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Hanultarin, a cytotoxic lignan as an inhibitor of actin cytoskeleton polymerization from the seeds of *Trichosanthes kirilowii*

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ABSTRACT

Bioactivity-directed fractionation of extracts from the seeds of *Trichosanthes kirilowii* led to the isolation of (−)-1-*O*-feruloylsecoisolariciresinol (2), named hanultarin, In addition, four known lignans were also isolated, including (−)-secoisolariciresinol (1), 1,4-*O*-diferuloylsecoisolariciresinol (3), (−)-pinoresinol (4), and 4-ketopinoresinol (5). Their structures were elucidated on the basis of spectroscopic data. Compounds **2** and **3** exhibited strong cytotoxic effects against human lung carcinoma A549 cells, melanoma SK-Mel-2 cells, and mouse skin melanoma B16F1 cells with IC_{50} ranges of 3–13 μg/mL. Compound **2** showed an inhibitory effect on the polymerization of the actin cytoskeleton in normal epidermal keratinocyte (HaCaT cells), suggesting unique biological properties of compound **2** compared to those of the other isolates.

1. Introduction

Trichosanthes is a member of the Cucurbitaceae family and is known as a folk medicine. It performs a variety of biological activities such as antibiotic, antituberculotic, expectorant, antidiabetic, abortifacient, antineoplastic, and anti-inflammatory activities.[1,2] While searching for bioactive metabolites from medicinal plants,[3–5] we found that the extract from the seeds of cucurbitaceous plant, *Trichosanthes kirilowii* (known by the Korean common name of hanultari) showed strong cytotoxic activity against the human lung carcinoma cell line, A549 (IC_{50} 145 μg/mL). The extract also demonstrated an inhibitory effect on the polymerization of the actin cytoskeleton in normal epidermal keratinocyte HaCaT cells.

Actin is the major component of the cytoskeleton and plays many important roles in cell growth, division, motility, signal transduction, cell–cell adhesion, and wound-healing processes. Polymerization of actin monomers into actin filaments occurs by nucleation and elongation pathways associated with actin-binding proteins. Actin filaments are assembled and disassembled as the cell divides, changes its shape, and adheres to tissue. These actin dynamics are associated with actin-binding proteins. Actin filaments and their regulatory proteins have become selective targets for cancer treatments.[6,7] In recent years, increasing numbers of natural products have been reported to interact with the actin cytoskeleton, such as latrunculin A, jasplakinolide, swinholide A, pectenotoxins, cryptotanshinone, and azadirachtin A.[8–12]

The plant *T. kirilowii* has been reported to possess many biologically active constituents. These compounds include trichosanic acid (punicic acid) as a conjugated linolenic acid with beneficial effects on lipid metabolism[13–16]; protein trichosanthin with abortifacient, antitumor, immunosuppressive, and anti-HIV activities[17–22]; and glycan trichosans with glucose-level reducing activity.[23] In addition, diterpene geranylgeranoic acid has been shown to perform cancer-preventing activities[24] and triterpenoids cucurbitadienol and karounidiol derivatives have shown anti-inflammatory activity.[25,26]

Bioassay-guided purification of the extract from *T. kirilowii* yielded cytotoxic lignan-type esters. One of them showed an inhibitory effect on actin polymerization in cells. Here, we report the isolation and structure-determination of five lignans from the seeds of *T. kirilowii* and their inhibitory effects on polymerization of the actin cytoskeleton below cytotoxic concentrations in normal epidermal keratinocyte HaCaT cells. Cytotoxicities against human lung cancer (A549), human melanoma (SK-Mel-2), and mouse melanoma (B16F1) cell lines were also examined.

2. Results and discussion

2.1. Isolation, structure elucidation, and stereochemistry

The powdered seeds (10.0 kg) of *T. kirilowii* were successively extracted with 80% aqueous methanol, ethyl acetate, and hexane.

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The combined extracts were successively partitioned between immiscible solvents (hexane, methylene chloride, and aqueous methanol). The methylene chloride layer (cytotoxicity: $IC_{50} \sim 95$ μg/mL against A549 cells) was fractionated on a silica gel flash column. The bioactive fractions were further subjected to a series of chromatography (silica gel column chromatography and C_{18} HPLC) to produce lignan derivatives, **1**–**5**, with yields of 8.56, 28.1, 40.2, 18.3, and 6.32 mg, respectively (Fig. 1).

The molecular formula of compound **1** was deduced to be $C_{20}H_{26}O_6$ as the high resolution time-of-flight mass spectrum (HRTOFMS) showed its protonated molecular ion $[M+H]^+$ at *m/z* 363.1808 (calcd, 363.1808). On the basis of extensive analysis of 2D NMR (COSY, HMBC, and HSQC), it was determined to be seco-isolariciresinol. Its spectral data were in good accordance with those of secoisolariciresinol reported in the literature.[27,28] From the optical rotation (−37.5, *c* 0.10, MeOH), compound **1** was determined to be (−)-(2*R*,3*R*)-secoisolariciresinol (literature values of its optical rotation: −16, *c* 0.1, methanol[27]; −28.2, *c* 0.33, methanol[28]).

Compound **2** was obtained as a light brownish oil. Its HRTOFMS displayed a protonated molecular ion $[M+H]^+$ at *m/z* 539.2294 (calcd, 539.2281), indicating its molecular formula as $C_{30}H_{34}O_9$. The UV spectra in MeOH showed maximum absorption bands at 326, 288, and 204 nm. The IR spectra showed the presence of a hydroxyl group (3409 cm^{-1}), ester (1702 cm^{-1}), double bond (1637 cm^{-1}), and aromatic ring (1602, 1513 cm^{-1}). The ^1H and COSY NMR spectra indicated the presence of two olefinic protons at δ 7.56 (1H, d, J = 16 Hz) and 6.35 (1H, d, J = 16 Hz) with *trans*-configuration; aromatic protons at δ 7.05 (1H, dd, J = 8.4, 1.6 Hz), 6.81 (1H, d, J = 8.4 Hz), and 7.17 (1H, d, J = 1.6 Hz); and methoxy protons at δ 3.88 (3H, s). Combining these data with ^{13}C and HSQC NMR spectral data, the partial substructure of compound **2** could be assigned as a 4-hydroxy-3-methoxyphenylcinnamoyl (i.e., feruloyl) group. The remaining part of the spectra showed two sets of aromatic ABX proton signals [δ 6.55 (1H, dd, J = 7.6, 1.6 Hz), 6.664 (1H, d, J = 7.6 Hz), and 6.62 (1H, d, J = 1.6 Hz); and δ 6.54 (1H, dd, J = 8.0, 2.0 Hz), 6.662 (1H, d, J = 8.0 Hz), and 6.58 (1H, d, J = 1.6 Hz)] and two methoxy group signals [δ 3.71 and 3.73 (each 3H, s)], suggesting the presence of two 4-hydroxy-3-methoxyphenyl groups. In addition, signals were observed for two sets of methylene protons [δ 2.71 (1H, dd, J = 14.0, 7.2 Hz) and 2.60 (1H, dd, 14.0, 8.0 Hz); and δ 2.61 (2H, dd, J = 13.2, 8.0 Hz)], and two methine protons [δ 2.24 (m) and 1.97 (m)]. Two sets of additional methylene protons were also observed at δ 4.33 (1H, dd, J = 11.2, 6.0 Hz) and 4.10 (1H, dd, J = 11.2, 6.4 Hz) and at 3.53 (1H, dd, J = 11.2, 6.8 Hz) and 3.67 (1H, dd, J = 11.2, 6.0 Hz). Assignments for all the carbons attached to the corresponding protons were established from the HSQC experiment. From the H–H spin coupling networks, coupling constants, and H–C one bond correlations, compound **2** was deduced to be composed of two 1,3,4-trisubstituted aromatic rings and a –CH_2–CH(CH_2O–)–CH(CH_2O–)–CH_2– moiety as additional partial substructures. The connectivity between the feruloyl, the two trisubstituted aromatic, and the aliphatic moieties was established from the HMBC spectrum. The carbonyl carbon of the feruloyl moiety at δ 169.1 was correlated with the *trans*-olefinic protons at δ 7.56 and 6.35 in that moiety. This carbon also showed long range C to H correlations with the methylene protons of the aliphatic moiety at δ *4.33* and 4.10. These spectral correlations were suggestive of an ester linkage between the two moieties. Good correlations between the two aromatic moieties and the aliphatic moiety were also observed in the HMBC spectrum (Fig. 2). Thus, the gross structure of compound **2** was deduced to be a monoferuloylated form of compound **1**, that is, 1-*O*-feruloylsecoisolariciresinol. The optical rotation of compound **2** was measured as −19.7 (*c* 0.36, methanol) which is the same polarity as compound **1**. Thus, the absolute structure of compound **2** was determined to be (−)-(2*R*,3*R*)-1-*O*-feruloylsecoisolariciresinol (^1H and ^{13}C NMR assignments are presented in Table 1). This compound was designated as hanultarin after the common Korean name of the plant.

The molecular formula of compound **3** was determined to be $C_{40}H_{42}O_{12}$, [$(M + H)^+$ at *m/z* 715.2746 (calcd, 715.2755)] from its HRTOFMS. Its spectral data were in good accordance with data in the literature for 1,4-*O*-diferuloylsecoisolariciresinol.[29] However, the optical rotation of 1,4-*O*-diferuloylsecoisolariciresinol was not reported. Compound **3** showed the same (−) value for optical rotation [−40.4, *c* 0.37, CH_3OH] as compound **1**, indicating identical stereochemistry at the chiral centers compared with compound **1**. Thus, the structure of compound **3** was deduced to be (−)-(2R,3R)-1,4-*O*-diferuloylsecoisolariciresinol (^1H and ^{13}C NMR assignments are presented in Table 1). Upon comparison of the spectral data and optical rotations in the literature, the structures of compounds **4** and **5** were determined to be (+)-pinoresinol[27] and 4-ketopinoresinol,[30] respectively.

It is of interest to note that there are no clear reports examining the presence of bioactive lignans in the genus *Trichosanthes*. Isolation of lignans **1**–**5** from this plant is the first occurrence of lignans from the *Trichosanthes* genus. Lignans are a diverse group of naturally occurring plant phenols and are widely distributed in vascular plants, especially in seeds such as flaxseeds. [31,32] Lignan **4** was re-

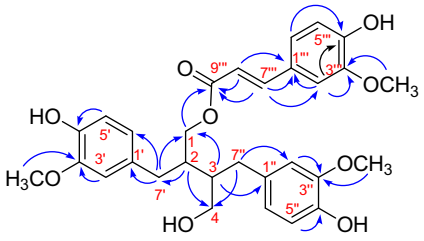
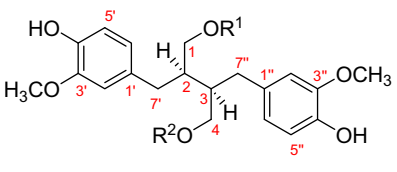
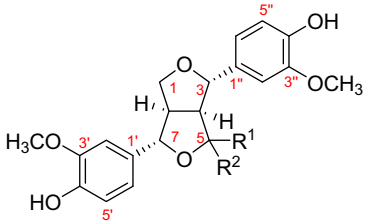


Figure 2. Significant HMBC correlations of compound **2** (H to C).



1 $R^1 = R^2 = H$
2 R^1 = feruloyl, $R^2 = H$
3 $R^1 = R^2$ = feruloyl



4 $R^1 = R^2 = H$
5 R^1 & $R^2 = O$

Figure 1. Chemical structure of the isolates **1**–**5**.

Table 1
^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data for the isolates **2** and **3**

Proton or carbon no.[a]	2		3	
	^{1}H NMR[b] (multiplicity, *J* in Hz)	^{13}C NMR[b]	^{1}H NMR[c] (multiplicity, *J* in Hz)	^{13}C NMR[d]
1	4.33 (dd, 11.2, 6.0) 4.10 (dd, 11.2, 6.4)	66.1	4.27 (dd, 10.8, 7.2) 4.07 (dd, 10.8, 4.8)	64.6
2	2.24 (m)	40.8	2.19 (m)	40.2
3	1.97 (m)	44.7	2.19 (m)	40.2
4	3.53 (dd, 11.2, 6.8) 3.60 (dd, 11.2, 6.0)	62.8	4.27 (dd, 10.8, 7.2) 4.07 (dd, 10.8, 4.8)	64.6
1′	—	133.1	—	131.3
2′	6.62 (d, 1.6)	113.2	6.65 (br s)	111.7
3′	—	148.76[e]	—	146.9
4′	—	145.5[e]	—	143.9
5′	6.664 (d, 7.6)	115.82	6.67 (d, 7.6)	114.5
6′	6.55 (dd, 7.6, 1.6)	122.6[e]	6.54 (d, 7.6)	121.5
7′	2.71 (dd, 14.0, 7.2) 2.60 (dd, 14.0, 8.0)	36.1 2.57 (dd, 14.0, 8.8)	2.75 (dd, 14.0, 6.0)	35.3
1″	—	133.6	—	131.3
2″	6.58 (d, 2.0)	113.4	6.65 (br s)	111.7
3″	—	148.75[e]	—	146.9
4″	—	145.6[e]	—	143.9
5″	6.662 (d, 8.0)	115.87	6.67 (d, 7.6)	114.5
6″	6.54 (dd, 8.0, 2.0)	122.7[e]	6.54 (d, 7.6)	121.5
7″	2.62 (2H, d, 7.6)	35.5	2.75 (dd, 14.0, 6.0) 2.57 (dd, 14.0, 8.8)	35.3
1‴	—	127.6	—	126.3
2‴	7.17 (d, 1.6)	111.6	7.29 (br s)	110.0
3‴	—	150.5	—	147.4
4‴	—	149.2	—	148.5
5‴	6.81 (d, 8.4)	116.4	6.77 (d, 8.4)	115.1
6‴	7.05 (dd, 8.4, 1.6)	124.1	7.09 (d, 8.4)	122.9
7‴	7.56 (d, 16.0)	146.7	7.52 (d, 15.6)	145.4
8‴	6.35 (d, 16.0)	115.5	6.47 (d, 15.6)	114.5
9‴	—	169.1	—	167.5
1⁗			—	126.3
2⁗			7.29 (s)	110.0
3⁗			—	147.4
4⁗			—	148.5
5⁗			6.77 (d, 8.4)	115.1
6⁗			7.09 (d, 8.4)	122.9
7⁗			7.52 (d, 15.6)	145.4
8⁗			6.47 (d, 15.6)	114.5
9⁗			—	167.5
OCH_3 at 3′	3.71 (s)	56.3	3.67 (s)	55.7
OCH_3 at 3″	3.73 (s)	56.3	3.67 (s)	55.7
OCH_3 at 3‴	3.88 (s)	56.5	3.80 (s)	55.9
OCH_3 at 3⁗			3.80 (s)	55.9

[a] The numberings 1⁗–9⁗ were given to the other 4-*O*-feruloyl group of compound **3**.
[b] CD_3OD
[c] DMSO-d_6.
[d] CD_3OD + $CDCl_3$.
[e,f,g] Interchangeable each other marked with the same letter.

ported to be derived biosynthetically from the dimerization of coniferyl alcohol. This lignan was shown to be transformed to compound **1** by a human intestinal bacterial mixture[27,33] or plant (*Arctium lappa* or *Forsythia intermedia*)-derived enzymes.[34,35] Lignans are one of the major class of phytoestrogens which can behave like estrogen or demonstrate antiestrogen effects such as protecting against hormone-dependent tumors (e.g., breast and prostate cancers).[36] Podophyllotoxins, currently used in cancer chemotherapy, are the best known examples of cytotoxic lignans.[37–39]

2.2. Cytotoxic activity of the isolates

The cytotoxicities of the isolates (**1**–**5**) were evaluated against human lung cancer (A549), human melanoma (SK-Mel-2), and mouse melanoma (B16F1) cell lines using a sulforhodamine B (SRB) assay method.[40] The results are summarized in Table 2. Hanultarin (**2**) and compound **3** were the most active against all the cell lines tested, with an IC_{50} range of 3–13 μg/mL, whereas lignans **1** and **4** were inactive below a concentration of 40 μg/mL. Compound **5** was active against SK-Mel-2 and against B16F1 with an IC_{50} range of 13–20 μg/mL. However, this compound was inactive against A549 cells.

2.3. Effects of the isolates 1–5 on cellular structures

The cellular effects of the isolates (**1**–**5**) were analyzed using normal epidermal keratinocytes, HaCaT cells. HaCaT cells were derived from normal epidermis, and the relatively large size of these cells allowed the detailed observation of intracellular structures such as microtubules and the actin cytoskeleton.[41] HaCaT cells were treated with the different isolates at a dose of near the IC_{50} of each compound and the cellular changes were analyzed using immunofluorescence analysis. Except for hanultarin (compound **2**), none of the isolates produced any noticeable changes in the cytoskeletal structure. Treatment with compound **2** produced aggregate-like structures of actin in a dose-dependent manner (Fig. 3), and at higher dose (12 μg/mL), almost all the actin fibers were disrupted to form aggregate-like structures (Fig. 3B). Similar aggregate-like structures could be observed in the case of cytochalasin D treatment, which is a well-known actin-depolymerizing agent (Fig. 3A). Detainment of the intact actin structure at a sublethal dose of compound **2** (1–3 μg/mL) also suggested that compound **2** might inhibit actin polymerization, rather than disrupting the already formed actin structures (Fig. 3B, arrows). Treatment with compound **3** induced cell dissociation with perturbations in actin accumulation at the sites of cell–cell contacts (Fig. 3A, arrow). However, a similar phenomenon is frequently observed in the process of cell death. Therefore, these results suggest that hanultarin (compound **2**) may have inhibitory effects on actin polymerization and that this compound may have unique cellular functions compared to the other isolates.

3. Experimental

3.1. General methods

The melting points were measured using a Fisher melting point apparatus and are reported as uncorrected values. HRTOFMS [positive electron spray ionization (ESI) mode] were measured using a Waters LCT Premier mass spectrometer coupled with a Waters

Table 2
Cytotoxicity assay data of the isolates **1**–**5** against A549, SK-Mel-2, and B16F1 cell lines

Compound	Cell line (IC_{50}, μg/mL)		
	A549[a]	SK-Mel-2[b]	B16F1[c]
1	>40	>40	>40
2	3.0	3.0	13
3	12	10	12
4	>40	>40	>40
5	>40	20	13
Cisplatin	10.1	7.0	5.0

[a] Human small lung cancer cell.
[b] Human melanoma cell.
[c] Mouse melanoma cell.

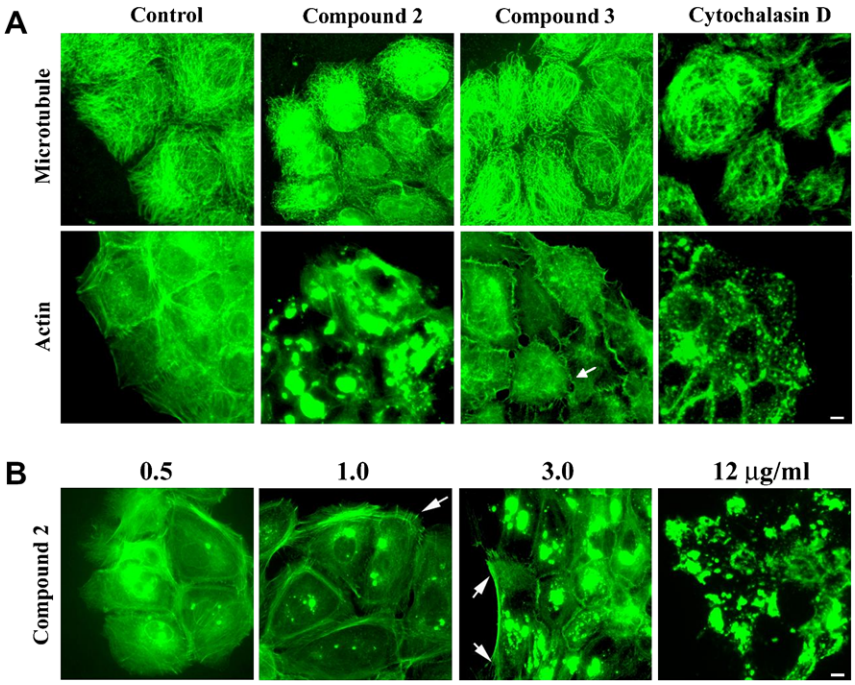


Figure 3. An inhibitory effect of hanultarin (compound **2**) on actin polymerization. Microtubules and actins were labeled with anti-α-tubulin antibody and fluorochrome-conjugated phalloidin, respectively. (A) HaCaT cells were treated with compounds **1**–**5** at about IC_{50} and cytochalasin D at 1 μg/mL, then subjected to immunofluorescence analysis. The doses of the isolates used for the experiment were as follow: 40 μg/mL for compounds, **1**, **4**, and **5**, 12 μg/mL for compound **3**, 3.5 μg/mL for compound **2**. Results were shown only for compounds **2** and **3**. (B) HaCaT cells were treated with compound **2** at the various doses of specified concentrations and then subjected to immunofluorescence analysis. The bars represent a length of 10 μm.

AQUITY HPLC system and data acquisition was achieved using MassLynx software, version 4.0. Optical rotations were measured using a Perkin-Elmer's 341-LC polarimeter. UV and IR spectra were measured using a Shimadzu UV-2401 PCR spectrometer and a Perkin-Elmer BXFT-IR spectrometer, respectively. NMR spectra were recorded on a Varian Mercury 400 spectrometer with standard pulse sequences operating at 400 and 100 MHz for ^{1}H and ^{13}C NMR, respectively. ^{1}H and ^{13}C spectra were referenced relative to methanol-d_4 (δ_H 3.30 and δ_C 49.15 ppm). 2D NMR spectra (COSY, NOESY, HSQC, and HMBC) were recorded using the manufacturer's software VNMR 6.1C. Flash column chromatography was carried out with silica gel 60 (70–230 mesh, Merck, 50 id × 220 mm). Medium pressure liquid chromatography (MPLC) was carried out on a C_{18} prepacked column (Ultra pack, ODS-S-50B, 26 id × 300 mm). Thin-layer chromatography (TLC) was performed on pre-coated silica gel plates (Kieselgel 60 F_{254}, 20 × 20 cm, 0.25 mm thick, Merck). Spots were detected under UV light at 254 and 365 nm or by heating after spraying with a solution of *p*-anisaldehyde-sulfuric acid in methanol. Preparative HPLC was performed on a Waters 600 model system with a photodiode array UV detector 996 using a C_{18} reverse phased silica gel column (Senshu pak, Pegasil ODS, 20 id × 250 mm) with a gradient elution of 30–60% aqueous MeCN over 60 min with a flow rate of 7 mL/min. Optical density was measured at 520 nm in a Tecan microplate reader. Anti-α-tubulin antibody and fluorochrome-conjugated phalloidin were purchased from Sigma. Fluorescence images were observed in a Axioscope fluorescence microscopy and captured with a Photometrics digital camera and MetaVue 5.0 software.

3.2. Plant material

The ripe yellow fruits of *T. kirilowii* were collected from mountainous areas near Kongju in October, 2004, and bulk seeds were purchased from a local medicinal plant market at Geumsan, Korea in August, 2005. The plant was identified by Dr. Eunkyu Lim at the Busong Clinic of Medicinal Herbs (Iksan, Korea). A voucher specimen (SM1375 and 1376) was deposited at the Natural Product Chemistry Lab, Department of Chemistry, Kongju National University, Korea.

3.3. Extraction and isolation

The seeds (10.0 kg) were pulverized and soaked with the following series of extraction solvents at room temperature for one week: 80% aqueous MeOH (20 L), EtOAc (18 L), and a mixture of EtOAc–hexane (6:1, 14 L). The extracts were pooled and evaporated under reduced pressure, which yielded brownish oily syrup (2.6 kg). This was suspended in a mixture of 30% aqueous MeOH (1.4 L) and extracted with hexane (1.2 L × 10). The concentrate of the resulting aqueous methanol layer yielded a brownish oily syrup (101 g), which was partitioned between H_2O (1.2 L) and CH_2Cl_2 (1.2 L × 5). The CH_2Cl_2 layer (30 g, $IC_{50} \sim 100$ μg/mL against A549 cells) was chromatographed on a flash column (silica gel) with elution with stepwise mixtures of CH_2Cl_2, MeOH, and H_2O of increasing polarity to yield 14 fractions. Of all the fractions subjected to cytotoxicity assays, two fractions, 3 (0.9 g) and 4 (6.9 g), showed cytotoxic activity (IC_{50} 20–40 μg/mL) against the lung carcinoma A549 cell line. These two fractions also demonstrated a disrupting effect on the actin cytoskeleton, forming aggregate-like structures at 50 μg/mL in normal epidermal keratinocyte, HaCaT cells.

Fraction 4 (6.9 g) was fractionated by silica gel chromatography with a gradient elution of hexane, EtOAc, and MeOH to give eight subfractions. Subfraction 7 (2.9 g, IC_{50}: 6–10 μg/mL against A549 cells) was further fractionated by using C_{18} MPLC (30–100% aqueous MeOH for 220 min, 8 mL/min) to give eight portions. Preparative C_{18} HPLC (30–40% aqueous MeCN for 75 min, 7 mL/min, UV 225 nm) of portion 3 (254 mg) resulted in compound **4** (18.3 mg) and **5** (6.32 mg) eluting at 31.2 and 36.6 min, respectively. The C_{18} HPLC (30–60% aqueous MeCN for 60 min, 7 mL/min, UV 225 nm) of portion 4 (354 mg) revealed compounds **1** (8.56 mg)

and **2** (28.1 mg) eluting at 25.2 and 37.2 min, respectively. Compound **3** (40.2 mg) was obtained from portion 5 (350 mg) by preparative C_{18} HPLC (25–70% aqueous MeCN for 50 min, 7 mL/min, UV 225 nm) with an elution time of 33.6 min.

3.3.1. (−)-(2*R*,3*R*)-Secoisolariciresinol (1)

Pale brownish oil, $[\alpha]_D^{20}$ −37.5 (*c* 0.10, CH_3OH); ^1H NMR (CD_3OD) δ 6.65 (2H, d, *J* = 8.0 Hz, H-5′ and H-5″), 6.58 (2H, d, *J* = 2.4 Hz, H-2′ and H-2″), 6.53 (2H, dd, *J* = 8.0, 2.4 Hz, H-6′ and H-6″), 3.72 (6H, s, OCH_3), 3.59 (4H, m, H-1 and H-4), 2.64 (2H, dd, *J* = 14.0, 7.2 Hz, H_a-7′ and H_a-7″), 2.54 (2H, dd, *J* = 14.0, 7.6 Hz, H_b-7′ and H_b-7″), and 1.91 (2H, m, H-3 and H-4); ^{13}C NMR (CD_3OD) δ 148.7 (C-3′, C-3″), 145.4 (C-4′, C-4″), 133.8 (C-1′, C-1″) 122.7 (C-6′, C-6″), 115.7 (C-5′, C-5″), 113.3 (C-2′, C-2″), 62.2 (C-1, C-4), 56.3 (OCH_3, OCH_3), 44.3 (C-2, C-3), and 36.2 (C-7′, C-7″); HRTOFMS $[M+H]^+$ *m*/*z* 363.1808 [calcd for $C_{20}H_{27}O_6$ (M + H), 363.1808].

3.3.2. Hanultarin [(−)-(2*R*,3*R*)-1-*O*-feruloylsecoisolariciresinol] (2)

Light brownish oil, $[\alpha^{20}]_D$ −19.7 (*c* 0.36, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 326.00 (4.40), 288.00 (4.35), and 204.00 (5.00) nm; IR (KBr) ν_{max} 3409, 2950, 1702, 1637, 1602, 1513 1467, 1429, 1387, 1244, 1210, 1157, and 1023 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); HRTOFMS $[M+H]^+$ *m*/*z* 539.2294 [calcd for $C_{30}H_{35}O_9$ (M + H), 539.2281].

3.3.3. (−)-(2*R*,3*R*)-1,4-*O*-Diferuloylsecoisolariciresinol (3)

Brownish powder, mp 181 °C, $[\alpha]_D^{20}$ −0.4 (*c* 0.37, CH_3OH); ^1H and ^{13}C NMR data (Table 1); HRTOFMS $[M+H]^+$ *m*/*z* 715.2746 [calcd for $C_{40}H_{43}O_{12}$ (M + H), 715.2755].

3.3.4. (+)-Pinoresinol (4)

Brownish oil, $[\alpha]_D^{20}$ −68.5 (*c* 1.83, CH_3OH); ^1H NMR (CD_3OD) δ 6.94 (2H, d, *J* = 2 Hz, H-6′ and H-6″), 6.80 (2H, dd, *J* = 8.0, 2.0 Hz, H-2′ and H-2″), 6.75 (2H, d, *J* = 8.0 Hz, H-3′ and H-3″), 4.70 (2H, d, *J* = 4.0 Hz, H-3 and H-7), 4.22 (2H, dd, *J* = 8.4, 5.5 Hz, H_a-1 and H_a-5), 3.80 (2H, dd, *J* = 8.4, 2.8 Hz, H_b-1 and H_b-5), 3.80 (6H, s, OCH_3), and 3.13 (2H, m, H-4 and H-8); ^{13}C NMR (CD_3OD) δ 149.0 (C-3′, C-3″), 147.2 (C-4′, C-4″), 133.7 (C-1′, C-1″), 120.0 (C-6′, C-6″), 116.0 (C-5′, C-5″), 111.0 (C-2′, C-2″), 87.6 (C-3, C-7), 72.7 (C-1, C-5), 56.5 (OCH_3, OCH_3), and 55.4 (C-4, C-8); HRTOFMS $[M+H]^+$ *m*/*z* 359.1510 [calcd for $C_{20}H_{23}O_6$ (M + H), 359.1495].

3.3.5. 4-Ketopinoresinol (5)

Brownish oil, $[\alpha]_D^{20}$ 0.0 (*c* 0.63, CH_3OH)^1H NMR (CD_3OD) δ 6.96 (1H, d, *J* = 2.0 Hz, H-2″), 6.84 (1H, dd, *J* = 8.0, 2.0 Hz, H-6″), 6.81 (1H, d, *J* = 8.0 Hz, H-5″), 5.38 (1H, d, *J* = 4.0 Hz, H-3), 3.66 (1H, dd, *J* = 8.8, 3.6, H-4), 4.03 (1H, dd, *J* = 9.2, 4.4 Hz, H_a-1), 4.29 (1H, dd, *J* = 9.2, 7.0 Hz, H_b-1), 3.86 (3H, s, OCH_3), 6.94 (1H, d, *J* = 1.6 Hz, H-6′), 6.82 (1H, dd, *J* = 8.4, 1.6 Hz, H-2′), 6.78 (1H, d, *J* = 8.4 Hz, H-3), 5.22 (1H, d, *J* = 4.0 Hz, H-7), 3.83 (1H, m, H-8), and 3.85 (3H, s, OCH_3); ^{13}C NMR (CD_3OD) δ 179.5 (C-5), 149.3 (C-3′), 149.1 (C-3″), 148.1 (C-4′), 147.4 (C-4″), 133.2 (C-1′), 132.4 (C-1″), 119.8 (C-6′), 119.5 (C-6″), 116.4 (C-5′), 116.1 (C-5″), 110.7 (C-2″), 110.6 (C-2′), 87.2 (C-7), 85.1 (C-3), 73.9 (C-1), 56.6 (OCH_3), 56.5 (OCH_3), 55.3 (C-4), and 54.6 (C-8); HRTOFMS $[M+H]^+$ *m*/*z*: 373.1302 [calcd for $C_{20}H_{20}O_7$ (M + H), 373.1287].

3.4. Cytotoxicity measurements

The cytotoxicity assay was done against B16-F1 (2×10^4 cells/mL), A-549 (1×10^5 cells/mL), and SK-Mel-2 (1×10^5 cells/mL) cell lines using a colorimetric SRB assay method.[3,40] Exponentially growing cells were harvested and suspended in the culture media (100 μL, RPMI-1640) in a 96-well plate. After 24 h of incubation at 37 °C in humidified 5% CO_2, the cells were treated with varying concentrations of test compounds (100 μL) and incubated further for 48 h under the same conditions. The cells were fixed with 50% trichloroacetic acid and stained for 30 min with SRB. The unbound dye was removed by 1% acetic acid, and the protein-bound dye was extracted with 10 mM tris base (pH 10.5) for 5 min. The optical density was measured at 520 nm in a microplate reader to determine cell growth inhibition. The results were expressed as the concentration at which there was 50% inhibition (IC_{50}).

3.5. Actin polymerization activity measurements (immunofluorescence analysis)

Normal epidermal HaCaT keratinocyte cells derived from normal epidermis were cultured using DMEM containing 10% fetal bovine serum (FBS).[41] Cells were treated with test compounds overnight and then subjected to immunofluorescence analysis. Briefly, treated cells were rinsed with ice-cold phosphate-buffered saline (PBS, pH 7.4) and fixed with 4% paraformaldehyde in PBS. After fixation, cells were permeabilized and then incubated with primary and secondary antibodies. Microtubules were labeled with anti-α-tubulin antibody and actin was labeled with fluorochrome-conjugated phalloidin. Stained cells were analyzed by fluorescence microscopy and fluorescence images were captured with a digital camera.

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