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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; externally, 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.

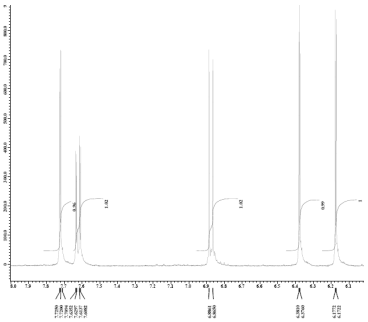

Fig. 1 ^{1}H NMR spectrum of compound **1**

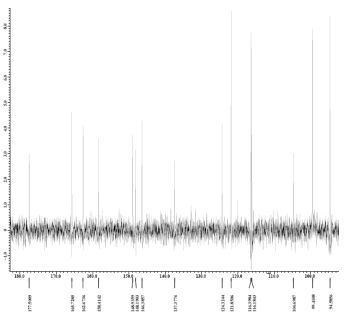

Fig. 2 ^{13}C NMR spectrum of compound **1**

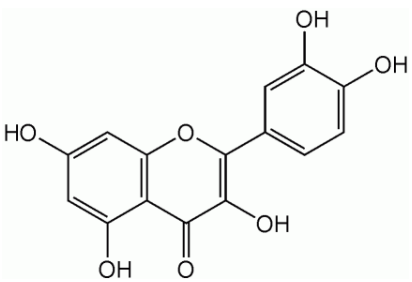

Fig. 3 Chemical structure of compound **1**

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.

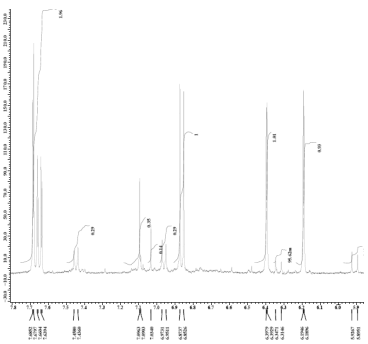

Fig. 4 ^{1}H NMR spectrum of compound **2**

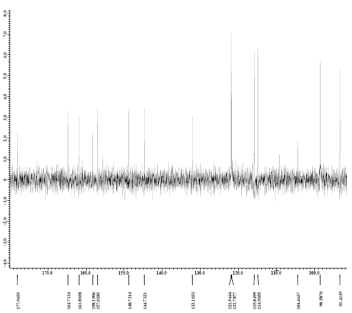

Fig. 5 ^{13}C NMR spectrum of compound **2**

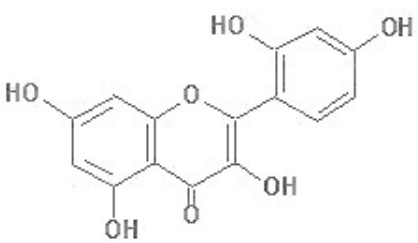

Fig. 6 Chemical structure of compound **2**

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.

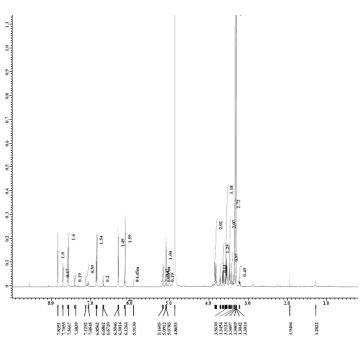

Fig. 7 ^{1}H NMR spectrum of compound **3**

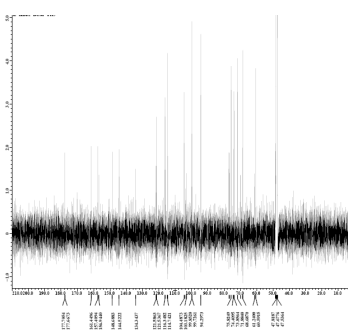

Fig. 8 ^{13}C NMR spectrum of compound **3**

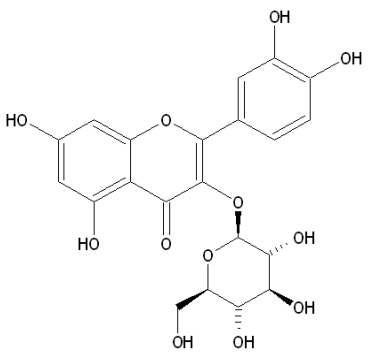

Fig. 9 Chemical structure of compound **3**

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.

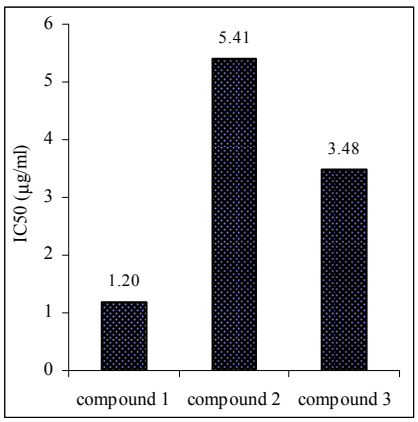

Fig. 10 The IC_{50} value (µg/ml) for the isolated compounds

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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