Phytochemical Investigation and Anti-Inflammatory Activity of the Leaves of Machilus japonica var. kusanoi

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Abstract: In a series of anti-inflammatory screenings of lauraceous plants, the methanolic extract of the leaves of Machilus japonica var. kusanoi (Hayata) J.C. Liao showed potent inhibition on both superoxide anion generation and elastase release in human neutrophils. Bioassay-guided fractionation of the leaves of M. japonica var. kusanoi led to the isolation of twenty compounds, including six new butanolides, machinolides A–F (1–6), and fourteen known compounds (7–20). Their structures were characterized by 1D and 2D NMR, UV, IR, CD, and MS data. The absolute configuration of the new compounds were unambiguously confirmed by single-crystal X-ray diffraction analyses (1, 2, and 3) and Mosher’s method (4, 5, and 6). In addition, lignans, (+)-eudesmin (11), (+)-methylpiperitol (12), (+)-pinoresinol (13), and (+)-galbelgin (16) exhibited inhibitory effects on N-formyl-methionyl-leucyl-phenylalanine/cytochalasin B (fMLP/CB)-induced superoxide anion generation in human neutrophils with IC50 values of 8.71 ± 0.74 µM, 2.23 ± 0.92 µM, 6.81 ± 1.07 µM, and 7.15 ± 2.26 µM, respectively. The results revealed the anti-inflammatory potentials of Formosan Machilus japonica var. kusanoi.

Keywords: Machilus japonica var. kusanoi; lauraceae; butanolide; lignan; anti-inflammatory activity

1. Introduction

Neutrophils play an important role in the human body against infections [1]. In response to immune stimulation, activated neutrophils generate a series of cytotoxic substances, such as the superoxide anion (O2•−), a precursor of other ROS, granule proteases, and bioactive lipids. The superoxide anion is known to cause damage to cells and tissues, stimulate macrophages, and trigger a cascade of inflammatory pathways [2]. Neutrophil elastase is one of the serine proteases stored in large amounts in neutrophil granules and is involved in the nonoxidative pathway of the intracellular
and extracellular immune response [3]. Neutrophil elastase is stimulated by neutrophils and causes the destruction of tissue in chronic inflammatory disease [2]. Besides, the persistent overexpression of neutrophils is involved in various conditions, such as rheumatoid arthritis, asthma, psoriasis, and ischemic heart disease.

Lauraceous plants are a dominant family in South and East Asia, consisting of aromatic trees and shrubs. They stand out, resulting in its economic benefits and diverse bioactivities. A previous investigation showed that some lauraceous plants exhibit bioactivities, such as cytotoxicity, anti-tuberculosis, anti-inflammatory, and antiplatelet activities [4]. Recently, we completed the anti-inflammatory screening of 174 methanolic extracts from 60 Taiwanese lauraceous plants. Among the screening results, the methanolic extract of the leaves of Machilus japonica var. kusanoi showed potent anti-inflammatory activity on both superoxide anion generation and elastase release in human neutrophils.

The Machilus genus comprises about 100 species with accepted names, mainly distributed in East Asia [5]. Previous studies of Machilus species identified various classes of chemical constituents, such as lignans, flavonoids, and terpenoids [4]. M. japonica var. kusanoi is a large evergreen tree endemic to Taiwan and is distributed in broad-leaved forests from lowlands up to 1400 m throughout the island [5]. Few investigations of M. japonica var. kusanoi have been published before. Only ten compounds were isolated from this plant [6–8], and only antimicrobial along with anti-α-glucosidase activity of this plant have been found previously [8,9]. Based on anti-inflammatory screening results and the rare investigation of the leaves from M. japonica var. kusanoi, the aims of this study are the isolation of components from the leaves of M. japonica var. kusanoi and the evaluation of their anti-inflammatory effects.

2. Results

After anti-inflammatory assay-guided fractionation of the leaves of M. japonica var. kusanoi, we successfully isolated six new butanolides (1–6) (Figure 1) and 14 known compounds (7–20) (Supplementary Materials, Figure S1). The phytochemical spectra of compounds 1 to 6 are available in the Supplementary Materials, Figures S2–S57. In particular, Mosher’s method and X-ray crystallographic analysis were applied to determine the absolute configuration of the new compounds. Moreover, anti-inflammatory effects of isolates on neutrophil pro-inflammatory responses were evaluated by the suppression of N-formyl-methionyl-leucyl-phenylalanine/cytochalasin B (fMLP/CB)-induced superoxide anion (O$_2^-$) generation and elastase release. The structure identification of the new compounds and anti-inflammatory activity results are illustrated below.

Compound 1 was obtained as colorless needles. Its molecular formula was determined as C$_{15}$H$_{26}$O$_4$ from high-resolution electrospray ionization mass spectroscopy (HRESIMS) data (m/z 295.18923 [M + Na]$^+$ (calcd. for 295.18853)), implying two degrees of unsaturation. The infrared spectroscopy (IR) spectrum showed typical absorptions of C=O (1736 cm$^{-1}$) for γ-lactone and hydroxy groups (3433 cm$^{-1}$). The $^1$H-NMR spectrum of 1 displayed signals of three oxymethines at δ$_H$ 3.59 (1H, m, H-11), 4.45 (1H, dd, J = 6.6, 3.2 Hz, H-4), and 4.31 (1H, dd, J = 4.8, 3.2 Hz, H-3), two methyl groups including one doublet methyl group at δ$_H$ 1.43 (3H, d, J = 6.6 Hz, H-5) and one triplet methyl group at δ$_H$ 0.91 (3H, t, J = 7.0 Hz, H-15), and alkyl side chains at δ$_H$ 1.66 (1H, m, H-6b), δ$_H$ 1.82 (1H, m, H-6a), and δ$_H$ 1.26–1.47 (14H, m, H-7–H-10, H-12–H-14) (Table 1). The γ-lactone was confirmed by IR spectrum, the $^3$H-$^1$H correlation spectroscopy (COSY) correlations between H-2/H-3/H-4/H-5 and the heteronuclear multiple bond correlation (HMBC) between H-2/C-1 (δ 177.5), C-3 (δ 71.2), H-3/C-1, and H-4/C-3 (Figure 2). The doublet methyl group (C-5) was connected to C-4, based on the COSY correlations between H-5/H-4, and HMBC correlations between H-5/C-3, C-4 (δ 78.8) (Figure 2). The HMBC showed correlations H-6/C-2 (δ 47.6), C-3, C-7 (δ 27.5), and C-8 (δ 29.4), which supported that the alkyl chain was located at C-2 (Figure 2). The key correlations in the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum (H-2 showed correlation with H-3, H-4, and no correlation with H-5; H-3 showed correlation with H-4 and no correlation with H-5).
confirmed that H-2, H-3, and H-4 were in the same phase (Figure 3). However, a remaining hydroxy group (δC 71.9) was located at a position of the alkyl chain which cannot be determined by NMR spectrum. Finally, the location of the remaining hydroxy group and the absolute configuration of 1 was further confirmed by single-crystal X-ray diffraction (Figure 4). The results proved that the stereochemistry of 1 should be shown as 2R,3S,4S,11R-form in the Oak Ridge thermal ellipsoid plot program (ORTEP) diagram. Thus, compound 1 was elucidated and named machinolide A.

![Structures of new compounds 1–6.](image)

Table 1. ¹H and ¹³C-NMR data of machinolides A–C (1–3).

| Position | δH (m, J in Hz) | δC | δH (m, J in Hz) | δC | δH (m, J in Hz) | δC |
|----------|-----------------|----|-----------------|----|-----------------|----|
| 1        | 177.5           |     | 177.4           |     | 177.4           |     |
| 2        | 2.57, dt (9.8, 4.8) 47.6 | 2.57, dt (10.2, 4.6) 47.6 | 2.57, dt (8.7, 4.8) 47.6 |
| 3        | 4.31, dd (4.8, 3.2) 71.2 | 4.31, dd (4.6, 3.2) 71.3 | 4.31, dd (4.8, 3.2) 71.3 |
| 4        | 4.45, qd, (6.6, 3.2) 78.8 | 4.45, qd (6.6, 3.2) 78.7 | 4.45, qd (6.6, 3.2) 78.7 |
| 5        | 1.43, d (6.6) 13.7 | 1.44, d (6.6) 13.7 | 1.44, d (6.6) 13.7 |
| 6        | 1.82, m 23.1 | 1.84, m 23.2 | 1.84, m 23.2 |
| 7        | 1.66, m 23.1 | 1.67, m 23.2 | 1.67, m 23.2 |
| 8        | 1.26–1.47, m 27.5 | 1.32–1.51, m 27.6 | 1.30–1.49, m 27.5 |
| 9        | 1.26–1.47, m 29.4 | 1.32–1.51, m 29.4 | 1.30–1.49, m 29.3 |
| 10       | 1.26–1.47, m 37.16 | 1.32–1.51, m 37.2 | 1.30–1.49, m 25.5 |
| 11       | 3.59, m 71.9 | 3.59, m 71.9 | 3.59, m 71.9 |
| 12       | 1.26–1.47, m 37.19 | 1.32–1.51, m 37.4 | 1.30–1.49, m 37.4 |
| 13       | 1.26–1.47, m 27.8 | 1.32–1.51, m 25.2 | 1.30–1.49, m 39.7 |
| 14       | 1.26–1.47, m 22.7 | 1.37, m 28.9 | 1.30–1.49, m 18.8 |
| 15       | 0.91, t (7.0) 14.0 | 2.07, m 33.7 | 0.93, t (7.2) 14.1 |
| 16       | 5.81, ddt (17.1, 10.2, 6.6) 138.9 | 5.00, ddt (17.1, 3.3, 1.5) 138.9 |
| 17       | 4.94, ddt (10.2, 3.3, 1.5) 114.4 | 5.00, ddt (17.1, 3.3, 1.5) 114.4 |

⁠a ¹H (400 MHz, CDCl₃) and ¹³C-NMR (100 MHz, CDCl₃). b ¹H (600 MHz, CDCl₃) and ¹³C-NMR (150 MHz, CDCl₃). c,d the data in the same column are interchangeable.
Figure 2. Key $^1$H-$^1$H COSY (—) and HMBC (H→C) correlations of machinolides A–F (1–6).

Figure 3. NOESY (H↔H) correlations of machinolides A–F (1–6).
Thus, the absolute configuration of C-2 was determined as R-form. The NOESY correlations of H-2/H-4, and H-3/H-4 confirmed that H-2, H-3, and H-4 were in the same phase (Figure 3). Hence, the absolute configuration of C-2 was determined and named machinolide B.

Compound 3 was isolated as colorless needles and assigned the molecular formula C_{15}H_{28}O_{4} through analysis of its HRESIMS data (m/z 273.20656 [M + H]⁺ (calcd. for 273.20658). All the spectra of 3 were similar to those of 1. However, electron ionization mass spectra (EIMS) showed the different fragments between 3 (m/z 215 (56), 186 (37)) and 1 (m/z 229 (39), 200 (24)), which suggests that the position of the hydroxy group in the alkyl chain was different. The hydroxy group of 3 was located at C-12 and the absolute configuration of 3 was assigned as 2R,3S,4S,12R-form, which were both determined by single-crystal X-ray diffraction (Figure 4). Therefore, compound 3 was named machinolide C, and its structure was further confirmed by COSY and HMBC experiments (Figure 2).

Compound 4 was obtained as a colorless oil. The ESIMS analysis of 4 showed the [M+H]⁺ ion at m/z 287, in agreement with the molecular formula of C_{15}H_{26}O_{3}, as confirmed by HRESIMS. Compound 4 had similar IR and ¹H-NMR spectra to those of 3, except for the presence of a ketone group at C-11 (δ 212.6) in the ¹³C-NMR spectrum (Table 2). The HMBC correlation between H-9, H-10, H-12/C-11, and H-12/C-11, C-13, C-14 supported the position of the ketone group and hydroxy group at C-11 and C-12, respectively (Figure 2). The planar structure of 4 was decided. The CD spectrum of 4 showed a negative cotton effect at 219.5 nm, which was similar to malleastrumolide A [10]. Thus, the absolute configuration of C-2 was determined as R-form. The NOESY correlations between H-2/H-3, H-2/H-4, and H-3/H-4 confirmed that H-2, H-3, and H-4 were in the same phase (Figure 3). Hence, the absolute configuration of 4 was determined as 2R,3S,4S-form. Based on the ¹³C-NMR-based empirical rules, the chemical shifts of C-3 and C-4 in 4 were similar to those of 2R,3S,4S-form compounds in the literature [11]. According to these two pieces of evidence, the absolute configuration of C-2, C-3, and C-4 in 4 was established to be 2R,3S,4S-form. The absolute configuration of C-12 was determined by Mosher’s method [12]. Based on the Δδ values of the (S)-MTPA and
(R)-MTPA esters in chloroform-$d_1$, the absolute configuration of C-12 was established as S-form (Figure 5). Accordingly, the absolute configuration of 4 was defined as 2R,3S,4S,12S. The structure of 4 was confirmed and named machinolide D.

Table 2. $^1$H and $^{13}$C-NMR data of machinolides D–F (4–6).

| Position | $^1$H (m, J in Hz) | $^1$H (m, J in Hz) | $^1$H (m, J in Hz) | $^1$H (m, J in Hz) |
|----------|--------------------|--------------------|--------------------|--------------------|
| 1        | 177.4              | 177.3              | 177.7              |                    |
| 2        | 2.55, dt (9.9, 5.0)| 47.5              | 2.56, dt (9.8, 4.5)| 47.5              |
| 3        | 4.31, dd (5.0, 3.0)| 71.3              | 4.30, br t (4.5)  | 71.3              |
| 4        | 4.45, qd (6.5, 3.0)| 78.8              | 4.54, qd (6.4, 2.9)| 78.7              |
| 5        | 1.44, d (6.5)      | 13.7              | 1.44, d (6.4)     | 13.7              |
| 6        | 1.80, m            | 23.1              | 1.84, m           | 23.1              |
| 7        | 1.67, m            | 1.67, m           | 1.64, m           | 23.1              |
| 8        | 1.35–1.55, m       | 27.4              | 1.33–1.58, m      | 27.4              |
| 9        | 1.35–1.55, m       | 29.0              | 1.33–1.58, m      | 29.0              |
| 10       | 1.35–1.55, m       | 23.2              | 1.67, m           | 23.2              |
| 11       | 1.35–1.55, m       | 37.7              | 2.46, m           | 37.7              |
| 12       | 1.35–1.55, m       | 212.6             | 212.4             | 212.7             |
| 13       | 1.35–1.55, m       | 76.3              | 4.17, dd (7.2, 3.6)| 76.5              |
| 14       | 1.35–1.55, m       | 39.5              | 1.84, m           | 33.1              |
| 15       | 1.35–1.55, m       | 18.2              | 1.33–1.58, m      | 24.0              |
| 16       | 0.95, t (6.9)      | 13.9              | 2.09, m           | 33.3              |
| 17       | 5.03, m            | 5.78, dd (17.2, 10.4, 6.8) | 138.1             | 1.24–1.52 m, 22.5 |
|          | 4.98, m            | 115.1             | 0.88, t (6.8)     | 14.0              |

$^a$ $^1$H (600 MHz, CDCl$_3$) and $^{13}$C-NMR (150 MHz, CDCl$_3$). $^b$ $^1$H (400 MHz, CDCl$_3$) and $^{13}$C-NMR (100 MHz, CDCl$_3$).

Figure 5. Results with the modified Mosher’s method ($\Delta\delta_{S-R}$) of machinolides D–F (4–6).

Compound 5 was isolated as a colorless oil. The ESIMS ($m/z$ 313 [M + H]$^+$) and HRESIMS ($m/z$ 335.18295 [M + Na]$^+$ (calcd. for 335.18290)) data were used to establish the molecular formula of compound 5 as C$_{17}$H$_{29}$O$_5$. The $^1$H-NMR spectrum of 5 was similar to that of 4, except for the presence of a terminal double bond at $\delta$H 5.78 (1H, ddt, J = 17.2, 10.4, 6.8 Hz, H-16), 4.98 (1H, m, H-17b), and 5.03 (1H, m, H-17a) in 4 (Table 2). The HMBC correlation between H-15/C-16, C-17, H-16/C-15, and the COSY correlation between H-16/H-17 supports the presence of a terminal double bond (Figure 2). The CD spectrum (a negative cotton effect at 217.5 nm) and NOESY correlation (Figure 3) of 5 were also similar to 4. Moreover, in accordance with the $^{13}$C-NMR-based empirical rules [11], the chemical shifts of C-3 and C-4 in 5 were similar to those of 2R,3S,4S-form compounds in the previous data [11], showing that the absolute configuration of 6 was 2R,3S,4S-form. The absolute configuration of C-12
in 5 was established as S-form by Mosher’s method (Figure 5). On the basis of the above results, the structure and absolute configuration of 5 were determined and named machinolide E.

Compound 6 was purified as a colorless oil. Its molecular formula of C_{17}H_{30}O_8, two protons more than 5, was determined by EIMS (m/z 315 [M + H]^+) and HRESIMS m/z 337.19849 [M + Na]^+ (calcd. for 337.19855). The difference between 6 and 5 is that the terminal double bond in 5 is replaced by the ethyl group in 6. The HMBC correlations between H-17/C-15, C-16, and the COSY correlation between H-16/H-17 (Figure 2) also supported the presence of the ethyl group. The absolute configuration of 6 was elucidated as 2R,3S,4S,12S-form by the CD spectrum, NOESY correlation, and Mosher’s method. As determined by the above observations, the structure of 6 was elucidated as a new compound and named machinolide F.

By comparison of the experiments and reported spectroscopic data ([α]_D, UV, IR, NMR, and MS), known compounds were identified as one apocarotenoid: amibenzoic acid (8) [14], one chlorophyll: pheophytin a (9) [15], one coumarin: isoaxifadin (10) [16], six lignans: (+)-eudesmin (11) [17], (+)-methylpiperitol (12) [18], (+)-pinoresinol (13) [19], (+)-syringaresinol (14) [20], (2S,5S)-diteratryl-(3R,4S)-dimethyldihydrofuran (15) [21], and (+)-galbelgin (16) [22], three sesquiterpenoids: β-eudesmol (17) [23], caryophyllene oxide (18), and clovane-2α,9β-diol (19) [24], and one steroid: β-sitosterol (20) [25].

In this study, eight isolates present in sufficient amounts (1, 2, 3, 6, 11–13, and 16) were evaluated for an inhibitory effect on fMLP/CB-induced superoxide anion (O_2^-•) generation and elastase release (Table 3). (+)-Eudesmin (11), (+)-methylpiperitol (12), (+)-pinoresinol (13), and (+)-galbelgin (16) displayed inhibitory activity on superoxide anions in fMLP/CB-stimulated human neutrophils with IC_{50} values of 8.71 ± 0.74 μM, 2.23 ± 0.92 μM, 6.81 ± 1.07 μM, and 7.15 ± 2.26 μM, respectively. LY294002 (Sigma-Aldrich), a potent phosphatidylinositol 3-kinase (PI3K) inhibitor, was used as a positive control to inhibit O_2^-• generation and elastase release, with IC_{50} values of 2.17 ± 0.53, and 6.38 ± 1.72 μM, respectively.

**Table 3.** Effect of compounds on superoxide anion generation and elastase release in fMLP/CB-stimulated human neutrophils.

| Compound            | Superoxide Anion IC_{50} (μM) | Elastase Release IC_{50} (μM) |
|---------------------|-------------------------------|-------------------------------|
| machinolide A (1)   | >10                           | >10                           |
| machinolide B (2)   | >10                           | >10                           |
| machinolide C (3)   | >10                           | >10                           |
| machinolide F (6)   | >10                           | >10                           |
| (+)-eudesmin (11)   | 8.71 ± 0.74                   | >10                           |
| (+)-methylpiperitol (12) | 2.23 ± 0.92                  | >10                           |
| (+)-pinoresinol (13) | 6.81 ± 1.07                   | >10                           |
| (+)-galbelgin (16)  | 7.15 ± 2.26                   | >10                           |
| LY294002 b          | 2.17 ± 0.53                   | 6.38 ± 1.72                   |

\(^{a}\) Concentration necessary for 50\% inhibition (IC_{50}). \(^{b}\) Positive control.

3. Discussion

Inflammation is triggered by infection or tissue injury. In our series of anti-inflammatory screenings of lauraceous plants, the leaves of *M. japonica* var. *kusanoi* stand out as a research candidate. Focusing on the anti-inflammatory activity results in this paper, the lignans, (+)-eudesmin (11), (+)-methylpiperitol (12), (+)-pinoresinol (13), and (+)-galbelgin (16) exhibited inhibitory activities on superoxide anion generation. (+)-Methylpiperitol (12) showed better anti-inflammatory activity than (+)-eudesmin (11), suggesting the methylenedioxy group may enhance the anti-inflammatory activity. (+)-Methylpiperitol (12) exhibited similar anti-inflammatory activity as (+)-pinoresinol (13), indicating the replacement of the methoxy group may not influence anti-inflammatory activity. The results suggested that the
furofuran-type lignan containing a methylenedioxy group showed the best anti-inflammatory activity in this study. More importantly, this is the first report on the anti-inflammatory activity of *M. japonica* var. *kusanoi*.

Butanolides (γ-butyrolactones) are four-carbon heterocyclic lactone ring structures reported from some specific families (Myristicaceae [26], Meliaceae [10], Actinomycetes [27–31]), especially in Lauraceae plants (*Machilus* sp. [32–34], *Lindera* sp. [35,36], *Litsea* sp. [37], *Cinnamomum* sp. [38–40], *Persea* sp. [41]). The characteristic butanolides in Lauraceae plants contain an alkyl side chain group at C-2, a hydroxy group at C-3, and one methyl group at C-4, with or without a double bond between C-2/C-3 and C-2/C-6. In this report, six new compounds, machinolides A–F (1–6), were butanolide compounds without a double bond between C-2/C-3 or C-2/C-6. This type of butanolide has not been isolated from *Machilus* before, which might improve our understanding of secondary metabolites from *Machilus* species. The chemical results can contribute to the chemotaxonomy of *Machilus* species.

Although the potency of the lignans exhibiting anti-inflammatory activity in this study was similar to bioactive lignans described in the literature [42], it is worth noting that most of the lignans with anti-inflammatory activity in this study have not been reported previously. Besides, there are no anti-inflammatory medicines act via inhibiting superoxide anion and neutrophil elastase. The research shows some lead compounds and will help develop novel anti-inflammatory drugs.

### 4. Materials and Methods

#### 4.1. General Experiment Procedures

Optical rotations were measured on a Jasco P-2000 polarimeter (Jasco, Kyoto, Japan), and IR spectra (ATR) were acquired with a Jasco FT/IR-4600 spectrometer. We recorded 1D (1H, 13C, DEPT) and 2D (COSY, NOESY, HSQC, HMBC) NMR spectra on a Varian Germini-2000 spectrometer (Varian, Inc. Vacuum Technologies, Lexington, MA, USA) operated at 200 (1H) and 50 MHz (13C), a Varian Unityplus-400 spectrometer (Varian, Inc. Vacuum Technologies, Lexington, MA, USA) operated at 400 (1H) and 100 MHz (13C), a Varian Mercuryplus-400 spectrometer (Varian, Inc. Vacuum Technologies, Lexington, MA, USA) operated at 400 (1H) and 100 MHz (13C), and a Varian VNMRS-600 spectrometer (Varian, Inc. Vacuum Technologies, Lexington, MA, USA) operated at 600 (1H) and 150 MHz (13C). Low-resolution mass spectra were obtained with POLARIS Q Thermo Finnigan (Thermo Fisher Scientific, Chicago, IL, USA), Waters ZQ 4000 (Waters, Milford, MA, USA), and VG Quattro GC/MS/MS/DS (Waters, Milford, MA, USA) mass spectrometers. EI/MS were taken on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan). HRESIMS were recorded on a Bruker APEX II mass spectrometer (Bruker, Karlsruhe, Germany) and VARIAN 901-MS (Varian, CA, USA). Silica gel (70–230 and 230–400 mesh; Silicycle, QC, Canada) was used for column chromatography (CC), and silica gel 60 F254 (Merck, Darmstadt, Germany) and RP-18 F254S (Merck, Darmstadt, Germany) were used for thin layer chromatography (TLC) and preparative TLC, respectively, visualized with a Ce2(SO4)3 aqueous solution. Further purification was performed by medium-performance liquid chromatography (MPLC; ceramic pump: VSP-3050; EYELA, Kyoto, Japan).

#### 4.2. Plant Material

The leaves of *Machilus japonica* var. *kusanoi* (Hayata) J.C. Liao were collected in March 2018 in Mudan Township, Pingtung County, Taiwan, and identified by I.-S.C. A voucher specimen (Chen 5480) was deposited with the herbarium of the College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan.

#### 4.3. Extraction and Isolation

Dried leaves (5.8 kg) of *M. japonica* var. *kusanoi* were extracted at room temperature with methanol (MeOH) (30 L) three times to yield a MeOH extract (730 g). The MeOH extract was suspended in water and partitioned with ethyl acetate (EtOAc) to give a water layer (265.4 g), EtOAc layer (390 g),
and precipitate (72 g). The EtOAc layer (390 g) was taken and 100 g were subjected to column chromatography (silica gel; n-hexane/EtOAc 100/0 to 0/100 EtOAc, then washed with 100% acetone and 100% methanol) to yield six fractions (Fr. 1–6). Fr. 3 (15.1 g) was subjected to open column (silica gel; n-hexane/acetone 6/1 to 2/1, column size: 3 × 70 cm) to yield 13 fractions (Fr.3-1–3-13). Fr. 3-9 was subjected to MPLC (RP-18; water/methanol 1:1; column size: 1.5 × 30 cm) to give seven fractions (Fr. 3-9-1–3-9-7). Fr. 3-9-1 was subjected to MPLC (silica gel; n-hexane/CH₂Cl₂/EtOAc 2/1 to 1/1; column size: 1 × 30 cm) to afford 14 fractions (Fr. 3-9-1-1–3-9-1-14) and compound 7 (30.5 mg). Fr. 3-9-1-11 was subjected to MPLC (silica gel; n-hexane/CH₂Cl₂/methanol 15/20/1; column size: 1 × 30 cm) to produce compound 6 (14.0 mg). Fr. 3-9-2 was subjected to MPLC (silica gel; n-hexane/CH₂Cl₂/EtOAc 2/1; column size: 1 × 30 cm) to obtain 10 fractions (Fr. 3-9-2-1–3-9-2-10). Fr. 3-9-2-9 was subjected to MPLC (silica gel; H₂O/methanol 1/1 to 2/3; column size: 1.5 × 30 cm) to gain 14 fractions (Fr. 3-10-2-1–3-10-2-14). Fr. 3-10-2-4 was subjected to MPLC (silica gel; CH₂Cl₂/EtOAc 3/1; column size: 1 × 30 cm) to obtain compound 4 (5.3 mg). Fr. 3-10-2-7 was subjected to MPLC (silica gel; CH₂Cl₂/EtOAc 3/1; column size: 1 × 30 cm) to produce compound 5 (0.9 mg). Fr. 3-10-2-13 was subjected to MPLC (silica gel; CH₂Cl₂/EtOAc 4/1; column size: 1 × 30 cm) to yield compound 2 (2.3 mg). Fr. 3-10-2-15 was subjected to MPLC (silica gel; CH₂Cl₂/acetone 15/1; column size: 1 × 30 cm) to afford five fractions (Fr. 3-10-2-15-1–3-10-2-15-5). Fr. 3-10-2-15-3 was subjected to MPLC (silica gel; CH₂Cl₂/EtOAc 3/1; column size: 1 × 30 cm) to give five fractions (Fr. 3-7-1–3-7-5). Fr. 3-7-3 was subjected to MPLC (silica gel; H₂O/methanol 2/3; column size: 1 × 30 cm) to produce nine fractions (Fr. 3-7-3-1–3-7-3-9). Fr. 3-7-3-5 was subjected to MPLC (silica gel; n-hexane/CH₂Cl₂/EtOAc 4/2/1; column size: 1 × 30 cm) to afford 10 fractions (Fr. 3-7-3-5-1–3-7-3-5-10). Fr. 3-7-3-5-4 was subjected to MPLC (silica gel; n-hexane/CH₂Cl₂/EtOAc 1/3/0.3; column size: 1 × 30 cm) to give compound 12 (4.6 mg). Fr. 3-7-3-7 was subjected to MPLC (silica gel; n-hexane/CH₂Cl₂/EtOAc 4/2/1; column size: 1 × 30 cm) to produce nine fractions (Fr. 3-7-3-7-1–3-7-3-7-9). Fr. 3-7-3-7-2 was subjected to MPLC (silica gel; n-hexane/CH₂Cl₂/EtOAc 6/2/1; column size: 1 × 30 cm) to obtain compounds 15 (0.2 mg) and 16 (2.5 mg). Fr. 3-7-3-7-6 was subjected to MPLC (RP-18; water/methanol 1/3; column size: 1 × 30 cm) to give compound 17 (2.9 mg). Fr. 3-11 was separated with Sephadex LH-20 (column size: 3 × 70 cm) and eluted with methanol to provide 11 fractions (3-11-1–3-11-11). Fr. 3-11-7 was subjected to MPLC (RP-18; water/acetone 3/2; column size: 1 × 30 cm) to give compound 13 (0.5 mg). Fr. 2 was subjected to column chromatography (silica gel; n-hexane/CH₂Cl₂/acetone 1/10 to 1/1) to yield ten fractions (Fr. 2-1–2-10). Fr. 2-4 was subjected to column chromatography (silica gel; n-hexane/acetone 40/1 to 20/1; column size: 2 × 30 cm) to yield nine fractions (Fr. 2-4-1–2-4-9). Fr. 2-4-3 was subjected to MPLC (RP-18; water/acetonitrile 1/5; column size: 1.5 × 30 cm) to produce nine fractions (Fr. 2-4-3-1–2-4-3-9). Fr. 2-4-3-3 was subjected to MPLC (silica gel; n-hexane/acetone 40/0.5; column size: 1 × 30 cm) to afford eight fractions (Fr. 2-4-3-3-1–2-4-3-3-8). Fr. 2-4-3-3-3 was subjected to HPLC to obtain two fractions (Fr. 2-4-3-3-3-1–2-4-3-3-3-2). Fr. 2-4-3-3-3-2 was further separated with prep. RP-18 TLC (water/acetonitrile = 1/10) to give compound 18 (1.9 mg). Fr. 2-7 was subjected to column chromatography (silica gel; n-hexane/CH₂Cl₂/acetone 20/4/1 to 12/4/1) to produce eight fractions (Fr. 2-7-1–2-7-8). Fr. 2-7-2 was subjected to MPLC (silica gel; n-hexane/CH₂Cl₂/acetone 12/4/1; column size: 2 × 30 cm) to give compound 20 (1.6 g). Fr. 2-8 was subjected to column chromatography (silica gel; n-hexane/CH₂Cl₂/acetone 16/16/1 to 8/16/1) to afford 14 fractions (Fr. 2-8-1–2-8-14). Fr. 2-8-7 was subjected to MPLC (silica gel; n-hexane/acetone 6/1; column size: 1 × 30 cm) to afford compound 11 (10.7 mg).
size: 1.5 × 30 cm) to produce seven fractions (Fr. 2-8-7-1–2-8-7-7). Fr. 2-8-7-5 was subjected to MPLC (silica gel; n-hexane/CH$_2$Cl$_2$/acetone 16/16/1; column size: 1 × 30 cm) to produce compound 9 (15.2 mg). Fr. 4 was subjected to column chromatography (silica gel; n-hexane/acetone 5/1 to 3/1) to yield six fractions (Fr. 4-1–4-6). Fr. 4-5 was separated with Sephadex LH-20 (column size: 3 × 70 cm) and eluted with methanol to provide six fractions (4-5-1–4-5-6). Fr. 4-5-4 was subjected to MPLC (RP-18; water/methanol 1/1; column size: 1.5 × 30 cm) to produce four fractions (Fr. 4-5-4-1–4-5-4-14). Fr. 4-5-4-1-1 was subjected to MPLC (silica gel; n-hexane/CH$_2$Cl$_2$/acetone 2/2/1; column size: 1 × 30 cm) to give compound 14 (1.6 mg). (Supplementary Materials, Figure S58)

4.3.1. Machinolide A (1)

Colorless needles; $[\alpha]_{D}^{25} -29.6$ (c 0.30, MeOH); IR $\nu_{\text{max}}$ (ATR): 3433 (OH), 1736 (y-lactone) cm$^{-1}$; $^1$H-NMR and $^{13}$C-NMR (Table 1); ESIMS m/z 273 [M + H]$^+$; EIMS m/z (rel. int.): 254 ([M – H$_2$O]$^+$, 3), 215 (56), 186 (37), 129 (99), 57 (100); HRESIMS m/z 295.18923 [M + Na]$^+$ (calcd. for C$_{15}$H$_{28}$NaO$_4$, 295.18853).

4.3.2. Machinolide B (2)

Colorless needles; $[\alpha]_{D}^{25} -42.1$ (c 0.092, MeOH); IR $\nu_{\text{max}}$ (ATR): 3436 (OH), 1737 (y-lactone) cm$^{-1}$; $^1$H-NMR and $^{13}$C-NMR (Table 1); ESIMS m/z 299 [M + H]$^+$; HRESIMS m/z 321.20370 [M + Na]$^+$ (calcd. for C$_{17}$H$_{30}$NaO$_4$, 321.20363).

4.3.3. Machinolide C (3)

Colorless needles; $[\alpha]_{D}^{25} -42.0$ (c 0.14, MeOH); IR $\nu_{\text{max}}$ (ATR): 3321 (OH), 1743 (y-lactone) cm$^{-1}$; $^1$H-NMR and $^{13}$C-NMR (Table 2); ESIMS m/z 273 [M + H]$^+$; EIMS m/z (rel. int.): 254 ([M – H$_2$O]$^+$, 3), 229 (39), 200 (24), 129 (80), 57 (100); HRESIMS m/z 273.20656 [M + H]$^+$ (calcd. for C$_{15}$H$_{26}$O$_4$, 273.20658).

4.3.4. Machinolide D (4)

Colorless oil; $[\alpha]_{D}^{27} -42.5$ (c 0.25, MeOH); IR $\nu_{\text{max}}$ (ATR): 3437 (OH), 1752 (y-lactone), 1708 (C=O) cm$^{-1}$; CD $\lambda_{\text{ext}}$ (MeOH) (Δc): 280 (+15.43), 219.5 (−54.44) nm; $^1$H-NMR and $^{13}$C-NMR (Table 3); ESIMS m/z 287 [M + H]$^+$; HRESIMS m/z 309.16726 [M + Na]$^+$ (calcd. for C$_{15}$H$_{26}$NaO$_5$, 309.16725).

4.3.5. Machinolide E (5)

Colorless oil; $[\alpha]_{D}^{25} -35.4$ (c 0.145, MeOH); IR $\nu_{\text{max}}$ (ATR): 3445 (OH), 1748 (y-lactone), 1713 (C=O) cm$^{-1}$; CD $\lambda_{\text{ext}}$ (MeOH) (Δc): 280.5 (+17.23), 217.5 (−52.40) cm$^{-1}$; $^1$H-NMR and $^{13}$C-NMR (Table 3); ESIMS m/z 313 [M + H]$^+$; HRESIMS m/z 335.18295 [M + Na]$^+$ (calcd. for C$_{17}$H$_{26}$NaO$_5$, 335.18290).

4.3.6. Machinolide F (6)

Colorless oil; $[\alpha]_{D}^{20} -39.4$ (c 0.85, MeOH); IR $\nu_{\text{max}}$ (ATR): 3440 (OH), 1751 (y-lactone), 1705 (C=O) cm$^{-1}$; CD $\lambda_{\text{ext}}$ (MeOH) (Δc): 280.5 (+17.34), 217.5 (−52.74) cm$^{-1}$; $^1$H-NMR and $^{13}$C-NMR (Table 3); ESIMS m/z 315 [M + H]$^+$; HRESIMS m/z 337.19849 [M + Na]$^+$ (calcd. for C$_{17}$H$_{30}$NaO$_5$, 337.19855).

4.4. X-Ray Crystallographic Data for Machinolide A (1), Machinolide B (2), and Machinolide C (3)

The absolute configurations of 1, 2, and 3 were determined from data collected on a Bruker D8 VENTURE single-crystal XRD equipped with Oxford Cryostream 800°. Crystallographic data for 1: C$_{15}$H$_{39}$O$_5$, M = 290.39, size 0.220 × 0.097 × 0.057 mm$^3$, orthorhombic, space group P2$_1$2$_1$2$_1$, a = 4.72807(10) Å, b = 12.9141(3) Å, c = 27.8178(6) Å, α = β = γ = 90°, V = 1698.53(6) Å$^3$, T = 200(2) K, Z = 4, $d_{\text{calc}}$ = 1.136 Mg/m$^3$, $\lambda$(Cu Kα) = 1.54178 Å, $F$(000) = 640, reflections collected/independent
A probability of less than 0.05 was considered significant. All assays were repeated at least three times. Results are presented as mean ± standard error of the mean (SEM). The Student’s t-test was used to compare the test compound with a DMSO (0.1%) control. A probability of less than 0.05 was considered significant.

5. Conclusions

Six new butanolides, machinolides A–F (1–6), together with 14 known compounds, were obtained from the leaves of *M. japonica var. kusanoi*. The absolute configurations of these new compounds were assigned by their CD spectrum, single-crystal X-ray diffraction analyses, and Mosher’s method. Hence, absolute configurations of all new compounds were determined as 2R,3S,4S-form in a furan ring, and the chiral center in the side chain group was R-form in 1 and 3, and S-form in 2, 4, 5, and 6. Besides, butanolides and lignans were major skeletons in this study. Bioactivity results indicated that lignans could reduce superoxide anion generation in fMLP/CB-stimulated human neutrophils, and the anti-inflammatory activities of those compounds were as potent as compounds in the literature [42]. Furthermore, the structure-activity relationship (SAR) discussion of anti-inflammatory activity compounds indicated that furofuran lignan with methylenedioxy was the most active structure. To our knowledge, this is the first report on anti-inflammatory activity from the leaves of *M. japonica var. kusanoi*. Figures S59–S64: The phytochemical spectra of compounds 1–6. Supplementary Materials: Figure S1: Structures of known compounds 7–20. Figures S2–S57: The phytochemical spectra of compounds 1–6. Figure S58: Extraction and isolation of the leaves from *M. japonica var. kusanoi*. Figures S59–S64: The phytochemical spectra of compounds 4a, 4b, 5a, 5b, 6a, and 6b. Table S1: Inhibitory effects...
of crude extracts from the leaves of *M. japonica* var. *kusanoi* on superoxide anion generation and elastase release in fMLP/CB-induced human neutrophils.

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**Sample Availability:** Samples of all compounds are available from the authors.

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