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Identification and quantification of flavonoids in *Carica papaya* leaf and peroxynitrite-scavenging activity

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ABSTRACT

Objective: To characterize the types, contents, and peroxynitrite-scavenging activities of flavonoids in the leaf of *Carica papaya* (*C. papaya*).

Methods: Chromatographic and spectroscopic techniques along with high performance liquid chromatography quantitative analysis and peroxynitrite-scavenging assay were performed to isolate and quantify flavonoid compounds in the flavonoid-rich fraction (BuOH fraction) derived from MeOH extract of *C. papaya* leaves and evaluate their peroxynitrite-scavenging activities.

Results: Seven flavonoids were isolated from the leaves of *C. papaya*, including quercetin 3-(2^G-rhamnosylrutinoside), kaempferol 3-(2^G-rhamnosylrutinoside), quercetin 3-rutinoside, myricetin 3-rhamnoside, kaempferol 3-rutinoside, quercetin, and kaempferol. All of the substances exhibited potent activities on peroxynitrite scavenging ($IC_{50} \leq 4.15$ μmol/L), which were stronger than the positive control, L-penicillamine (6.90 μmol/L). The content of kaempferol 3-(2^G-rhamnosylrutinoside) was significantly higher than other identified compounds (123.18 mg/g BuOH fraction and 7.23 mg/g MeOH extract).

Conclusions: The results of the present study demonstrate the potent antioxidant flavonoids of *C. papaya* leaf, with kaempferol 3-(2^G-rhamnosylrutinoside) as the major one.

1. Introduction

Papaya [*Carica papaya* Linn. (*C. papaya*)] is one of the most cultivated plants in tropical countries and the most popular and economically important species among the Caricaceae family [1]. Although only the fruits are generally used as commercial produces, in several Asian Pacific countries, the leaves are also used as traditional medicines for treatment of asthma, colic, fever, beriberi (India), malaria and dengue fever (Sri Lanka, Pakistan and Malaysia), and cancer (Vietnam and Australia) [2]. In Indonesia, the leaves are consumed as a vegetable, tea, and traditional medicine (called *jamu*) for many purposes such as increasing the appetite and breast milk production, reducing fever, and also for preventing and curing malaria [3].

Many *in-vitro* and *in-vivo* studies have demonstrated the medicinal properties of the extracts of papaya leaves including anti-dengue [4,5], anti-plasmodial [6], anti-cancer [2,7], anti-bacteria [8], hepatoprotection [9], anti-inflammatory [10] and antioxidant [11]. To date, few studies concerned in the investigation of the composition and biological activities of the constituents of *C. papaya* leaf. By using high performance liquid chromatography (HPLC)-based activity profiling, Julianti *et al.* [6] reported that flavonoids and alkaloids were

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the active constituents of *C. papaya* leaf and suggested the alkaloid carpaine as the major anti-plasmodial compound. Furthermore, Julianti *et al.* [3] also developed and validated a quantitative assay for determination of carpaine in papaya leaves.

In case of flavonoids, several studies have performed the identification of the flavonoid constituents in *C. papaya* leaves using ultra performance liquid chromatography-time of flight-electrospray ionization-mass spectrometry methods. Afzan *et al.* [12] identified four flavonoids in the *C. papaya* leaf extract including quercetin 3-(2^G-rhamnosylrutinoside), kaempferol 3-(2^G-rhamnosylrutinoside), quercetin 3-rutinoside, and kaempferol 3-rutinoside. Nguyen *et al.* [13] tentatively identified kaempferol β-D-glucopyranoside, luteolin β-D-glucopyranoside, myricetin 3-rhamnoside, quercetin and rutin. Tan *et al.* [9] found apigenin, kaempferol, quercetin, myricetin, isorhamnetin, catechin, hesperitin and naringenin. Using HPLC, Andarwulan *et al.* [14] detected the presence of quercetin, kaempferol and apigenin.

Although many flavonoid compounds have been identified from the leaf of *C. papaya*, there is no report on the quantitative analysis and biological activity evaluation of its flavonoids. Therefore, this study was aimed to isolate and quantify the flavonoids in *C. papaya* leaf along with evaluation of their biological activities. Since the biological actions of flavonoids are generally related to their free radical scavenging activity [15], peroxynitrite-scavenging assays were employed to deliver more information of the identified compounds and as a comparative study.

2. Materials and methods

2.1. Plant material

Leaves of *C. papaya* were collected from a papaya farm near Pelaihari City, South Kalimantan Province, Indonesia. Plant species was identified and authenticated at the Department of Agronomy, Lambung Mangkurat University, and the voucher specimen (No. C-23) was deposited in the herbarium of Laboratory of Natural Products, Department of Agro-industrial Technology, Lambung Mangkurat University. The collected leaves of *C. papaya* were immediately stored for 6 days in a dark room for airy drying and dried completely in an oven at 40 °C. Prior to extraction process, the dried plant material was coarsely powdered using a rotary grinder.

2.2. Reagents and instruments

For isolation and purification, two stationary phases of column chromatography, Silica gel 60 (0.063–0.200 mm, Merck, Germany) and octadecylsilane (ODS, 12 nm, S-75 μm, YMC Co., Ltd., Japan) were used. Thin layer chromatography (TLC) plates were silica gel 60G F254 and RP-18 F254s purchased from Merck KGaA (Darmstadt, Germany). All compounds were detected under UV (254 and 365 nm).

Melting points of the isolated compounds were determined on an Electrothermal 9100 melting point apparatus. Optical rotation was measured on a Perkin Elmer Model 341 polarimeter at 20 °C. IR spectra were measured on a Jasco FT/IR-4200 spectrometer in potassium bromide disks. The fast atom bombardment mass spectra were obtained on a VG ZabSpec instrument. The 1hydrogen-nuclear magnetic resonance (^{1}H-NMR) spectra (δ ppm, J in Hz) were recorded in dimethyl sulfoxide (DMSO)-d_6 on a Brucker AM-500 spectrometer (500 MHz), while carbon-13 NMR (^{13}C-NMR) spectra were recorded in the same solvent on a Brucker AM-500 spectrometer at 125 MHz with tetramethylsilane as an internal standard.

HPLC system used for quantification was the Varian HPLC system consisted of Prostar 210 pumps, Prostar 325 UV–vis detector, Shiseido Capcell PAK C18 column (5 μm, 4.6 mm × 250 mm, Japan). Column temperature was constantly maintained using a MetaTherm temperature controller. Two solvents as mobile phases CH_3CN and H_2O were the HPLC grade purchased from J.T. Baker Co. (Phillipsburg, NJ, USA). The reagents of diethylenetriaminepentaacetic acid (Sigma Co., St. Louis, MO, USA), dihydrorhodamine 123 (Molecular Probes, Eugene, OR, USA), and peroxynitrite (Cayman Chemicals Co., Ann Arbor, MI, USA) were used for the peroxynitrite-scavenging assay.

2.3. Isolation and identification

Dried leaves of *C. papaya* (750 g) were extracted three times with MeOH (6 L) under reflux at 70 °C for 5 h. The extract was filtered and concentrated on a vacuum rotary evaporator to give a viscous mass (86 g). This MeOH extract (80 g) was suspended in 800 mL H_2O and fractioned with 800 mL $CHCl_3$ three times. The residual aqueous layer was then fractionated with 800 mL BuOH three times, and the BuOH-soluble portion was concentrated to give the BuOH fraction (25.1 g). The BuOH fraction (24.0 g) was subjected to silica gel column chromatography (ø 50 mm × 35 cm, SiO_2) using $CHCl_3$–MeOH–H_2O (70:30:10, lower phase) as a mobile phase with flow rate of 4.5 mL/min. Totally, 186 fractions (each 50 mL) were collected and monitored by TLC and under UV 254 nm for combining into eight fraction groups (A–H). Fractions D, E and G were selected for next chromatographic steps in order to isolate several single compounds. Isolation procedures of single compounds from those three fractions were described in concise as below.

An ODS column (ø 20 mm × 32 cm) with MeOH–H_2O (65:35) as mobile phase was used to isolate three compounds from group D (#82–101, 2.38 g). By modulating the flow rate of 2 mL/min, 28 fractions (the volume of each fraction obtained by the column chromatography was 20 mL) were obtained and divided into three groups (D1, D2 and D3). Fraction group D3 (#25–28, 0.77 g) was purified to produce compound **7** (kaempferol, 25 mg). Using the same ODS column and MeOH−H_2O (60:40) mobile phase on a lower flow rate (1 mL/min), fraction group D2 (#06–24, 1.15 g) was chromatographed again, and resulted 22 fractions (D2a, D2b and D2c). From these fractions, two compounds were obtained; group D2a (#08–13) produced compound **5** (kaempferol 3-rutinoside, 44 mg), and group D2c (#18–22) produced compound **6** (quercetin, 19 mg).

Fraction group E (#102–114, 1.24 g) was subjected to ODS column (ø 20 mm × 32 cm) using MeOH−H_2O (55:45) mobile phase, and eluted at 1.5 mL/min. By this procedure, 20

fractions were produced and were classified into three groups (E1, E2 and E3). Fraction group E2 (#06–11) was selected by TLC as a single compound (compound **4**, myricetin 3-rhamnoside). Evaporation and recrystallization of group E2 produced a total of 35 mg of that compound.

More polar fractions, fraction group G (#127–154, 3.15 g) was chromatographed on the same ODS column (ø 20 mm × 32 cm) using MeOH−H_2O (50:50). By collecting every 10 mL for each fraction at flow rate of 1.5 mL/min, 32 fractions were obtained. Based on TLC analysis, those 32 fractions were grouped into six groups, G1 (#01–06), G2 (#07–10), G3 (#11–15), G4 (#15–21), G5 (#22–27) and G6 (#28–32). Purification and recrystallization of fraction group G2 and G4 produced a couple of flavonoid triglycosides, quercetin 3-(2^G-rhamnosylrutinoside) (compound **1**, 32 mg) and kaempferol 3-(2^G-rhamnosylrutinoside) (compound **2**, 76 mg). While, from fraction group G6, compound **3** (quercetin 3-rutinoside, 26 mg) was obtained.

2.4. HPLC quantitative analysis

The HPLC method used for quantitative analysis was described as follows. Two mobile phases, solvent A (H_2O with 0.1% acetic acid, v/v) and solvent B (CH_3CN with 0.1% acetic acid, v/v) were used in this method. Gradient elution of the mobile phases (A:B, v/v) was programmed as 85:15 at 0 min → 35:65 at 35 min (constant for 5 min) → 0:100 at 42 min (constant for 4 min) → 85:15 at 49 min (constant for 6 min). The flow rate and column temperature was set constantly at 1.0 mL/min and 40 °C, respectively. The detection wavelength was fixed at 254 nm and monitored during 40 min for each sample.

The standard stock solution (1 000 μg/mL) was prepared by dissolving each standard compound in MeOH and preserved at less than 4 °C. As standard compounds, quercetin 3-(2^G-rhamnosylrutinoside) and kaempferol 3-(2^G-rhamnosylrutinoside) were obtained from the isolation, and other compounds were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). The working standard solution was prepared by serial dilutions of the standard stock solution. Regression equation was determined by plotting the peak area (*y*) *vs*. concentration (*x*, μg/mL) at six minimum concentration solutions.

2.5. Peroxynitrite-scavenging assay

Assay for peroxynitrite-scavenging activity was performed using a modified method of Kooy *et al.* [16]. This method is to monitor high fluorescent rhodamine 123 rapidly formed from non-fluorescent dihydrorhodamine (DHR) 123. Rhodamine buffer (pH 7.4) consisted of 50 mmol/L sodium phosphate dibasic, 50 mmol/L sodium phosphate monobasic, 90 mmol/L sodium chloride, 5 mmol/L potassium chloride, and 100 μmol/L diethylenetriamine pentaacetic acid. Final concentration of DHR 123 was 5 μmol/L. Buffer solution was prepared and preserved in ice bath prior to use. Tested compounds were dissolved in 10% DMSO to prepare 0.4, 2.0, and 10.0 μg/mL concentrations.

The final intensity was measured with or without the treatment of 10 μmol/L peroxynitrite in 0.3 mol/L NaOH. The fluorescence intensity was measured at the excitation and emission of 480 nm and 530 nm by microplate fluorescence reader FL (Bio-Tek Instruments Inc., Winooski, VT, USA). Peroxynitrite-scavenging activity was determined by subtracting the background fluorescence from the final fluorescence intensity, which is measured by detection of DHR 123 oxidation. L-penicillamine was used as a positive control, and the data were expressed as mean ± SD.

3. Results

Isolation was performed on combination of two chromatography columns, silica gel column (for normal phase) and an ODS column (for reversed phase). Structures of the isolated compounds were determined by comparison of their physical [appearance, color and melting point (mp)] and spectroscopic data (UV–vis, IR, ^{1}H and ^{13}C-NMR) with those reported in the literatures.

Compound ***1***: Quercetin 3-*O*-[α-L-rhamnopyranosyl (1 → 2)][α-L-rhamnopyranosyl (1 → 6)]-β-D-glucopyranoside

Yellowish powder; mp 176–178 °C; UV (MeOH) λ_{max} nm (log ε): 356.0 (4.05), 256.0 (4.26); IR ν_{max} (KBr, cm^{-1}): 3 435 (br., O−H), 2 988 (aromatic C−H), 1 656 (α,β-unsaturated ketone), 1 599 (aromatic C=C), 1 459 (CH_2), 1 362 (CH_3), 1 064 (glycosidic C−O); ^{1}H-NMR (500 MHz, DMSO-d_6) δ: Quercetin – 6.21 (1H, d, *J* = 1.8 Hz, H-6), 6.42 (1H, d, *J* = 1.8 Hz, H-8), 7.56 (1H, d, *J* = 10.2 Hz, H-2′), 6.86 (1H, d, *J* = 8.4 Hz, H-5′), 7.60 (1H, dd, *J* = 1.8 Hz, 10.2 Hz, H-6′), Glc – 5.50 (1H, d, *J* = 7.2 Hz, anomeric H), Rha – 5.35 (1H, d, *J* = 1.6 Hz, anomeric H), 1.00 (3H, d, *J* = 6.6 Hz), Rha – 5.08 (1H, br. s, anomeric H), 1.02 (3H, d, *J* = 6.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: Quercetin – 157.32 (C-2), 133.05 (C-3), 177.69 (C-4), 156.92 (C-5), 94.22 (C-6), 164.71 (C-7), 99.22 (C-8), 161.69 (C-9), 104.43 (C-10), 121.42 (C-1′), 116.35 (C-2′), 144.86 (C-3′), 147.24 (C-4′), 115.86 (C-5′), 122.75 (C-6′), Glc–99.53 (C-1″), 77.92 (C-2″), 77.51 (C-3″), 68.70 (C-4″), 76.06 (C-5″), 67.33 (C-6″), Rha–101.21 (C-1‴), 71.08 (C-2‴), 72.34 (C-3‴), 72.34 (C-4‴), 71.08 (C-5‴), 17.74 (C-6‴), Rha–101.21 (C-1⁗), 70.80 (C-2⁗), 72.30 (C-3⁗), 72.30 (C-4⁗), 70.80 (C-5⁗), 18.14 (C-6⁗). The NMR data were in good agreement with the data of the same compounds reported by Kazuma *et al.* [17].

Compound ***2***: Kaempferol 3-*O*-[α-L-rhamnopyranosyl (1 → 2)][α-L-rhamnopyranosyl (1 → 6)]-β-D-glucopyranoside

Yellowish powder; mp 172–174 °C; UV (MeOH) λ_{max} nm (log ε): 345.0 (4.24), 265.0 (4.33); IR ν_{max} (KBr, cm^{-1}): 3 434 (br., O−H), 2 988 (aromatic C−H), 1 655 (α,β-unsaturated ketone), 1 599 (aromatic C=C), 1 459 (CH_2), 1 362 (CH_3), 1 063 (glycosidic C−O); ^{1}H-NMR (500 MHz, DMSO-d_6) δ: Kaempferol – 6.42 (1H, d, *J* = 1.8 Hz, H-6), 6.21 (1H, d, *J* = 1.8 Hz, H-8), 7.96 (1H, d, *J* = 10.2 Hz, H-2′), 6.86 (1H, d, *J* = 8.4 Hz, H-3′), 6.86 (1H, d, *J* = 8.4 Hz, H-5′), 7.96 (1H, d, *J* = 10.2 Hz, H-6′), Glc – 5.51 (1H, d, *J* = 7.2 Hz, anomeric H), Rha – 5.35 (1H, d, *J* = 1.6 Hz, anomeric H), 0.98 (3H, d, *J* = 6.6 Hz), Rha – 5.07 (1H, br. s, anomeric H), 1.02 (3H, d, *J* = 6.0 Hz); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: Kaempferol – 160.30 (C-2), 133.05 (C-3), 177.69 (C-4), 156.92 (C-5), 94.22 (C-6), 164.71 (C-7), 99.22 (C-8), 161.69 (C-9), 104.43 (C-10), 121.42 (C-1′), 131.15 (C-2′), 115.56 (C-3′), 157.04 (C-4′), 115.76 (C-5′), 131.15 (C-6′), Glc–99.03 (C-1″), 77.82 (C-2″), 77.51 (C-3″), 68.70 (C-4″),

76.06 (C-5″), 67.33 (C-6″), Rha–101.21 (C-1‴), 71.08 (C-2‴), 72.34 (C-3‴), 72.34 (C-4‴), 71.08 (C-5‴), 17.74 (C-6‴), Rha–101.21 (C-1⁗), 70.80 (C-2⁗), 72.30 (C-3⁗), 72.30 (C-4⁗), 70.80 (C-5⁗), 18.14 (C-6⁗). The NMR data were in good agreement with the data of the same compounds reported by Kazuma *et al.* [17].

Compound ***3***: Quercetin 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside

Yellowish powder, mp 187–189 °C, ^{1}H and ^{13}C-NMR: literature [18].

Compound ***4***: Myricetin 3-*O*-α-L-rhamnopyranosyl

Yellowish powder, mp 205–207 °C, ^{1}H and ^{13}C-NMR: literature [19].

Compound ***5***: Kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside

Yellowish powder, mp 183–185 °C, ^{1}H and ^{13}C-NMR: literature [20].

Compound ***6***: Quercetin

Yellowish powder; mp 314–316 °C, ^{1}H and ^{13}C-NMR: literature [19].

Compound ***7***: Kaempferol

Yellowish powder, mp 275–277 °C, ^{1}H and ^{13}C-NMR: literature [19].

To determine the content of the identified compounds, HPLC quantitative analysis was performed. HPLC analytical method was established for the purpose of simultaneous determination of the flavonoids. Regression equation was determined by plotting the peak area (*y*) *vs*. concentration (*x*, μg/mL) at six concentration solutions. Linear regression, linearity, limit of detection (LOD), and limit of quantification (LOQ) are shown in Table 1.

Content of each compound in MeOH extract and BuOH fraction of *C. papaya* leaves is presented in Table 2. Concentration of kaempferol 3-(2^{G}-rhamnosylrutinoside) was much higher than other flavonoids both in MeOH extract and BuOH fraction (7.23 and 123.18 mg/g, respectively). Figure 1 shows the HPLC chromatograms of standard compounds, BuOH fraction and MeOH extract. HPLC chromatogram of $CHCl_3$ was not shown because the peaks of all flavonoids were not detected (under LOD).

To provide more pharmacological information of the identified compounds and as a comparative study, the seven isolated compounds were evaluated for their antioxidant activity using a peroxynitrite-scavenging assay. Table 3 shows the peroxynitrite-scavenging activities of the seven flavonoid compounds together with L-penicillamine as a positive control. The activity was expressed by IC_{50} value with μg/mL and μmol/L units.

Table 2

Content of compounds in MeOH extract of *C. papaya* leaf and its BuOH fraction (mg/g of lyophilized extract or fractions).

Compounds	MeOH extract	BuOH fraction
Quercetin 3-(2^{G}-rhamnosylrutinoside) (1)	3.11	25.53
Kaempferol 3-(2^{G}-rhamnosylrutinoside) (2)	7.23	123.18
Quercetin 3-rutinoside (3)	0.97	14.54
Myricetin 3-rhamnoside (4)	0.81	9.78
Kaempferol 3-rutinoside (5)	0.52	10.15
Quercetin (6)	< LOQ	0.55
Kaempferol (7)	< LOQ	0.19
Total	12.64	183.92

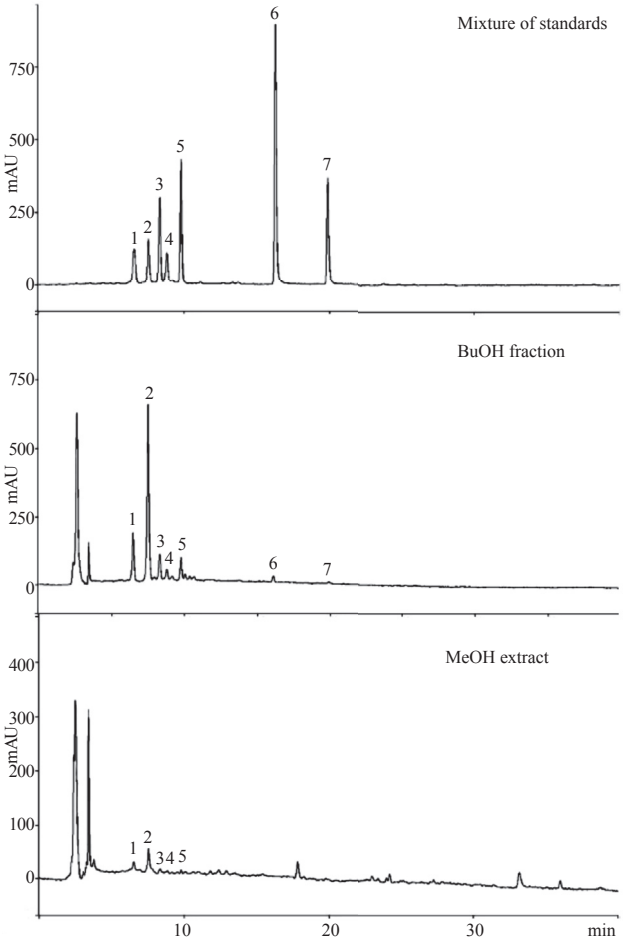

Figure 1. HPLC chromatograms of mixture of standards, BuOH fraction, and MeOH extract of *C. papaya* leaf.
1: Quercetin 3-(2^{G}-rhamnosylrutinoside); 2: Kaempferol 3-(2^{G}-rhamnosylrutinoside); 3: Quercetin 3-rutinoside; 4: Myricetin 3-rhamnoside; 5: Kaempferol 3-rutinoside; 6: Quercetin; 7: Kaempferol.

Table 1

Regression equation and linearity of the compounds.

Compounds	t_R (min)	Linear range	Linear regression[a]	R^{2}[b]	LOD[c] (μg/mL)	LOQ[d] (μg/mL)
Quercetin 3-(2^{G}-rhamnosylrutinoside) (1)	6.56	6.25–200.00	$y = 97.669x + 39.17$	0.9992	0.83	2.76
Kaempferol 3-(2^{G}-rhamnosylrutinoside) (2)	7.54	6.25–200.00	$y = 84.015x + 28.11$	0.9994	1.10	3.65
Quercetin 3-rutinoside (3)	8.32	3.13–100.00	$y = 142.93x + 36.77$	0.9996	0.58	1.94
Myricetin 3-rhamnoside (4)	8.81	3.13–100.00	$y = 101.18x + 60.48$	0.9998	0.59	1.96
Kaempferol 3-rutinoside (5)	9.78	3.13–100.00	$y = 131.70x + 30.95$	0.9995	0.68	2.25
Quercetin (6)	16.25	1.56–50.00	$y = 659.28x + 87.33$	0.9998	0.05	0.17
Kaempferol (7)	19.85	1.56–50.00	$y = 456.72x + 56.83$	0.9996	0.14	0.46

[a]: *y*, peak area at 254 nm; *x*, concentration of the standard (μg/mL); [b]: R^{2}, correlation coefficient for six data points in the calibration curves ($n = 3$); [c]: LOD (S/N = 3); [d]: LOQ (S/N = 10).

Table 3
Peroxynitrite-scavenging activities of seven compounds identified in *C. papaya* leaf.

Compounds	Peroxynitrite-scavenging (%)			IC_{50}	
	0.4 μg/mL	2.0 μg/mL	10.0 μg/mL	(μg/mL)	(μmol/L)
Quercetin 3-(2^G-rhamnosylrutinoside) (1)	36.65 ± 1.32	41.49 ± 0.53	74.36 ± 0.74	1.60	2.11
Kaempferol 3-(2^G-rhamnosylrutinoside) (2)	26.45 ± 0.23	52.60 ± 0.06	80.86 ± 0.96	1.83	2.47
Quercetin 3-rutinoside (3)	48.89 ± 1.10	81.06 ± 1.02	97.30 ± 0.21	0.57	0.94
Myricetin 3-rhamnoside (4)	27.22 ± 3.85	51.47 ± 0.36	86.06 ± 0.19	1.90	4.09
Kaempferol 3-rutinoside (5)	25.93 ± 3.41	38.97 ± 2.34	71.34 ± 1.71	2.47	4.15
Quercetin (6)	47.44 ± 8.90	82.68 ± 3.79	94.82 ± 1.49	0.51	1.69
Kaempferol (7)	28.95 ± 6.18	92.50 ± 1.45	99.41 ± 0.11	0.92	3.21
L-penicillamine	39.95 ± 0.69	65.51 ± 0.82	84.72 ± 0.44	1.03	6.90

Values are represented as mean ± SD, $n = 3$.

4. Discussion

From the BuOH fraction obtained from MeOH extract of *C. papaya* leaves, seven flavonoids (quercetin 3-(2^G-rhamnosylrutinoside), kaempferol 3-(2^G-rhamnosylrutinoside), quercetin 3-rutinoside, myricetin 3-rhamnoside, kaempferol 3-rutinoside, quercetin and kaempferol) were successfully isolated through combination of normal and reversed systems of open column chromatography. Extraction with MeOH was reasonable, because MeOH has capability to effectively produce greater quantities of flavonoids due to more favorable partitioning kinetics [21]. BuOH was selected as solvent for fractionation because it has appropriate polarity to yield a flavonoid-rich fraction [22]. As shown in Table 2, among the isolated compounds, kaempferol 3-(2^G-rhamnosylrutinoside) was obtained in the highest amount (66.97%).

Quantitative analysis is an important tool to provide information of the composition and level of the active components contained in a plant material [23], in which the major ones are generally responsible for some particular pharmacological effects including antioxidant effect [20]. From the linear regression equation (Table 1), the linearities of seven compounds were more than 0.999, represented by the values of R^2 (correlation coefficient). This result indicated that the present HPLC method was sufficient to generate linear regressions with good linearity. As shown in Table 2, the content of kaempferol 3-(2^G-rhamnosylrutinoside) is much higher than other flavonoids, either in MeOH extract or in BuOH fraction (7.23 and 123.18 mg/g, respectively). From the HPLC chromatogram of BuOH fraction (Figure 1), the peak area of kaempferol 3-(2^G-rhamnosylrutinoside) was also dominant compared to other flavonoids. It can be suggested that kaempferol 3-(2^G-rhamnosylrutinoside) is the major flavonoid of *C. papaya* leaf.

Flavonoids have been frequently reported as the active substances associated with antioxidant properties and health benefits [24]. The association of flavonoids with health benefits is explained by their antioxidant properties that can neutralize free radicals through their inherent redox properties. Flavonoids work as radical scavengers of lipid peroxidation chain reactions. They donate an electron to reactive free radical species in the body, neutralizing their potentially damaging chain reactions in cell chemistry and forming stable phenolic radical products in the process [21].

In order to evaluate the antioxidant activities of the isolated compounds, peroxynitrite-scavenging assays were performed. As shown in Table 3, all of the flavonoids exhibited potent activities on peroxynitrite scavenging. Their activities were stronger than L-penicillamine (IC_{50} = 6.90 μmol/L) as the positive control. In particular, activity of the most abundance flavonoid [kaempferol 3-(2^G-rhamnosylrutinoside), IC_{50} = 2.47 μmol/L] was little bit stronger than kaempferol or kaempferol 3-rutinoside (IC_{50} = 3.21 and 4.15 μmol/L, respectively). Compared to quercetin or its glycosides, kaempferol and its glycosides relatively showed weaker activities. As reported by Heijnen *et al.* [25], quercetin with a catechol structure in its B-ring, as well as a 2,3-double bond in conjunction with a 4-carbonyl group in C-ring, allows for delocalization of the phenoxyl radical electron to the flavonoid nucleus. The combined presence of a 3-hydroxy group with a 2,3-double bond additionally increases the resonance stabilization for electron delocalization; hence, it has a higher antioxidant value.

By this experiment, we know that antioxidant flavonoids are abundant in *C. papaya* leaf, and kaempferol 3-(2^G-rhamnosylrutinoside) was the major one. These results are important to understand more about the composition of flavonoids in *C. papaya* leaf and provide more physical and chemical evidences especially to the previous studies reported the existence of such flavonoids in *C. papaya* leaf by liquid chromatography-electrospray ionization-mass spectrometry-based detection methods [12–14].

Seven flavonoids including quercetin 3-(2^G-rhamnosylrutinoside), kaempferol 3-(2^G-rhamnosylrutinoside), quercetin 3-rutinoside, myricetin 3-rhamnoside, kaempferol 3-rutinoside, quercetin and kaempferol were isolated from the leaves of *C. papaya*. From the HPLC quantitative analysis, kaempferol 3-(2^G-rhamnosylrutinoside) contained the highest amount among the flavonoids both in MeOH extract (7.23 mg/g) and in BuOH fraction (123.18 mg/g). The present study reveals that antioxidant flavonoids of *C. papaya* leaf are comprised of quercetin, kaempferol, and their certain glycosides, and suggests that kaempferol 3-(2^G-rhamnosylrutinoside) is an important flavonoid of *C. papaya* leaf because of its abundance and strong antioxidant activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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