Flavonoid Compounds from Krokot Herb (*Lygodium microphyllum*) and their Antioxidant Activity against DPPH

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Abstract. Six flavonoid compounds, kaempferol (1), quercetin (2), acacetin (3), quercetin-3-*O*-β-D-glucopyranoside (4), kaempferol-3-*O*-β-D-glucopyranoside (5), and isorhamnetin-3-*O*-β-D-glucopyranoside (6), were isolated from the methanol extract of the *Lygodium microphyllum* herb. The chemical structures of compounds 1-6 were identified on the basis of spectroscopic evidence and comparison with previously reported spectral data. Compounds 1-6 were isolated for first time from this plant. Their antioxidant activity against DPPH (2,2-Diphenyl-1-picrylhydrazyl) was evaluated. Among the compounds, quercetin (2) showed high antioxidant activity with IC_{50} values of 6.94 ± 0.03 μg/mL.

Keywords: *Antioxidant; DPPH; flavonoids; Lygodium microphyllum; Lygodiaceae.*

1 Introduction

The fern contains many useful secondary metabolites, including flavonoids, steroids, alkaloids, phenols and triterpenoids [1]. One of the thousands of fern species is the *Lygodiaceae* family, which has only one genus *Lygodium* [2]. The *Lygodium* genus is a group of ferns that propagate around other plants and are different from other kinds of ferns in that they have fleshy rhizomes that crawl over the ground. They can only live in the open because they like sunlight. Some *Lygodium* species are invasive and can become problematic in forest areas. Their fast growth and lack of predators makes these plants dominant, displacing wildlife, threatening biodiversity, and enhancing the human-animal conflict [3]. One of the invasive species from the *Lygodium* genus is *L. microphyllum*. Phytochemical studies on the *Lygodium* genus have shown it to contain a compound with an unique structure and diverse biological activities,

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such as flavonoids [4], phenolic glycoside [5], naphthoquinone [6], steroids [7], and phenylpropanoid glycoside [8].

In our continuing search for novel biologically active compounds from Indonesia *Lygodium* plants, we isolated two flavonol, quercetin and quercetin-3-*O*-β-D-glucopyranoside from the ethyl acetate extract of the *L. microphyllum* herb [9]. In further screening for antioxidant compounds from the *L. microphyllum* herb we found that the methanol extract showed a significat antioxidant activity against DPPH radical-scavenging. In this paper we report the isolation and structure elucidation of flavonoid compounds from the *L. microphyllum* herb together with their antioxidant activity against DPPH radical-scavenging.

2 Materials and Methods

2.1 General

Melting points were measured on an electrothermal melting point apparatus and were not corrected. IR spectra were measured on a Perkin-Elmer 1760X spectrophotometer, FT-IR on KBr, mass spectra were recorded with a Waters XEVO Qtof HR-TOFMS mass spectrometer and a Waters UPLC MS/MS H-Class chromatographic system. ^{1}H and ^{13}C NMR spectra were obtained by JEOL NMR 500 MHz, JEOL NMR ECZR 600 MHz using TMS as an internal standard. Chromatographic separation was carried out on silica gel 60 (Merck), octa desyl silane (ODS, Fuji silysia). Preparative MPLC was used with a Buchi Pump Controller C-610, Buchi Pump Modules C-605, and a SiliCycle FLH-R10030B-ISO04 Siliasep column (Butchi, Switzerland). A TLC plate filled with silica gel GF254 (Merck, 0.25 mm) and detection was obtained with the appearance of 10% H_2SO_4 in ethanol followed by heating and irridiation under ultraviolet-visible light at a wavelength of 257 and 364 nm.

2.2 Plant Material

L. microphyllum herb was collected from forest areas in Samarinda, East Kalimantan in June 2014. The plant was identified by staff at the Faculty of Forestry, University of Mulawarman, Samarinda and a sample specimen (No. 09/1117.4.1.8/LL/2011) was stored at the Faculty of Forestry, University of Mulawarman, Samarinda.

2.3 Extraction and Isolation

The *L. microphyllum* herb (3.5 kg) was extracted with methanol at room temperature for 4 days. The methanol extract was evaporated using a rotary evaporator to give a dark brown residue (133.5 g). The dark brown residue was

dissolved in 520 mL of water (4:1) and partitioned successively with *n*-hexane, ethyl acetate, and *n*-butanol. Evaporation of the solvents resulted in *n*-hexane (59 g) ethyl acetate (72 g) and *n*-butanol (54 g) extracts. All of the extracts were tested for antioxidant activity against DPPH radical scavenging and showed antioxidant activity for *n*-hexane, ethyl acetate, and *n*-butanol with IC_{50} values of 20.36 ± 0.05, 12.36 ± 0.03 and 10.42 ± 0.03 µg/mL, respectively. The EtOAc and *n*-BuOH showed the strongest antioxidant activity and showed rich contents of flavonoid compounds using TLC analysis under UV light at wavelenght 254 and 367 nm as well as with $AlCl_3$ as a sprayer reagent. Subsequent phytochemical analysis was therefore focused on the EtOAc and *n*-BuOH extract.

The EtOAc soluble fraction (15.50 g) was fractionated by column chromatography on silica gel 60 using a gradient of *n*-hexane-EtOAc-MeOH as eluen to give eight fractions (A–H). Fraction A (85 mg) was chromatographed on a MPLC on silica gel, eluted with a gradient of $CHCl_3$–MeOH (10:0–1:1), to give seven subfractions (A01–A07). Subfraction A02 (45 mg) was separated on preparative TLC silica gel GF_{254}, eluted with $CHCl_3$–EtOAc–HOAc (9:1:0.5), to give **1** (2.1 mg) and **2** (2.6 mg). Fraction B (75 mg) was chromatographed on a ODS column, eluted with H_2O–MeOH (1:1), to give five subfractions (B01–B05). Subfraction B01(110 mg) was chromatographed on a MPLC of silica gel, eluted with a gradient of $CHCl_3$–MeOH (10:0–6:4), to give five subfractions (B01A–B01E). Subfraction B01B (32 mg) was separated on preparative TLC silica gel GF_{254}, eluted with $CHCl_3$–EtOAc–HOAc (9:1:0.5), to give **4** (4 mg). Subfraction B02 (25 mg) was separated on preparative TLC silica gel GF_{254}, eluted with $CHCl_3$–EtOAc–HOAc (9:0.5:0.5), to give **3** (1.8 mg). The *n*-BuOH soluble fraction (30 g) was fractionated by column chromatography on silica gel 60 using a gradient $CHCl_3$–MeOH to give six fractions (I–N). Fraction K (2 g) was chromatographed on a Sphadex LH-20 column, eluted with MeOH, to give five subfractions (K01–K05). Subfraction K05 (42 mg) was chromatographed on a ODS column, eluted with H_2O–MeOH (1:1), to give **5** (7.5 mg) and **6** (5.5 mg).

2.3.1 Kaempferol (1)

Yellow powder, m.p. 276-278 °C, UV (MeOH) λ_{max} nm (log ε) 353 (4.08); 266 (4.20); IR (KBr) ν_{max} 3343, 1640, 1478, 1051, 827 cm^{-1}. ^{1}H-NMR (acetone-d_6, 500 MHz): δ_H 8.17 (2H, d, J = 9.0 Hz, H-2′ and H-6′), 7.03 (1H, d, J = 9.0 Hz, H-3′ and H-5′), 6.54 (1H, d, J = 1.9 Hz, H-8), 6.26 (1H, d, J = 1.9 Hz, H-6); ^{13}C-NMR (acetone-d_6, 125 MHz), see Table 1; HR- TOFMS (negative ion mode) *m/z* 285.2503 $[M-H]^-$, (calcd. $C_{15}H_{10}O_6$, *m/z* 286.2487).

Table 1 ^{13}C-NMR Data for Compounds **1**-**6**.

Position	**1***	**2***	**3***	**4****	**5****	**6****
C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C
2	157.8 (s)	157.8 (s)	160.5 (s)	157.0 (s)	159.9 (s)	158.3 (s)
3	136.7 (s)	136.8 (s)	107.6 (d)	134.1 (s)	133.2 (s)	135.3 (s)
4	176.6 (s)	176.6 (s)	175.8 (s)	178.9 (s)	177.5 (s)	180.2 (s)
5	162.3 (s)	162.4 (s)	159.6 (s)	161.3 (s)	161.2 (s)	163.0 (s)
6	99.2 (d)	99.2 (d)	96.0 (d)	100.4 (d)	98.7 (d)	99.4 (d)
7	165.1 (s)	165.0 (s)	162.3 (s)	163.2 (s)	164.2 (s)	166.9 (s)
8	94.5 (d)	94.5 (d)	95.9 (d)	94.7 (d)	93.8 (d)	94.7 (d)
9	157.8 (s)	148.4 (s)	159.4 (s)	157.6 (s)	156.2 (s)	158.0 (s)
10	104.1 (s)	104.2 (s)	106.0 (s)	102.7 (s)	103.9 (s)	105.4 (s)
1′	123.3 (s)	121.5 (s)	121.2 (s)	121.6 (s)	120.9 (s)	122.8 (s)
2′	130.5 (s)	116.2 (d)	127.6 (d)	116.0 (d)	130.9 (d)	113.9 (d)
3′	116.4 (d)	147.0 (s)	115.8 (d)	144.7 (s)	115.1 (d)	148.1 (s)
4′	162.3 (s)	145.8 (s)	160.6 (s)	148.8 (s)	156.4 (s)	150.6 (s)
5′	116.4 (d)	116.0 (d)	115.8 (d)	114.6 (d)	115.1 (d)	116.0 (d)
6′	130.5 (d)	124.5 (d)	127.6 (d)	121.7 (d)	130.9 (d)	123.8 (d)
1″	-	-	-	103.5 (d)	100.9 (d)	158.3 (s)
2″	-	-	-	74.3 (d)	74.2 (d)	135.3 (s)
3″	-	-	-	76.8 (d)	76.4 (d)	180.2 (s)
4″	-	-	-	69.8 (d)	69.9 (d)	163.0 (s)
5″	-	-	-	77.0 (d)	77.5 (d)	99.4 (d)
6″	-	-	-	61.1 (t)	60.8 (t)	166.9 (s)
4′-OCH$_3$	-	-	55.8 (q)	-	-	-
5′-OCH$_3$	-	-	-	-	-	56.8 (q)

*) measured in acetone-d_6, 125 MHz., ** measured in pyridine-d_6, 150 MHz.

2.3.2 Quercetin (2)

Yellow powder, m.p. 315-317 °C, UV (MeOH) λ_{max} nm (log ε) 370 (3.86); 255 (3.79); IR (KBr) ν_{max} 3410, 1602, 1693 cm^{-1}. ^{1}H-NMR (acetone-d_6, 500 MHz): δ_H 7.73 (1H, d, J = 2.5 Hz, H-2′), 7.62 (1H, dd, J = 8.5, 2.5 Hz, H-6′), 6.87 (1H, d, J = 8.5 Hz, H-5′), 6.38 (1H, d, J = 2.5 Hz, H-8), 6.17 (1H, d, J = 2.5 Hz, H-6); ^{13}C-NMR (acetone-d_6, 125 MHz), see Table 1; ESI- TOFMS (negative ion mode) *m/z* 301.40 [M-H]$^-$, (calcd. $C_{15}H_{10}O_7$, *m/z* 302.04).

2.3.3 Acacetin (3)

Yellow needle-like crystal. ^{1}H-NMR (acetone-d_6, 500 MHz): δ_H 7.80 (2H, d, J = 7.8 Hz, H-2′, H-6′), 6.70 (2H, d, J = 7.8 Hz, H-3′, H-5′), 6.41 (1H, d, J = 2.5 Hz, H-8), 6.40 (1H, d, J = 2.5 Hz, H-6), 6.50 (1H, s, H-3), 4.10 (3H, s, 4′-OCH_3); ^{13}C-NMR (DMSO-d_6, 125 MHz), see Table 1; HR-TOFMS (negative ion mode) *m/z* 285.0783 [M+H]$^+$, (calcd. $C_{16}H_{12}O_5$, *m/z* 284.0685).

2.3.4 Quercetin-3-*O*-β-D-glucopyranoside (4)

Yellow powder, UV (MeOH): λ_{max} (log ε) 264 (3.85), 350 (3.58) nm; IR (KBr) ν_{max} 3310, 1662, 1602, 1040 cm^{-1}. ^{1}H-NMR (pyridine-d_5, 600 MHz): δ_H 7.68 (1H, d, *J* = 2.4 Hz, H-2′), 7.55 (1H, dd, *J* = 8.4, 2.4 Hz, H-6′), 6.82 (1H, d, *J* = 8.4 Hz, H-5′), 6.23 (1H, d, *J* = 2.4 Hz, H-8), 6.07 (1H, d, *J* = 2.4 Hz, H-6), 5.11 (1H, d, *J* = 7.2 Hz, H-1″), 3.68 (2H, d, *J* = 5.0 Hz, H-6″), 3.46 (1H, d, *J* = 9.0 Hz, H-2″), 3.39 (1H, d, *J* = 9.6 Hz, H-3″), 3.32 (1H, d, *J* = 9.6 Hz, H-4″), 3.19 (1H, m, H-5″); ^{13}C-NMR (pyridine-d_5, 150 MHz), see Table 2; HR-TOFMS (negative ion mode) *m/z* 463.0883 $[M-H]^-$, (calcd. $C_{21}H_{20}O_{12}$, *m/z* 464.0955).

2.3.5 Kaempferol-3-*O*-β-D-glucopyranoside (5)

Yellow powder, IR (KBr) ν_{max} 3433, 2896, 1654, 1266, 839 cm^{-1}. ^{1}H-NMR (pyridine-d_5, 600 MHz): δ_H 8.08 (2H, d, *J* = 9.1 Hz, H-2′, H-6′), 6.88 (2H, d, *J* = 9.1 Hz, H-3′, H-5′), 6.43 (1H, d, *J* = 2.0 Hz, H-8), 6.21 (1H, d, *J* = 2.0 Hz, H-6), 5.45 (1H, d, *J* = 7.7 Hz, H-1″), 4.26 (1H, dd, *J* = 9.0, 7.7 Hz, H-2″), 3.56 (1H, t, *J* = 9.0 Hz, H-3″), 3.23 (1H, t, *J* = 9.0 Hz, H-4″), 3.17 (1H, dt, *J* = 4.9, 2.0 Hz, H-5″), 3.08 (2H, d, *J* = 4.9 Hz, H-6″); ^{13}C-NMR (pyridine-d_5, 150 MHz), see Table 2; HR-TOFMS (negative ion mode) *m/z* 447.3783 $[M-H]^-$, (calcd. $C_{21}H_{20}O_{11}$, *m/z* 448.3769).

2.3.6 Isorhamnethin-3-*O*-β-D-glucopyranoside (6)

Yellow powder, UV (MeOH): λ_{max} (log ε) 256 (3.81), 354 (3.52) nm. ^{1}H-NMR (pyridine-d_5, 600 MHz): δ_H 7.95 (1H, d, *J* = 2.0 Hz, H-2′), 7.62 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′), 6.93 (1H, d, *J* = 8.0 Hz, H-5′), 6.41 (1H, d, *J* = 2.0, H-8), 6.22 (1H, d, *J* = 2.0 Hz, H-6), 5.43 (1H, d, *J* = 7.8 Hz, H-1″), 3.97 (3H, s ,5′-OCH_3), 3.60 (2H, d, *J* = 5.2 Hz, H-6″), 3.56 (1H, t, *J* = 9.0 Hz, H-3″), 3.28 (1H, dd, *J* = 9.0, 7.8 Hz, H-2″), 3.23 (1H, t, *J* = 9.0 Hz, H-4″), 3.17 (1H, dt, *J* = 9.0, 5.2 Hz, H-5″); ^{13}C-NMR (pyridine-d_5, 150 MHz), see Table 2; ESI-TOFMS (positive ion mode) *m/z* 479.13 $[M+H]^+$, (calcd. $C_{22}H_{22}O_{12}$, *m/z* 478.1160).

2.4 Determination of DPPH Radical-scavenging Activity

Antioxidant activity determination was conducted according to the method described in [10] using the 2,2 diphenyl-1-picrylhydrazyl-hydrate (DPPH) radical scavenging method with L-ascorbic acid as positive control. The test samples were dissolved in DMSO and mixed with 20 mg/L of (DPPH) methanol solution to give final concentrations of 10, 50, 100, 200, 400 and 800 µg/mL. After 30 minutes at room temperature, the absorbance values were measured at 517 nm and converted into a percentage of antioxidant activity as follows:

$$\%\ inhibition\ = \frac{absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of\ control} \quad (1)$$

The IC_{50} value was determined by probit values and plotted against the logarithmic values of concentrations of the test samples and a linear regression curve. This is the amount of sample necessary to decrease the absorbance of DPPH by 50%. All analyses were carried out in triplicate and the results were expressed as the mean ± standard deviation (SD) and compared using the Waller-Duncan test. A value of $p < 0.05$ was considered statistically significant.

3 Results and Discussions

The phytochemical test for the EtOAc and *n*-BuOH extract showed the presence of flavonoids. By using DPPH radical scavenging assay to guide separations, the EtOAc and *n*-BuOH fractions were separated by vacuum liquid chromatography over silica gel by gradient elution. The fractions were repeatedly subjected to MPLC, reverse-phase column chromatography, Sephadex LH-20 and preparative TLC on silica gel GF_{254} and yielded six antioxidant flavonoid compounds **1**-**6** (Figure 1).

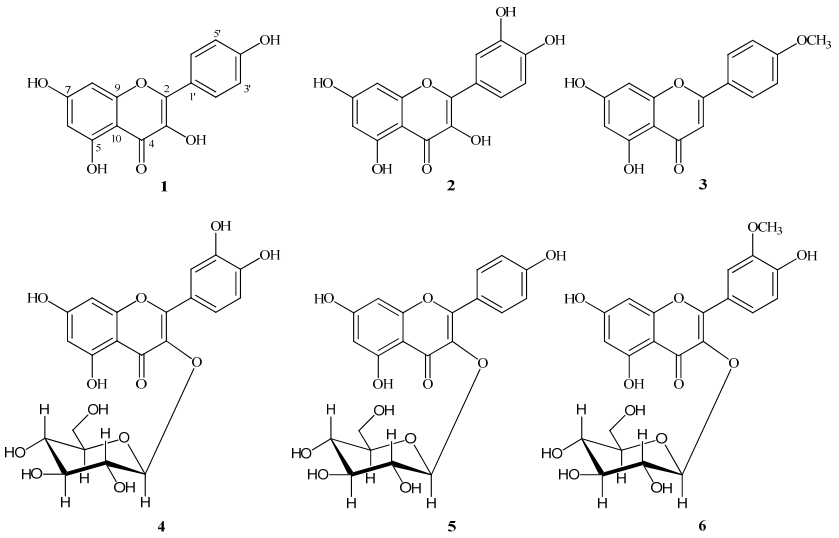

Figure 1 Structures of compounds **1**-**6.**

3.1 Compound 1

This was obtained as a yellow powder. The HR-TOFMS spectrum showed $[M-H]^-$ *m/z* 285.2503 (calcd. *m/z* 286.2487), which corresponds to the molecular formula of $C_{15}H_{10}O_6$ with eleven degrees of unsaturation. The UV (MeOH) λ_{max}

nm (log ε) 353 (4.08), 266 (4.20), suggests the presence of flavonoid compound. The IR spectra showed absorption peaks at 3343 cm^{-1} (OH), 1640 cm^{-1} (conjugated carbonyl), 1478 cm^{-1} (C = C aromatic), 1051 cm^{-1} (asymmetric C-O-C stretch) and 827 cm^{-1} (substituted benzene). The ^{1}H-NMR spectrum of compound **1** shows a characteristic AA′BB′ pattern of ring B at δ_H 8.17 (2H, d, J = 9.0 Hz) and 7.03 (2H, d, J = 9.0 Hz), corresponding to a *p*-hydroxyphenyl group. The resonances of two *meta*-coupling protons of ring A were observed at δ_H 6.26 (1H, d, J = 1.9 Hz) and δ_H 6.54 (1H, d, J = 1.9 Hz). The ^{13}C-NMR and DEPT 135° spectra showed the presence of six sp^2 methines, nine sp^2 quaternary carbons and a ketone group, C-4, resonating at δ_C 176.6 ppm. These functionalities account for eight of the total 11 degrees of unsaturation, and the remaining three degrees of unsaturation are consistent with the flavonoid structure. The full assignments of ^{13}C-NMR data of compound **1** are shown in Table 1. Comparison of the NMR data of **1** to those of kaempferol [11], shows high similarity, consequently, compound **1** was identified as a kaempferol.

3.2 Compound 2

This was obtained as a yellow amorphous powder. The molecular formula was established to be $C_{15}H_{10}O_7$ from its ESI-TOFMS spectral data (*m/z* 301.40 $[M-H]^-$), and ^{13}C-NMR data (Table 1), thus requiring eleven degrees of unsaturation. The UV spectrum of **2** shows λ_{max} at 255 and 340 nm, which is typical for a flavonol structure [11]. The IR spectrum of **2** shows absorption bands corresponding to hydroxyl (3410 cm^{-1}), carbonyl (1693 cm^{-1}) and double bond aromatic (1602 cm^{-1}) groups. The ^{1}H-NMR spectrum of **1** shows the presence of *meta*-coupled aromatic protons at δ_H 6.17 (1H, d, J = 2.5 Hz) and 6.38 (1H, d, J = 2.5 Hz) corresponding to H-6 and H-8, respectively. The ^{1}H-NMR spectrum of **2** also shows the presence of an aromatic region exhibiting an ABX system at δ_H 7.73 (1H, d, J = 2.5 Hz, H-2′), 7.62 (1H, dd, J = 2.5, 8.5 Hz, H-6′), and 6.87 (1H, d, J = 8.5 Hz, H-5′) due to a 3′, 4′ disubstitution of ring B. A total of fifteen carbon signals were observed in the ^{13}C-NMR spectrum. These were assigned by DEPT and HMQC experiments to fourteen sp^2 carbons and a carbonyl signal at δ_C 176.6. The degree of unsaturation accounted for eight out of the total of eleven double bond equivalents. The remaining three degrees of unsaturation were consistent with a flavonol structure. A detailed comparison of the NMR data of **2** with those of quercetin [12] revealed that the structures of the two compounds were very similar, therefore, compound **2** was identified as a quercetin.

3.3 Compound 3

This was obtained as a yellow powder. The HRTOFMS spectrum showed $[M+H]^+$ *m/z* 285.0783 (calcd. *m/z* 284.0685), which corresponds to the molecular formula of $C_{16}H_{12}O_5$ with eleven degrees of unsaturation. The ^{1}H-

NMR spectrum of compound **3** shows a characteristic AA′BB′ pattern of ring B at δ_H 7.80 (2H, d, J = 8.0 Hz) and δ_H 6.70 (2H, d, J = 8.0 Hz), corresponding to H-2′/H-6′ and H-3′/H-5′, respectively. The resonances of two *meta*-coupling protons of ring A were observed at δ_H 6.40 (1H, d, J = 2.5 Hz) and δ_H 6.41 (1H, d, J = 2.5 Hz). In ^{1}H-NMR also a singlet olefinic signal was observed at δ_H 6.50 (1H, H-3) and a methoxy group at δ_H 4.10 (3H, 4′-OCH_3). The ^{13}C-NMR and DEPT spectra show the presence of fifteen sp^2 carbons consisting of seven sp^2 methines, eight sp^2 quaternary carbons and a ketone at δ_C 175.8 ppm. These functionalities account for eight of the total of eleven degrees of unsaturation, and the remaining three degrees of unsaturation were consistent with a flavonoid structure. The full assignments of the ^{13}C-NMR data of compound **3** are shown in Table 1. Comparison of the NMR data of **3** with acacetin [13] shows high similarity, consequently, compound **3** was identified as an acacetin.

3.4 Compound 4

This was obtained as a yellow powder. The HR-TOFMS spectrum shows *m/z* 463.0883 $[M-H]^-$ (calcd. $C_{21}H_{20}O_{12}$, *m/z* 464.0955) and thus required twelve degrees of unsaturation. The ^{1}H and ^{13}C-NMR spectrum of **4** resembles that of **2** except for the presence of oxygenated carbons. A methylene signal was present, which occurred as a doublet at δ_H 3.68 (1H, d, J = 5 Hz) together with an anomeric signal proton at δ_H 5.11 (1H, d, J = 7.2 Hz) as well as four oxygenated methines, resonating at δ_H 3.46 (1H, d, J = 9.0 Hz, H-2″), 3.39 (1H, d, J = 9.0 Hz, H-3″), 3.32 (1H, d, J = 9.6 Hz, H-4″), and 3.19 (1H, m, H-5″), which is typical for a glucose moiety. In ^{13}C-NMR also a typical anomeric carbon resonating at δ_C 103.5 was observed, which can be assigned to the one sugar units of β-D-glucopyranoside. The HMBC spectrum shows correlations between H-1″ at δ_H 5.11 (1H, d, J = 7.2 Hz) with C-3 (δ_C 134.1 ppm), which indicate that the sugar group is attached at C-3 of the flavonoid structure. Comparing the NMR data of **4** (Table 2) with the literature data [14], compound **4** was identified as a quercetin-3-*O*-β-D-glucopyranoside.

3.5 Compound 5

This was obtained as a yellow powder. The HR-TOFMS spectrum shows *m/z* 447.3783 $[M-H]^-$ (calcd. $C_{21}H_{20}O_{11}$, *m/z* 448.3769) and thus required twelve degrees of unsaturation. The ^{1}H and ^{13}C-NMR spectrum of **5** resembles that of **1** except for the presence of oxygenated carbons. A methylene signal was present, which occurred as a doublet at δ_H 3.08 (1H, d, J = 5.2 Hz) together with an anomeric signal proton at δ_H 5.45 (1H, d, J = 7.75 Hz) as well as four oxygenated methines, resonating at δ_H 4.26 (1H, dd, J = 7.75, 9.0 Hz, H-2″), 3.56 (1H, t, J = 9.0 Hz, H-3″), 3.23 (1H, t, J = 9.0 Hz, H-4″), and 3.17 (1H, dt, J = 5.4, 9.0 Hz, H-5″), which is typical for a glucose moiety.

In ^{13}C-NMR a typical anomeric carbon resonating at δ_C 100.9 was also observed, which can be assigned to the one sugar units of a β-D-glucopyranoside. The HMBC spectrum shows correlations between H-1″ at δ_H 5.45 (1H, d, J = 7.75 Hz) with C-3 (δ_C 133.2 ppm), which indicate that the sugar group was attached at C-3 of flavonoid structure. Comparing the NMR data of **5** (Table 2) with the literature data [15], compound **5** was identified as kaempferol-3-*O*-β-D-glucopyranoside.

3.6 Compound 6

This was obtained as a yellow powder. The ESI-TOFMS spectrum showed *m/z* 479.13 $[M+H]^+$ (calcd. $C_{22}H_{22}O_{12}$, *m/z* 478.11) and thus required tvelve degrees of unsaturation. The ^{1}H and ^{13}C-NMR spectrum of **6** had high similarity with **4**. Both were attached to a flavonoid skeleton as quercetin derivatives and sugar moieties in C-3, which is a β-D-glucopyranoside group. The main difference between **6** and **4** is the presence of a methoxy group in **6** attached at C-5′ of the flavonoid skeleton. Comparing the NMR data of **6** (Table 2) with the literature data [16], compound **6** was identified as an isorhamnetin-3-*O*-β-D-glucopyranoside.

Table 2 Antioxidant Activity of Compounds 1-6 Against DPPH Radical-scavenging.

Compounds	IC_{50} (µg/mL)
1	14.78 ± 0.07
2	6.94 ± 0.03
3	16.18 ± 0.025
4	82.55 ± 0.18
5	70.48 ± 0.025
6	65.28 ± 0.022
L-ascorbic acid*	0.15 ± 0.01

*) positive control

Antioxidant activity determination of compounds **1**-**6** was conducted according to the method described in [9] with L-ascorbic acid as positive control. The antioxidant activities of compounds **1**-**6** are shown in Table 2. Compounds **1**-**6** showed weaker activity compared with standard compound. Among the isolated compounds, compound **2** showed the strongest activity with an IC_{50} value of 6.94 ± 0.03 µg/mL. Previous structure-activity studies of flavonoids have pointed to the importance of the number and location of OH groups, which could be more important for antiradical efficacy [15]. The glycoside derivatives of compound **4**-**6** showed weaker antioxidant activity than the nonglycoside derivatives, which suggests that sugar moiety can decrease antioxidant activity only in flavonoid derivatives.

4 Conclusions

Six flavonoid compounds, kaempferol (**1**), quercetin (**2**), acacetin (**3**), quercetin-3-*O*-β-D-glucopyranoside (**4**), kaempferol-3-*O*-β-D-glucopyranoside (**5**), and isorhamnetin-3-*O*-β-D-glucopyranoside (**6**), were isolated from the methanolic extract of *Lygodium microphyllum* herb. Compound **2** showed the strongest antioxidant activity, with IC_{50} values of 6.94 ± 0.03 µg/mL. Glycosilation will affect antioxidant activity in flavonoid derivatives.

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