FULL PAPER

Three New Abietane-type Diterpenes from the Bark of *Cryptomeria japonica*

by **Chi-I Chang**[a]), **Chien-Chih Chen**[b])[c]), **Sheng-Yang Wang**[d]), **Che-Yi Chao**[e])[f]), **Louis Kuoping Chao**[g]), **Jih-Jung Chen**[h]), **Horng-Huey Ko**[i])[1]), **Cheng-Chi Chen**[j])[1]), and **Yueh-Hsiung Kuo***[k])[l])

[a]) Department of Biological Science and Technology, National Pingtung University of Science and Technology, Pingtung 912, Taiwan

[b]) Department of Nursing, Hungkuang University, Taichung 443, Taiwan

[c]) Department of Biotechnology, Hungkuang University, Taichung 443, Taiwan

[d]) Department of Forestry, National Chung-Hsing University, Taichung 402, Taiwan

[e]) Department of Health and Nutrition Biotechnology, Asia University, Taichung 413, Taiwan

[f]) Department of Medical Research, China Medical University Hospital, China Medical University, Taichung 404, Taiwan

[g]) Department of Cosmeceutics, China Medical University, Taichung 404, Taiwan

[h]) Graduate Institute of Pharmaceutical Technology, Tajen University, Pingtung 907, Taiwan

[i]) Department of Fragrance and Cosmetic Science, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan

[j]) Department of Chemistry, National Taiwan University, Taipei 106, Taiwan

[k]) Department of Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung 404, Taiwan (phone: +886-4-2205-3366 ext. 5701, e-mail: kuoyh@mail.cmu.edu.tw)

[l]) Department of Biotechnology, Asia University, Taichung 413, Taiwan

Three new abietane-type diterpenoids, 7*β*-acetoxy-12-methoxyabieta-8,11,13-triene-6*α*,11-diol (**1**), 7*α*-acetoxy-12-methoxyabieta-8,11,13-triene-6*α*,11-diol (**2**), and 6*α*-acetoxy-12-methoxyabieta-8,11,13-triene-7*α*,11-diol (**3**), as well as two known abietane-type diterpenoids, 12-methoxyabieta-8,11,13-triene-6*α*,7*β*,11-triol (**4**) and 6*α*-acetoxy-12-methoxyabieta-8,11,13-triene-7*β*,11-diol (**5**), were isolated from the MeOH extract of the bark of *Cryptomeria japonica.* Their structures were determined by analysis of spectroscopic data and comparison of NMR data with those of related metabolites.

Keywords: Chinese herbs, Cupressaceae, *Cryptomeria japonica*, Abietanes, Diterpenoids.

Introduction

Cryptomeria, belonging to the cypress family Cupressaceae, consists of only one species, *Cryptomeria japonica* D. Don. It is endemic to Japan, known as sugi (Japanese cedar) [1], and commonly grown on plantations within Asia. Its wood exhibits aromatic, soft, lightweight but strong, waterproof, and reddish-pink in color properties and is resistant to natural decay. Sugi is a commercially important softwood species in Japan, and is planted for many construction purposes as well as for interior paneling and other usage. Phytochemical investigations on the leaves and heartwood of the plant indicated the presence of diverse terpenoids [2 – 20]. A variety of biological activities including antifungal [21], cytotoxic [22], anti-inflammatory [23], and insect antifeedant [24], and repellent [18] activities have been reported for the crude extracts or pure compounds from this plant. The bark of *C. japonica* has also been used as a substrate in soil-less cultured horticultural crops, and the replacement of rock wool with processed bark can reduce losses caused by soil-borne plant pathogens [25][26]. In a previous phytochemical research, *Yoshikawa et al.* reported eight new abietane-type diterpenes [27][28] from the acetone extract of its bark, some of which exhibited unique incorporated skeleton structures of an abietane diterpene and a cadinane sesquiterpene or a 1,10-secocadinane sesquiterpene. To search for the novel biologically active phytochemicals from the bark of *C. japonica*, we conducted a study on the chemical ingredients of the bark of *C. japonica* and identified a cytotoxic C_{35}-terpene, cryptotrione, with an unprecedented skeleton possessing an abietane diterpene with a unique bicyclic sesquiterpene [29]. In a continuation of our investigation of new bioactive phytochemicals from the same extracts, we further report three new abietane-type diterpenoids, 7*β*-acetoxy-12-methoxyabieta-8,11,13-triene-6*α*,11-diol (**1**), 7*α*-acetoxy-12-methoxyabieta-8,11,13-triene-6*α*,11-diol (**2**), and 6*α*-acetoxy-12-methoxyabieta-8,11,13-triene-7*α*,11-diol (**3**), together with two known abietane-type diterpenoids,

[1]) Equal contribution to this article.

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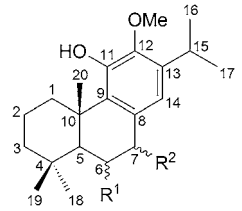
12-methoxyabieta-8,11,13-triene-6α,7β,11-triol (**4**) [30] and 6α-acetoxy-12-methoxyabieta-8,11,13-triene-7β,11-diol (**5**) [28] (*Fig. 1*). This report deals with the extraction, isolation, and structure elucidation of compounds **1** – **3**.

Results and Discussion

The UV spectrum of **1** showed absorption bands at λ_{max} 281 nm (3.78), which were attributed to the benzenoid moiety. Its IR spectrum exhibited absorption bands for OH and ester groups at 3436 and 1719 cm^{-1}, respectively. The ^{1}H-NMR spectrum of **1** (*Table*) showed resonances for three tertiary-linked Me groups (δ(H) 1.15, 1.21, and 1.48 (3*s*, Me(19), Me(18), and Me(20))), two O-bearing CH groups (δ(H) 4.14 – 4.18 (*m*, H–C(6)) and 5.93 (*d*, *J* = 8.0, H–C(7))), an aromatic H-atom (δ(H) 6.48 (*s*, H–C(14))), an ^{i}Pr group attached on the aromatic ring (δ(H) 1.20 (*d*, *J* = 6.8, 3 H), 1.22 (*d*, *J* = 6.8, 3 H), and 3.16 (*sept.*, *J* = 6.8, 1 H)), a phenolic H-atom (δ(H) 6.04 (*s*, HO–C(11)), disappeared on D_2O exchange), one MeO group (δ(H) 3.74 (*s*)), and an AcO Me group (δ(H) 2.18 (*s*)). A total of 23 C-atom signals were observed in the ^{13}C-NMR spectrum of **1** and were differentiated by DEPT experiments as five Me, three aliphatic CH_2, and two aliphatic CH groups, along with two aliphatic quaternary C-atoms, one MeO, one AcO group, two O-bearing CH, one olefinic CH groups, five quaternary olefinic, and one CO C-atoms. The HR-EI-MS of **1** showed a molecular ion at *m*/*z* 390.5179, which corresponded to the molecular formula, $C_{23}H_{34}O_5$, indicating seven degrees of unsaturation. A typical downshifted H_β–C(1) signal at δ (H) 3.20 (br. *d*, *J* = 13.6) and 23 C-atom signals in the ^{13}C-NMR spectrum including three C-atom signals attributing from one AcO and one MeO group each hinted that **1** was a 11-hydroxydehydroabietane diterpene [28]. The OH, MeO, and ^{i}Pr groups were located at C (11), C(12), and C(13) in the benzene ring, which were assured by the HMBCs (*Fig. 2*) between HO–C(11) (δ(H) 6.04)/C(9) (δ(C) 132.8) and C(11) (δ(C) 145.5); MeO–C



	R^1	R^2
1	α-OH	β-AcO
2	α-OH	α-AcO
3	α-AcO	α-OH
4	α-OH	β-OH
5	α-AcO	β-OH

Fig. 1. Structures of compounds **1** – **5** from *Cryptomeria japonica*.

Table. ^{1}H- and ^{13}C-NMR Data for compounds **1** – **3**

Position	**1**[a])		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
1	1.30 – 1.34 (*m*)[b]), 3.20 (br. *d*, *J* = 13.6)[c])	36.9	1.40 – 1.44 (*m*)[b]), 3.07 (*dt*, *J* = 12.8, 4.0)	36.6	1.44 – 1.48 (*m*)[b]), 3.08 (dt, *J* = 13.2, 4.0)	36.6
2	1.48 – 1.52 (*m*), 1.64 – 1.68 (*m*)	19.3	1.48 – 1.52 (*m*), 1.52 – 1.56 (*m*)	19.2	1.44 – 1.48 (*m*), 1.50 – 1.54 (*m*)	19.4
3	1.35 – 1.39 (*m*), 1.45 – 1.49 (*m*)	43.3	1.27 – 1.31 (*m*), 1.45 – 1.49 (*m*)	42.8	1.28 – 1.32 (*m*), 1.46 – 1.50 (*m*)	42.7
4		34.1		33.5		33.3
5	1.62 – 1.66 (*m*)[b])	54.2	1.94 (*d*, *J* = 11.2)	50.2	2.23 (*d*, *J* = 11.6)	47.3
6	4.14 – 4.18 (*m*)	72.7	4.31 (*ddd*, *J* = 11.2, 6.8, 3.6)	69.7	5.49 (*dd*, *J* = 11.6, 3.6)	73.3
7	5.93 (*d*, *J* = 8.0)	80.5	6.00 (*d*, *J* = 3.6)	75.1	4.69 (br. *s*)	70.5
8		129.2		129.0		130.8
9		132.8		132.7		131.3
10		42.0		41.8		42.3
11		145.5		146.3		145.8
12		143.7		144.7		144.1
13		138.3		138.9		138.6
14	6.48 (*s*)	116.0	6.70 (*s*)	119.1	6.71 (*s*)	118.7
15	3.16 (*sept.*, *J* = 6.8)	26.8	3.16 (*sept.*, *J* = 7.2)	26.6	3.17 (*sept.*, *J* = 6.8)	26.6
16	1.20 (*d*, *J* = 6.8)	24.1	1.19 (*d*, *J* = 7.2)	23.7	1.21 (*d*, *J* = 6.8)	24.0
17	1.22 (*d*, *J* = 6.8)	23.8	1.20 (*d*, *J* = 7.2)	23.7	1.22 (*d*, *J* = 6.8)	23.9
18	1.21 (*s*)	36.6	1.18 (*s*)	35.8	1.14 (*s*)	35.7
19	1.15 (*s*)	23.0	1.16 (*s*)	22.8	0.99 (*s*)	23.2
20	1.48 (*s*)	21.3	1.37 (*s*)	20.3	1.41 (*s*)	20.8
OH	6.04 (*s*)		6.07 (*s*)		6.04 (*s*)	
MeO	3.74 (*s*)	61.8	3.74 (*s*)	61.7	3.73 (*s*)	61.8
COO*Me*	2.18 (*s*)	21.8	2.16 (*s*)	21.6	2.16 (*s*)	22.2
*C*OOMe		172.1		171.5		169.7

[a]) Spectra recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$. [b]) Overlapped with other signals. [c]) The *J* values are in Hz in parentheses.

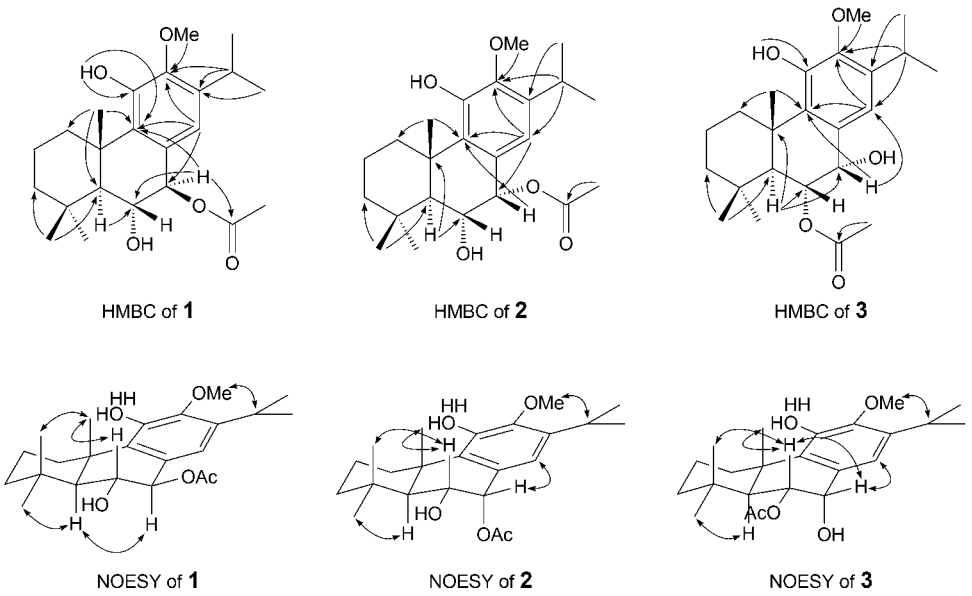


Fig. 2. Selected HMBC and NOESY correlations of compounds **1** – **3**.

(12)/C(12) (δ(C) 143.7); and H–C(15) (δ(H) 3.16)/C(13) (δ(C) 138.3) and C(14) (δ(C) 116.0). The HMBCs between H–C(5) (δ(H) 1.62 – 1.66)/C(4) (δ(C) 34.1), C(6) (δ(C) 72.7), and C(7) (δ(C) 80.5) and H–C(7) (δ(H) 5.93)/C(6), C(9) (δ(C) 132.8), and AcO–C(7) (δ(C) 172.1) suggested that a OH group and an AcO group were attached on C(6) and C(7), respectively. The OH group and AcO group were in α and β orientation, respectively, which was proven by the axial–axial coupling constant of H–C(7), 8.0 Hz [28] and NOE correlations (*Fig. 2*) between H–C(5)/H–C(7) and H–C(6)/Me(20) (δ(H) 1.48). From the above evidences, compound **1** was thus formulated as 7β-acetoxy-12-methoxyabieta-8,11,13-triene-6α,11-diol.

The HR-EI-MS of **2** revealed a molecular ion at *m/z* 390.5192, and the molecular formula was determined to be $C_{23}H_{34}O_5$, representing seven degrees of unsaturation. The IR spectrum displayed absorption bands for OH (3436 cm^{-1}) and ester (1719 cm^{-1}) groups. The ^{1}H- and ^{13}C-NMR data were similar to those of **1**, except for the signals of C(5) – C(7). An allylic *ABX* coupling system (δ(H) 1.94 (*d*, *J* = 11.2, H–C(5)), δ(H) 4.31 (*ddd*, *J* = 11.2, 6.8, 3.6, H–C(6)), and 6.00 (*d*, *J* = 3.6, H–C(7))) was observed and the coupling constant *J* = 6.8 Hz in H–C(6) was proved between H–C(6) and HO-C(6) due to disappearing on the D_2O exchange. The axial–equatorial coupling between H–C(6) and H–C(7) with a *J* value, 3.6 Hz, and the NOE correlation (*Fig. 2*) between H–C(7) and H–C(14) (δ(H) 6.70), together with downshift of H–C(5) ascribing to receiving anisotropic effect from AcO–C(7) suggested that the AcO group at C(7) was in α-axial orientation in **2**, instead of in β-equatorial orientation in **1**. Thus, compound **2** was identified as 7α-acetoxy-12-methoxyabieta-8,11,13-triene-6α,11-diol.

Compound **3** was also assigned as $C_{23}H_{34}O_5$ by the molecular ion of HR-EI-MS at *m/z* 390.5191, an isomer of compound **2** with seven degrees of unsaturation, showing almost similar IR absorption bands. Comparison of the ^{1}H- and ^{13}C-NMR spectra of **3** with those of **2** revealed that these two compounds were structurally very resemble in rings *A* and *C*. The only difference was that the OH and AcO groups attached on C(7) and C(6), respectively, in **3**, instead of on C(6) and C(7) in **2**. The HMBCs (*Fig. 2*) between H–C(5) (δ(H) 2.23)/C(6) (δ(C) 73.3) and C(7) (δ(C) 70.5) and H–C(7) (δ(H) 4.69)/C(9) (δ(C) 131.3) and C(14) (δ(C) 118.7) confirmed this proposal structure. The OH and AcO groups were all in α orientation, which was deduced from a small *J* value, 3.6 Hz, of axial–equatorial coupling between H–C(6) (δ(H) 5.49, *dd*, *J* = 11.6, 3.6) and H–C(7) (δ(H) 4.69, br. *s*) and the NOE correlations (*Fig. 2*) between H–C(6)/Me(20) (δ(H) 1.41) and H–C(7)/H–C(14) (δ(H) 6.71). Thus, compound **3** was thus elucidated as 6α-acetoxy-12-methoxyabieta-8,11,13-triene-7α,11-diol.

Compounds **1** – **5** are structurally and biogenetically related to the family of taiwaniaquinoids with an unusual 6-5-6-abeoabietane skeleton, which were first isolated from the leaves of *Taiwania cryptomerioides* by *Cheng* [31]. Some of them exhibited antitumor activities [32], and thus attracted remarkable attention from the synthetic researchers [33][34]. Biosynthetically, the abietane-6,7-diol could undergo pinacol rearrangement to generate the uncommon 6-5-6 tricyclic skeleton of the taiwaniaquinoids with a *trans*-5,10-bicyclic motif. The *cis*-abietane-6,7-diol could be a suitable precursor for a cationic rearrangement, and *trans*-abietane-6,7-diol may form an epoxide but not a suitable precursor for above rearrangement.

Compounds **1** – **5** were evaluated their tyrosinase, elastase, and collagenase inhibitory activities [35 – 37], as well as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity [38]. All the compounds showed weak inhibitory activities against tyrosinase, elastase, and collagenase enzymes with the EC_{50} values of more than 500 μM. Meanwhile, at the final concentration of 125 μM, they exhibited below 10% of DPPH radical-scavenging effect.

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Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/hlca.201600137.

Experimental Part

General

Optical rotations were measured using a *JASCO DIP-180* digital spectropolarimeter. The UV spectra were obtained on a *Shimadzu UV-1601PC* spectrophotometer. The IR spectra were recorded on a *Nicolet 510P* FT-IR spectrometer. The NMR spectra were obtained in $CDCl_3$ on a *Varian Mercury plus* 400 NMR spectrometer. Chemical shift values are given in ppm with reference to solvent (TMS as standard) and coupling constants (*J*) are given in Hz. The 2D-NMR spectra were recorded using standard pulse sequences. EI-MS and HR-EI-MS were recorded on *Finnigan TSQ-700* and *JEOL SX-102A* mass spectrometers, resp. TLC analyses were performed using Si gel *60 F_{254}* plates (*Merck*). Column chromatography was carried out on Si gel (230 – 400 mesh *ASTM*, *Merck*). Semiprep. HPLC was performed using a normal phase column (*Purospher STAR Si*, 5 μm, 250 × 10 mm; *Merck & Co., Inc.*) on a *LDC Analytical-III* system. The absorbance was measured on a microplate spectrophotometer (*SpectraMax Plus384*, *Molecular Devices*, CA). Quercetin, L-tyrosine, KH_2PO_4, DPPH, and *tert*-butyl hydroxytoluene (BHT) were purchased from *Acros Organics* (Morris Plains, NJ, USA). *N*-suc-$(Ala)_3$-nitroanilide (SANA), *N*-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA), collagenase, elastase, tyrosinase, oleanolic acid, and DMSO were obtained from *Sigma–Aldrich, Inc.* (St. Louis, MO, USA).

Plant Material

The bark of *C. japonica* D. Don was collected in Sitou, Taiwan in June 2000. The plant material was identified by Prof. *Shao-Shun Ying*, Department of Forestry, National Taiwan University.

Extraction and Isolation

The air-dried bark of *C. japonica* (16.0 kg) was extracted with MeOH (3 × 100 l) at r.t. The combined MeOH extract was evaporated under reduced pressure to afford a crude extract (480 g), which was suspended in H_2O (1 l), and then partitioned between H_2O and AcOEt. The AcOEt fraction (430 g) was subsequently chromatographed over a SiO_2 column (120 × 10 cm) using solvent mixtures of hexane and AcOEt with increasing polarity as eluents to obtain 11 fractions. *Fr. 3* from hexane/AcOEt (9:1) elution (47.8 g) was further purified through a SiO_2 column (7 × 60 cm), eluted with hexane/CH_2Cl_2 (1:0 – 0:1) to obtain nine fractions, *3A* – *3H*. Further purification of subfraction *3F* by HPLC gave **1** (0.9 mg, t_R = 37.1 min) using hexane/AcOEt (9:1). *Fr. 4* from hexane/AcOEt (4:1) elution (92.4 g) was further purified through a SiO_2 column (7 × 60 cm), eluted with CH_2Cl_2/AcOEt (100:1 – 0:1) to obtain 16 fractions, *4A* – *4P*. Further purification of subfraction *4C* by HPLC gave **3** (1.0 mg, t_R = 24.1 min) using hexane/AcOEt (4:1). Subfraction *4D* was subjected to HPLC eluted with hexane/AcOEt (4:1) to yield **2** (0.8 mg, t_R = 26.2 min). HPLC of subfraction *4E* eluted with hexane/AcOEt (4:1) to yield **5** (1.2 mg, t_R = 28.1 min). *Fr. 5* from hexane/AcOEt (7:3) elution (21.6 g) was further purified through a SiO_2 column (5 × 45 cm), eluted with CH_2Cl_2/AcOEt (100:1 – 0:1) to obtain 15 fractions, *5A* – *5O*. Further purification of subfraction *5D* by HPLC gave **1** (1.5 mg, t_R = 41.1 min) using hexane/AcOEt (7:3).

(6α,7β)-6,11-Dihydroxy-12-methoxyabieta-8,11,13-trien-7-yl Acetate (**1**). Gum. $[\alpha]_D^{25}$ = +4.7 (*c* = 0.9, $CHCl_3$). UV (MeOH): 281 (3.78). IR (KBr): 3436, 1719, 1414, 1381, 1301, 1248, 1016, 983, 738. ^{1}H- and ^{13}C-NMR: *Table*. EI-MS: 390 (4, M^+), 372 (7, $[M - H_2O]^+$), 330 (100, $[M - AcOH]^+$), 315 (47, $[M - AcOH - Me]^+$), 287 (22), 245 (33). HR-EI-MS: 390.5179 (M^+, $C_{23}H_{34}O_5^+$; calc. 390.2406).

(6α,7α)-6,11-Dihydroxy-12-methoxyabieta-8,11,13-trien-7-yl Acetate (**2**) Gum. $[\alpha]_D^{25}$ = +4.1 (*c* = 0.4, $CHCl_3$). UV (MeOH): 279 (3.47). IR: 3436, 1719, 1560, 1420, 1321, 1248, 1016, 739. ^{1}H- and ^{13}C-NMR: *Table*. EI-MS: 390 (5, M^+), 372 (3, $[M - H_2O]^+$), 330 (100, $[M - AcOH]^+$), 315 (67, $[M - AcOH - Me]^+$), 297 (16), 287 (30), 273 (17), 245 (74). HR-EI-MS: 390.5192 (M^+, $C_{23}H_{34}O_5^+$; calc. 390.2406).

(6α,7α)-7,11-Dihydroxy-12-methoxyabieta-8,11,13-trien-6-yl Acetate (**3**) Gum. $[\alpha]_D^{25}$ = +29.9 (*c* = 0.8, $CHCl_3$). UV (MeOH): 281 (3.66). IR: 3449, 1719, 1566, 1414, 1367, 1248, 1016, 744. ^{1}H- and ^{13}C-NMR: *Table*. EI-MS: 390 (5, M^+), 372 (3, $[M - H_2O]^+$), 330 (100, $[M - AcOH]^+$), 315

(67, $[M - AcOH - Me]^+$), 297 (15), 287 (29), 273 (15), 245 (68). HR-EI-MS: 390.5191 (M^+, $C_{23}H_{34}O_5^+$; calc. 390.2406).

Tyrosinase Inhibition Assay

Inhibition of tyrosinase was determined as previously described by *Tadtong et al.* [35]. Briefly, compounds **1** – **5** were dissolved in solvent to varying concentrations (20, 100, and 500 μM). The components were added into a 96-well plate as follows: 120 μl of phosphate buffer (20 mM, pH 6.8), 20 μl of sample soln., and 20 μl of mushroom tyrosinase soln. (500 U/ml in 20 mM phosphate buffer). The mixed solution was incubated at 25 °C for 15 min before adding 20 μl of L-tyrosine soln. (0.85 mM) to each well, and then incubated for a further 10 min at 25 °C. The enzyme activity was measured by the absorbance at 470 nm. Quercetin was used as a positive control. The percent tyrosinase inhibition was calculated by: Tyrosinase inhibition (%) = $[(A - B) - (C - D)]/(A - B) \times 100$, where A is the absorbance with the enzyme, but without the test sample, B is the absorbance without both the enzyme and test sample, C is the absorbance with both the enzyme and test sample, and D is the absorbance with the test sample, but without the enzyme.

Elastase Inhibition Assay

Elastase inhibition was carried out using the method of *Kransoe et al.* [36] with some modifications. In brief, 1 mM SANA was prepared in a 0.1M *Tris*·HCl buffer (pH 8.0), and 200 μl of SANA soln. was added to 20 μl of the sample soln. (final concentrations were 20, 100, and 500 μM). The soln. was mixed thoroughly by vortexing and preincubated for 10 min at 25 °C, and then 20 μl of elastase soln. (0.03 U/ml) was added. The mixture was incubated for further 10 min at 25 °C, and the absorbance was measured at 410 nm. Oleanolic acid was used as a positive control, whereas negative control was performed without any inhibitor. The percent activity of elastase was calculated according to the following equation: Elastase inhibition [%] = $(1 - B/A) \times 100$, where A is the enzyme activity without the test sample and B is the enzyme activity in the presence of test sample.

Collagenase Inhibition Assay

Collagenase inhibition activity was determined as described by *Van Wart et al.* [37]. *Clostridium histolyticum* collagenase (ChC; 0.8 U/ml) and FALGPA (2 mM) were dissolved in 50 mM tricine buffer, containing 400 mM NaCl and 10 mM $CaCl_2$ (pH 7.5). A quantity of 20 μl of test sample soln. (final concentrations were 20, 100, and 500 μM) was preincubated with 90 μl of the enzyme in the buffer for 15 min, and then adding 60 μl of FALGPA soln. The mixture was incubated for 20 min at 25 °C, and the collagenase activity was measured at 340 nm. Oleanolic acid was used as a positive control, whereas negative control was performed without any inhibitor. The percent activity of collagenase was calculated by the same formula described in the elastase inhibition assay.

DPPH Radical-Scavenging Assay

2,2-Diphenyl-1-picrylhydrazyl radical-scavenging activity was performed according to the method of *Shimada et al.* [38] with slight modification. A quantity of 150 μl of DPPH soln. (400 μM) was added to 50 μl of test sample in MeOH (final concentration was 25 – 1000 μM) in each well of a 96-well plate. After mixing gently and incubating in the dark for 90 min, the absorbance was measured at 517 nm against MeOH without DPPH as the blank reference. BHT was used as positive control. The percent DPPH scavenging effect was calculated as the inhibition rate (%) = [1 – (absorbance of sample/absorbance of control)] × 100.

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