Antiproliferative Constituents in Plants 9.[1] Aerial Parts of *Lippia dulcis* and *Lippia canescens*

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The antiproliferative constituents in the MeOH extracts of the aerial parts of *Lippia dulcis* TREV. and *Lippia canescens* KUNTH (Verbenaceae) were investigated. Activity-guided chemical investigation of the MeOH extracts resulted in the isolation of the three bisabolane-type sesquiterpenes [(+)-hernandulcin (1), (−)-epihernandulcin (2), and (+)-anymol (3)] and four phenylethanoid glycosides [acteoside (4), isoacteoside (5), martynoside (6), and a new diacetylmartynoside (7)] from the former, and four phenylethanoid glycosides [acteoside (4), isoacteoside (5), arenarioside (8), and leucosceptoside A (9)] and three flavones [desmethoxycentaureidin (10), eupafolin (11), and 6-hydroxyluteolin (12)] from the latter. Antiproliferative activity of the isolated compounds against murine melanoma (B16F10), human gastric adenocarcinoma (MK-1), and human uterine carcinoma (HeLa) cells was estimated. (+)-Anymol (3), acteoside (4), isoacteoside (5), arenarioside (8), eupafolin (11), and 6-hydroxyluteolin (12) had GI_{50} values of 10—16 μM against B16F10 cell. Desmethoxycentaureidin (10) and eupafolin (11) showed high inhibitory activity against HeLa cell growth (GI_{50} 9 μM, and 6 μM, respectively).

Key words *Lippia dulcis*; *Lippia canescens*; antiproliferative activity; sesquiterpene; phenylethanoid glycoside; flavone

In the previous paper in this series, we reported the identification of acteoside and isoacteoside as the antiproliferative constituents in the leaves of *Clerodendron bungei* and leaves and bark of *Clerodendron trichotomum* (Verbenaceae).[2] As a continuation of the search for the antiproliferative constituents in the Verbenaceae plants, we examined the constituents of *Lippia dulcis* TREV. and *L. canescens* KUNTH.

L. dulcis is a creeping sweet herb endemic to tropical America, and the tea or infusion of the leaves is reported to have been used as a traditional medicine for the treatment of coughs and bronchitis and as an emmenagogue.[3] The preliminary screening of the antiproliferative activity of the MeOH extract of this plant revealed that it inhibits murine melanoma (B16F10) cell growth (GI_{50}: 9 μg/ml).

The constituents of this plant were investigated by Compadre *et al.*[4] and Kaneda *et al.*[5] and the isolation and structures of the sweet sesquiterpenes [(+)-hernandulcin and (+)-4β-hydroxyhernandulcin], a nonsweet sesquiterpene [(−)-epihernandulcin], a monoterpene (6-methyl-5-hepten-2-one), and acteoside have been reported. The major constituents of the volatile oil of the leaves were identified by GC/MS analysis.[6] The constituents responsible for inhibition of tumor cell growth, however, have not been clarified.

L. canescens is indigenous to Peru, was introduced to Japan in the beginning of the 1920s and has been utilized as a ground cover, although its chemical constituents have not been investigated except for the comment on the presence of flavones.[7] Although the antiproliferative activity of its MeOH extract is much lower (GI_{50}: 67 μg/ml against B16F10), its constituents were investigated to compare them with those of *L. dulcis*. This paper deals with the constituents of *L. dulcis* and *L. canescens* and their antiproliferative activity against murine melanoma (B16F10), human gastric adenocarcinoma (MK-1), and human uterine carcinoma (HeLa) cells.

MATERIALS AND METHODS

Instruments and Reagents The instruments and reagents used in this study were the same as those described in the previous paper.[2]

Plant Materials *L. dulcis* was raised in the greenhouse of Fukuoka University, harvested in April 2000, and dried at room temperature. *L. canescens* was cultivated in Fukuoka Municipal Botanical Gardens, and the aerial part was collected in July 2000.

Tumor Cell Lines MK-1 cells were provided by Professor M. Katano, Faculty of Medicine, Kyushu University, and HeLa and B16F10 cells were supplied by the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University.

Measurement of Antiproliferative Activity against Tumor Cell Lines The inhibition of the cellular growth was estimated using the MTT assay as described by Mosmann.[8] The detailed procedure was described in the previous paper.[2] The GI_{50} values (μg/ml) of the MeOH extracts and fractions are the average of two determinations, and the GI_{50} values (μM) of the isolated compounds are the means of four determinations.

Extraction and Isolation From the Aerial Part of *L. dulcis*: The air-dried and powdered material (128 g) was extracted with MeOH (1 l) under reflux for 1 h. After decantation of MeOH, the residue was extracted twice for 30 min in the same manner. The MeOH solutions were combined and concentrated *in vacuo* to give a dark green resin (21.7 g). The GI_{50} values were 9 μg/ml for B16F10, 64 μg/ml for MK-1, and $>$100 μg/ml for HeLa. The MeOH extract was partitioned between AcOEt and H_2O. The AcOEt layer was concentrated *in vacuo* to give a dark green resin (fr. I, 5.0 g, GI_{50} values: 11 μg/ml for B16F10, 32 μg/ml for MK-1, and 58 μg/ml for HeLa). The aqueous layer was passed through a column of styrene polymer, Diaion HP-20, and the column was washed with H_2O, and then with MeOH. The H_2O eluate was concentrated *in vacuo* to give a brown resin (fr. II, 8.8 g, GI_{50} values: $>$100 μg/ml for the three tumor cell lines). The MeOH eluate (fr. III, 5.8 g) had GI_{50} values of 25 μg/ml for B16F10, 42 μg/ml for MK-1, and 68 μg/ml for HeLa. Fraction I was separated into four fractions (fr. I-1—I-4) by silica

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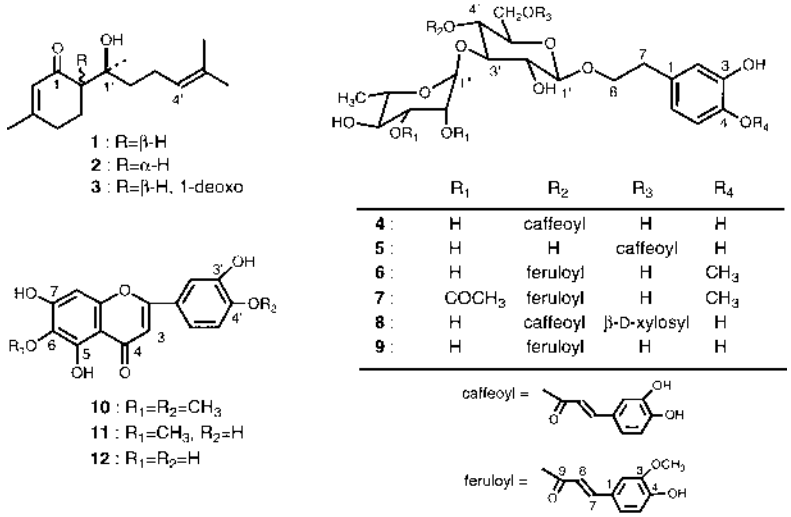

	R_1	R_2	R_3	R_4
4 :	H	caffeoyl	H	H
5 :	H	H	caffeoyl	H
6 :	H	feruloyl	H	CH_3
7 :	$COCH_3$	feruloyl	H	CH_3
8 :	H	caffeoyl	β-D-xylosyl	H
9 :	H	feruloyl	H	H

gel column chromatography using $CHCl_3$–MeOH–H_2O (10 : 1 : 0.1). Fraction I-1 was further chromatographed on silica gel using the solvent systems benzene–acetone (100 : 1—4 : 1) and hexane–AcOEt (9 : 1—1 : 2) to give (+)-hernandulcin[4] (**1**, 177 mg), (−)-epihernandulcin[4] (**2**, 82 mg), and (+)-anymol[9,10] (**3**, 17 mg). Fractions I-2 and I-3 were purified by YMC gel column (ODS, 30—50% MeCN) and HPLC (KC-PACK SM-A, 35% MeCN) to give martynoside[11] (**6**, 13 mg) and compound **7** (9 mg), respectively. Fractions I-4 and III were combined and subjected to YMC gel column chromatography (30% MeCN) and then to HPLC (40% MeOH) to afford acteoside[12] (**4**, 2.56 g) and isoacteoside[2] (**5**, 128 mg). Identification of the known compounds was performed by analyses of MS and NMR spectra and comparison of the physical data with those reported.

Compound **7**, a solid, $[\alpha]_D$ −74.3° (c=0.4, MeOH), high resolution (HR)-FAB-MS m/z: 759.2480 (Calcd for $C_{35}H_{44}O_{17}Na$: 759.2476). ^{1}H-NMR (pyridine-d_5) δ: (phenylethanoid moiety) 7.14 (d, J=2 Hz, H-2), 6.89 (d, J=8 Hz, H-5), 6.74 (dd, J=8, 2 Hz, H-6), 2.96 (2H, t, J=7 Hz, H-7), 3.88, 4.26 (1H each, dt, J=10, 7 Hz, H-8), 3.72 (3H, s, 4-OMe), (feruloyl moiety) 7.32 (d, J=2 Hz, H-2), 7.16 (d, J=8 Hz, H-5), 7.26 (dd, J=8, 2 Hz, H-6), 8.00 (d, J=16 Hz, H-7), 6.73 (d, J=16 Hz, H-8), 3.78 (3H, s, 3-OMe), (sugar moiety) 4.81 (d, J=8 Hz, H-1′), 3.94 (dd, J=8, 9 Hz, H-2′), 4.47 (t, J=9 Hz, H-3′), 5.68 (dd, J=9, 10 Hz, H-4′), 4.02 (m, H-5′), 4.12 (dd, J=12, 6 Hz, H-6′a), 4.21 (dd, J=12, 2 Hz, H-6′b), 6.06 (d, J=2 Hz, H-1″), 6.13 (dd, J=2, 3 Hz, H-2″), 5.81 (dd, J=3, 10 Hz, H-3″), 4.18 (dd, J=10, 8 Hz, H-4″), 4.53 (dq, J=8, 6 Hz, H-5″), 1.67 (3H, d, J=6 Hz, H-6″), 1.99 (3H, s, 2″-OAc), 1.90 (3H, s, 3″-OAc). ^{13}C-NMR (pyridine-d_5) δ: (phenylethanoid moiety) 132.2 (C-1), 117.4 (C-2), 148.1 (C-3), 147.2 (C-4), 112.7 (C-5), 119.9 (C-6), 35.9 (C-7), 71.0 (C-8), 56.0 (4-OMe), (feruloyl moiety) 126.5 (C-1), 111.6 (C-2), 148.9 (C-3), 151.2 (C-4), 116.8 (C-5), 123.7 (C-6), 146.5 (C-7), 114.9 (C-8), 166.7 (C-9), 55.8 (3-OMe), (sugar moiety) 104.2 (C-1′), 75.2 (C-2′), 81.6 (C-3′), 70.1 (C-4′), 76.2 (C-5′), 62.1 (C-6′), 100.0 (C-1″), 71.1 (C-2″), 73.2 (C-3″), 70.7 (C-4″), 70.2 (C-5″), 18.8 (C-6″), 170.20, 20.6 (2″-OAc), 170.22, 20.7 (3″-OAc).

From the Aerial Parts of *L. canescens*: The fresh aerial part of *L. canescens* (540 g) was homogenized in MeOH and filtered. The residue was extracted with MeOH again. The MeOH solutions were combined and concentrated *in vacuo* to give a dark green resin (22.0 g). The GI_{50} values are 67 μg/ml for B16F10 and >100 μg/ml for MK-1 and HeLa. The MeOH extract was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was concentrated *in vacuo* to give a dark green resin (fr. I, 4.0 g, GI_{50} values: 48 μg/ml for B16F10, 59 μg/ml for MK-1, and >100 μg/ml for HeLa). The aqueous layer was passed through a column of Diaion HP-20, and the column was washed with H_2O and then MeOH. The H_2O eluate and the MeOH eluate were separately concentrated *in vacuo* to give a brown resin (fr. II, 12.8 g) from the former, and a dark brown resin (fr. III, 3.1 g) from the latter. Fraction II showed no antiproliferative activity, while fr. III had GI_{50} values of 35 μg/ml for B16F10, 31 μg/ml for MK-1, and 10 μg/ml for HeLa. From fr. III, desmethoxycentaureidin[13] (**10**, 49 mg) precipitated as pale yellow crystals from MeOH. The residue of fr. III was subjected to column chromatography on silica gel ($CHCl_3$–MeOH–H_2O, 10 : 1 : 0.1—7 : 3 : 0.5), YMC gel (30—60% MeOH), Sephadex LH-20 (40—60% MeOH), and HPLC (KC-PACK SM-A, 40% MeOH) to give compounds **4** (88 mg), **5** (15 mg), arenarioside[14] (**8**, 30 mg), leucosceptoside A[15] (**9**, 6 mg), **10** (171 mg), eupafolin[16] (**11**, 55 mg), and 6-hydroxyluteolin[17] (**12**, 15 mg). Identification of the known compounds was performed by analyses of MS and NMR spectra and comparison of the physical data with those reported.

RESULTS AND DISCUSSION

Antiproliferative activity-guided fractionation of the MeOH extract of the aerial part of *L. dulcis* resulted in the isolation of three bisabolane-type sesquiterpenes, (+)-hernandulcin (**1**), (−)-epihernandulcin (**2**), and (+)-anymol (**3**), and four phenylethanoid glycosides, acteoside (**4**), isoacteoside (**5**), martynoside (**6**), and a new compound, diacetylmartynoside (**7**). Compound **7** had a molecular formula of $C_{35}H_{44}O_{17}$ base on a quasimolecular ion peak at m/z 759.2480 (Calcd for $C_{35}H_{44}O_{17}Na$: 759.2476) in its HR-FAB-MS. ^{1}H- and ^{13}C-NMR spectra indicated the presence of two acetyl groups in addition to the same functional groups as those of martynoside (**6**). Comparing the ^{1}H-NMR spectrum

Table 1. GI_{50} (μM) Values of Compounds **1**—**12**

Compound	B16F10	MK-1	HeLa
Sesquiterpenes			
(+)-Hernandulcin (**1**)	59	178	254
(−)-Epihernandulcin (**2**)	85	203	297
(+)-Anymol (**3**)	14	90	113
Phenylethanoid glycoside esters			
Acteoside (**4**)	11	35	50
Isoacteoside (**5**)	10	32	40
Martynoside (**6**)	>160	>160	>160
Diacetylmartynoside (**7**)	>136	>136	>136
Arenarioside (**8**)	16	34	34
Leucosceptoside A (**9**)	28	33	42
Flavones			
Desmethoxycentaureidin (**10**)	64	24	9
Eupafolin (**11**)	16	29	6
6-Hydroxyluteolin (**12**)	13	26	30

Values are the means of four determinations.

with that of **6**, it was found that the signals of H-2 (δ 4.78) and H-3 (δ 4.50) of the rhamnopyranosyl group of **6** were shifted downfield to δ 6.13 (dd, J=2, 3 Hz) and δ 5.81 (dd, J=3, 10 Hz) in **7**, and in the HMBC spectrum of **7**, the corresponding proton signals showed three-bond connectivity with the acetyl carbonyl carbon signals at δ 170.20 and 170.22, respectively. From this spectral evidence, **7** was determined to be 2″,3″-di-*O*-acetyl martynoside.

On the other hand, from the MeOH extract of the aerial part of *L. canescens*, four phenylethanoid glycosides, acteoside (**4**), isoacteoside (**5**), arenarioside (**8**), and leucosceptoside A (**9**), and three flavones, desmethoxycentaureidin (**10**), eupafolin (**11**), and 6-hydroxyluteolin (**12**), were isolated. The antiproliferative activity of the isolated compounds was determined by the MTT assay, and their GI_{50} values (μM) are listed in Table 1.

Considering the GI_{50} values (B16F10: 11 μM; MK-1: 35 μM; HeLa: 50 μM) and the yield (2%), acteoside (**4**) is responsible for the activity of the MeOH extract of the aerial part of *L. dulcis*. (+)-Anymol (**3**) showed high activity against B16F10 comparable to that of acteoside (**4**), although its contribution to the overall activity is slight judging from its low content. The low activity of the MeOH extract from *L. canescens* would be due to the low contents of active constituents, although individual phenylethanoid glycoside and flavone show moderate activity.

Among the six phenylethanoid glycosides, acteoside (**4**), isoacteoside (**5**), and arenarioside (xylopyranosylacteoside) (**8**), which have a 3,4-dihydroxyphenethyl group and ester-linked caffeic acid, showed almost the same high activity followed by leucosceptoside A which has ferulic acid instead of caffeic acid. Martynoside (**6**) and diacetylmartynoside (**7**), which have a 3-hydroxy-4-methoxyphenethyl group and ferulic acid, showed very low activity. These data indicate that the activity is largely dependent on the substituents of the phenethyl group, slightly influenced by the substituents of the phenylpropanoic acid, and not influenced by the structure of the sugar moiety. This is consistent with our previous results.[2)]

The inhibitory activity of three flavones suggests that HeLa cells are more sensitive to these flavones than the other two tumor cells. The presence of a 3′,4′-dihydroxy group in ring B plays an important role in the high activity against B16F10, and introduction of the methoxyl group appears to lower the activity. However, this is not the case in the activity against HeLa cells. It appears that the substitution patterns at rings A and B have different influences on the activity against different tumor cell species.

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