Isolation and Identification of Quercetin Derivatives and their α-Glucosidase Inhibitory Acitivities from *Bryophyllum pinnatum*

Fajriah Sofa*, Darmawan Akhmad, Megawati Megawati and Hanafi Muhammad
Research Center for Chemistry, Indonesian Institute of Sciences, Kawasan PUSPIPTEK Serpong Tangerang Selatan, Banten, 15314, INDONESIA
*sofa002@lipi.go.id; sofafajriah@gmail.com

Abstract

In our ongoing investigation on chemical constituents from Indonesian medicinal plants, we have isolated and evaluated antidiabetic effects on α-glucosidase of quercetin derivatives from Bryophyllum pinnatum leaves. The methanolic extract was first separated into n-hexane, ethyl acetate and water-soluble fractions. The ethyl acetate fraction was further purified with silica gel column chromatography techniques based on bioassay guided fractionation which led to 3',4'-dimethoxy quercetin and two flavonoid glycosides, quercetin 3-O-α-L-rhamnoside (1) and quercetin-3-O-α-L-arabinopyranosyl (1→2)α-L-rhamnopyranoside (2).

The chemical structures were determined by extensive spectroscopic analyses, namely, UV/Vis, FTIR, LCMS, 1D and 2D NMR. All isolated compounds have α-glucosidase inhibitory activity with IC_{50}103.20, 83.83 and 110.52 μg/mL respectively.

Keywords: *Bryophyllum pinnatum*, α-glucosidase inhibitors, quercetin derivatives.

Introduction

Diabetes mellitus, among the most prevalent diseases worldwide, is a chronic metabolic disorder characterized by hyperglycemia and accompanied by various chronic vascular complications. Therefore, the control of postprandial blood glucose surges is critical for the treatment of diabetes[2]. Synthetic amylase and α-glucosidase inhibitors, for example, acarbose, are widely applied in the treatment of patients with type II diabetes. They are, however, reported to cause various side-effects. Consequently, safer natural amylase and α-glucosidase inhibitors are desired and many compounds have been reported to be derived from plant sources[3-7].

Bryophyllum pinnatum (synonims: *B. calycinum, Kalanchoe pinnata* (Lam.) Pers. (family Crassulaceae) is a medicinal plant widely used in folk medicine for the treatment of gastric ulcers, pulmonary infections and rheumatoid arthritis[8,9]. Previous studies in the literature on the chemical composition of *B. pinnatum* demonstrate that bufadienolides, terpenoids and flavonoids are the main secondary metabolites of this species[10,11]. Thus, in our ongoing study, we isolate and identify two flavonoid glycosides from *B. pinnatum* leaves and test their α-glucosidase inhibitory activity using α-glucosidase enzyme including 3',4'-dimethoxy quercetin from previous research.

Material and Methods

Plant Material: *B. pinnatum* leaves material samples were collected from Ballitro, Bogor, West Java, Indonesia. The plant was identified by staff at the Research Center for Biology, Indonesian Institute of Sciences (LIPI).

General Experiment: ^{1}H- and ^{13}C-NMR spectra were recorded with JEOL JNM ECA-500 spectrometer, operating at 500 MHz (^{1}H-) and 125.76 MHz (^{13}C-), using TMS (Tetra Methyl Silane) as an internal standard. MS were obtained with a Mariner Biospectrometry spectrometer using System ESI (Electrospray Ionisation) and positive ion mode. The FTIR spectrum was recorded with shimadzu Prestige-21 and the UV/Vis spectrum was recorded with Hitachi U-2000. A melting point experiment was obtained using Fisher Sicentific apparatus. Column chromatography was carried out using Merck Silica gel 60 (.70 - 230 mesh ASTM) and TLC (Thin Layer Chromatography) analysis on precoated Silica gel plates (Merck Kieselgel 60 F 254, 0.25 mm).

Extraction, Isolation and Identification: The dried leaves (1.7 kg) of *B. pinnatum* were extracted thoroughly using a macerator with methanol. The methanol extracts (100 g) were concentrated using a vacuum rotary evaporator and then partitioned with *n*-hexane-water (1:1), followed by ethyl acetate-water (1:1). The ethyl acetate fraction (4.05 g) was further separated using a vacuum column chromatography technique based on bioassay guided fractionation and obtained nine fractions. Each fraction was tested for its α-glucosidase inhibitory activity and the active fractions were further purified using chromatography techniques on silica gel.

Fraction 6 was isolated using column chromatography with a gradient of *n*-hexane-EtoAc solvent,and obtained compound 3',4'-dimethoxy quercetin[12], but the α-glucosidase inhibitory activity was not done. Fraction 7 (0.5 g) was further purified using a column chromatography tecnique on silica gel with gradien elution (*n*-hexane-ethyl acetate) and a yellow crystal was obtained of compound 1 (100 mg). On the other hand, fraction 9 (50 mg) underwent recrystallization using chloroform methanol (1:1) and yielded compound 2 (20 mg). Compounds 1 and 2 were identified using UV/Vis, FT-IR, LC-MS and FT-NMR. Compounds 1 and 2, along with 3',4'-dimethoxy quercetin

were examined in terms of their α-glucosidase inhibitory activity using α-glucosidase enzyme.

Inhibition assay for α-glucosidase activity: α-Glucosidase inhibitory activity evaluation of the extracts was performed using an established procedure[13]. α-Glucosidase enzyme solution was dissolved in a phosphate-buffer solution (pH 7) containing 200 mg albumin serum. Prior to its application, 1 mL of the enzyme solution was diluted 25 times with the buffer solution. The reaction mixture comprised 250 µL of 20 mM *p*-nitrophenyl α-D-glucopyranose as the substrate and 490 µL of 100 mM phosphate buffer (pH 7).

10 µL of the extract dissolved in DMSO was prepared. The reaction mixture was then water-bath incubated at 37°C for 5 minutes. The enzyme solution (250 µL) was added and the solution was incubated for 15 minutes. The enzyme reaction was stopped by the addition of 1000 µL, 200 mM sodium carbonate solution.

The resulted *p*-nitrophenol from the reaction was measured at λ 400 nm. As positive control, the reaction of 1% of quercetin solution was measured. The commercial α-glucosidase anti-diabetic drug, glucobay, was available in the laboratory only in theform of sustain release tablets. Therefore, quercetin was selected for positive control for *in vitro* evaluation. Sample concentrations for activity evaluation were 6.25, 12.5, 25, 50, 100 µg/mL at concentrations of 0.78, 1.56, 3.12, 6.25, 12.5 µg/mL respectively for quercetin.

Results and Discussion

Isolation and Identification: The crude methanolic extract of *B. pinnatum* leaves demonstrated strong inhibitory activity with IC_{50} 32.06 µg/mL. Partitioning of the methanolic extract using water, *n*-hexane and ethyl acetate yielded 20.77 g of *n*-hexane soluble extract (BPH), 4.05 g of EtOAc-soluble extract (BPE) and 50 g of water soluble extract (BPW). BPE demonstrated the highest inhibitory activity against α-glucosidese (IC_{50} 18.46 µg/mL) compared with BPH (IC_{50} 83.20 µg/mL) and BPW (IC_{50} 41.55 µg/mL).

Due to its high α-glucosidese inhibitory activity, BPE was selected for further investigation. BPE was subjected to silica gel column chromatography using *n*-hexane, ethyl acetate and MeOH gradient to yield nine fractions (fraction 1 (0.06%); 2 (0.27%); 3 (2.23%); 4 (1.84%); 5 (6.86%); 6 (6.86%); 7 (16.37%); 8 (9.75%); 9 (30.95%)).

The nine collected fractions were subjected to α-glucosidese inhibitory activity assay. Fractions 6, 7 and 9 had significant inhibitory activity as shown in fig. 1. Fraction 6 was investigated in previous research[12] and resulted in 3',4'-dimethoxy-quercetin, but its α-glucosidese inhibitory activity was not evaluated. The active fraction 7 was further purified using a column chromatography tecnique on silica gel using gradien elution (*n*-hexane-ethyl acetate) to obtain compound 1.

On the other hand, fraction 9 underwent recrystallization using chloroform methanol and yielded compound 2. The isolates were successfully identified by the consistence of the spectral data with those in reference.

Compound 1 was isolated as a yellow crystal (mp. 178-179°C) with molecular weight *m/z* 448.92 $[M + H]^+$ corresponding to the molecular formula $C_{21}H_{20}O_{11}$. The UV spectrum displayed typical absorption bands for flavonoid skeleton at λ_{max} 210, 255 and 349 nm[7], with IR absorption bands at 3530, 2951 and 1602 cm^{-1}, due to hydroxyl, aliphatic and unconjugated carbonyl functionalities respectively.

A typical flavonoid signal was observed in the ^{1}H-NMR spectra of compound 1. Singlet signals at δ 12.71 indicate the presence of 5-OH in flavonoids. The ^{1}H-NMR spectrum of compound 1 (Acetone-d_6, 500 MHz) indicates a flavonoid with an ABX system ring at δ 7.36 (1H, dd, *J* = 1.53 and 8.41 Hz), 6.96 (1H, d, *J* = 8.41) and 7.47 (1H, d, *J* = 1.53) in the Bring. The existence of H-6 and -8 was confirmed by two doublet signals (δ 6.24 (*J* = 2.29 Hz) and 6.44 (*J* = 2.29 Hz) respectively).

The presence of a rhamnoside unit was suggested by a characteristic methyl doublet at δ 0.88 (*J* = 6.12 Hz) and a broad doublet corresponding to the anomeric proton at δ 5.49 with a coupling constant *J* = 1.3 Hz indicated that the rhamnose unit has α-configuration. The ^{13}C NMR spectrum (Acetone-d_6, 125 MHz), combined with HMQC experiments, indicated 21 carbons comprising 10 quartenary carbons, 10 methine carbons and a methyl carbon. A terminal methyl group at d 0.88 (H-6") indicated a ^{2}J HMBC correlation with C-5 (δ 70.5). Furthermore, HMBC long-range correlation (fig. 1) was observed between H-1" (δ 5.49) and C-3 (δ 134.9) in agreement with the *O*-rhamnopyranoside unit at C-3. Hence, compound 1 compared with prior work in the literature[14,15] was elucidated as quercetin 3-O-α-L-rhamnoside, trivially referred to as quercitrin.

Compound 2 was obtained as amorphous yellow powder with molecular weight *m/z* 580.62 $[M + H]^+$, consistent with the molecular formula $C_{26}H_{28}O_{15}$. UV absorption at 210, 255, 347 nm suggested the presence of a flavonoid skeleton[7]. IR absorption for an unconjugated carbonyl was noted at 1605 cm^{-1}, while other bands were assigned to hydroxyl (3532 cm^{-1}) and aliphatic (2912 and 2868 cm^{-1}) groups. The NMR spectra (1D and 2D) of compound 2 were similar to compound 1 (table 2),and showed a quercetin aglycone and a rhamnopyranosyl unit. The presence of a second carbohydrate unit which was identified in compound 2 did not appear in compound 1.

Five additional ^{13}C signals (CD_3OD, 125 MHz) were observed at δ, namely, 106.5, 72.9, 71.4, 68.5 and 66.1.This indicates the presence of a second carbohydrate unit which was identified as an arabinopyranosyl group from analysis of the $^{1}H–^{1}H$ coupling constants and by comparison with ^{13}C NMR spectroscopic data in the literature[11]. The large coupling constant (7.64 Hz) corresponding to the anomeric H- 1''' (δ 4.19) at HMBC spectrum indicated a di-axial relation with H-2''', consistent with the presence of an α-arabinopyranose moiety (fig. 2).

According to the NMR spectroscopic data, the α-arabinopyranosyl moiety was inferred to be linked to the 2''-position of 3-*O*-rhamnopyranosyl unit (δ 81.5 (C-2'')). This substitution pattern agrees with the deshielding effect observed for the C-2'' signal (+10 ppm) when compared to the C-2 signal of an unsubstituted 3-*O*-rhamnosyl unit[11]. According to these data, compound 2 was concluded to be quercetin-3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (fig. 3).

α-Glucosidase Inhibitory Activity: The isolated flavonoid glycoside 1 and 2, along with 3',4'-dimethoxy quercetin, were carried out for α-glucosidase inhibitory activity. The results were presented as a measure of the compound's effectiveness in inhibiting α-glucosidase function. The IC_{50} calculation demonstrates that compound 1 has the highest inhibition among all those isolated from *B. pinnatum* leaves in this study followed by compound 2 and 3',4'-dimethoxy quercetin (table 3).

Upon comparison of flavonoids 1 and 2, both contain a quercetin aglycone. The difference in their activity profile is related to the presence of the arabinosyl unit linked at the inner rhamnosyl unit in flavonoid 2 and it decreased the α-glucosidase inhibitory activity[11]. However, all isolated compounds have weaker activities compared to quercetin as positive control. It was suggested that position 3-OH is an important group to increase the activity[16], besides the methyl group in 3',4'-dimethoxy quercetin decreasing the α-glucosidase inhibitory activity.

Conclusion

Bioassay-guided isolation on α-glucosidase inhibitory activity afforded a 3',4'-dimethoxy quercetin and two flavonoid glycosides:quercetin 3-O-α-L-rhamnoside (1) and quercetin-3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (2). All isolated coompounds have α-glucosidase inhibitory activity with IC_{50}103.20, 83.83 and 110.52 μg/mL respectively.

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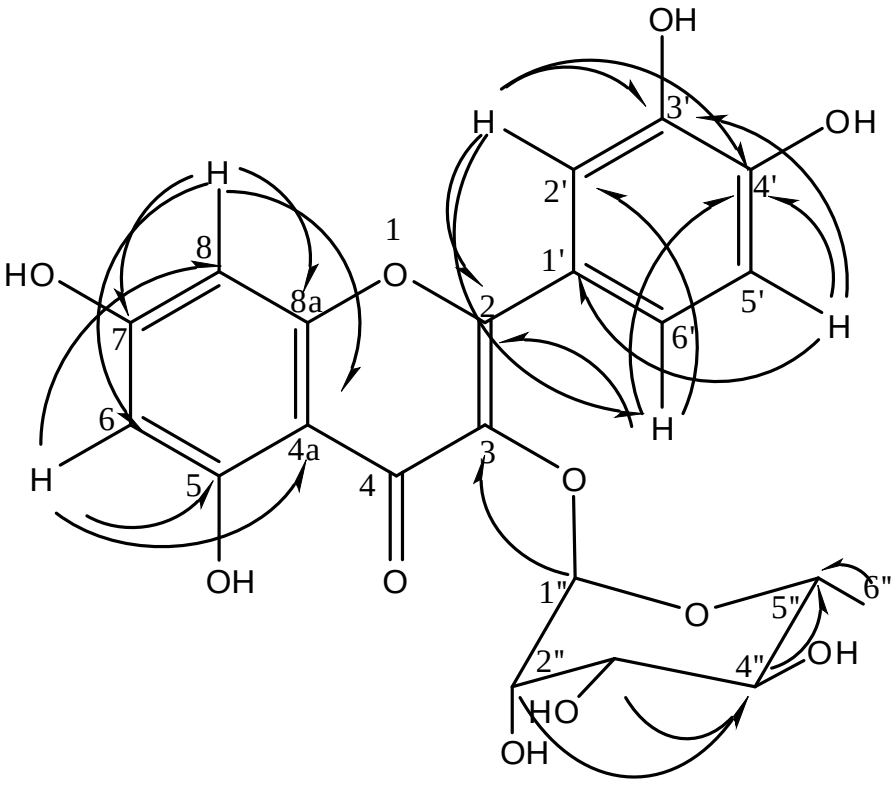


Figure 1: Selected HMBC correlation of compound 1

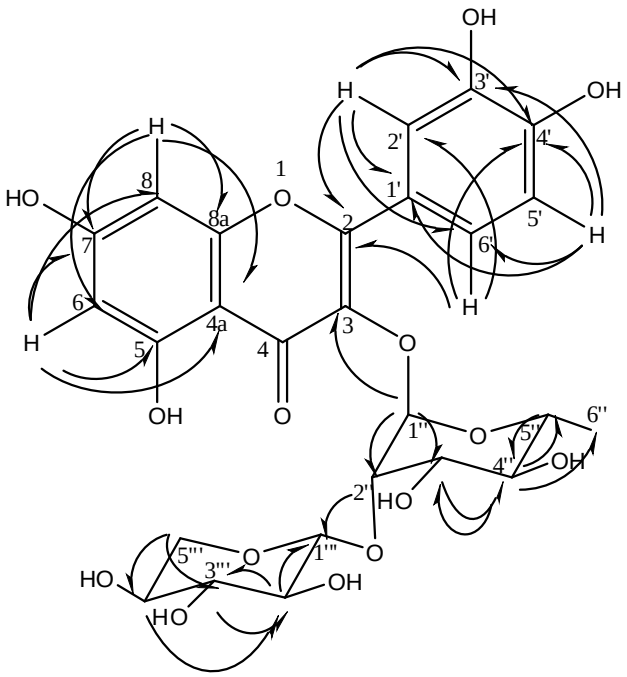
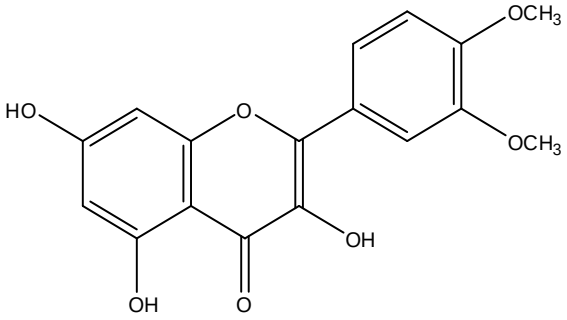
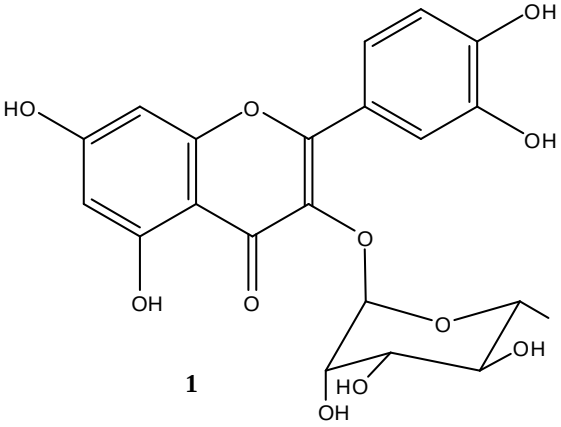


Figure 2: Selected HMBC correlation of compound 2



3',4'-dimethoxy quercetin



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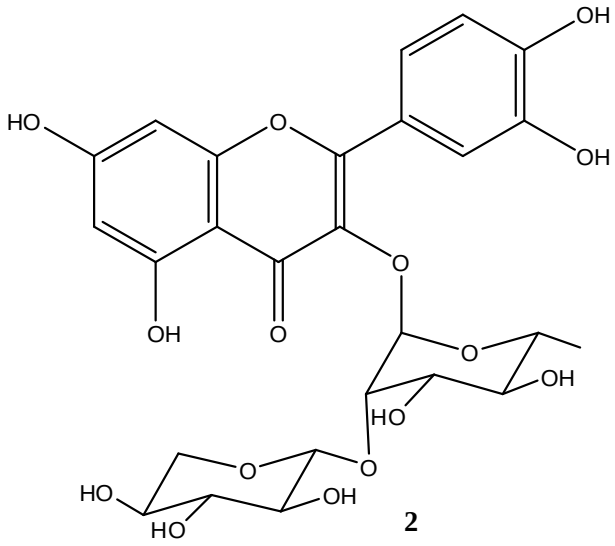

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Figure 3: Isolated compounds from *Bryophyllum pinnatum* leaves: 3',4'-dimethoxy quercetin,quercetin 3-O-α-L-rhamnoside (1) and quercetin-3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (2).

Table 1
α-Glucosidase inhibitory activity for fractions 1-9 from ethyl acetate fraction of *B. pinnatum* leaves

Fraction	IC_{50} (μg/mL)	Fraction	IC_{50} (μg/mL)
1	NA	6	160.31
2	NA	7	83.83
3	NA	8	126.13
4	NA	9	91.40
5	207.88		

Table 2
^{1}H- and ^{13}C-NMR spectroscopic data for compound 1 and 2

Proton and Carbon No.	δ_H-NMR (ppm), multiplicity, *J* (Hz)		δ_C-NMR (ppm)	
	Compound 1 (Acetone-d_6)	Compound 2 (CD_3OD)	Compound 1 (Acetone-d6)	Compound 2 (CD_3OD)
2			157.6	157.8
3			134.9	135.5
4			178.5	178.4
4a			104.9	104.4
5			162.4	161.9
6	6.24, *d*, 2.29	6.18, *d*, 1.72	98.7	98.6
7			164.1	164.7
8	6.44, *d*, 2,.9	6.34, *d*, 1.72	93.7	93.5
8a			157.1	157.2
1’			122.0	122.6
2’	7.47, *d*, 1.53	7.35, *d*, 1.72	115.9	115.5
3’			145.0	145.2
4’			148.2	148.5
5’	6.96, *d*, 8.41	6.9, *d*, 8.02	115.3	115.2
6’	7.36, *dd*, 1.53; 8.41	7.29,*dd*, 1.72; 8.02	121.8	121.4
1”	5.49, *d*, 1,53	5.35, *d*, 1.53	101.9	101.9
2”	4.18, *dd*, 1.5; 1.5	4.17, *m*	70.6	81.4
3”	3.68, *dd*, 3.82; 3.82	3.85, *dd*, 3.44; 3.44	71.3	70.4
4”	3.32, *dd*, 8.41; 9.17	3.33, *dd*, 9.74; 9.74	70.5	72.4
5”	3.37, *dq*, 9.17; 6.12	3.88, *dq*, 9.74; 6.12	72.2	70.6
6”	0.88, *d*, 6.12	1.0,*d*, 6.12	16.9	16.4
1”’		4.19, *d*, 7.64	157.6	106.5
2”’		3.51, *dd*, 7.64; 9.17	134.9	71.4
3”’		3.46, , *dd*, 3.06; 9.17	178.5	72.9
4”’		3.72,*m*	104.9	68.5
5”’		3.63, *dd*, 2.29;13.003.36, *br d*, 13.00	162.4	66.1

Table 3
α-Glucosidase inhibitory activity for isolated compounds from ethyl acetate fraction of *B. pinnatum* leaves

Sample	IC_{50} (μg/mL)
3',4'-dimethoxy quercetin	103.2
Quercetin 3-O-α-L-rhamnoside (1)	83.83
Quercetin-3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (2)	110.52
Quercetin (positive control)	5.67

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