t, 8.7, H-5′′), 3.48 (1H, m, H-3′′), 3.54 (1H, m, H-6a′′), 3.55 (1H, m, H-2′′), 3.64 (1H, d, H-6b′′), 3.85 (1H, m, H-4′′), 5.09 (1H, d, 7.70, H-1′′), 6.13 (1H, d, 2.0, H-6), 6.30 (1H, d, 2.0, H-8), 6.86 (1H, d, 8.5, H-5'), 7.58 (1H, dd, 8.5, H-6'), 7.83 (1H, d, 2.2, H-2'). The ^{13}C-NMR (150MHz, CD_3OD): δ 60.59 (C-6′′), 68.68 (C-4′′), 73.85 (C-2′′), 75.82 (C-3′′), 77.02 (C-5′′), 94.26 (C-8), 99.85 (C-6), 103.18 (C-1′′)104.36 (C-10), 114.78 (C-5'), 116.34 (C-2'), 121.53 (C-6'), 121.79 (C-1'), 134.40 (C-3), 144.52 (C-3′),148.68 (C-4'), 156.44 (C-2), 157.40 (C-9),161.44 (C-5), 165.50 (C-7), 177.70 (C-4). From these results, compound 3 was considered quercetin-3-*O*-glucopyranoside and its chemical structure is shown in Fig 9.

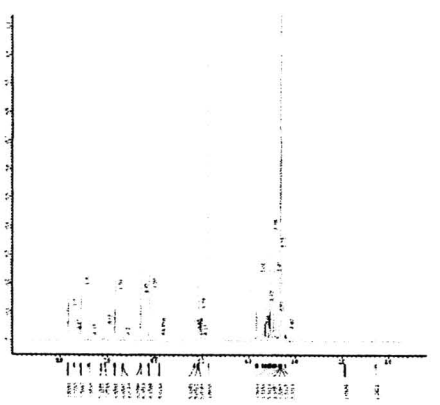

Fig. 7 ^{1}H NMR spectrum of compound **3**

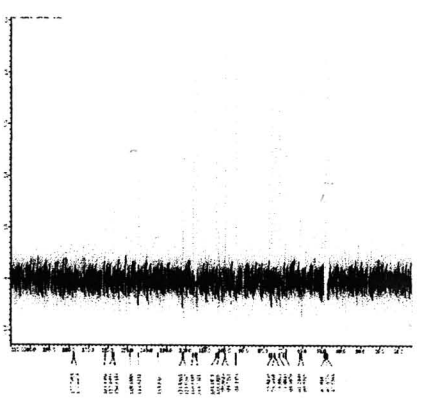

Fig. 8 ^{13}C NMR spectrum of compound **3**

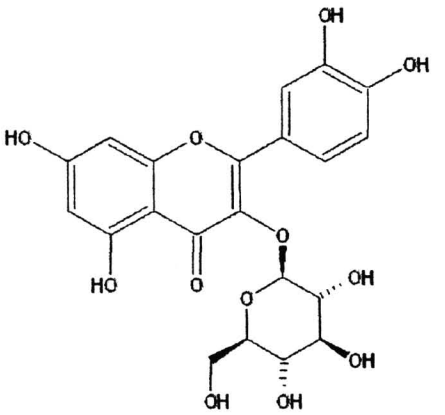

Fig. 9 Chemical structure of compound **3**

Many attempts have been reported in the literature to delineate the structure-activity relationship underlying the natural antioxidant activity of certain phenolic compounds. This may involve the neutralization of free radicals initiating oxidation processes, or the termination of radical chain reactions, due to their hydrogen donating ability [16]. In this study, the comparative biological activity of three compounds isolated from guava leaves was evaluated as their antioxidant capacity to scavenge DPPH free radicals. It was found that all three isolated principles, quercetin, quercetin-3-*O*-glucopyranoside and morin had antioxidant activity but at different levels as shown in Fig 10 with the IC_{50} of 1.20±0.02, 3.58±0.05 and 5.41±0.20 μg/ml, respectively. It was clearly seen that quercetin is the most active principle in Thai guava leaves, followed by quercetin-3-*O*-glucopyranoside and morin, respectively. This result could be explained by the higher antioxidant activity being related to the greater number of hydroxyl groups on the flavonoid nucleus. The antioxidant activity of flavonoids was considered dependent on the presence of ortho

phenolic functions [17]. This finding is in accordance with the results reported by Bors et al., 1990 [18] and confirms that the *O*-dihydroxybenzene (catechol) structure is an important feature for enhanced radical-scavenging activity.

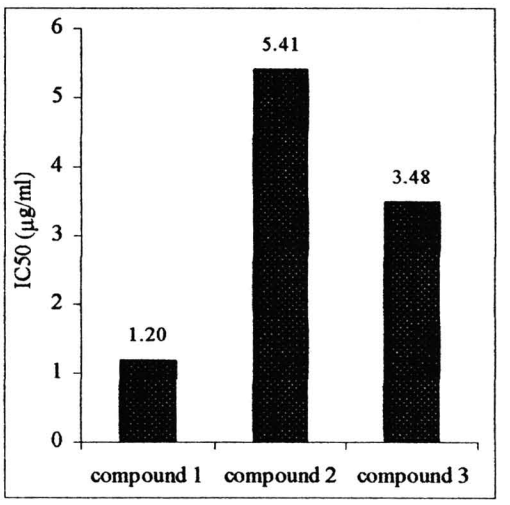

Fig. 10 The IC_{50} value (µg/ml) for the isolated compounds

Conclusion

The methanolic extract of Thai guava leaves has high antioxidant activity. The active principles isolated from the methanolic extracts are three flavonoids with different levels of antioxidant power. The structure elucidation study reveals that the three active principles are quercetin, quercetin-3-*O*-glucopyranoside and morin

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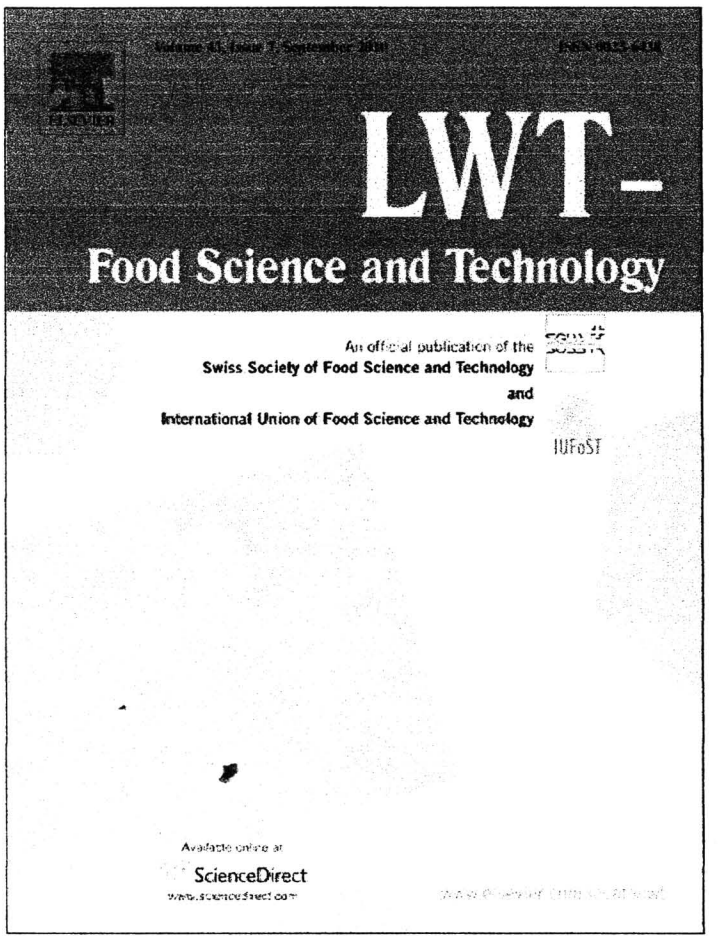
Reprint

เรื่อง

Factors influencing antioxidant activities and total phenolic content of guava leaf extract



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Factors influencing antioxidant activities and total phenolic content of guava leaf extract

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ABSTRACT

The aim of this study is to investigate the influence of certain factors on the yield, antioxidant activity (AA) and total phenolic content (TPC) of guava leaf extract. The effects of pretreatment of leaf sample prior to extract, extraction method, and the leaf age were investigated. Folin–Ciocalteau was used to determine the TPC. Trolox equivalent antioxidant capacity (TEAC) and equilibrium concentration (EC) were used for evaluation of AA. Results indicated that ultrasonication is the most suitable method for guava leaf extraction as it yielded the extract with the significantly highest TPC and AA. Blanching followed by ice water cooling (BCD) was suggested for the pretreatment process of guava leaves. The study of leaf maturity demonstrated that the highest activity was from the young leaves. Hot water was the best solvent to extract the active principles. The extract of BCD pretreated young leaves, extracted by hot water exhibited the highest TPC and AA with the TEAC and EC values of 24.30 ± 0.50 and 20.41 ± 0.67 mM/mg, respectively. These values are 1.88 and 8.72 times higher than the synthetic antioxidant butylated hydroxy toluene (BHT) and 1.75 and 1.21 times higher than vitamin E, respectively. It was concluded that pretreatment and drying process, method of extraction and leaf maturity played important roles on the bioactive compounds and their antioxidant power of guava leaf extract.

1. Introduction

Antioxidants which are used nowadays are obtained mainly from two major routes; chemical synthesis and natural living source extraction. According to scientific research, severe toxicity caused by chemical synthetic antioxidants such as genotoxicity, carcinogenicity (Ito et al., 1986; Williams, Iatropoulos, & Whysner, 1999), or hepatotoxicity (Safer & Al-Nughamish, 1999) has been increasingly reported. Hence, the use of synthetic antioxidants is tending to decrease and needs replacement with other safer compounds. Meanwhile, natural antioxidants, derived mostly from their plants have been reported for high potential in prophylacsis and treatment of many degenerative diseases caused by chain oxidative reactions such as atherosclerosis, coronary heart disease, aging and cancer (Finkel & Holbrook, 2000). An inverse relationship has been reported between consumption of natural antioxidants and mortality from such degenerative diseases (Govindarajan, Vijayakumar, & Pushpangadan, 2005). Therefore, the search for non-toxic high potential natural antioxidants is of increasing interest. Many authors present their work about antioxidant activities from different plant sources (Cai, Luo, Sun, & Corke, 2004; Okonogi, Duangrat, Anuchpreeda, Tachakittirungrod, & Chowwanapoonpohn, 2007). Guava (*Psidium guajava*), one of the most effective edible plants, has long been used as a traditional medicine. It has been demonstrated to have several biological activities such as antidiabetic (Oh et al., 2005), anticough, antibacterial (Jaiarj et al., 1999) and antispasmodic actions (Lozoya et al., 2002). Recently, it has been reported to possess high potential for antioxidant activity (Guo et al., 2003). Besides the plant cultivar, other factors have been revealed to influence the quality of plant extracts. The use of high temperature in the drying process or in the extraction of the plant sample is generally expected in order to prevent the compounds from deteriorating due to moisture content or existing enzyme, resulting in extended shelf life or higher activity of the active compounds. For example lycopene, a heat-stable natural antioxidant found in tomato fruits (Nicoli, Anese, & Parpinel, 1999; Stahl & Sies, 1992) demonstrated a higher antioxidant activity through thermal treatments of tomato fruits (Chen, Wu, Tsai, & Liu, 2000). However, enzymatic and/or non-enzymatic processes that may occur during the drying process may lead to significant changes in the composition of photochemicals (Capecka, Marczezek, & Leja, 2005). In addition, the high temperature may accelerate the degradation

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reaction of certain compounds (Burg & Fraile, 1995; Tomaino et al., 2005), resulting in decreasing shelf life or activity of the active principles. Several vitamins, for example, show rapid degradation with the increasing temperature. The age of the plant materials is also one of important factors that should be considered. Klavsen and Madsen (2008) revealed that some mechanisms and photosysthesis in *Littorella uniflora* were depended on the leaf age of plant. Hence, to obtain plant extracts with the highest potential, there is a need to understand precisely those factors which might affect the interested activity. Although guava has been reported by many authors for its promising antioxidant activity, no research has been carried out to investigate the factors which affect antioxidant activity in guava. This study was the first to determine the influence of parameters in the extraction process on the antioxidant activity and total phenolic content of guava. Those parameters were involved mainly in the difference between extraction methods, the pretreatment and drying process. Since our previous study found that the leaf was the most effective part of this plant (Tachakittirungrod, Okonogi, & Chowwanapoonpohn, 2007), in this study the influence of leaf maturity was also investigated as well as the polarity of solvent used in the extraction process.

2. Materials and methods

2.1. Chemicals

Gallic acid, 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ferrous sulfate ($FeSO_4 \cdot 7H_2O$), ferric chloride ($FeCl_3 \cdot 6H_2O$), and potassium persulfate were from Sigma (MO, USA). Trolox was obtained from Aldrich Chemical Company (Steinheim, Germany) and 2,4,6-tri(2-pyridyl)-S-triazine (TPTZ) was from Fluka Chemicals (Buchs, Switzerland). Ethanol, ethyl acetate and hydrochloric acid were from Merck (Darmstadt, Germany). All other reagents were of the highest quality grade available.

2.2. Plant materials

Plants generally exhibit chemical composition change according to the cultivar and environmental conditions such as climate and soil (Thaipong, Boonprakob, Cisneros-Zevallos, & Byrne, 2005). To fix the variations of cultivar, only one flesh clone of guava (*P. guajava*) harvested from Sopa garden (Chiang Mai, Thailand) was used throughout this study. The harvesting time was also limited to the second half of July, 2007 in order to overcome the variations due to the environmental condition.

2.3. Extract preparation

2.3.1. Effect of pretreatment

In this study, the middle age leaf (ML) was used according to its location majority in the plant and its stage of maturity. The sample was divided into three groups and each was treated before drying as followed: blanching in boiling water for 30 s and after that immersion in ice water for 15 min (BCD); blanching in boiling water for 30 s and then exposure to 30 °C for 15 min (BD); and no treatment group (FD). After that each group was divided into two categories according to the drying conditions of 30 °C for 72 h (drying #1) and 50 °C for 20 h (drying #2). Each dried leaf sample was pulverized into fine powder then subjected to extraction process by ultrasonication for 10 min × 3 using ethanol as the extraction solvent. The solvent was removed by using rotary evaporation under vacuum at 45 °C. The obtained extracts were kept in light-protected containers at 4 °C until total phenolic content and antioxidant activity were analyzed.

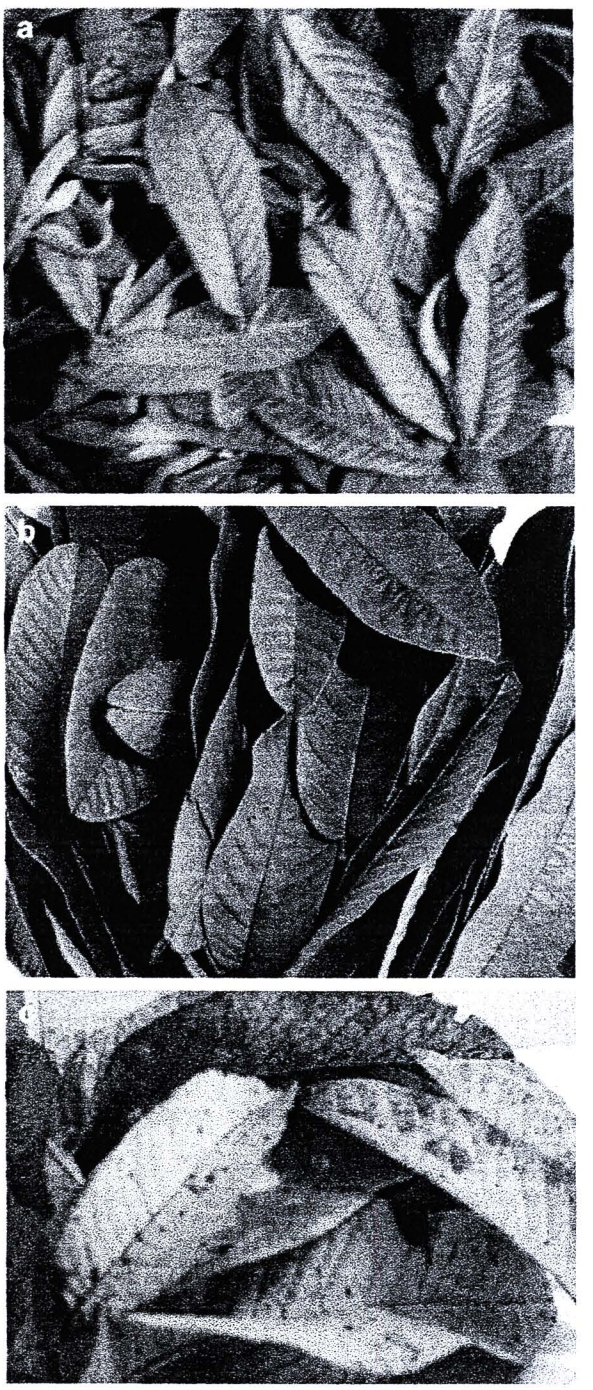

Fig. 1. Macroscopic characters of guava leaves in different stage of maturity; young age leaf (a), middle age leaf (b) and old age leaf (c).

2.3.2. Effect of extraction method

In this study, the extraction solvent used was ethanol and the guava leaf sample was the BCD with drying #2 pretreated leaf powder of ML sample. Four different extraction techniques were used: maceration at ambient temperature for 24 h × 3 without stirring; maceration at ambient temperature for 24 h × 3 with 200 rpm stirring; ultrasonication at ambient temperature for 10 min × 3; soxhlet extraction at the boiling point of ethanol for 4 h. The crude extract was obtained by evaporation of the solvent under vacuum at 45 °C. The extracts were kept in light-protected

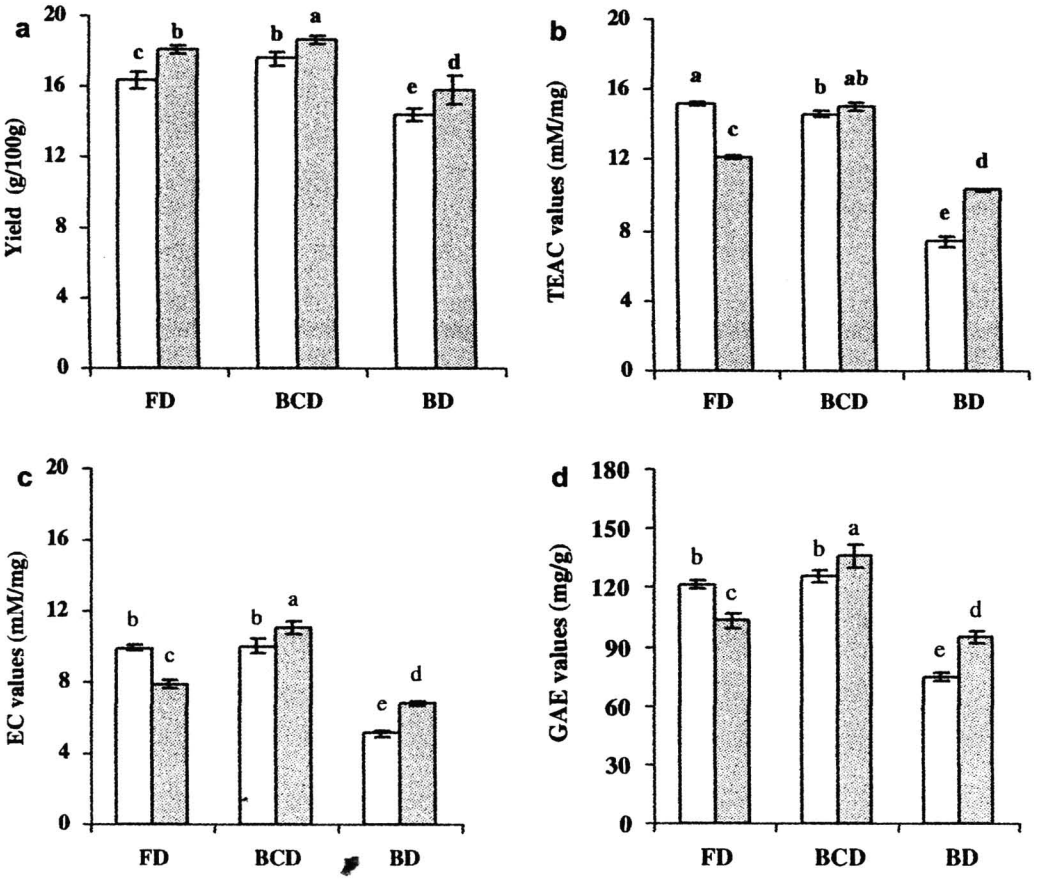

Fig. 2. The yield (a), free radical scavenging activity (b), reducing power (c), and total phenolic content (d) of guava leaf extracts obtained from various pretreatments; blanching for 30 s then immersing in ice water for 15 min (BCD), blanching for 30 s then exposing to 30 °C for 15 min (BD), no treatment (FD), drying at 30 °C for 72 h (white column), and drying at 50 °C for 20 h (gray column). Different lower letters on the histograms imply the significant differences from three replications ($p < 0.05$).

containers at 4 °C until total phenolic content and antioxidant activity were analyzed.

2.3.3. *Effect of leaf maturity and polarity of solvent*

In this study, guava leaves were divided into three groups according to the leaf stage of maturity. The young age leaf (YL) referred to the ones from the apex of the branch, with light green color. The middle age leaf (ML) regarded to the complete mature stage leaves with an intense green color. The old age leaf (OL) was mostly from the branch position close to the stem with yellow color. The macroscopic features of the three leaf categories used in this study are shown in Fig. 1. Pretreatment of BCD with drying #2 was applied to the leaf samples before individual batch extraction by ultrasonication for 10 min × 3 for each solvent. Three solvents with different polarities: ethyl acetate, absolute ethanol and hot water were used for extraction. The extract was obtained after evaporation of the filtrate of each solvent under vacuum at 45 °C. The extracts were kept in light-protected containers at 4 °C until total phenolic content and antioxidant activity were analyzed.

2.4. *Determination of total phenolic content*

Guava leaves have been reported to contain many different kinds of phenolic compounds (Matsuo, Hananure, Shimoi, Nakamura, & Tomita, 1994). The determination of individual phenolic compounds by HPLC is of complicated performance and presented only 50–60 percent of total phenolic content (Ferreira et al., 2002; Scalbert & Williamson, 2000). In this study, the determination of total phenolic content was done by the Folin–Ciocalteu method which was introduced by Sato et al. (1996), with some modification. Briefly, crude extracts were dissolved in absolute ethanol at a final concentration of 0.2 mg/mL prior to test. An aliquot of 20 μL of the ethanolic solution of each sample was mixed with 45 μL of Folin–Ciocalteu reagent. After 3 min, 135 μL of 2 g/100 mL sodium carbonate was added. The mixture was allowed to stand for 2 h at room temperature before the absorbance was measured spectrophotometrically at 750 nm. The experiment was done in triplicate. Gallic acid (0–40 μg/mL) was used as the standard for the calibration curve. The total phenolic content of the sample was expressed as gallic acid equivalent (GAE) to 1.0 g of extract.

2.5. *Determination of antioxidant activity*

Many compounds are known to exist in guava extract (Huang & Zhang, 2004). The interaction of those compounds contributes to overall antioxidant activity and it is difficult to measure total antioxidant activity on the basis of individual components (Pinelo, Manzocco, Numez, & Nicoli, 2004). Therefore, the antioxidant activity expressed in this study was in the form of total activity. Two potential methods, ABTS and FRAP, based on different principles were selected for measurement of antioxidant activity of the test extracts. ABTS measures the ability of the natural antioxidants to scavenge the free radicals whereas the FRAP measures the total reducing capacity of the test compounds. Therefore, different mechanisms of antioxidant actions can be expected from these two methods. The values of TEAC and EC from ABTS and FRAP methods

Table 1
The yield, antioxidant activities, and total phenolic content of the extracts obtained from different extraction methods.

Extraction method/ positive control	Yield (g/100 g)	TEAC (mM/mg)	EC (mM/mg)	GAE (mg/g)
Maceration without stirring	15.6 ± 0.8bc	9.41 ± 0.26d	5.63 ± 0.23d	80.28 ± 1.58d
Maceration with stirring	16.4 ± 0.6b	13.18 ± 0.30c	7.75 ± 0.10c	94.08 ± 3.54c
Ultrasonication	18.7 ± 1.0a	15.06 ± 0.23a	11.03 ± 0.35a	136.02 ± 5.55a
Soxhlet	14.7 ± 0.3c	14.01 ± 0.20b	8.44 ± 0.20b	125.94 ± 3.08b
BHT	–	12.91 ± 0.20	2.34 ± 0.07	–
Vitamin E	–	13.87 ± 0.06	16.86 ± 0.22	–

Values are mean ± SD ($n = 3$) followed by different letters imply the significant differences ($p < 0.05$) between values in the same column. Butylated hydroxyl toluene (BHT) and vitamin E are the positive controls.

respectively indicate the power of activity; the higher the values, the higher the antioxidant activity.

2.5.1. ABTS assay

The ABTS assay was determined according to our previous procedure with some modifications (Tachakittirungrod et al., 2007). Briefly, the free radical ABTS solution was diluted with absolute ethanol to obtain the absorbance of 0.7 ± 0.1 units at 750 nm. The crude extracts were diluted appropriately with absolute ethanol to obtain 20–80% inhibition of the blank absorbance. An aliquot of 20 μL of the ethanolic solution of each sample was added to 180 μL of ABTS free radical cation solution. The mixture was left to stand for 5 min at ambient temperature and the absorbance was immediately recorded spectrophotometrically at 750 nm using a microtiter plate reader (Biorad 680, USA). All measurements were carried out in triplicate. Standardized trolox dissolved in absolute ethanol at a final concentration from 0 to 50 μM was used as the calibration curve. The results were expressed as millimolar concentration of a trolox solution whose antioxidant capacity is equivalent to 1.0 mg of extract.

2.5.2. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the method introduced by Guo et al. (2003), with some modification. Briefly, the FRAP reagent was prepared from a sodium acetate buffer (0.3 mol/L, pH 3.6), 10 mmol/L TPTZ solution in 40 mmol/L HCl and 20 mmol/L $FeCl_3$ solution in volume proportions of 10:1:1, respectively. An aliquot of 20 μL of the ethanolic solution of each extract was mixed with 180 μL of FRAP reagent. The absorbance of the reaction mixture was then recorded at 595 nm after 5 min by a microtiter plate reader. The assay was carried out in triplicate. The standard curve was constructed using $FeSO_4$ solution (0–80 μM). The reducing power was expressed as an equivalent capacity (EC) which was the ability to reduce ferric ions expressed as mM $FeSO_4$ equivalents per gram of the extract.

2.6. Phenolic compound analysis by high performance liquid chromatography (HPLC)

Phenolic compounds in the extracts obtained from guava leaf at any maturity stage were identified using a method introduced by Wu, Hsieh, Wang, and Chen (2009), with some modification. Briefly, HPLC analyses were performed using an HP1100 system with a thermostatically controlled column oven and a UV detector set at 280 nm (Hewlett-Packard, Palo Alto, CA). A reversed phase column Zorbax SB-C18 (250 × 4.6 mm i.d., 5 μm, Algilent, USA) was connected with a Zorbax SB-C18 guard column (125 × 4.6 mm i.d., 5 μm, Algilent, USA). A gradient of acetic acid-water (2:98, v/v) as solvent A and acetic acid-water (0.5:95.5, v/v) with acetonitrile (50:50, v/v) as solvent B was used to elute samples at ambient temperature. The elution gradient program with a ratio of B to A was as follows: from 0 to 1 min (0:100–5:95, v/v), from 1 to 15 min (5:95–10:90, v/v), from 15 to 35 min (10:90–35:65, v/v), from 35 to 40 min (35:65–40:60, v/v), from 40 to 55 min (40:60–55:45, v/v), from 55 to 65 min (55:45–80:20, v/v) and hold for 5 min, from 70 to 80 min (80:20–100:0, v/v) at a flow rate of 1 ml/min and 20 μL of sample was injected. Samples and mobile phases were filtrated through a 0.45 μm Millipore filter, type GV (Millipore, Bedford, MA) prior to HPLC injection. Each sample was analyzed in triplicate. Phenolic standards of interest such as gallic acid, catechin, ellagic acid, morin, and quercetin were used for identification of phenolic compounds in guava leaf extracts. The identified phenolic compounds were quantified on the basis of their peak area and compared with calibration curves obtained with the corresponding standards and then expressed as mg/g of extract.

Table 2
The extract yield and content of gallic acid, ellagic acid, and quercetin in the extracts obtained from different leaf maturities and solvents.

Extracts	Yield (g/100 g)	Gallic acid (mg/g)	Ellagic acid (mg/g)	Quercetin (mg/g)
Young age leaf				
Ethyl acetate extract	4.6 ± 0.6e	1.96 ± 0.14d	3.67 ± 0.17ef	13.86 ± 0.44c
Ethanol extract	10.6 ± 0.7c	3.67 ± 0.12b	13.82 ± 0.34c	26.12 ± 0.98a
Hot water extract	21.0 ± 0.7a	6.27 ± 0.23a	36.68 ± 1.16a	14.73 ± 0.70bc
Middle age leaf				
Ethyl acetate extract	7.7 ± 0.7d	0.93 ± 0.02f	2.74 ± 0.45f	12.25 ± 1.68d
Ethanol extract	18.7 ± 0.5b	1.90 ± 0.13d	2.61 ± 0.67f	12.08 ± 1.08d
Hot water extract	18.1 ± 0.9b	2.43 ± 0.11c	17.51 ± 0.85b	11.07 ± 0.20d
Old age leaf				
Ethyl acetate extract	11.2 ± 0.5c	1.17 ± 0.06e	4.76 ± 0.83de	16.07 ± 0.31b
Ethanol extract	20.1 ± 0.8a	1.96 ± 0.20d	5.21 ± 0.31d	14.47 ± 0.81c
Hot water extract	17.8 ± 1.0b	3.72 ± 0.22b	13.20 ± 0.21c	10.85 ± 0.15d

Values are mean ± SD ($n = 3$) followed by different letters imply the significant differences ($p < 0.05$) between values in the same column.

2.7. Statistical analysis

To determine statistical difference between means ($p < 0.05$), ANOVA and Duncan's test were calculated using SPSS statistical software package v. 10. Results were expressed as mean values ± SD.

3. Results and discussion

3.1. Effect of pretreatment

The pretreatment is the performance of the fresh guava leaf samples before extraction. In this study, the pretreatment was varied in blanching and drying conditions. Six different extracts were obtained according to the various pretreatment conditions and the yields are presented in Fig. 2(a). The results indicated that each treatment yielded different amounts of extract ranging from 14.5 to 18.7 g/100 g of dried leaf powder. The highest yield was obtained from the extract pretreated with blanching – ice water immersing (BCD) with drying at 50 °C (#2). It was noted that the drying process affected the yield of the extracts. Drying at 50 °C for 20 h (drying #2) yielded much higher amounts than drying at 30 °C for 72 h (drying #1). The difference in antioxidant activity and total

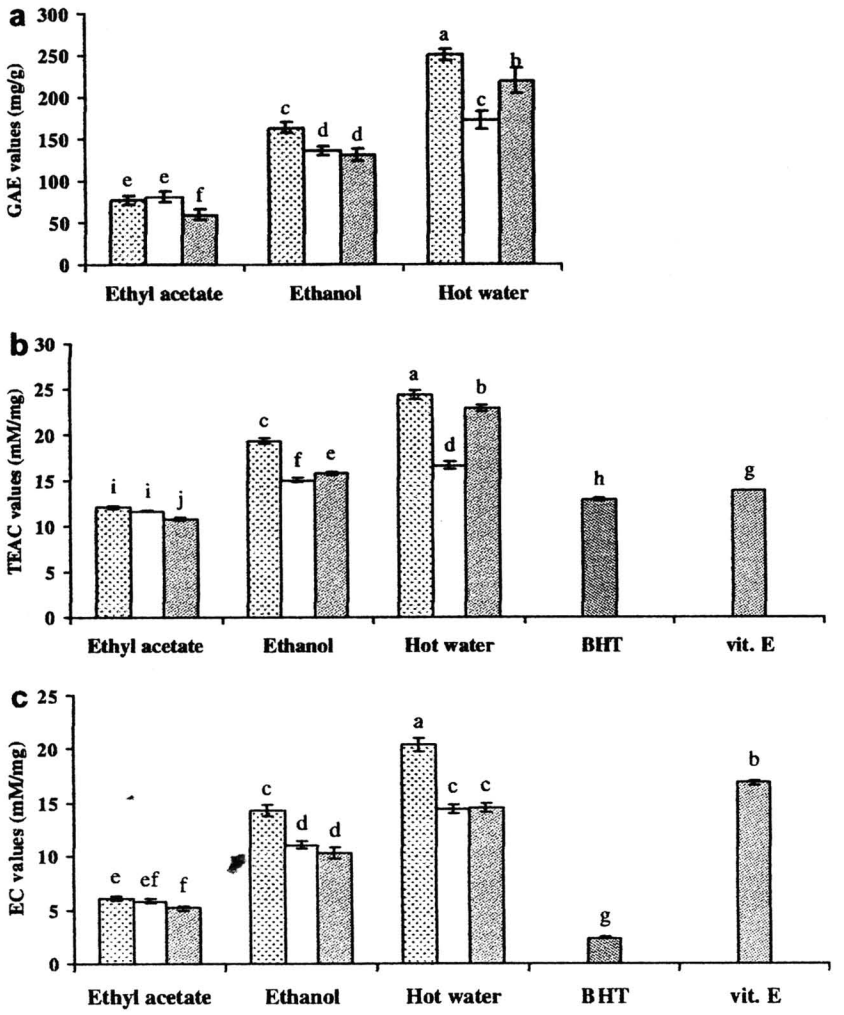

Fig. 3. Total phenolic content (a), free radical scavenging activity (b), and reducing power (c) of guava leaf extracts obtained from different extraction solvents and leaf stage of maturity; young age leaf (dotted column), middle age leaf (white column) and old age leaf (gray column) in comparison with two positive controls, butylated hydroxyanisole (BHT) and vitamin E (vit. E). Different lower letters on the histograms imply the significant differences from three replications ($p < 0.05$).

phenolic content among the extracts of these conditions was obvious as shown in Fig. 2. High TEAC values were obtained from the extract of BCD with either drying #1 or drying #2 and FD drying #1 as shown in Fig. 2(b). This result was similar to the reducing power as EC values are shown in Fig. 2(c). The extract from BD with drying #1 yielded the lowest scavenging and reducing activities. When total phenolic content was analyzed, results showed that this BD treated sample possessed the least content of total phenolic compounds whereas the BCD treated leaves with drying #2 showed the highest total phenolic content as shown in Fig. 2(d). This result suggested that a suitable pretreatment condition is a necessary process for guava leaves. The high temperature was considered necessary to stop certain enzymes that cause degradation of the active antioxidant principles in guava leaves. However, the heat-labile substances may be destroyed by high temperature (Capecka et al., 2005; Castro et al., 2008). The results of BCD and BD pretreatment indicate that long heating could affect the heat-labile active compounds in guava leaves. The BCD pretreatment, in which the heat was stopped promptly after blanching by an ice water bath, prevented those heat-labile antioxidants from degradation caused by prolonged heat. Hence, the antioxidant activity and total phenolic content of the guava extract with BCD pretreatment was much higher than that of BD pretreatment. This BCD pretreatment therefore was considered to be a necessary process for high quality guava leaf extract. Differences between drying #1 and drying #2 showed some influence on antioxidant activity and total phenolic content of the FD and BD pretreated samples, this effect was only slightly observed with the BCD samples. Interestingly, when all activities of crude extracts were considered with the pretreatment process, it was found that the extract of no blanching (FD) drying at 30 °C (#1) had significantly higher activities than that of FD drying at 50 °C (#2), whereas for the extract of both types of blanching (BCD and BD) drying #2 revealed significantly higher activities than those with drying #1. From these results, it is suggested that a drying temperature of 50 °C might stimulate the activation of enzymes, such as a moderately heat-stable enzyme polyphenol oxidase (PPO), which then led to significant changes in the phenolic compounds of guava leaves. These results agree with Liu et al. (2007) who reported that activation of PPO in litchi pericarp at near 50 °C was higher than that of 30 °C. Capecka et al. (2005) published that the drying process could increase total phenolics in oregano and peppermint. In addition, Zheng and Wang (2001) suggested that it is difficult to explain these effects because antioxidant activities might be affected from synergistic and antagonistic actions of constituents in plant extracts. However, the results of our present study suggested that to obtain

(Wang & Weller, 2006). This result led to the lowest yield when compared with other methods and the extract showed lower activity when compared to ultrasonication technique (Table 1).

Considering the effects of stirring, the results revealed that the extract obtained by stirring showed significant higher yield and activity than that without stirring. Furthermore, stirring demonstrated less potential to achieve the extract with the strongest activities when compared with ultrasonication and soxhlet techniques. Regarding the length of time, ultrasonication, soxhlet and both macerations required the extraction time as follows: 30 min, 4 h, and 72 h, respectively. This indicated that ultrasonication was the most efficient process not only for obtaining the most optimizing activities but also for reducing time consumption. Hence, ultrasonication is considered to be the extraction method of choice for guava leaves.

3.3. *Effect of extraction solvent and leaf maturity*

The crude extracts obtained from individual solvent extraction of three different leaf maturity; young (YL), middle (ML) and old age leaves (OL) yielded in the range of 4.6–21.0 g/100 g of dried guava leaf powder as shown in Table 2. The solvents used in this experiment possessed distinct polarity. The extracts obtained from water had the highest polarity and those from ethyl acetate and ethanol were of the lowest and moderate polarity respectively. The hot water extract of YL showed the highest yield when compared to the others indicating that the polar compounds existed in YL more than in the other two groups. The ethanolic and hot water extracts from ML showed similar relatively high yields of 18.7 and 18.1 g/100 g respectively. This result indicated that both high and moderate polar compounds existed in high quantities in ML. The GAE values which indicated total phenolic content of these extracts were demonstrated in Fig. 3. The results revealed that different solvents could have different potentials to extract the active phenolic compounds from the guava leaves. Hot water was the most effective solvent among the three kinds of solvent used. According to the active polyphenolic compounds in guava leaves identified by Chen and Yen (2007) and the results from this study, it is obvious that hot water is a better solvent to extract the polyphenolic compounds existing in guava leaves. When the leaf maturity was compared, it was found that hot water extract of YL possessed the highest total phenolic content followed by that of OL and ML, respectively. GAE values of the ethanol extracts revealed that total phenolic content of YL > OL and ML whereas that of YL and ML > OL in ethyl acetate extract as shown in Fig. 3(a). The antioxidant activity of all samples expressed as TEAC and EC values is shown in Fig. 3(b) and (c), respectively. Results revealed that the extracts obtained from hot water possessed the highest activity. Comparing of leaf age, the hot water extract of YL exhibited the highest antioxidant power followed by that of OL and ML respectively. This might be in accordance with the increased total phenolic content in the respective extracts. The TEAC and EC values of the hot water extract obtained from YL were found to be 24.30 ± 0.50 and 20.41 ± 0.67 mM/mg, respectively. These values are 1.88 and 8.72 times higher than the synthetic antioxidant butylated hydroxy toluene (BHT) and 1.75 and 1.21 times higher than vitamin E, respectively. This result indicates the extremely high antioxidant potential of young guava leaves.

3.4. *HPLC analysis of phenolic compounds in guava leaf extract at different maturity*

Phenolic compounds of YL, ML, and OL extracted from various solvents were determined using HPLC. The sample peaks were identified by matching retention time (t_R) of phenolic standards of interest, namely gallic acid ($t_R = 6.45$), catechin ($t_R = 24.14$), ellagic acid ($t_R = 39.85$), morin ($t_R = 55.25$), and quercetin ($t_R = 59.77$) and quantified by external standard methods. The calibration curves of the standards were linear with R^2 values of 0.9973, 0.9991, 0.9873, 0.9908, and 0.9918 for gallic acid, catechin, ellagic acid, morin, and quercetin, respectively. The chromatogram of these standards is depicted in Fig. 4(a). Results indicate that certain phenolic compounds; gallic acid, ellagic acid, and quercetin exist in guava leaf extracts at different amounts depending on the leaf age and the extracted solvent used, as demonstrated in Table 2. This result was in agreement with Hsieh, Lin, Yen, and Chen (2007) and Wu et al. (2009), where gallic acid and quercetin were identified in guava leaf extract. Among the three compounds, ellagic acid showed the highest content in the hot water extracts. Additionally, the amount of gallic acid and ellagic in water extracts was significantly higher

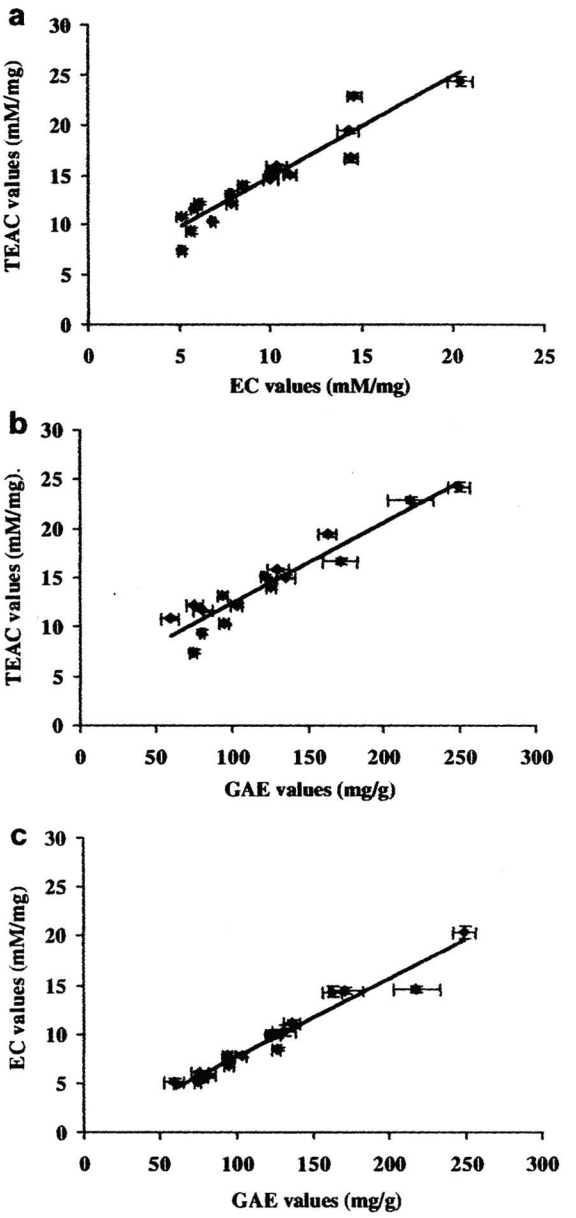

Fig. 5. Correlation between free radical scavenging activity *versus* reducing power (a), free radical scavenging activity *versus* total phenolic content (b), and reducing power *versus* total phenolic content (c) of guava leaf extracts. The calibration equation of each was $y = 1.0045x + 4.7486$ ($R^2 = 0.8940$), $y = 0.0823x + 4.2292$ ($R^2 = 0.9062$), and $y = 0.0793x - 0.1910$ ($R^2 = 0.9496$), respectively.

than that of ethanol and ethyl acetate extracts in all (guava) leaf ages. In contrast, the highest amount of quercetin was found in ethanol extract of YL and the least in the water extract of OL. It was noted that the hot water extract of YL revealed the highest total phenolic contents when considering the sum of content of gallic acid, ellagic acid, and quercetin and its chromatogram is shown in Fig. 4(b). It was reported that a number of phenolic compounds including gallic acid, ellagic acid, and quercetin showed high antioxidant activities (Gulsen, Makris, & Kefalas, 2007; Iacopini, Baldi, Storchi, & Sebastiani, 2008; Srivastava, Jagan Mohan Rao, & Shivanandappa, 2007); therefore, medicinal plant extracts consisting of high amounts of these compounds could act as a potent natural antioxidant.

3.5. Correlation of antioxidant activities and total phenolic content

The correlation between TEAC and EC values of all samples is shown in Fig. 5(a). It was found to have a high correlation, indicated that guava leaf extract possessed antioxidant mechanisms in both ways; the free radical scavenging and the reducing power. Moreover, this result suggests that the antioxidant activity of the obtained guava leaf extracts might be mainly from the major principles of the same group. To clarify this, the correlation of total phenolic content against free radical scavenging activity and reducing power of all samples was determined and the results were shown in Fig. 5(b) and (c) respectively. It was demonstrated that very high linear correlation was obtained in both curves. The R^2 of GAE *versus* TEAC was 0.9062 whereas that *versus* EC was 0.9496. These results explained that guava leaf extracts from all conditions possess higher antioxidant capacities with higher total phenolic contents. From this point of view, it could be considered that the attributed phenolic compounds in guava leaf extracts were the major group that contributed strongly to the antioxidant activities of the extracts.

4. Conclusion

This study demonstrated the effects of extraction process parameters and leaf maturity on antioxidant activity and total phenolic content of guava leaf extracts. The results suggest that the pretreatment process of the guava leaf sample prior to extraction and the extraction method were the most important factors that affected the amount of active principles and antioxidant activity of the extracts. Ultrasonication was found to be the best method, followed by soxhlet extraction and maceration. In addition, the maturity stage of guava leaves and the extraction solvent were other important factors. Young guava leaves were the best, followed by the old and middle leaves, respectively. It was demonstrated that guava leaf extract could exhibit antioxidant activity through both mechanisms of free radical scavenging and reducing power, which contributed to the total phenolic compounds existing in the extracts. It was concluded that to obtain the most effective natural antioxidant from guava, the young leaves should be used with the best pretreatment condition of BCD with drying #2. Hot water was the best solvent to extract the active principles. Finally, ultrasonication was suggested to be the extraction method of choice. The knowledge gained from this study is expected to be beneficial for producing the extraction of natural antioxidants from guava leaves, not only in the small scale but also for large-scale production in commercial industry.

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