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Two novel flavonoids from the leaves of *Rhododendron dauricum* L. with their inhibition of TNF-α production in LPS-induced RAW 264.7 cells

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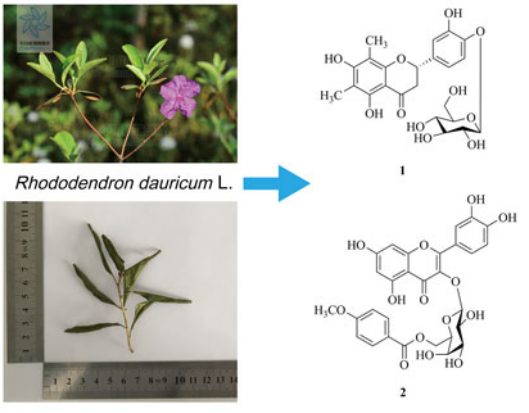
ABSTRACT

Two new flavonoids, (2*S*)-6,8-dimethyl-5,7,3′,4′-tetrahydroxyflavanone 4′-O-β-D-glucopyranoside (**1**) and quercetin 3-O-β-D-(6″-*p*-methoxybenzoyl)-galactopyranoside (**2**), together with ten known flavonoids (**3**–**12**) were isolated from the leaves of *Rhododendron dauricum* L. The structures of the flavonoids were characterized from spectroscopic data (1D and 2D NMR and HR-ESI-MS). The isolated flavonoids were evaluated for their inhibitory effects on the production of tumour necrosis factor (TNF)-α in LPS-stimulated RAW 264.7 cells. Compound **11** exhibited inhibitory activity against TNF-α production with an IC_{50} value of 46.2 ± 1.2 μM.



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Rhododendron dauricum; Ericaceae; flavonoid; TNF-α

1. Introduction

Rhododendron dauricum L. belongs to the Ericaceae family and is mainly distributed in northeast China, north Mongolia, Korea, Japan and Russia. The dried leaves and

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flowers of *R. dauricum* have been used as traditional Korean ethnic medicines to treat acute and chronic bronchitis, asthma and cough (Popescu and Kopp 2013). Previous chemical studies have verified that plants from the *Rhododendron* genus are enriched with a large number of terpenoids, steroids, coumarins, phenols, volatile oils and flavonoids (Iwata et al. 2004; Qiang et al. 2011; Shakeel et al. 2013). Flavonoids and terpenoids are considered to be the major chemical components from the species of the *Rhododendron* genus. We have previously discovered 13 triterpenoids, including one new pentacyclic triterpenoid from the leaves of *R. dauricum* (Li et al. 2019). Pharmacological research has suggested that the flavonoids isolated from *R. dauricum* exhibit broad biological activity, including anti-oxidative, anti-inflammatory and neuroprotective effect, as well as vasodilation and myocardial preservation effects (Zhang et al. 2010; Lou et al. 2015; Cui et al. 2019). Tumour necrosis factor (TNF)-α, a pro-inflammatory cytokine, is generally recognised as one of the key players involved in immunity and inflammation. Overexpression of the pro-inflammatory cytokine TNF-α is associated with several diseases, including diabetes, septic shock, tumorigenesis, cardiovascular diseases, rheumatoid arthritis and inflammatory bowel disease (Du et al. 2014). Thus, inhibition of TNF-α production may help to prevent inflammatory diseases. In our continued search for novel flavonoids and active anti-inflammatory compounds from the leaves of *R. dauricum*, two new flavonoids (**1**–**2**) together with ten known flavonoids (**3**–**12**) were isolated from the ethanol extract of this plant. Herein, we describe the isolation and structure elucidation of these twelve compounds (**1**–**12**), as well as the inhibitory activity against TNF-α production in LPS-induced RAW 264.7 cells.

2. Results and discussion

Compound **1** was obtained as a yellow amorphous powder, with a molecular formula of $C_{23}H_{26}O_{11}$ based on positive HR-ESI-MS (obsd. $[M+H]^+$ at *m/z* 479.1535, theor. $C_{23}H_{27}O_{11}$ at *m/z* 479.1548). The ^{1}H-NMR spectrum of compound **1** showed proton signals for an 1,3,4-trisubstituted aromatic ring at δ_H 7.32 (1H, d, $J = 2.0$ Hz, H-2′), 6.99 (1H, dd, $J = 8.0$, 2.0 Hz, H-6′) and 6.78 (1H, d, $J = 8.0$ Hz, H-5′), an oxygenated methine signal at δ_H 5.64 (1H, dd, $J = 10.2$, 5.3 Hz, H-2), one methylene proton at δ_H 2.91 (2H, m, H-3), two methyl protons at δ_H 2.06 (3H, s, 8-CH_3) and 2.02 (3H, s, 6-CH_3) and a sugar moiety at δ_H 4.80 (1H, d, $J = 7.9$ Hz, H-1″), 3.85 (1H, m, H-6″a), 3.72 (1H, m, H-6″b), 3.45 (2H, m, H-2″, H-3″), 3.40 (1H, m, H-5″) and 3.38 (1H, m, H-4″). The ^{13}C-NMR spectrum showed 23 carbon signals, including a carbonyl group at δ_C 198.5 (C-4), 12 carbons from two benzene rings at δ_C 164.2 (C-7), 160.3 (C-5), 159.4 (C-9), 152.4 (C-4′), 150.5 (C-3′), 128.0 (C-1′), 118.8 (C-6′), 116.7 (C-5′), 116.4 (C-2′), 104.8 (C-6), 104.2 (C-8) and 103.2 (C-10), an oxygenated methine carbon at δ_C 75.5 (C-2), a methylene carbon at 42.9 (C-3) and two methyl carbons at δ_C 8.2 (6-CH_3) and 7.4 (8-CH_3). The remaining six carbon resonances were characteristic of a glucopyranosyl moiety at δ_C 103.5 (C-1″), 78.1 (C-5″), 78.0 (C-3″), 75.0 (C-2″), 71.4 (C-4″) and 62.5 (C-6″). The observed ^{1}H and ^{13}C-NMR data suggested that the skeleton of compound **1** was a glycosylated flavanone. The absolute configuration of the sugar was determined to be D-glucose by comparison of the retention time and optical rotation value with an authentic sample

of D-glucose after acid hydrolysis of compound **1** (Zong et al. 2016; Zhang et al. 2018). The coupling constant ($J = 7.9$ Hz) of the anomeric proton confirmed a β-glucosidic linkage (Orhan 2003). The aglycone was identified as 5,7,3′,4′-tetrahydroxy-6,8-dimethyl flavanone based on the close similarity of the NMR data with myrciacitrin III (**3**, Matsuda et al. 2002), except for the relative positions of the OH groups in the B ring, and sugar moiety. The glycosidic linkage was established based on the key HMBC correlation (Supplementary material, Figure S1) between the anomeric proton signal H-1″ (δ_H 4.80) and the oxygenated C-4′ (δ_C 152.4), which indicated that the β-D-glucose linkage was at the C-4′ position of the B-ring. The HMBC correlations from the methyl group protons 6–CH_3 (δ_H 2.02) to C-5 (δ_C 160.3), C-6 (δ_C 104.8) and C-7 (δ_C 164.2), and 8–CH_3 (δ_H 2.06) to C-7 (δ_C 164.2), C-8 (δ_C 104.2) and C-9 (δ_C 159.4) indicated the location of the two methyl groups at C-6 and C-8. The configuration of compound **1** was considered to be the same as myrciacitrin III ([α]25D : –104.2, EtOH) (Matsuda et al. 2002) based on the similarity of chemical shifts in the ^{13}C-NMR data and specific optical rotation ([α]25D : –197.6, MeOH). According to the overall analysis, the structure of compound **1** was identified as (2*S*)-6,8-dimethyl-5,7,3′,4′-tetrahydroxyflavanone 4′-O-β-D-glucopyranoside.

Compound **2** was obtained as a yellow amorphous powder, with a molecular formula of $C_{29}H_{26}O_{14}$ based on HR-ESI-MS (obsd. $[M+H]^+$ at *m/z* 599.1396, theor. $C_{29}H_{27}O_{14}$ at *m/z* 599.1395). The ^{1}H-NMR spectrum showed that compound **2** had a trisubstituted aromatic ring with an ABX-spin coupling system at δ_H 7.73 (1H, d, $J = 2.1$ Hz, H-2′), 7.58 (1H, dd, $J = 8.5$, 2.1 Hz, H-6′) and 6.86 (1H, d, $J = 8.5$ Hz, H-5′), and an aromatic ring with an AX spin coupling system at 6.35 (1H, d, $J = 2.0$ Hz, H-8) and 6.20 (1H, d, $J = 2.0$ Hz, H-6). In addition, an 1,4-disubstituted aromatic ring with protons at δ_H 7.67 (2H, d, $J = 8.8$ Hz, H-2‴, 6‴), 6.78 (2H, d, $J = 8.8$ Hz, H-3‴, 5‴) and a methoxy group protons at δ_H 3.86 (3H, s, 4‴-OCH_3) suggested the presence of a *p*-methoxybenzoyl group. A sugar moiety was present as indicated by proton signals at δ_H 5.31 (1H, d, $J = 7.9$ Hz, H-1″), 4.47 (1H, m, H-6″a), 4.33 (1H, m, H-6″b), 3.90 (1H, m, H-4″), 3.89 (1H, m, H-2″), 3.87 (1H, m, H-5″) and 3.63 (1H, m, H-3″). The ^{13}C-NMR spectrum revealed 29 carbon signals, including signals from two carbonyl groups at δ_C 179.5 (C-4) and δ_C 167.5 (C-7‴), 18 carbons from three benzene rings at 165.8 (C-7), 164.9 (C-4‴), 163.0 (C-5), 158.3 (C-9), 149.8 (C-4′), 145.8 (C-3′), 132.3 (C-2‴, 6‴), 123.1 (C-6′, 1‴), 122.8 (C-1′), 117.4 (C-2′), 116.0 (C-5′), 114.5 (C-3‴, 5‴), 105.5 (C-10), 99.9 (C-6), and 94.7 (C-8), a double bond at 158.7 (C-2) and 135.3 (C-3), a methoxy group at δ_C 55.9 (4‴-OCH_3) and a galactose moiety at δ_C 104.4 (C-1″), 75.0 (C-5″), 74.8 (C-3″), 73.0 (C-2″), 70.4 (C-4″), and 64.6 (C-6″). The coupling constant ($J = 7.9$ Hz) of the anomeric proton confirmed a β-galactosidic linkage (Lou et al. 2015). Acid hydrolysis of compound **2** gave D-galactose, which was identified by the same method as for compound **1**. The ^{1}H and ^{13}C-NMR spectra suggested that the skeleton of compound **2** was a glycosylated flavonoid, and the NMR spectroscopic data were found to be similar to quercetin 3-O-β-D-galactopyranoside (**7**, Ye and Huang 2006). Thus, compound **2** was identified as a quercetin-3-O-β-D-galactopyranoside derivative containing a *p*-methoxybenzoyl group. In the HMBC spectrum of compound **2** (Supplementary material, Figure S1), connectivity between the *p*-methoxybenzoyl moiety and the D-galactose unit was indicated

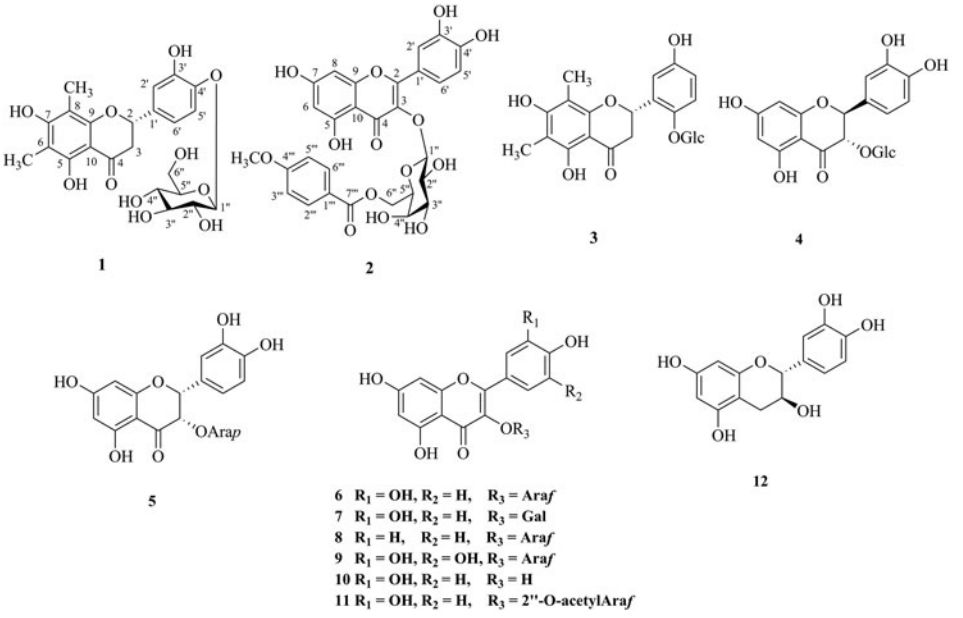

Figure 1. Structure of compounds 1-12 from *Rhododendron dauricum*.

by the cross peak between H-6″ (δ_H 4.47, 4.33) and C-7‴ (δ_C 167.5). The HMBC correlation between H-1″ (δ_H 5.31) and C-3 (δ_C 135.3) revealed that the D-galactose moiety was attached to the C-3. According to the overall analysis, the structure of compound **2** was identified as quercetin 3-O-β-D-(6″-*p*-methoxybenzoyl)-galactopyranoside.

The structures of the ten known flavonoids were determined to be myrciacitrin III (**3**, Matsuda et al. 2002), (2*S*,3*S*)-taxifolin 3-O-β-D-glucoside (**4**, Seo et al. 2017), *trans*-taxifolin 3-O-α-L-arabinopyranoside (**5**, De Abreu et al. 2011), avicularin (**6**, Marzouk et al. 2007), quercetin 3-O-β-D-galactopyranoside (**7**, Ye and Huang 2006), kaempferol 3-O-α-L-arabinofuranoside (**8**, Alvarez et al. 2016), myricetin 3-O-α-L-arabinofuranoside (**9**, Kadota et al. 1990), quercetin (**10**, Bitis et al. 2010), quercetin 3-O-α-L-(2″-O-acetyl) arabinofuranoside (**11**, Camacho-Luis et al. 2008) and (+)-catechin (**12**, Badral et al. 2017) (Figure 1). The structures were elucidated by comparing the experimental spectroscopic data with reported values.

All the isolated flavonoids were screened for inhibitory effects on the level of TNF-α in LPS-activated RAW 264.7 cells. First, cell viability was determined by the MTT assay. Compounds **1**–**12** did not display significant cytotoxicity toward RAW 264.7 cells at 100 μM (Supplementary material, Figure S12). These compounds were examined further at concentrations of 10, 30 and 100 μM to explore the effects on the production of TNF-α. Compound **11** displayed inhibitory activity with an IC_{50} value of 46.2 ± 1.2 μM (Table 1). Compound **11** showed better inhibitory activity than the other flavonoids tested. Analysis of the structure-activity relationship showed that the acetyl group at the C-2″ position of compound **11** was likely responsible for the improved inhibitory of TNF-α production compared with the other compounds.

Table 1. Inhibitory effects of compounds **1**–**12** on TNF-α levels in LPS-stimulated RAW 264.7 cells[a].

Compounds	IC_{50} (μM)
1	76.9 ± 3.0
2	>100
3	72.75 ± 1.7
4	69.5 ± 1.9
5	49.6 ± 2.3
6	>100
7	>100
8	93.7 ± 2.1
9	>100
10	54.7 ± 1.8
11	46.2 ± 1.2
12	>100
Dexamethasone[b]	3.4 ± 0.4

[a]Data are the mean ± SD of three experiments.
[b]Dexamethasone was used as the positive control.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a Rudolph Autopollautomatic polarimeter. The ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV 300 MHz and AV 500 MHz spectrometers (Burker, Fällanden, Switzerland) using TMS as an internal standard. HR-ESI-MS was performed using a Bruker microTOF QIImass spectrometer (Bruker Daltonics, Fremont, CA, USA). Column chromatography was carried out with normal-phase silica gel (200–300 mesh, Branch of Qingdao Haiyang Chemical Co., Ltd, Qingdao, P.R.China), and reversed-phase silica gel LiChroprep RP-18 gel (40–63 μm, Merck KGaA, Darmstadt, Germany). Preparative MPLC was performed on an automatic flash chromatography device (CHEETAH MP-200, Bonna-Agela Technologies Co., Ltd.) and columns were packed with C_{18} (20–45 μm, 40–60 μm) silica. TLC was conducted on pre-coated silica gel GF_{254} (200 × 200 mm, Qingdao Haiyang Chemical Co., Ltd.) or RP-18 F_{254} (Merck) glass plates. All other chemicals and solvents were of analytical grade and used without further purification.

3.2. Plant material

The leaves of *R. dauricum* L. were collected from the surrounding region of Yanji city, Jilin province and identified by one of the authors (Prof. Gao Li). A voucher specimen (YB-RD-1610) was deposited at the College of Pharmacy, Yanbian University.

3.3. Extraction and isolation

The dried leaves of *R. dauricum* (17.0 kg) were extracted with 95% EtOH (85 L × 3) at room temperature for a week. The EtOH extract (3.8 kg) was suspended in H_2O and partitioned with petroleum ether, EtOAc and *n*-BuOH. The EtOAc fraction (569.0 g) was subjected to silica gel column chromatography with CH_2Cl_2–MeOH (25:1 to 5:1) to obtain 11 fractions (Fr.E1–E11). Fraction E9 (104 g) was purified by silica gel column chromatography and eluted with CH_2Cl_2–MeOH (18:1 to 10:1) to give seven sub-

fractions (Fr.E9-1–E9-7). Fraction E9-3 (3.2 g) was further separated by MPLC (C_{18}, 40–60 μm) eluted with MeOH–H_2O (1:3 to 1:0) to give compounds **10** (12.6 mg) and **12** (2.3 mg). Fraction E9-7 (1.4 g) was purified by MPLC (C_{18}, 20–45 μm) with MeOH–H_2O (1:4 to 1:0) as the elution solvent to afford compounds **2** (4.5 mg) and **11** (16.4 mg). Fraction E10 (55 g) was purified by MPLC (C_{18}, 40–60 μm) with MeOH–H_2O (1:19 to 1:0) to give six sub-fractions (Fr.E10-1–E10-6). Fraction E10-3 (6 g) was purified by silica gel column chromatography eluted with CH_2Cl_2–MeOH (20:1 to 5:1) followed by MPLC (C_{18}, 20–45 μm) with MeOH–H_2O (1:5 to 1:0) to give compounds **5** (196.9 mg) and **8** (8.4 mg). Fraction E10-5 (12.1 g) was separated using MPLC (C_{18}, 40–60 μm) with MeOH–H_2O (1:4 to 1:0) to obtain compounds **9** (10.2 mg) and **7** (2.3 g). Fraction E11 (9 g) was purified by MPLC (C_{18}, 40–60 μm) with MeOH–H_2O (1:9 to 3:2) to give three sub-fractions (Fr.E11-1–E11-3). Fraction E11-2 (6.10 g) was subjected to silica gel column chromatography and eluted with CH_2Cl_2–MeOH (20:1 to 12:1) to yield compound **4** (122.1 mg) and three sub-fractions (Fr.E11-2-2–E11-2-4). Fraction E11-2-4 (4.51 g) was purified by MPLC (C_{18}, 40–60 μm) with MeOH–H_2O (1:9 to 1:0) to give compounds **1** (46.7 mg), **3** (1.9 mg), and **6** (3.42 g).

(2*S*)-6,8-Dimethyl-5,7,3′,4′-tetrahydroxyflavanone 4′-O-β-D-glucopyranoside (1): Yellow amorphous powder, [α]25D : –197.6 (*c* 0.06, MeOH). HR-ESI-MS: *m/z* 479.1535 $[M+H]^+$ (calcd. for $C_{23}H_{27}O_{11}$, *m/z* 479.1548) .^{1}H-NMR (300 MHz. methanol-d_4); δ: 7.32 (1 H, d, $J=2.0$ Hz, H-2′), 6.99 (1 H, dd, $J=8.0$, 2.0 Hz, H-6′), 6.78 (1 H, d, $J=8.0$ Hz, H-5′), 5.64 (1 H, dd, $J=10.2$, 5.3 Hz, H-2), 4.80 (1 H, d, $J=7.9$ Hz, H-1″), 3.85 (1 H, m, H-6″a), 3.72 (1 H, m, H-6″b), 3.45 (2 H, m, H-2″, H-3″), 3.40 (1 H, m, H-5″), 3.38 (1 H, m, H-4″), 2.91 (2 H, m, H-3), 2.06 (3 H, s, 8-CH_3), 2.02 (3 H, s, 6-CH_3); ^{13}C-NMR (75 MHz, methanol-d_4), δ: 198.5 (C-4), 164.2 (C-7), 160.3 (C-5), 159.4 (C-9), 152.4 (C-4′), 150.5 (C-3′), 128.0 (C-1′), 118.8 (C-6′), 116.7 (C-5′), 116.4 (C-2′), 104.8 (C-6), 104.2 (C-8), 103.5 (C-1″), 103.2 (C-10), 78.1 (C-5″), 78.0 (C-3″), 75.5 (C-2), 75.0 (C-2″), 71.4 (C-4″), 62.5 (C-6″), 42.9 (C-3), 8.2 (6-CH_3), 7.4 (8-CH_3).

Quercetin 3-O-β-D-(6″-*p*-methoxybenzoyl)-galactopyranoside (2): Yellow amorphous powder, [α]25D : +16.7 (*c* 0.04, MeOH). HR-ESI-MS: *m/z* 599.1396 $[M+H]^+$ (calcd. for $C_{29}H_{27}O_{14}$, *m/z* 599.1395). ^{1}H-NMR (500 MHz, methanol-d_4) δ: 7.73 (1 H, d, $J=2.1$ Hz, H-2′), 7.67 (2 H, d, $J=8.8$ Hz, H-2‴, 6‴), 7.58 (1 H, dd, $J=8.5$, 2.1 Hz, H-6′), 6.86 (1 H, d, $J=8.5$ Hz, H-5′), 6.78 (2 H, d, H-3‴, 5‴), 6.35 (1 H, d, $J=2.0$ Hz, H-8), 6.20 (1 H, d, $J=2.0$ Hz, H-6), 5.31 (1 H, d, $J=7.9$ Hz, H-1″), 4.47 (1 H, m, H-6″a), 4.33 (1 H, m, H-6″b), 3.90 (1 H, m, H-4″), 3.89 (1 H, m, H-2″), 3.87 (1 H, m, H-5″), 3.86 (3 H, s, 4‴-OCH_3), 3.63 (1 H, m, H-3″). ^{13}C-NMR (125 MHz, methanol-d_4) δ: 179.5 (C-4), 167.5 (C-7‴), 165.8 (C-7), 164.9 (C-4‴), 163.0 (C-5), 158.7 (C-2), 158.3 (C-9), 149.8 (C-4′), 145.8 (C-3′), 135.3 (C-3), 132.3 (C-2‴, 6‴), 123.1 (C-6′, 1‴), 122.8 (C-1′), 117.4 (C-2′), 116.0 (C-5′), 114.5 (C-3‴, 5‴), 105.5 (C-10), 104.4 (C-1″), 99.9 (C-6), 94.7 (C-8), 75.0 (C-5″), 74.8 (C-3″), 73.0 (C-2″), 70.4 (C-4″), 64.6 (C-6″), 55.9 (4‴-OCH_3).

3.4. Determination of absolute configuration of the sugars

Acid hydrolysis was carried out to obtain the free sugar residues. Compounds **1** and **2** (3 mg each) were treated with 1.0 M HCl at 85 °C for 2 h and the reaction mixture was partitioned with EtOAc. The EtOAc residue was dissolved in dry pyridine after the

addition of L-cysteine methyl ester hydrochloride and heated at 60 °C for 2 h. Trimethylsilylimidazole was added followed by heating at 60 °C for 2 h, and then the mixture was evaporated. The dried product was partitioned with *n*-hexane and H_2O. The *n*-hexane layer was analysed by GC, and the sugars were confirmed to be D-glucose and D-galactose by comparison of the retention times with those of authentic samples. The absolute configurations of the sugar units were also elucidated by analyzing the optical rotation values using a polarimeter after the acid hydrolysis. The hydrolysates of compounds **1** and **2** were detected at t_R 21.83 (positive optical rotation) and 22.74 min (positive optical rotation) indicating D-glucose and D-galactose, respectively.

3.5. Cell viability assay

Cell viability was measured by the MTT assay. RAW 264.7 cells (1×10^5 cells/well) were seeded into 96-well plates for 24 h at 37 °C. After incubation, the cells were treated with compounds **1**–**12** (100 μM) for 24 h, added with 20 μL MTT (0.5 mg/mL) was added and the mixture was incubated for an additional 3 h. The absorbance was measured at 540 nm on a micro-plate reader.

3.6. TNF-α production assay

The levels of TNF-α were analysed by the ELISA method using commercially available kits. RAW 264.7 cells (1×10^5 cells/well) were incubated in 96-well plates and treated with compounds **1**–**12** at 10, 30 and 100 μM for 2 h before stimulation with LPS (1 μg/mL). After that, the supernatants were collected after 24 h stimulation. Dexamethasone was used as a positive control. The absorbance was examined with a micro-plate reader at 450 nm.

3.7. Statistical analysis

All data were expressed as the mean ± standard deviation (SD), and analysed using the GraphPad Prism Software (San Diego, CA, USA); p values < 0.05 were deemed to indicate statistical significance.

4. Conclusion

In summary, two new flavonoids (**1**–**2**) and ten known flavonoids (**3**–**12**) were isolated and identified from the leaves of *R. dauricum*. Among the isolates, five compounds (**1**–**3**, **9, 11**) were found in the Ericaceae family for the first time. Compound **11** exhibited TNF-α inhibitory activity with an IC_{50} value of 46.2 ± 1.2 μM. This study demonstrated that new flavonoid compounds remain undiscovered in this plant and that some of these isolates may have potential use in the treatment of inflammatory diseases.

Disclosure statement

No potential conflict of interests was reported by the authors.

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