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Inhibitory effects of flavonol glycosides from *Cinnamomum osmophloeum* on inflammatory mediators in LPS/IFN-γ-activated murine macrophages

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Abstract—Four kaempferol glycosides were isolated from the leaves of *Cinnamomum osmophloeum* Kaneh, a Taiwan endemic tree. These compounds namely, kaempferitrin (**1**), kaempferol 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside (**2**), kaempferol 3-*O*-β-D-apiofuranosyl-(1→2)-α-L-arabinofuranosyl-7-*O*-α-L-rhamnopyranoside (**3**), and kaempferol 3-*O*-β-D-apiofuranosy-(1→4)-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside (**4**). The structure of compound **2** was determined by spectroscopic analyses and acid hydrolysis. The isolates **1**–**4** were evaluated as inhibitors of some macrophage functions involved in the inflammatory process. These four compounds inhibited lipopolysaccharide (LPS) and interferon (IFN)-γ-induced nitric oxide (NO), and cytokines [tumor necrosis factor (TNF)-α and interleukin (IL)-12] in a dose-dependent manner. The concentration of 50% inhibition (IC_{50}) of NO by compounds **1**, **3**, **4** were 40, 15, 20 μM, respectively. In parallel, these concentrations were approximately in a similar manner to that observed for TNF-α and IL-12 production. However, compound **2** inhibited NO and cytokines production by 30% at 100 μM concentration. On the other hand, compounds **3** and **4** showed no inhibitory effect on the production of NO from macrophages, when inducible NO synthase was already expressed by the stimulation with LPS and IFN-γ. Taken together, our results provide evidence that isolates of *C. osmophloeum* possess an anti-inflammatory potential which constitutes a previously unrecognized biological activity.

1. Introduction

The genus *Cinnamomum* includes 250 species, inclusive of shrubs and trees. Many plants of this genus have long been used in folk medicine for their interesting biological activities such as anti-diabetic, anti-inflammatory, intestinal infections, astringent, and diuretic.[1] *Cinnamomum osmophloeum* Kaneh. (Lauraceae) is an endemic tree that grows in Taiwan's natural hart wood forest at an elevation between 400 and 1500 m. This plant has been of interest to researchers because the chemical constituents of its leaf essential oils were similar to those of *Cinnamomum cassia* bark oil, commercially known as cinnamon oil, commonly used in food and beverages in many countries. The chemical constituents of leaf essential oils were different from various *C. osmophloeum* clones found in different regions of Taiwan.[2] This plant species was not only important as a spice, but also essential oils have various medicinal properties such as sweet constituents,[3] anti-bacterial,[4,5] anti-termitic,[6] anti-mite,[7] chemical polymorphism,[8] anti-cancer,[9] and mosquito larvicidal activity.[10] However, despite its essential oils use, there is no report on the pharmacological properties of *C. osmophloeum* and its secondary metabolites. It is increasingly being acknowledged that medicinal plants and their foods, and beverages contain non-nutritional constituents that may possess biological activities compatible with beneficial health effects, such as anti-inflammatory and anti-carcinogenic properties.[11] Because of rapid development and employment of modern analytical equipment and technology, natural active components as inhibitors of inflammatory mediators have been studied and identified from medicinal plants.[12]

Nitric oxide (NO) is an endogenous free radical species that produced from L-arginine by nitric oxide synthase (NOS), a family of ubiquitous enzymes. Molecular cloning and sequencing analyses have revealed the existence

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of at least three main types of NOS isoforms. Both neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed,[13] whereas inducible NOS (iNOS) is expressed in response to interferon (IFN)-γ, lipopolysaccharide (LPS), and a variety of pro-inflammatory cytokines.[14] Following exposure to LPS or cytokines, iNOS can be induced in various cells, such as macrophages, kupffer cells, smooth muscle cells, and hepatocytes. iNOS activation catalyzes the formation of a large amount of NO, which plays a key role in a variety of pathophysiological processes including various forms of circulatory shock, inflammation, and carcinogenesis.[15] Therefore, the amount of NO produced by iNOS may be a reflection of the degree of inflammation, and therefore provide a means of assessing the effects of compounds on the inflammatory process.

Cytokines are soluble hormone-like protein mediators produced by diverse cell types in response to various stimuli including other cytokines. Tumor necrosis factor (TNF)-α is a pro-inflammatory cytokine, LPS and phagocytosis of microbes are potent stimuli for TNF-α production by macrophages. This cytokine is generally assumed to play an important role in chronic inflammatory diseases such as multiple sclerosis or rheumatoid arthritis.[16] Over production of TNF-α is associated with a wide range of pathologic conditions and has therefore led to much recent effort to find ways to down regulate its production or inhibit its effects in vivo. On the other hand, interleukin (IL)-12 exerts multiple biological activities mainly through T and natural killer cells by inducing their production of interferon (IFN)-γ, which augments their cytotoxicity, and by enhancing their proliferation potential. However, IL-12 production is critical for the development of T-helper type 1 cells and initiation of cell-mediated immune responses.[17] Recent evidences revealed to a critical role for IL-12 in the pathogenesis of rodent models of Th1-mediated autoimmune diseases such as type-1 diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, and acute graft-versus-host.[18] Thus, pharmacological control of IL-12 production may be a key strategy in modulating specific immune-mediated diseases dominated by type-1 cytokine responses. To develop new and potential safer types of anti-inflammatory mediators, in the present study we describe the isolation of four kaempferol glycosides, namely kaempferitrin (**1**), kaempferol 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside (**2**), kaempferol 3-*O*-β-D-apiofuranosyl-(1→2)-α-L-arabinofuranosyl-7-*O*-α-L-rhamnopyranoside (**3**), and kaempferol 3-*O*-β-D-apiofuranosyl-(1→4)-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside (**4**) from *C. osmophloeum* leaves. The inhibitory effects of the isolates **1**–**4** were evaluated on the production of NO and cytokines (TNF-α, and IL-12) in LPS/IFN-γ-activated murine peritoneal macrophages.

Flavonoids are prominent plant secondary metabolites that are consumed by humans as dietary constituents in amounts exceeding 0.1 g/day, suggesting that their ingestion may play a significant role in health and disease.[19] These compounds possess a common phenylbenzopyrone structure (C6–C3–C6), and they are categorized according to the saturation level and opening of the central pyran ring, mainly into flavones, flavanols, isoflavones, flavonols, flavanones, and flavanonols. Flavonoids, and their sugar derivatives display a remarkable spectrum of biological activities including anti-inflammatory.[19–22] Importantly, a flavonoid derivative flavopiridol has been found to inhibit cyclin-dependent kinases (Cdk's), induce apoptosis, suppress inflammation, and modulate the immune response,[23] and is the first compound with this activity to have entered the clinic as an anti-cancer drug. Although not considered nutrients and thus essential for life, flavonoids have beneficial health effects and can be considered possible chemopreventive or therapeutic agents against inflammatory diseases.

2. Results

The leaves of *C. osmophloeum* were extracted with MeOH under reflux. The water suspended methanol extract was defatted with *n*-hexane, and then partitioned with chloroform and *n*-butanol. The chloroform soluble fraction was subjected to repeated column chromatography to give compound **1**. The *n*-butanol extracts were chromatographed by conventional isolation procedures to afford compounds **2**–**4**. The compounds **1**–**4** were isolated from this plant for the first time, and **1**, **3**, and **4** were identified by comparison of their physical and spectral data with those reported in the literature.[24–26] Compound **2** was obtained as yellow amorphous powder. This kaempferol glycoside was previously identified.[27] However, as the spectral data of the compound were not reported so far these have been recorded here (vide experimental). The interesting point mentioned here is that compounds **1** and **2** were obtained from the *C. osmophloeum* leaves in appreciable quantities (0.2428% and 0.0228% (w/w), respectively).

In order to investigate anti-inflammatory properties of compounds **1**–**4** and to elucidate the underlying mechanisms of action, we used murine peritoneal excluded macrophages, which can produce NO, TNF-α, and IL-12, upon stimulation with LPS (2 μg/mL) plus IFN-γ (10 U/mL), thus providing a suitable model for studying inflammatory response in culture cells. LPS/IFN-γ, a known potent macrophage activator, was used as a positive control. In the macrophages, the potential toxicity of compounds **1**–**4** was assessed by MTT assay after 24 h incubation. Cell viability was not affected by compounds **1**–**4** up to the concentration range studied (data not shown). Thus, these concentrations of compounds **1**–**4** were chosen in the subsequent experiments. At these concentrations, compounds **1**–**4** caused a dose-dependent inhibition of LPS/IFN-γ-stimulated peritoneal excluded macrophages NO production, as indicated by the nitrite concentrations in supernatants (Fig. 2, open column). Among these kaempferol glycosides tested, although all exhibited some activity compound **3** was significantly more active in inhibiting NO production. At 20 μM concentration compound **3** reduced 69% of NO production of the positive control, whereas com-

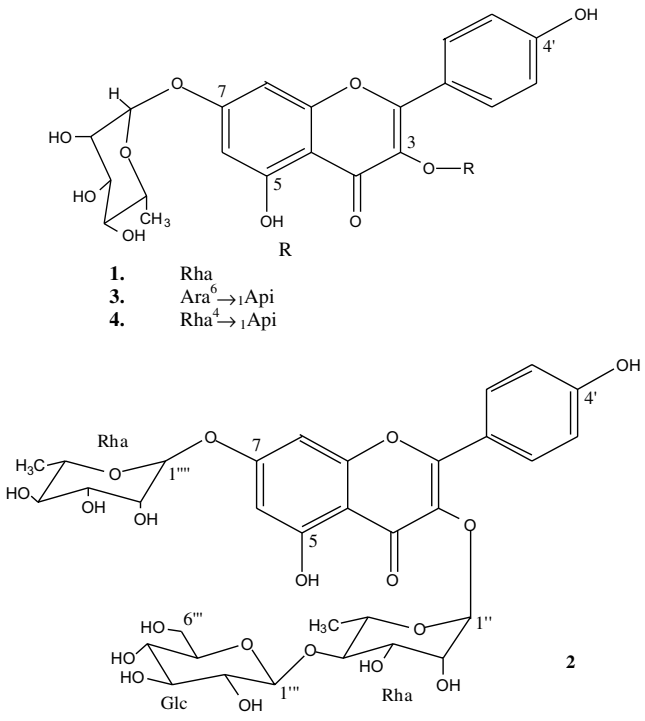

Figure 1. Chemical structures of kaempferol glycosides (**1**–**4**), isolated from *Cinnamomum osmophloeum* leaves.

pound **2** reduced only 9%. The 50% inhibition concentration (IC_{50}) value of compounds **1**, **3**, **4** were 40, 15, 20 μM, respectively. However, no detectable level of NO measured when macrophages were cultured with compounds **1**–**4** in the absence of LPS and IFN-γ.

We further evaluated the inhibitory effects of compounds **1**–**4** on the production of TNF-α, and IL-12 another mediators of inflammation in LPS/IFN-γ treated mouse peritoneal macrophages. Macrophages were incubated in the presence of compounds **1**–**4** and the quantities of these cytokines secreted into the culture supernatants were then monitored by ELISA. Addition of compounds **1**–**4** to LPS/IFN-γ stimulated peritoneal macrophage cultures inhibited the TNF-α (Fig. 2, striped columns), and IL-12 (Fig. 2, dotted columns) production in a manner similar to that reported above for NO production. Compound **3** was the most potent TNF-α and IL-12 inhibitor, with significant inhibition at 10 μM, and 41% of TNF-α production and 35% of IL-12 production of the positive control at 20 μM, whereas the relatively inhibitory effect of compound **1** at 50 μM was 44% (TNF-α), 42% (IL-12); and compound **4** at 40 μM of 21% (TNF-α), 24% (IL-12), respectively.

In the next experiment, the compounds **1**–**4** were investigated whether the reduction of nitrite accumulation was a result from inhibition of early activation stage or from inhibition of its enzymatic activity. Therefore, we pretreated macrophages with LPS (2 μg/mL) for 24 h, a time when inducible synthase was already induced. After washing the medium, compounds **1**–**4** were added individually to the cells already treated with LPS and nitrite production was measured after a further 24 h. As shown in Figure 3, compounds **1** and **2** failed to reduce the secretion of NO from macrophages that had been pretreated with LPS for 24 h (Fig. 3A and B, respectively). Importantly, the inhibitory effect of 20 μM compound **3** was similar no matter macrophages

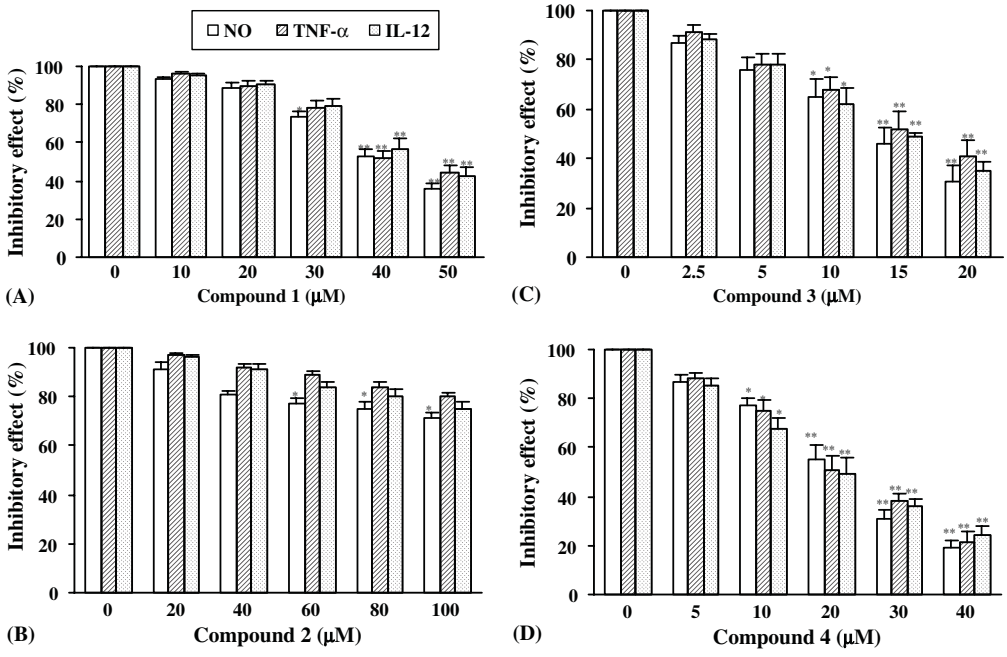

Figure 2. Effect of kaempferol glycosides **1**–**4**, on the production of nitric oxide (open column), tumor necrosis factor (TNF)-α (striped columns) and interleukin (IL)-12 (dotted columns). Macrophages were stimulated with LPS (2 μg/ml) plus IFN-γ (10 U/mL) in the presence of various concentrations of compounds **1** (panel A), **2** (panel B), **3** (panel C), and **4** (panel D) as indicated. Supernatants were collected after 24 h (data represent the mean ± SD of three separate experiments). Values are significant when compared with the absence of test compounds **1**–**4**, $*p < 0.05$; $**p < 0.01$.

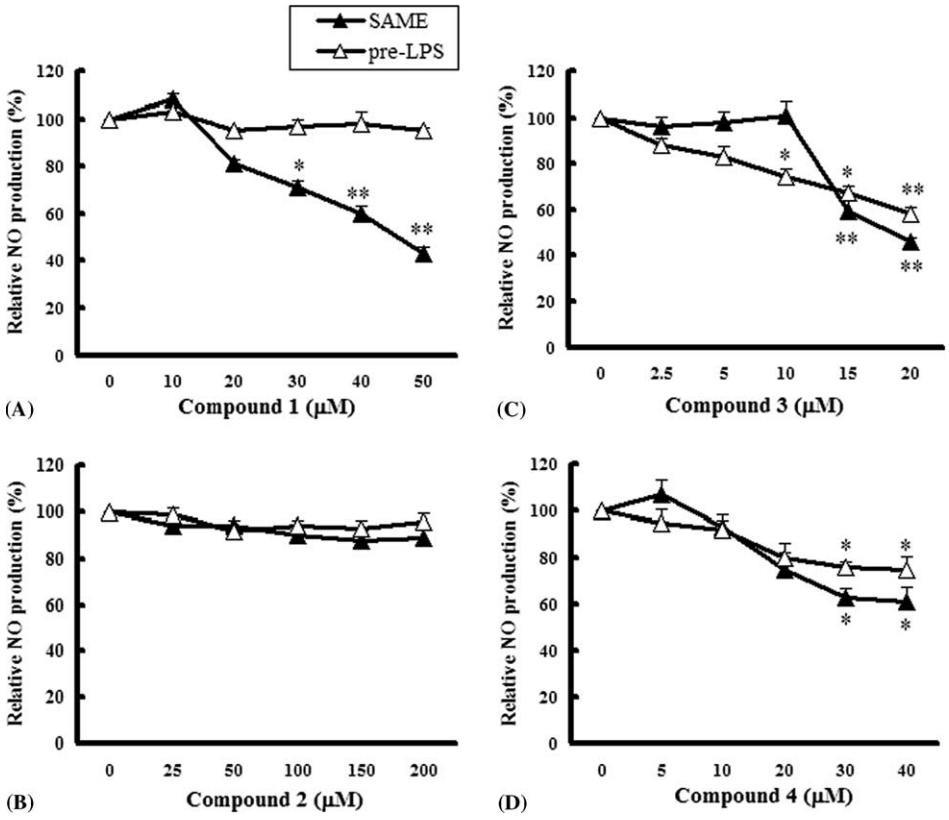

Figure 3. Effects of kaempferol glycosides **1**–**4** on NO synthesis by LPS (2 μg/ml)/IFN-γ (10 U/mL) stimulated or pre-activated macrophages. Different concentrations of test compounds **1** (panel A), **2** (panel B), **3** (panel C), and **4** (panel D) were added to LPS/IFN-γ stimulated macrophages at the same time (open triangle) or after 24 h (black triangle). Supernatants were collected after further 24 h, and determined the NO (data represent the mean ± SD of three replications). Values are significant when compared with the absence of test compounds **1**–**4**, $*p < 0.05$; $**p < 0.01$.

were pre-activated or not (Fig. 3C). In addition, the 40% inhibitory effect of compound **4** was decreased to about 20% when macrophages had been pre-activated (Fig. 3D).

3. Discussion

3.1. Structure elucidation of compound 2

Compound **2** was obtained as a yellow amorphous powder, and was assigned the molecular formula $C_{33}H_{40}O_{19}$ by analyses of HR-EIMS at *m*/*z* 740.6679 $[M]^+$ and ^{13}C NMR spectrum which showed signals for all the 33 carbons of the molecule. In addition, DEPT experiments also indicated the presence of two methyls, one methylene, 21 methines, as well as nine non-protonated carbons in the molecule. The UV spectra in methanol and with diagnostic reagents of **2** were in accord with 3,7-di-*O*-substituted flavonols with free hydroxyl groups at the 5 and 4′-positions.[28] The IR spectra of **2** showed strong absorption bands at 3416 (OH), 2990 (C-H), 1660 (C=C aromatic), 1620 (C=O), and a broad band at 1140–1000 cm^{-1} indicating its glycosidic nature.

The ^1H NMR spectrum of **2** displayed the expected signals in the aromatic region, that is, two *ortho* coupled doublets at δ 6.94 (2H, d, $J = 9$ Hz) and 7.80 (2H, d, $J = 9$ Hz) corresponding to the protons of the B-ring and the two *meta* coupled doublets at δ 6.46 (1H, d, $J = 1.8$ Hz) and 6.79 (1H, d, $J = 1.8$ Hz) for 6 and 8 protons of ring-A. The chemical shift of unsubstituted kaempferol usually appears C-3 and C-7 at 136.4 and 163.7, respectively.[29] However, the ^{13}C NMR spectrum of compound **2** exhibited an upfield shift for C-3 and C-7, by 1.7 and 2 ppm, respectively, revealed glycosylation at both positions. The diequatorial coupling anomeric protons at δ 5.55 (1H, d, $J = 1.2$ Hz) and 5.21 (1H, d, $J = 1.2$ Hz) were attributed to two α-L-rhamnosyl moieties directly linked to the aromatic rings at the 7 and 3 positions, respectively. As the signals of H-1″ and H-1‴ showed ^3J correlations with the signals of C-3 (δ 134.70) and C-7 (δ 161.71) in the HMBC spectrum (Table 1). The presence of two rhamnosyl moieties of 7 and 3 positions were also inferred by two methyl signals at δ 1.12 (3H, s, $J = 6$ Hz) and 0.92 (3H, s, $J = 6$ Hz), respectively. A third diaxial coupling anomeric proton resonated an upfield at δ 4.30 (1H, d, $J = 7.8$ Hz) was assigned to a β-D-glucose moiety involved in the interglycosidic linkage.[24] Attachment of this glucose to C-4″ position of 3-*O*-rhamnose was determined based on downfield shift of C-4″ by 8.6 ppm, and the upfield shift of the C-3″ and C-5″ by 2.3 and 3.1 ppm, respectively.[24,30] In addition, significant signals of C-4″ (δ 81.96) and to HMBC correlations for the pairs of H-1‴/C-4″, as well as H-4″/C-1‴ were also supported the interglycosidic conformation (Table 1). Furthermore, the triglycosidic nature of compound **2** was also con-

Table 1. ^{1}H and ^{13}C NMR spectral data for compound **2**[a]

Position	^{1}H NMR ($J_{H,H}$ in Hz)	^{13}C NMR	HMBC (H→C)
2		157.84	
3		134.70	
4		177.93	
5		160.94	
6	6.46 (d, 1.8)	99.49	5,7,8,10
7		161.71	
8	6.79 (d, 1.8)	94.63	6,7,9,10
9		156.12	
10		105.75	
1′		120.23	
2′	7.80 (d, 9)	130.74	2,3′,4′,6′
3′	6.94 (d, 9)	115.54	1′,2′,4′,5′
4′		160.34	
5′	6.94 (d, 9)	115.54	1′,3′,4′,6′
6′	7.80 (d, 9)	130.74	
5-OH	12.58 (s)		
4′-OH	10.31 (s)		
1″	5.21 (d, 1.2)	101.99	
2″	4.05 (s)	69.82	3″
3″	3.27–3.45 (m)	69.76	2″,5″
4″	3.27–3.45 (m)	81.96	1‴,5″
5″	3.27–3.45 (m)	69.03	4″
6″	0.92 (d, 6)	17.37	4″,5″
1‴	4.30 (d, 7.8)	104.74	4″,5‴
2‴	3.03 (dd, 7.8, 8.4)	74.47	4‴
3‴	2.98 (t, 8.4)	76.99	2‴,4‴
4‴	3.07 (t, 9.6)	70.33	2‴,6‴
5‴	3.15 (ddd, 3.0, 6.0, 9.6)	76.64	3‴
6‴(A)	3.75 (dd, 3.0, 11.4)	60.98	
(B)	3.62 (dd, 6.0, 11.4)		5‴
1⁗	5.55 (d, 1.2)	98.40	7,2⁗,3⁗
2⁗	3.84 (s)	70.12	1⁗,3⁗
3⁗	3.27–3.45 (m)	70.23	2⁗
4⁗	3.27–3.45 (m)	71.58	5⁗
5⁗	3.27–3.45 (m)	69.82	3⁗,6⁗
6⁗	1.12 (d, 6)	17.95	4⁗,5‴

[a] All NMR data were recorded using DMSO-d_6; δ values in ppm. All assignments were confirmed by ^{1}H–^{1}H COSY, HMBC and HMQC spectra.

formed by the ^{13}C NMR, which exhibited three anomeric carbons at δ 101.99, 104.74, and 98.40 for two rhamnosyls and one glucosyl residue, respectively. Using a combination of homo and hetero nuclear two-dimensional NMR techniques (^{1}H–^{1}H COSY, NOESY, HMQC, and HMBC), complete assignment of the ^{1}H and ^{13}C signals of **2** were successfully performed. These findings were confirmed by acid hydrolysis of **2**, which afforded kaempferol, glucose, and rhamnose. In addition, compound **2** was also subjected to mild hydrolysis with 1 N HCl and the intermediate product obtained was identified as kaempferol 3,7-di-*O*-rhamnoside, which was also isolated as a major constituent from the *C. osmophloeum* leaves in this study. Together with these observations along with the molecular formula $C_{33}H_{40}O_{19}$ have led to the conclusion that compound **2** was kaempferol 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside (Fig. 1).

3.2. Biological activity

Experimental and clinical studies demonstrate that exposure to endotoxin (LPS) and IFN-γ results in the release of various inflammatory mediators although particular attention has been paid to the pro-inflammatory cytokines, for example, TNF-α, IL-12, and the reactive oxygen (ROS) and nitrogen species, such as NO, which currently are considered to be a key mediator of tissue injury and mortality in septic shock.[31] We have shown here that compounds **1**–**4** inhibit the production of NO in murine peritoneal macrophages stimulated by LPS and IFN-γ in a dose-dependent manner. Cell viability was not affected after a continuous 24 h exposure to compounds **1**–**4**, therefore these inhibitory effects did not occur through cytotoxicity. LPS and IFN-γ have been reported to stimulate NO production through independent signal pathways. The former stimulates cells by activating NF-kB and the latter by the interferon regulatory factor-1 (IRF-1).[32,33] Based on the results obtained with inflammatory stimuli, the order of potency for the inhibition of NO production by peritoneal macrophages was compounds **3** > **4** > **1** > **2**. This study suggests that *C. osmophloeum* leaves are able to show anti-inflammatory activity in LPS/IFN-γ-activated murine macrophages. Accordingly, the compounds **3** and **4** reduced while compounds **1** and **2** did not change the level of NO from macrophages when iNOS had been induced. These results exclude the possibility that compounds **1** and **2** directly affects the iNOS activity but suggest that these primarily interferes with the LPS/IFN-γ induced expression of certain genes that regulate the production NO independent of upstream of NF-kB mobilization.

Flavonoids extracted from natural sources inhibit inflammatory responses in a variety of in vivo and in vitro models at numerous levels of intervention. Specifically, flavonoids inhibit the release of histamine from basophils,[34] the production of pro-inflammatory eicosanoids,[35] and the production of pro-inflammatory cytokines including TNF-α.[36] Glycosylation commonly occurs in the metabolism of flavonoids, and flavonoid glycosides have been shown to possess more-hydrophilic properties than do flavonoid aglycones. Kim et al. reported that flavonoid glycosides were metabolized to aglycones by human intestinal microflora producing α-rhamnosidase, *exo*-β-glucosidase, *endo*-β-glucosidase, and/or β-glucuronidase. Accordingly, compounds **1**–**4** may be transformed to their respective aglycones (kaempferol), by bacteria producing α-rhamnosidase and β-glucosidase.[37] Furthermore, in a recent in vivo study revealed that compound **1** led to a significant hypoglycemic effect in normal and in alloxan-induced diabetic rats.[38] These data indicated that in vivo metabolic activity may be involved in converting flavonoid glycosides to flavonoid aglycones, and that this might increase their inhibitory activities. However, direct evidence is still sought. From these observations, we are anticipating that compounds **1**–**4** may possess strong in vivo anti-inflammatory activity than the results reported here.

The regulation of cytokine production is a potential target for toxicants that are capable of modifying the immune function. In vitro studies using established cell lines have been useful for characterizing the effects of

natural compounds on the immune function alteration.[39] Administration of TNF-α induces shock, whereas treatment of mice with neutralizing monoclonal antibodies *anti*-TNF-α prevents the mortality caused by lipopolysaccharide challenge.[40] The compounds **3** and **4**, out of the four compounds tested, had significant inhibitory effects on TNF-α production, while the rest showed weak inhibitory activity. However, the protective effect of compounds **1**–**4** in endotoxic shock may be due to not only the inhibitory action on TNF-α production, but also to the suppression of other cytokine such as IL-12, as well as of NO production. Moreover, IL-12 is a potent inducer of interferon-γ, another cytokine related to lethality in endotoxic shock.[41]

4. Conclusion

In conclusion, the present investigation results in the isolation of four kaempferol glycosides for the first time from *C. osmophloeum* leaves. All the isolates were showed a dose-dependent inhibition on the production of NO and cytokines (TNF-α, IL-12), from LPS/IFN-γ-activated macrophages. The compound **3** showed the highest inhibitory activity. In addition, it showed similar inhibitory effect at the same time or post-activation. The kaempferol glycosides reported here are the major class of constituents of *C. osmophloeum*, especially compounds **1** and **2** with high contents in the leaves (0.2428%, and 0.0228% (w/w), respectively). Thus, consumption of *C. osmophloeum* leaves may reduce the oxidative stress of nitric oxide and increase the protective effects against chronic inflammatory diseases.

5. Experimental

5.1. General

Chemicals and solvents were reagent grade and used without further purification. The column fractions were monitored by thin layer chromatography (TLC) on precoated Merck Silicagel $60F_{254}$ aluminum plates; the spots were visualized by exposure to UV radiation and/or by spraying with 10% H_2SO_4 in ethanol and then with saturated alcoholic $FeCl_3$, followed by heating at 110 °C. Melting points were determined on a Kofler hot stage apparatus. Chemical structures of the compounds as shown in Figure 1 were determined by the following spectral data. Specific optical rotations were recorded on a Jasco DIP-370 polarimeter. UV spectra were recorded on a Varian Cary Win UV-50 spectrophotometer. IR spectra were determined in KBr discs on a Perkin–Elmer FT-IR paragon 500 spectrometer. ^1H, ^{13}C, COSY, HMBC, HMQC, and NOESY NMR spectra were recorded on a Varian Unity Inova-600 VXR-300/51 spectrometer operating at 599.949 MHz, 150.872 MHz, respectively, in DMSO-d_6; all chemical shifts were given in ppm from tetramethylsilane as an internal standard. ESI-MS were recorded on a Thermo-Finnigan LCQ Advantage system. HR-EIMS was obtained on VG 70–250S spectrometer by a direct inlet system. Column chromatography (CC) separations were carried out by using silica gel 60 (0.063–0.200 mm) supplied by E. Merck.

Lipopolysaccharide (LPS, from *Escherichia coli* 055: B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Griess reagent were purchased from Sigma Chemical (St. Louis, MO, USA). RPMI-1640 medium, Hank's balanced salt solution (HBSS), penicillin, streptomycin, L-glutamine and fetal calf serum were purchased from Gibco BRL (Grand Island, NY, USA).

5.2. Plant material

The leaves of *C. osmophloeum* were collected in March 2003 from the Yuli, Hualien county in Eastern Taiwan. The authenticity of the plant was confirmed by Forest Bureau Council of Agriculture, Taiwan. The samples were shade dried and milled to powder form, which were then kept in air-tight brown bottle until use.

5.3. Extraction and isolation

The air-dried leaves of *C. osmophloeum* (1.4 kg) were powdered and extracted with methanol (5 L × 8) under reflux. The combined extracts were concentrated under reduced pressure to give dark brown syrup about 58 g (4.12% based on the dry weight of the leaves). The crude extract was then suspended in H_2O, defatted with *n*-hexane, and then portioned with chloroform, *n*-butanol successively. The concentrated chloroform layer was chromatographed on a silica gel column by eluting with a gradient of hexane and acetone (8:2 to 100% acetone). Twenty column fractions were collected and analyzed by TLC (hexane–ethyl acetate, 1:9). Fractions with similar TLC patterns were combined, and rechromatographed on a silica gel column to give compound **1** [3.4 g, 0.2428% (w/w)]. The concentrated *n*-butanol layer was subjected to repeated column chromatography on a silica gel and eluted with a gradient of $CHCl_3$ and MeOH (9:1 to 100% MeOH) to give pure compounds **2** (320 mg), **3** (15 mg), and **4** (8 mg) in yields of 0.0228%, 0.0011%, and 0.0006% (w/w), respectively.

5.4. Spectral data of compound 2

Yellow amorphous powder (MeOH), mp 224–226 °C; $[\alpha]_D^{25}$ −65 (*c* 0.001, MeOH); UV (MeOH) λ_{max} (log ε) 265 (4.31), 349 (4.24), +NaOMe 276, 395, +NaOAc 267, 350, +NaOAc/H_3BO_3 266, 350, +$AlCl_3$ 269, 302 (sh), 340, 385 (sh), +$AlCl_3$/HCl 270, 301(sh), 342, 386 nm; IR (KBr) ν_{max} 3416 (OH), 2990 (C–H), 1660 (C=C aromatic), 1620 (C=O), 1140–1000 cm^{-1}; for ^1H and ^{13}C NMR (DMSO-d_6) see Table 1; HR-EIMS *m*/*z* 740.6679 $[M]^+$ (calcd for $C_{33}H_{40}O_{19}$, 740.6676).

5.5. Acid hydrolysis of 2 and determination of the absolute configuration of sugars

A solution of **2** (10 mg) in 1,4-dioxane (8 mL) and 5% aqueous H_2SO_4 (4 mL) were heated at 90 °C for 2 h. After cooling to room temperature, water was added to the reaction mixture, and extracted with EtOAc.

The organic layer was evaporated to dryness in vacuo. The residue was purified by silica gel CC to give kaempferol (3.0 mg). The aqueous layer containing the sugar mixture was neutralized by passage through an Amberlite IRA-96SB column, then analyzed by HPLC (Capcell Pak NH_2 UG-80, 4.6×250 mm; eluent, 85% MeCN; flow rate, 1 mL/min) according to identify L-rhamnose (t_R 7.7 min, negative peak), and D-glucose (t_R 15.6 min, positive peak).

5.6. Mice

Female Balb/c mice supply by Animal Center of the College of Medicine, National Taiwan University and maintained in the Animal Center of China Medical University. The animal room was on a 12 h light and dark cycle with a constant temperature and humidity. All mice were 8 weeks old, and used to obtain cells from peritoneal exudate. The experiments were conducted in accordance with the ethical guidelines for investigation in laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the Center of Pharmaceutical Chemistry.

5.7. Macrophage cultures

Mouse peritoneal excluded macrophages were obtained from mice by lavage with 10 mL of cold HBSS per mouse at 3 days after intraperitoneal injection of 2 mL 3% thioglycollate in saline (1.5 mL per mouse, Difco, Detroit, MI). Cells were seeded in 96-well cluster plates at a density of 2×10^6 cells/mL and incubated at 37 °C in humidified 5% CO_2/95% air to allow macrophages adherence. Two hours later, the non-adherent cells were removed by washing with warmed PBS and the remaining cells (90% macrophages, judged by non-specific esterase stain) were used for further experiments. Test compounds **1**–**4** were dissolved in DMSO and added directly to the culture media. Control cells were treated only with solvent, the final concentration of which never exceeded 0.1% (v/v), and this concentration did not have any noticeable effect on the assay systems. Cell-free supernatants were harvested after 24 h incubation with the stimuli and assayed for nitrite and cytokines. Mitochondrial respiration-dependent MTT assay was employed to determine their cytotoxicity.[42] MTT in PBS (0.1 mg) was added into each well and then incubated at 37 °C for 4 h. The MTT formazan (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) crystals were dissolved using acidified isopropanol (0.1 HCl) and mixed at room temperature. After 20 min, index of cell viability was calculated by measuring the optical density (OD) of color produced by MTT dye reduction with a microplate reader (BIO-RAD, model 3550, USA) at 570 nm ($OD_{570–620}$). The mean OD value of the content of four wells was used for assessing the cell viability expressed as % of control.

5.8. NO determination

The production of NO was estimated from the accumulation of nitrite (NO_2^-), the metabolic end product of NO metabolism, in the medium using the Griess reagent as described previously.[43] Cells were incubated with medium containing various concentrations of test compounds (**1**–**4**) in the presence or absence of LPS (2 μg/mL) plus IFN-γ (10 U/mL) for 24 h. Equal volumes of culture supernatant or serum and Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid, and 0.1% α-naphthylethylenediamine dihydrochloride in distilled water) were mixed and incubated for 15 min at room temperature. Absorbance was measured at 540 nm on a spectrophotometer. The absorbance was referred to a nitrite standard curve to determine the nitrate concentration in supernatants.

5.9. Cytokines determination

TNF-α, and IL-12 concentrations in supernatants from macrophage cultures were determined by enzyme-linked immunosorbent assay (ELISA) using antibody from PharMingen, according to manufacturer's instruction.[44] Cells were incubated with LPS (2 μg/mL) plus IFN-γ (10 U/mL) in the presence of different concentrations of compounds **1**–**4** for 24 h. The supernatants were collected and stored at −80 °C before analysis. Standards were prepared from recombinant mouse TNF-α and IL-12 separately (PharMingen, San Diego, CA). The sensitivity of TNF-α and IL-12 were 15.6 pg/mL. Cell viability was assessed by trypan blue dye exclusion method and was always greater than 95%.

5.10. Statistical analysis

All experiments data are shown as means ± SD and accompanied by the number of experiments. For in vitro data, statistical analysis was performed using a one-way ANOVA followed by Dunnetts post-hoc test, and the significant difference was set at $*p < 0.05$; $**p < 0.01$.

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