Rutin (8) - Yellow amorphous powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ_H: 1.00 (3H, d, J = 6.0 Hz, H-5'''), 3.04-3.72 (10H, m, sugar H), 4.39 (1H, s, H-1'''), 5.34 (1H, d, J = 7.5 Hz, Glc H-1), 6.20 (1H, d J = 1.2 Hz, H-6), 6.39 (1H, d, J = 1.2 Hz, H-8), 6.84 (1H, d, J = 8.5 Hz, H-5'), 7.53 (1H, d, J = 2.5 Hz, H-2'), 7.55 (1H, dd, J = 2.5, 8.5 Hz, H-6'), 12.59 (1H, s, 5-OH); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 156.6 (C-2), 133.3 (C-3), 177.3 (C-4), 161.2 (C-5), 98.7 (C-6), 164.2 (C-7), 93.6 (C-8), 156.4 (C-9), 104.1 (C-10), 121.6 (C-1'), 115.3 (C-2'), 144.7 (C-3'), 148.4 (C-4'), 116.3 (C-5'), 121.2 (C-6'), 101.2 (C-1''), 74.1 (C-2''), 76.5 (C-3''), 70.6 (C-4''), 75.9 (C-5''), 67.0 (C-6''), 100.7 (C-1'''), 70.4 (C-2'''), 70.0(C-3'''), 71.9 (C-4'''), 68.2 (C-5'''), 17.7 (C-6''').

Daidzein (9) - White amorphous powder; ^{1}H NMR (400 MHz, DMSO-*d*6) δ_H: 6.79 (2H, d, J = 8.4 Hz, H-3',-5'), 6.84 (1H, d, J = 2.1 Hz, H-8), 6.92 (1H, dd, J = 8.8, 2.1 Hz, H-6), 7.37 (2H, d, J = 8.4 Hz, H-2', 6'), 7.95 (1H, d, J = 8.8 Hz, H-5), 8.27 (1H, s, H-2), 9.52 (1H, s, H-4'), 10.75 (1H, s, H-7); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 102.2 (C-8), 115.1 (C-3', 5'), 115.2 (C-6), 116.8 (C-4a), 122.7 (C-1'), 123.6 (C-3), 127.4 (C-5), 130.2 (C-2', 6'), 152.9 (C-2), 157.3 (C-4'), 157.6 (C-8a), 162.7 (C-7), 174.8 (C-4).

Measurements of nitric oxide in lipopolysaccharide-stimulated BV2 microglia cells

BV-2 cells were maintained in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin. To measure NO production, BV-2 cells were dispensed in wells of a 96-well plate (2 at 10^4 cells/well). After 24 h, the cells were pretreated with compounds for 30 min and stimulated with 100 ng/mL LPS for 24 h. Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant was harvested and mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using an Emax microplate reader (molecular devices). Sodium nitrite was used as a standard to calculate the nitrite concentration. Cell viability was measured using a MTT assay. N^G-Monomethyl-L-arginine (L-NMMA), a well-known NO synthase inhibitor, was tested as a positive control.

Statistical analysis

Data were evaluated for statistical significance by ANOVA test using a computerized statistical package. The data were considered to be statistically significant if the $P \leq 0.05$.

RESULTS AND DISCUSSION

Nine compounds were isolated and their structures were identified as kaempferol (1), afzelin (2), nicotiflorin (3), kaempferol-3-*O*-β-glucopyranosyl-7-*O*-α-rhamnoside (4), rhoifolin (5), quercetin (6), isoquercitrin (7), rutin (8) and daidzein (9) [Figure 1]. Among them, seven flavonoids (2–5 and 7–9) were isolated from *L. cuneata* for the first time. Moreover, compounds 4, 5, 7 and 9 are reported from the genus *Lespedeza* for the first time. The present phytochemical investigation has further enriched our knowledge about the chemistry of *L. cuneata* and has identified compounds 2–5 and 7–9 could be potential chemotaxonomic markers for the species.

Many flavonoids have been intensively studied on their inhibitory effects on inflammatory NO production.[21] Among the studied flavonoids, the bioactivity of various flavonoids is related to the number of hydroxyl groups on the flavonoid B-ring and the presence of the sugar moiety.[22] In the light of this, it appears that interactions might be structure-dependent, meaning that different flavonoids are likely to express different cellular outcomes. Thus, the effect of the isolated flavonoids on NO production in LPS-stimulated BV2 cells were evaluated to estimate the structure-dependency.

Nitric oxide inhibitory activities of the isolated compounds (1–9) and *L. cuneata* extract were evaluated by examining the inhibition of NO production in LPS-activated microglia BV-2 cells [Table 2]. Among the tested compounds, 1 and 6 significantly inhibited NO production with the IC_{50} values of 28.01 and 26.97 μM, respectively, which displayed more potent activity than L-NMMA, a well-known NOs inhibitor. None of the isolates (1–9) showed cytotoxicity at the concentrations up to 50 mM. The result showed that compounds with the sugar moiety (2–5 and 7–8) did not show the inhibition

Table 2: Inhibitory effect on NO production of *L. cuneata* extract and compounds 1-9 in LPS-activated BV-2 cells

Compounds	IC_{50}[a] (μM)	Cell viability[c] (%)
1	28.01	124.1±2.0
2	>500	92.5±6.4
3	>500	101.0±1.7
4	>500	98.6±1.8
5	>500	92.8±8.9
6	26.97	131.5±13.4
7	>500	112.2±7.6
8	>500	115.5±3.2
9	>500	114.4±6.9
Extract	>500	104.0±1.8
L-NMMA[c]	38.17	103.3±3.2

[a]IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells. [b]Cell viability after treatment with 50 μM of each extract was expressed as a percentage (%) of the LPS only treatment group. The results are averages of three independent experiments, and the data are expressed as mean±SD. [c]L-NMMA as a positive control

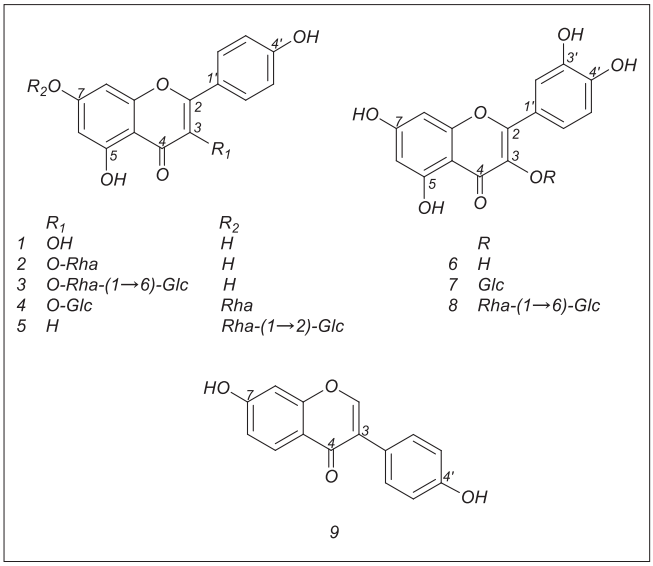

Figure 1: Chemical structures of compounds 1–9

of NO production compared to the aglycones (1 and 6). As results, we suggested that the presence of a hydroxyl group attached at C-3 on the C-ring may be the functional group responsible for the NO inhibitory properties of flavonoids.

CONCLUSION

From the aerial part of *L. cuneata*, nine flavonoids were isolated by chromatographic methods. The isolated compounds were elucidated by ESI-Q-TOF-MS and several NMR techniques. Among the isolates, compounds **1** and **6** exhibited significant NO inhibitory activities in LPS-stimulated microglial BV-2 cells. Compared to the other inactive compounds, these two active compounds have two free hydroxyl groups at both C3 and C7 positions. These results suggest the possible contribution of these hydroxyl groups to the NO inhibitory activity of flavonoids. Taken together, compounds 1 and 6 might be promising candidates for the treatment of various inflammatory diseases.

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