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Chemical composition and antioxidant activity of *Psidium guajava* L. leaves

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ABSTRACT

Guava, *Psidium guajava* L., is well-known tropic tree which is abundantly grown for fruit. It belongs to phylum Magnoliophyta, class Magnoliopsida and Myrtaceae family and has been found a lot in the Mekong Delta of Viet Nam. Antioxidant activity of different extracts from *Psidium guajava* L. leaves revealed that ethyl acetate and *n*-butanol extracts have the good activities. Processing of these fractions using repeated column and thin layer chromatographic techniques resulted in the isolation of four compounds, which have identified their chemical structures by spectroscopic techniques including avicularin (**1**), kaempferol (**2**), gallic acid (**3**), and protocatechuic acid (**4**). All the isolated compounds showed antioxidant activity using DPPH and ABTS scavenging assays. These results suggest that the leaves of guava would be useful natural sources of antioxidants.

1. INTRODUCTION

Vietnamese traditional medicine plays a major role in the healthcare system and many of plants, especially those found in the Mekong Delta, have been proved to possess medicinal functions. Among them, *Psidium guajava* L., a species belonging to the Myrtaceae family, has been found to be rich in nutrients, including vitamins and minerals that are significant for human health (Barbalho et al., 2012). The leaves of *Psidium guajava* L. are found to contain flavonoids, polyphenols, tannins, glycosides and terpenoids (Naseer et al., 2018; Biswas et al., 2013). It has been used traditionally as a medicinal plant throughout the world for a number of ailments. Guava extracts have the potential to act as powerful antioxidants against hepatic diseases as well as cancer. The vitamins present in guava help the body improve immunity (Ngbolua et al., 2018). There were several researches on pharmaceutical

application of *Psidium guajava* such as anti-inflammatory, antifungal, antibacterial, antidiarrheal, antioxidant, antidiabetic effects (Rawi et al., 2011; Mahfuzul et al., 2007). Therefore, in this study, the antioxidant activity of fractionated extracts and chemical investigation have been studied.

2. EXPERIMENT

2.1. Chemicals and instruments

Solvents utilized including *n*-hexane, chloroform, ethyl acetate, *n*-butanol, methanol (purity $\geq 99.0\%$), and ethanol 96% were purchased from Chemsol company (Viet Nam).

NMR spectra were recorded on a Bruker AM500 FTNMR spectrometer (Bruker, Karlsruhe, Germany) using TMS as an internal standard, Institute of Chemistry - Vietnam Academy of

Science and Technology, Hanoi, Viet Nam. TLC was performed on silica gel 60 F₂₅₄ (0.063–0.200 mm, Merck, Germany). The zones were detected using UV at 254 or 365 nm or a solution of FeCl₃/EtOH or H₂SO₄/EtOH. Column chromatography was performed on silica gel (240-430 mesh, Merck, Germany), ODS (70-230 mesh, Merck, Germany), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.2. Sample treatment and preparation

The leaves of *Psidium guajava* L. were collected in August 2020 from Vinh Long city and authenticated by Dr. Dang Minh Quan. A voucher specimen is kept at the Department of Biology, School of Education, Can Tho University, under the number: Psi200920.

The sample was then washed away from muds and dust; the rotten and damaged parts were also discarded. The raw materials were left to dry in the shade at room temperature for some days and then dried in an oven at about 50°C until well-dried.

2.3. Extraction and isolation

The well-dried leaves of *Psidium guajava* L. were ground into powder (5 Kg) which was then soaked in 96% ethanol at room temperature for four times (4×20 L) and filtered. The filtrate was concentrated under reduced pressure to give brown residue as crude ethanol extract (500 g). This crude extract was then extracted on flash column chromatography successively with *n*-hexane, ethyl acetate, *n*-butanol, and methanol, respectively to yield the corresponding extracts of *n*-hexane (110 g), ethyl acetate (75 g), *n*-butanol (145 g), and methanol extract (152 g).

The ethyl acetate extract was subjected to flash column chromatography (CC) on silica gel and eluted with various proportions of *n*-hexane and ethyl acetate (eluting with a linear gradient from 100:0 to 0:100, v/v) to obtain 15 fractions (EE1-15).

Fraction EE5 was further separated on a silica gel column, eluted with CHCl₃:MeOH (from 50:1 to 1:1, v/v) to yield eight subfractions (EE5.1-8). Finally, compound **1** (50 mg) was obtained from subfraction EE5.6.

Fraction EE6 was separated by a silica gel column and eluted with CHCl₃:MeOH (from 50:1 to 5:1, v/v) to yield 11 subfractions (EE6.1-11). Subfraction EE6.2 was further chromatographed on silica gel CC, eluted with CHCl₃:MeOH (from 10:1

to 1:1, v/v) to obtain eight subfractions (EE6.2.1-8). At last, compound **2** (20 mg) was obtained from subfraction EE6.2.2.

Similarly, subfraction EE7 was subjected on silica gel CC to collect subfraction EE7.7 and then was repeatedly purified by silica gel CC to obtain compound **3** (18 mg).

The *n*-butanol extract was subjected to a silica gel column and eluted with EtOAc:MeOH (from 100:1 to 10:1, v/v) to obtain seven fractions (BE1-7). Fraction BE7 was further separated on a silica gel CC and eluted with EtOAc:MeOH (from 50:1 to 0:100, v/v) to yield 12 subfractions (BE7.1-12). Subfraction BE7.8 was subjected repeatedly many times on silica gel CC to yield subfraction BE 7.8.4 and then further separation of this subfraction by silica gel CC gave compound **4** (22 mg).

Compound **1** was obtained as a yellow powder, m.p. 216-218°C.

¹H-NMR (500 MHz, CD₃OD), δ_H (ppm): 7.54 (1H, *s*, H-2'); 7.51 (1H, *d*, 8.5 Hz, H-6'); 6.92 (1H, *d*, 8.5 Hz, H-5'); 6.41 (1H, *s*, H-8); 6.23 (1H, *d*, 8.5 Hz, H-6); 5.49 (1H, *s*, H-1''); 4.35 (1H, *d*, 2.5 Hz, H-2''); 3.92 (1H, *m*, H-4''); 3.89 (1H, *t*, 4.5 Hz, H-3''); 3.52 (2H, *m*, H-5'').

¹³C-NMR (125 MHz, CD₃OD), δ_C (ppm): 180.0 (C-4); 166.1 (C-7); 163.1 (C-5); 159.4 (C-2); 158.6 (C-9); 149.9 (C-4'); 146.4 (C-3'); 134.9 (C-3); 123.1 (C-6'); 123.0 (C-1'); 116.9 (C-2'); 116.5 (C-5'); 109.6 (C-1''); 105.6 (C-10); 99.9 (C-6); 94.8 (C-8); 88.1 (C-4''); 83.3 (C-2''); 78.7 (C-3''); 62.6 (C-5'').

Compound **2** was obtained as light yellow crystals, m.p. 275-277°C.

¹H-NMR (500 MHz, CD₃OD), δ_H (ppm): 8.10 (2H, *d*, 9.0 Hz, H-2', 6'); 6.93 (2H, *d*, 9.0 Hz, H-3', 5'); 6.41 (1H, *d*, 2.5 Hz, H-8); 6.20 (1H, *d*, 2.0 Hz, H-6).

¹³C-NMR (125 MHz, CD₃OD), δ_C (ppm): 177.4 (C-4); 165.6 (C-7); 162.5 (C-5); 160.6 (C-4'); 158.3 (C-9); 148.1 (C-2); 137.1 (C-3); 130.7 (C-2', 6'); 123.8 (C-1'); 116.3 (C-3', 5'); 104.6 (C-10); 99.3 (C-6); 94.5 (C-8).

Compound **3** was characterized as a colorless solid, m.p. 158-160°C.

¹H-NMR (500 MHz, CD₃OD), δ_H (ppm): 7.10 (1H, *s*, H-2); 7.10 (1H, *s*, H-6).

¹³C-NMR (125 MHz, CD₃OD), δ_C (ppm): 170.6 (C-7); 146.4 (C-3,5); 139.5 (C-4); 122.3 (C-1); 110.3 (C-2,6).

Compound **4** was characterized as a light brown solid, m.p. 201-203°C.

¹H-NMR (500 MHz, acetone-*d*₆), δ_H (ppm): 7.53 (1H, *d*, 2.0 Hz, H-2); 7.48 (1H, *dd*, 8.5 and 2.0 Hz, H-6); 6.90 (1H, *d*, 8.5 Hz, H-5);

¹³C-NMR (125 MHz, acetone-*d*₆), δ_C (ppm): 167.6 (C-7); 150.7 (C-4); 145.6 (C-3); 123.6 (C-6); 123.1 (C-1); 117.5 (C-2); 115.7 (C-5).

2.4. Antioxidant activity

2.4.1. DPPH assay

In a 96-well microtiter plate, 50 μL of each sample was added to 6×10⁻⁵ M methanol solution of DPPH. After mixing with a vortex mixer, the mixture was incubated for 30 min at room temperature and the absorbance was measured at 517 nm. The DPPH radical scavenging activity was recorded as a percentage in comparison to the control. Vitamin C was used as a positive standard (Sharma et al., 2009).

2.4.2. ABTS^{•+} assay

The free radical 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS^{•+}) was produced by reacting ABTS solution in methanol (7 mM) with aqueous potassium persulfate solution (2.45 mM). The resulting mixture was allowed to stand in the dark for 12-16 hours before use. For aqueous extract ABTS was diluted with PBS (7.4 pH) to an absorbance of 0.700 ± 0.002 at 734 nm and trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) was used as standard for calibration curve (Nenadis et al., 2004)

2.5. Statistical analysis

The variation in a set of data has been estimated by performing one-way analysis of variance (ANOVA). Results were calculated from three independent experiments and repeated five times at each experiment, and are shown as mean ± SD, n = 3.

3. RESULTS AND DISCUSSION

3.1. Structural elucidation

The structures of isolated compounds were characterized by NMR spectra and by comparison with literature data.

The ¹H-NMR data of compound **1** indicated that ring A is 5,7-disubstituted, as shown by two *meta*-located protons at δ_H [6.41 (1H, *s*, H-8) and 6.23 (1H, *s*, H-6)]. The observation of an ABX system at δ_H [7.54 (1H, *s*, H-2'), 7.51 (1H, *d*, 8.5 Hz, H-6') and 6.92 (1H, *d*, 8.5 Hz, H-5')] has suggested a 3',4'-disubstituted ring B. A 3-*O*-substituted quercetin structures were indicated for compounds **1** due to the corresponding anomeric protons at δ_H 5.49 (1H, *s*) characteristic for arabinofuranosyl moiety. The structure of compound **1** was characterized as quercetin 3-*O*-α-L-arabinofuranoside (avicularin) by comparison with literature data (Dawidar et al., 2014).

Compound **2** was isolated as yellow crystals, m.p. 275-277°C. The ¹H-NMR spectrum of compound **2** appeared four signals of six aromatic protons in which there were two couples of chemical shift equivalent protons with ortho-coupling signals at δ_H [8.10 (2H, *d*, 9.0 Hz) and 6.93 (2H, *d*, 9.0 Hz)]; two meta-coupling signals at δ_H [6.41 (1H, *d*, 2.5 Hz) and 6.20 (1H, *d*, 2.0 Hz)]. ¹³C-NMR and DEPT spectra also exhibited signals of total 15 carbons of a flavone backbone. These carbons consisted of two signals of two couples of chemical shift equivalent carbons at δ_C 116.3 (2C) and 130.7 (2C), related to two couples of chemical shift equivalent protons in its ¹H-NMR spectrum. It proved that the four-hydroxyl substituted flavone had a symmetric aromatic ring. Moreover, the 1D-NMR spectral data of compound **2** were similar to those of kaempferol notified in the literature (Abdullah et al., 2012). From this evidence compound **2** was determined as 3,5,7,4'-tetrahydroxyflavone or kaempferol.

The chromatographic and physical properties along with NMR spectral data of compounds **3** and **4** were completely in agreement with the corresponding published data for gallic acid and protocatechuic acid (Khanh et al., 2016; Silva et al., 2015).

Four compounds **1-4** were isolated and identified from the leaves of *Psidium guajava* L., including avicularin (**1**), kaempferol (**2**), gallic acid (**3**) and protocatechuic acid (**4**) by analysis of their NMR spectra and HR-ESI-MS, and comparison with literature data (Figure 1).

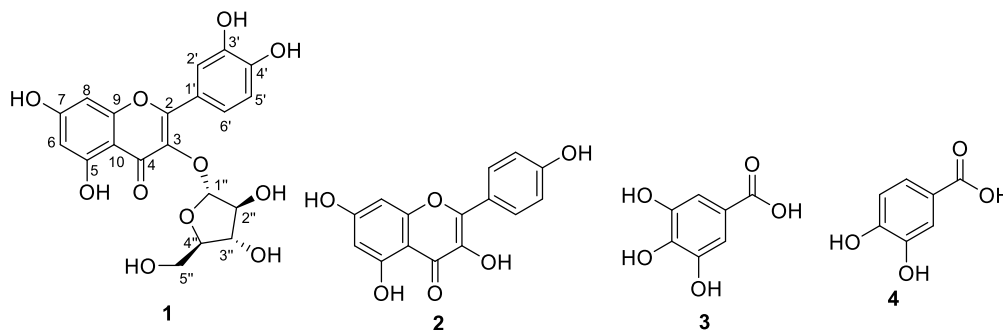


Figure 1. Chemical structures of compounds 1–4

3.2. *In vitro* antioxidant activity results

The free radical scavenging activities of isolated compounds were measured by DPPH and ABTS assays. The results are presented in Table 1. Ethyl acetate and *n*-butanol extracts of *P. guajava* L. exhibited high scavenging potential with $IC_{50} = 16.75 \mu\text{g/mL}$ and $15.64 \mu\text{g/mL}$; $11.76 \mu\text{g/mL}$ and $13.67 \mu\text{g/mL}$ for both DPPH and ABTS methods, respectively. All four isolated compounds exhibited the good ability of scavenging DPPH and ABTS radicals. Kaempferol exhibited the highest scavenging potential with $IC_{50} = 15.74 \mu\text{g/mL}$ and $13.09 \mu\text{g/mL}$ for DPPH and ABTS assays, respectively.

Table 1. IC_{50} values of fractionated extracts and isolated compounds

Extracts and isolated compounds	IC_{50} ($\mu\text{g/mL}$)	
	DPPH	ABTS
<i>n</i> -Hexane extract	368.62 ± 33.15	357 ± 28.18
Ethyl acetate extract	16.75 ± 2.75	15.64 ± 3.75
<i>n</i> -Butanol extract	11.76 ± 2.43	13.67 ± 3.03
Methanol extract	20.83 ± 3.15	24.37 ± 3.85
Avicularin	20.64 ± 1.35	22.83 ± 0.72
Kaempferol	15.74 ± 11.29	13.09 ± 5.87
Gallic acid	21.88 ± 4.73	18.43 ± 3.22
Protocatechuic acid	19.09 ± 2.68	14.47 ± 0.32
Vitamin C	3.82 ± 0.74	-
Trolox	-	3.47 ± 0.07

Antioxidant substrates from plants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation, and by other mechanisms. Natural antioxidant defense systems protect biomolecules against reactive oxygen species (ROS) induced damage. This protective effect can be enhanced by the use of antioxidant micronutrients (vitamins C and E, β -carotene), and by non-nutrient ingredients like phenolic and flavonoid compounds from plants.

A number of studies have demonstrated a correlation between the antioxidant effect and the structures of flavonoids and phenolic compounds. Polyphenolic compounds have received extensive attention because of their beneficial physiological role, including antioxidant, antimutagenic, and for other diseases caused by oxidative stress. The phenolic compounds are very important plant constituents because of their scavenging ability due to their hydroxyl groups. Flavonoids are a large group of ubiquitous molecules and possess antioxidant activities (Heim et al., 2002; Seyoum et al., 2006). The present study found a correlation between the isolated flavonoids as well as phenolic compounds and the antioxidant activity. These results revealed that the leaves of *Psidium guajava* L. has a powerful antioxidant activity and might be a good candidate for development as a novel natural antioxidant.

4. CONCLUSION

The results of this study revealed that from the leaves of *Psidium guajava* L., grown in Vinh Long city, four compounds have isolated and identified: avicularin (1), kaempferol (2), gallic acid (3), and protocatechuic acid (4). The structures of these compounds have been elucidated by modern spectroscopic method NMR and in comparison with the literature data. Furthermore, the antioxidant potentials of fractionated extracts and four isolated compounds were also evaluated through the DPPH and ABTS assays. The results indicated that ethyl acetate and *n*-butanol extracts have good antioxidant activities and all isolated compounds exhibited the ability of scavenging DPPH and ABTS radicals.

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