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Rotenoids and flavonoids with anti-invasion of HT1080, anti-proliferation of U937, and differentiation-inducing activity in HL-60 from *Erycibe expansa*

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Abstract—Principal rotenoids (deguelin, tephrosin, rotenone, and 12a-hydroxyrotenone) (3–30 μM) isolated from the stems of *Erycibe expansa* significantly inhibited invasion of human fibrosarcoma HT1080 cells through Matrigel-coated filters and release of proMMPs-2 and 9. In addition, deguelin and tephrosin showed differentiation-inducing activity in human promyelocytic leukemia HL-60 cells. Furthermore, effects of various constituents isolated from the ethyl acetate-soluble fraction on proliferation of human leukemia U937 cells were examined. As a result, most of isoflavones and several flavans as well as rotenoids showed moderate or substantial anti-proliferative activities.

1. Introduction

Metastatis of cancer, which is the major cause of death in cancer patients, occurs through a complex multistep process consisting of invasion into the circulation from the primary tumor, immigration to distant organs, adhesion to endothelial cells, and infiltration into the tissue. Therefore, its blockade has been considered to enhance survival of cancer patients,[1] and a few compounds were pre-clinically developed, such as the matrix metalloproteinases (MMPs) inhibitor marimastat.[2]

The Convolvulaceae plant, *Erycibe expansa*, is widely distributed in the Southeast Asian countries. The stems of *E. expansa* have been used for the treatments of hepatitis and hepatic dysfunction in the traditional Thai medicine. However, the chemical constituents and pharmacological effects of this natural medicine are left uncharacterized. We previously reported the methanolic (MeOH) extract from the stems of *E. expansa* was found to show hepatoprotective effect on D-galactosamine (D-GalN)-induced cytotoxicity in primary cultured mouse hepatocytes and inhibitory effects on overproduction of nitric oxide (NO) in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages, and six new compounds named erycibenins A–F were isolated from the ethyl acetate (EtOAc)-soluble fraction together with four known rotenoids, various isoflavones, etc.[3,4] As a continuing study, the MeOH extract and EtOAc-soluble fraction from the stems of *E. expansa* were found to inhibit invasion of human fibrosarcoma HT1080 cells through Matrigel-coated filters in vitro. In the present study, effects of rotenoids and/or flavonoids isolated from *E. expansa* on invasion of HT1080 cells, proliferation of HT1080 cells and human leukemia U937 cells, and differentiation-inducing activity of several rotenoids in human promyelocytic leukemia HL-60 cells were examined.

2. Results and discussion

2.1. Inhibitory effects of rotenoids (1–4) and an isoflavone (5) on invasion of HT1080 cells through Matrigel-coated filter

Tumor invasion through the extracellular matrix (ECM) and basement membrane (BM) is an essential step in the tumor metastasis. Recently, Transwell chamber precoated with ECM components and a reconstituted BM

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(Matrigel)-coated filter has been used for in vitro assay of invasion of tumor cells.[5] In the present study, effects on the invasion of HT1080 cells through Matrigel-coated filters were examined. As a result, mean percentage value of the invased cells through Matrigel-coated filters without test compound was 3.6 ± 0.4% (independent eight experiments) after 24 h incubation. The MeOH extract and its EtOAc-soluble fraction from the stems of *E. expansa* inhibited invasion of HT1080 cells in a concentration-dependent manner (Table 1), and only a weak cytotoxic effect was observed using MTT assay after 24 h incubation (Table 2).

Next, effects of four principal rotenoids [deguelin (**1**), tephrosin (**2**), rotenone (**3**), 12a-hydroxyrotenone (**4**)] (Fig. 1) isolated from the EtOAc-soluble fraction on the invasion of the cells were examined. As shown in Table 3, compounds **1**–**4** significantly inhibited the invasion at 3–30 μM, and only a weak cytotoxic effect was observed using MTT assay and calcein-AM assay (Cell Counting Kit-F™) after 24 h incubation. But, substantial anti-proliferative activities of **1**–**4** were observed after 48 and 72 h incubation (Table 4). Among them, compounds **1** and **4** showed stronger inhibition of the invasion of HT1080 cells.

Recently, phosphoinositide 3-kinase (PI3K)-Akt signaling was revealed to regulate cell growth, survival, and cancer cell invasion via increased motility and MMPs production, and the PI3K-Akt signaling network is considered as a potential target for novel anti-cancer therapies.[6] Deguelin (**1**) was reported to inhibit PI3K in premalignant and malignant human bronchial epithelial cells, increase apoptosis of human bronchial cells, and enhance chemosensitivity of leukemia cells.[7] While, rotenone (**3**) was reported to be an inhibitor of mitochondrial complex I and be able to induce apoptosis via enhancing the amount of mitochondrial reactive oxygen species production.[8] As expected from the previous reports,[6,7] compound **1** inhibited the invasion of HT1080 cells.

In the previous study, Yan and Han reported that genistein (**5**) inhibited the invasion of HT1080 cells.[9] In agreement with the report, compound **5** showed significant inhibition of the invasion of HT1080 cells at 30 μM, but the effect was weaker than those of rotenoids (**1**–**4**).

A reference compound, paclitaxel (0.03–1 μM), also strongly inhibited the invasion before exhibition of cytotoxic effects. Recently, with regard to the mechanism of action, microtubule-dependent traffic of MMP-containing vesicles and exocytosis were reported to be critical steps for invasive behavior in melanoma cells and paclitaxcel inhibits secretion of MMPs-2 and 9 and a cell invasion assay.[10]

Table 1. Inhibitory effects of MeOH ext. and EtOAc-soluble fraction from *E. expansa* on invasion of HT1080 cells

	Inhibition (%)			
Concn (μg/mL)	0	10	30	100
MeOH ext.	0.0 ± 12.4	−20.9 ± 9.2	−0.4 ± 2.2	34.6 ± 5.3*
EtOAc-soluble fraction	0.0 ± 4.7	−2.0 ± 9.9	36.0 ± 6.2**	53.9 ± 1.0**

Results are expressed as percentage inhibition of invasion (means ± SEM, $n = 4$). Significantly different from the control: $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 2. Effects of MeOH ext. and EtOAc-soluble fraction from *E. expansa* on proliferation of HT1080 cells by MTT assay

		Inhibition (%)			
Concn (μg/mL)		0	10	30	100
MeOH ext.	24 h	0.0 ± 0.9	2.1 ± 0.5	6.1 ± 1.0**	14.6 ± 1.8**
EtOAc-soluble fraction	24 h	0.0 ± 2.9	7.2 ± 1.6	9.4 ± 1.1**	13.7 ± 2.1**

Each value represents the means ± SEM ($n = 4$). Significantly different from the control: $^{*}p < 0.05$, $^{**}p < 0.01$.

2.2. Inhibitory effects of rotenoids (1–4) on release of proMMPs-2 and 9

MMPs are synthesized as pre-proenzymes and most of them are secreted from the cells as proenzymes (proMMPs), and then proMMPs are activated by other proteinases extracellularly or on the cell surface in a stepwise manner.[11] Since PI3K-Akt signaling regulates cancer cell invasion via production of MMPs,[6] effects of **1**–**4** on production of proMMPs-2 and 9 were examined using a gelatin zymography analysis. In the present study, compounds **1**–**4** substantially inhibited secretion of proMMPs-2 and 9 (Fig. 2). These findings suggest that inhibition of proMMPs production is involved in the inhibition of invasion of HT1080 cells by **1**–**4**, and modes of action of **2**–**4** were similar to that of a PI3K-Akt inhibitor **1**, although their mechanisms of action need to be studied further.

2.3. Differentiation-inducing activity of deguelin (1) and tephrosin (2) in HL-60 cells

In the previous study, genistein (**5**) was reported to induce a differentiation of human leukemia HL-60 cells.[12,13] In the present study, differentiation-inducing effects of two rotenoids [deguelin (**1**) and tephrosin (**2**)] on HL-60 cells comparing with that of compound **5** were examined using NBT reduction test. The cells proliferated from 2.0×10^5 cells/mL to 8.1×10^6 cells/mL without test compound after 72 h and anti-proliferative effects of **1** (10 and 30 μM), **2** (10 and 30 μM), and **5** (30 μM) were observed (Tables 5). The percentage of NBT-positive HL-60 cells in the survival cells increased from 3.4% (vehicle) to 22.2% and 8.4% at 30 μM, and the effect of compound **1** was stronger than those of compounds **2** and **5**. 1,25-Dihydroxyvitamin D_3, a reference compound, strongly induced the differentiation at low concentrations.

Compounds **1** and **5** were previously reported to induce apoptosis for human leukemia cells.[7,13] A strong differentiation-inducing agent, 1,25-dihydroxyvitamin D_3, is known to induce apoptosis of human leukemia cells as

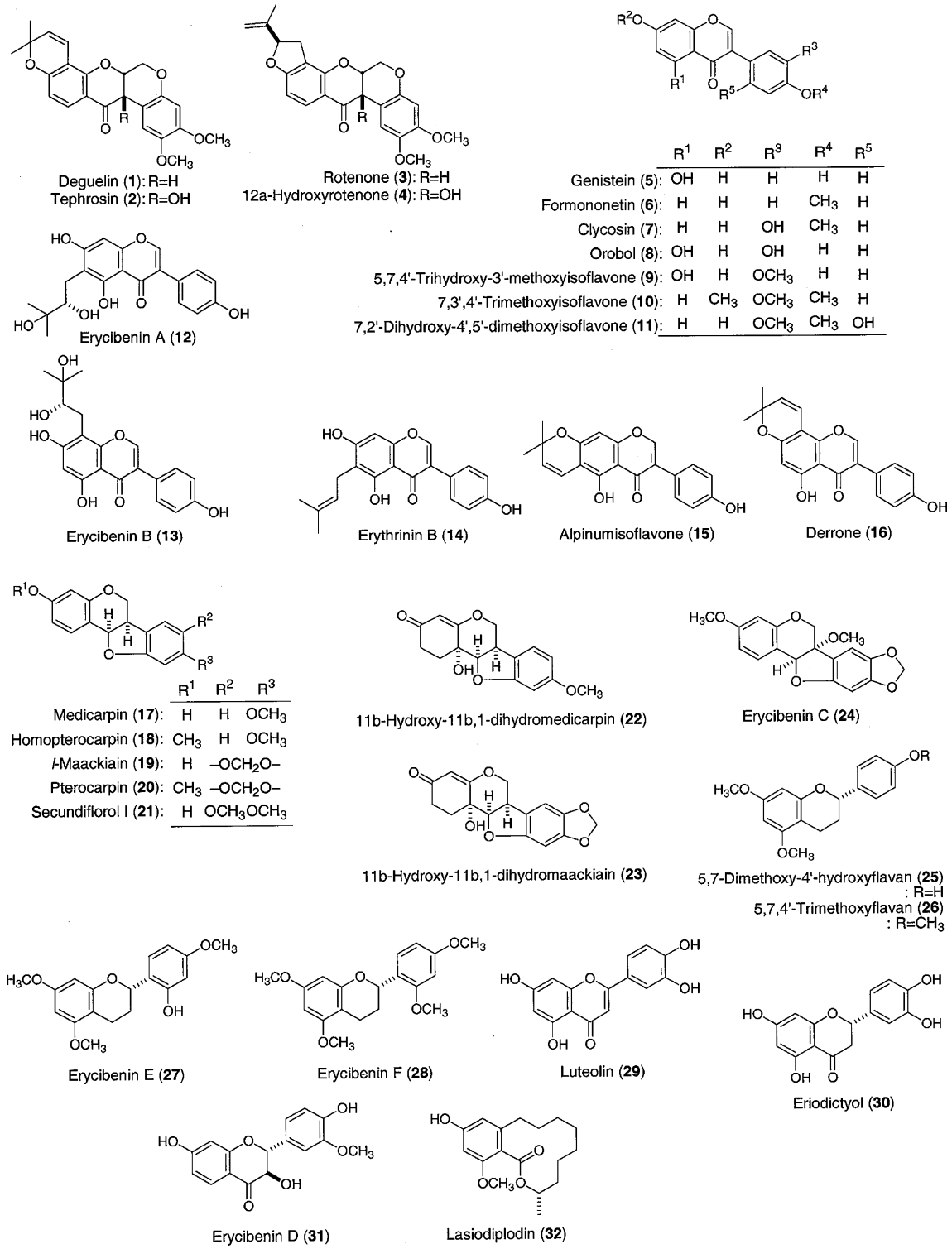

Figure 1. Chemical structures of the isolated compounds from *E. expansa*.

HL-60 cells at the lower concentration, but it inhibits apoptosis of the cells at the higher concentration at which the differentiation-inducing activity is observed. This reason is considered to depend on the anti-apoptotic effect caused by the strong differentiation-inducing activity.[14] Since there are complicated relations between apoptosis and differentiation, further studies will be necessary.

2.4. Effects of isolated constituents (1–32) on proliferation of U937 cells

Finally, effects of all isolated constituents (**1**–**32**) on proliferation of U937 cells were examined using Cell Counting Kit-8™ (WST-8 assay), and IC_{50} value of each compound was presented in Table 6. A reference compound, campthothecin, showed strong cytotoxic effect,

Table 3. Inhibitory effects of rotenoids (**1**–**4**) and an isoflavone (**5**) on invasion of HT1080 cells

	Inhibition (%)				
Concn (μM)	0	1	3	10	30
Deguelin (**1**)	0.0 ± 7.3	38.0 ± 14.4	50.2 ± 11.6*	58.0 ± 7.5**	57.9 ± 10.5**
Tephrosin (**2**)	0.0 ± 14.3	−9.3 ± 33.3	29.6 ± 10.9	49.2 ± 10.9	67.6 ± 2.1*
Rotenone (**3**)	0.0 ± 9.2	0.6 ± 9.8	19.6 ± 5.4	53.7 ± 5.9**	51.6 ± 7.4**
12a-Hydroxyrotenone (**4**)	0.0 ± 9.1	3.3 ± 7.3	30.6 ± 4.2**	64.9 ± 3.5**	54.6 ± 4.0**
Genistein (**5**)	0.0 ± 15.2	—	5.5 ± 11.1	35.1 ± 9.8	43.2 ± 5.8*
	0	0.03	0.1	0.3	1
Paclitaxel	0.0 ± 11.4	50.5 ± 1.6**	79.8 ± 0.4**	87.8 ± 0.6**	89.2 ± 0.2**

Each value represents the means ± SEM ($n = 4$). Significantly different from the control: $^{*}p < 0.05$, $^{**}p < 0.01$.

and the IC_{50} values after 24, 48, and 72 h were 0.68, 0.032, and 0.015 μM, respectively. The four rotenoids showed substantial cytotoxic effects (IC_{50} = 0.07–7.3 μM at 72 h), and rotenone (**3**) and 12a-hydroxyrotenone (**4**) showed the stronger cytotoxic effects than deguelin (**1**) and tephrosin (**2**). Most of isoflavone constituents [e.g., genistein (**5**), formononetin (**6**), clycosin (**7**), 5,7,4′-trihydroxy-3′-methoxyisoflavone (**9**), 7,2′-dihydroxy-4′,5′-dimethoxyisoflavone (**11**), erycibenin A (**12**), erythrinin B (**14**), alpinumisoflavone (**15**), derrone (**16**)], a flavone [luteolin (**29**)], and lasiodiplodin (**32**) showed moderate cytotoxic effects (IC_{50} = 13–74 μM at 72 h). All pterocarpane constituents [medicarpin (**17**), homopterocarpin (**18**), *l*-maackiain (**19**), pterocarpin (**20**), secundiflorol I (**21**), 11b-hydroxy-11b,1-dihydromedicarpin (**22**), 11b-hydroxy-11b,1-dihydromaackiain (**23**), erycibenin C (**24**)], a flavanone [eriodictyol (**30**)], and a flavanonol [erycibenin D (**31**)] showed no cytotoxic effect (IC_{50} > 100 μM at 72 h). Among flavan constituents, 5,7-dimethoxy-4′-hydroxyflavan (**25**) and erycibenin E (**27**) having the 5,7-dimethoxyl group and a hydroxyl group at the B-ring showed substantial cytotoxic effects, but the methylated compounds, 5,7,4′-trimethoxyflavan (**26**) and erycibenin F (**28**), showed weaker activity, suggesting that the hydroxyl group at the B-ring is important in 5,7-dimethoxyflavan's structure. A flavone constituent, luteolin (**29**), was reported to inhibit proliferation of HL-60 cells moderately.[15] In agreement with the previous report, compound **29** moderately inhibited the proliferation of U937 cells.

In conclusion, principal rotenoids [deguelin (**1**), tephrosin (**2**), rotenone (**3**), and 12a-hydroxyrotenone (**4**)] isolated from the stems of *E. expansa* significantly inhibited invasion of HT1080 cells through Matrigel-coated filters and release of proMMPs-2 and 9 before exhibition of anti-proliferative effects. These findings suggest that the inhibition of proMMPs release is involved in the inhibition of invasion of HT1080 cells by rotenoids, and modes of action of **2**–**4** were similar to that of a PI3K-Akt inhibitor **1**. In addition, compounds **1** and **2** were found to show differentiation-inducing activity in HL-60 cells. Furthermore, effects of all constituents isolated from the ethyl acetate-soluble fraction on proliferation of U937 cells were examined. As a result, most of isoflavones (**5**–**9**, **11**, **12**, and **14**–**16**) and several flavans (**25**–**27**) as well as rotenoids (**1**–**4**) showed moderate or substantial anti-proliferative activities. Anti-metastatic and differentiation-inducing activities of active compounds (**1**–**4**) including animal models and their mechanisms of action need to be studied further, but this information may be useful in the design of new lead compounds for development of anti-tumor agents.

3. Material and methods

3.1. Materials

3.1.1. Isolation. Isolation of test compounds from *E. expansa* was described in our previous reports.[3,4] Briefly, the methanolic extract from the dried stems of *E. expansa* was partitioned into a mixture of ethyl acetate (EtOAc) and water to provide the EtOAc-soluble portion and H_2O-soluble portion. The EtOAc-soluble portion (1.33% from the dried stems) was further subjected to silica gel and ODS column chromatographies and finally HPLC to give four rotenoids [deguelin (**1**, 0.0027% from the dried stems), tephrosin (**2**, 0.0020%), rotenone (**3**, 0.0062), 12a-hydroxyrotenone (**4**, 0.0019%)], 12 isoflavones [genistein (**5**, 0.0016%), formononetin (**6**, 0.00024%), clycosin (**7**, 0.00059%), orobol (**8**, 0.00054%), 5,7,4′-trihydroxy-3′-methoxyisoflavone (**9**, 0.0012%), 7,3′,4′-trimethoxyisoflavone (**10**, 0.00030%), 7,2′-dihydroxy-4′,5′-dimethoxyisoflavone (**11**, 0.00027%), erycibenin A (**12**, 0.00034%), erycibenin B (**13**, 0.00032%), erythrinin B (**14**, 0.0014%), alpinumisoflavone (**15**, 0.00025%), derrone (**16**, 0.00019%)], seven pterocarpanes [medicarpin (**17**, 0.00033%), homopterocarpin (**18**, 0.00023%), *l*-maackiain (**19**, 0.00044%), pterocarpin (**20**, 0.00024%), secundiflorol I (**21**, 0.0010%), 11b-hydroxy-11b,1-dihydromedicarpin (**22**, 0.00030%), 11b-hydroxy-11b,1-dihydromaackiain (**23**, 0.00050%), erycibenin C (**24**, 0.00080%)], five flavans [5,7-dimethoxy-4′-hydroxyflavan (**25**, 0.00028%), 5,7,4′-trimethoxyflavan (**26**, 0.00018%), erycibenin E (**27**, 0.00026%), erycibenin F (**28**, 0.00027%), a flavone [luteolin (**29**, 0.00048%)], a flavanone [eriodictyol (**30**, 0.00055%)], a flavanonol [erycibenin D (**31**, 0.00010%)], and an aromatic compound [lasiodiplodin (**32**, 0.00014%)].

3.1.2. Reagents for bioassay. Minimum essential medium Eagle's (MEM) and RPMI1640 were purchased from Sigma–Aldrich (MO, USA); fetal calf serum (FCS) was from Roche Diagnostics (Basel, Switzerland);

Table 4. Effects of rotenoids (**1**–**4**) and an isoflavone (**5**) on proliferation of HT1080 cells by MTT and calcein-AM assay

	Inhibition (%)				
Concn (μM)	0	1	3	10	30
MTT assay					
Deguelin (**1**)					
24 h	0.0 ± 6.4	6.3 ± 2.6	8.6 ± 3.7	10.7 ± 2.6	10.9 ± 1.0
48 h	0.0 ± 4.0	78.3 ± 0.9**	96.5 ± 0.5**	97.7 ± 0.1**	97.3 ± 0.1**
72 h	0.0 ± 3.1	98.2 ± 0.1**	98.7 ± 0.2**	98.8 ± 0.2**	98.9 ± 0.1**
Tephrosin (**2**)					
24 h	0.0 ± 1.6	−1.9 ± 1.8	0.8 ± 0.6	2.0 ± 0.4	3.8 ± 0.4
48 h	0.0 ± 1.2	96.4 ± 0.5**	97.9 ± 0.2**	98.1 ± 0.2**	98.0 ± 0.1**
72 h	0.0 ± 1.4	98.7 ± 0.2**	98.7 ± 0.1**	98.9 ± 0.1**	98.6 ± 0.0**
Rotenone (**3**)					
24 h	0.0 ± 6.4	9.4 ± 1.9	9.9 ± 2.4	9.5 ± 2.2	0.7 ± 1.0
48 h	0.0 ± 4.0	95.9 ± 0.3**	97.5 ± 0.1**	97.3 ± 0.2**	96.9 ± 0.0**
72 h	0.0 ± 3.1	98.6 ± 0.3**	98.5 ± 0.1**	98.4 ± 0.1**	97.8 ± 0.2**
12a-Hydroxyrotenone (**4**)					
24 h	0.0 ± 1.6	−2.0 ± 0.8	0.8 ± 1.2	2.4 ± 1.0	5.5 ± 1.6*
48 h	0.0 ± 1.2	95.8 ± 0.1**	96.9 ± 0.0**	96.4 ± 0.2**	96.0 ± 0.2**
72 h	0.0 ± 1.4	98.3 ± 0.2**	98.7 ± 0.1**	98.3 ± 0.3**	98.5 ± 0.2**
Genistein (**5**)					
24 h	0.0 ± 4.5	—	−6.2 ± 2.7	−1.2 ± 2.0	12.5 ± 1.9*
48 h	0.0 ± 2.8	—	3.8 ± 1.1	9.3 ± 0.7**	25.3 ± 0.8**
72 h	0.0 ± 3.8	—	−0.9 ± 4.3	1.7 ± 4.0	22.5 ± 5.4**
	0	0.03	0.1	0.3	1
Paclitaxel					
24 h	0.0 ± 0.6	4.7 ± 1.5*	12.6 ± 1.2**	12.2 ± 0.8**	10.3 ± 1.0**
48 h	0.0 ± 1.2	43.7 ± 1.1*	48.7 ± 0.6**	50.0 ± 0.7**	50.3 ± 0.6**
72 h	0.0 ± 1.4	65.6 ± 0.5**	79.8 ± 0.4**	81.4 ± 0.3**	79.9 ± 0.1**
Cell Counting Kit-F (Calcein-AM)					
Deguelin (**1**)					
24 h	0.0 ± 1.4	10.5 ± 1.2	−0.9 ± 3.9	−1.8 ± 5.1	17.0 ± 1.4**
48 h	0.0 ± 1.6	35.2 ± 1.6**	44.2 ± 4.3**	51.0 ± 2.1**	40.4 ± 2.7**
72 h	0.0 ± 0.6	95.3 ± 0.2**	98.5 ± 0.0**	98.5 ± 0.1**	98.5 ± 0.1**
Tephrosin (**2**)					
24 h	0.0 ± 5.6	5.0 ± 2.4	7.0 ± 2.3	5.9 ± 2.3	13.7 ± 3.0**
48 h	0.0 ± 1.1	54.0 ± 5.6**	63.0 ± 2.6**	67.7 ± 1.4**	83.2 ± 2.1**
72 h	0.0 ± 2.4	97.8 ± 0.1**	97.9 ± 0.1**	98.2 ± 0.0**	98.3 ± 0.0**
Rotenone (**3**)					
24 h	0.0 ± 1.4	16.2 ± 3.2**	17.1 ± 1.9**	23.0 ± 1.7**	26.8 ± 2.7**
48 h	0.0 ± 1.6	35.2 ± 1.6**	44.2 ± 4.3**	51.0 ± 2.1**	40.4 ± 2.7**
72 h	0.0 ± 0.6	97.4 ± 0.3**	95.3 ± 0.4**	91.4 ± 0.6**	93.4 ± 0.3**
12a-Hydroxyrotenone (**4**)					
24 h	0.0 ± 5.6	17.2 ± 2.0**	21.8 ± 2.0**	24.4 ± 2.1**	29.2 ± 2.3**
48 h	0.0 ± 1.1	52.2 ± 2.2**	50.6 ± 2.7**	47.6 ± 1.4**	53.6 ± 1.5**
72 h	0.0 ± 1.6	97.3 ± 0.0**	97.1 ± 0.1**	94.9 ± 0.3**	95.0 ± 0.2**
Genistein (**5**)					
24 h	0.0 ± 0.9	—	2.8 ± 0.2*	6.1 ± 0.5**	17.3 ± 0.7**
48 h	0.0 ± 1.7	—	2.0 ± 0.5	5.1 ± 0.6**	17.6 ± 0.4**
72 h	0.0 ± 2.5	—	1.5 ± 2.0	6.0 ± 1.7	17.2 ± 2.5**
	0	0.03	0.1	0.3	1
Paclitaxel					
24 h	0.0 ± 4.9	−0.5 ± 2.1	10.8 ± 3.6*	12.4 ± 1.1**	10.3 ± 0.7*
48 h	0.0 ± 4.6	46.2 ± 2.5**	64.4 ± 0.3**	70.9 ± 1.6**	70.0 ± 0.6**
72 h	0.0 ± 3.7	59.8 ± 2.8**	87.0 ± 0.8**	87.6 ± 0.5**	87.7 ± 1.0**

Each value represents the means ± SEM ($n = 4$). Significantly different from the control: $^{*}p < 0.05$, $^{**}p < 0.01$.

Matrigel (BD Matrigel™) was from BD Biosciences (NJ, USA); Cell Culture Insert™ was from BD Falcon (NJ, USA); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), Cell Counting Kit-8™, and Cell Counting Kit-F™ were from Dojindo Lab. (Kumamoto, Japan); phorbol 12-myristate 13-acetate (PMA), nitrotetrazolium blue chloride (NBT), and other reagents were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 96-Well microplates and 24-well multiplates were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan); 96-well black microplates were from Nunc (Roskild, Denmark).

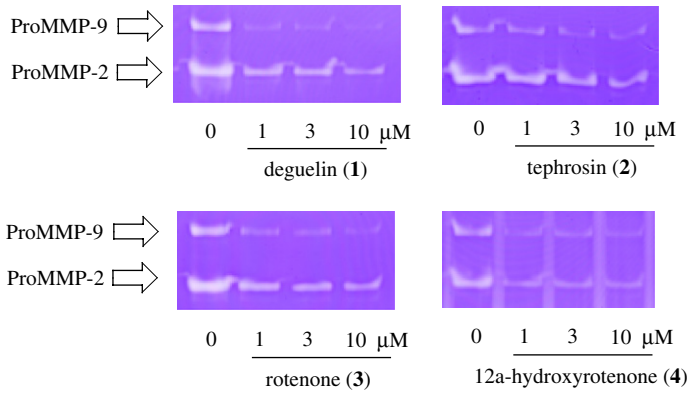

Figure 2. Gelatin zymography of the culture medium of HT1080 with rotenoids (**1**–**4**).

Table 5. Differentiation-inducing effects of rotenoids (**1** and **2**) and an isoflavone (**5**) in HL-60 cells using NBT reduction test

Test compounds	Concn (μM)	NBT-positive cells[a] (%)	No. of cells[b] (×10^5 cells/mL, 72 h)
Control	—	3.4 ± 0.9	8.1
Deguelin (**1**)	10	12.5 ± 1.3*	2.1
	30	22.2 ± 3.9*	0.3
Tephrosin (**2**)	10	5.9 ± 1.0	4.5
	30	8.4 ± 0.9*	1.0
Genistein (**5**)	10	4.1 ± 1.2	8.3
	30	11.2 ± 1.5*	5.3
1,25-Dihydroxyvitamin D_3	0.01	15.6 ± 2.7*	8.0
	0.02	27.9 ± 1.4*	6.8

[a] Each value represents the means ± SEM ($n = 4$). Significantly different from the control: $^*p < 0.05$.
[b] The cell number before treatment of test compound was 2.0×10^5 cells/mL.

3.1.3. Cell culture. Human fibrosarcoma HT1080 (Cell No.JCRB9113), human promyelocytic leukemia HL-60 (Cell No. JCRB0085), and human leukemia U937 (Cell No. JCRB9113) were obtained from Health Science Research Resources Bank (Osaka, Japan). These cells were maintained in MEM and RPMI1640 supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

3.1.4. Invasion Assay. The invasion assay of HT1080 cells was performed using Cell Culture Insert™ and 24-well multiplates. The upper side of each filter of Cell Culture Insert™ was pre-coated with Matrigel (25 μg/filter). Briefly, 100 μL of 0.25 mg/mL Matrigel in PBS solution was added onto each filter (pore size 8 μm), incubated for 4 h at 37 °C, and dried at room temperature. Cell Culture Insert™ with Matrigel-coated filters was inserted into the 24-well multiplates with 700 μL/well MEM supplemented with FCS [FCS (+)]. A mixture of HT1080 cells (1×10^6 cells/mL) suspended in 100 μL MEM without FCS [FCS (−)] and test compound solution in 100 μL MEM [FCS (−)] was then added onto the filters and incubated for 24 h. After incubation, the cells crossing the filters were collected after treatment of trypsin solution (0.25% trypsin and 0.02% EDTA in PBS), and the invaded cells were resuspended in RPMI1640 [FCS (−), phenol red (−)] and seeded onto 96-well black microplates. After incubation for 4 h at 37 °C in 5% CO_2 atmosphere, Cell Counting Kit-F™ was used for counting of the invaded cells according to the manufacturer's instruction. The test compound was dissolved in dimethylsulfoxide (DMSO) and final concentration of DMSO in the medium was 0.1%. Paclitaxel was used as a reference compound.

3.2. Cytotoxicity

Proliferation of HT1080 cells was determined by both MTT colorimetric assay and a Cell Counting Kit-F, and proliferation of U937 cells was determined by WST-8 colorimetric assay.

3.2.1. MTT assay. After 20, 44, or 68 h incubation of HT1080 cells (5×10^4 cells/100 μL/well) with test compounds in MEM [FCS (+)] in 96-well microplates, 10 μL of MTT (5 mg/mL in PBS) solution was added to each well. After a further 4 h in culture, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced by the cells. The optical density of the formazan solution was measured with a microplate reader (Model 550, Bio-Rad) at 570 nm (reference: 655 nm).

3.2.2. Calcein-AM Assay. After 24, 48, or 72 h incubation of HT1080 cells (5×10^4 cells/100 μL/well) with test compounds in MEM [FCS (+)] in 96-well black microplates, the medium was exchanged for RPMI1640 [FCS (−), phenol red (−)], and then 10 μL of calcein-AM in PBS solution (Cell Counting Kit-F™) was added to each well. After 30 min at rt, the fluorescence intensi-

Table 6. Effects of compounds **1**–**32** on proliferation of U937 cells

	IC_{50} (μM)		
	24 h	48 h	72 h
Rotenoids			
Deguelin (**1**)	42	16	5.8
Tephrosin (**2**)	30	23	7.3
Rotenone (**3**)	13	0.2	0.07
12a-Hydroxyrotenone (**4**)	3.6	0.07	0.09
Isoflavones			
Genistein (**5**)	>100	72	48
Formononetin (**6**)	50	44	45
Clycosin (**7**)	49	48	43
Orobol (**8**)	73	>100	>100
5,7,4′-Trihydroxy-3′-methoxyisoflavone (**9**)	68	85	74
7,3′,4′-Trimethoxyisoflavone (**10**)	>100	>100	>100
7,2′-Dihydroxy-4′,5′-dimethoxyisoflavone (**11**)	>100	72	48
Erycibenin A (**12**)	46	25	21
Erycibenin B (**13**)	>100	>100	>100
Erythrinin B (**14**)	28	35	32
Alpinumisoflavone (**15**)	35	32	28
Derrone (**16**)	26	20	13
Pterocarpanes			
Medicarpin (**17**)	>100	>100	>100
Homopterocarpin (**18**)	>100	>100	>100
l-Maackiain (**19**)	>100	>100	>100
Pterocarpin (**20**)	>100	>100	>100
Secundiflorol I (**21**)	>100	>100	>100
11b-Hydroxy-11b,1-dihydromedicarpin (**22**)	>100	>100	>100
11b-Hydroxy-11b,1-dihydromaackiain (**23**)	>100	>100	>100
Erycibenin C (**24**)	>100	>100	>100
Flavans			
5,7-Dimethoxy-4′-hydroxyflavan (**25**)	39	40	10
5,7,4′-Trimethoxyflavan (**26**)	29	21	18
Erycibenin E (**27**)	37	4.3	2.9
Erycibenin F (**28**)	>100	>100	>100
Flavones			
Luteolin (**29**)	36	28	20
Flavanones			
Eriodictyol (**30**)	>100	>100	>100
Flavanonols			
Erycibenin D (**31**)	>100	>100	>100
Others			
Lasiodiplodin (**32**)	36	28	20
Camptothecin	0.68	0.032	0.015

ty of each well was measured with a microplate reader (ex: 485 nm, em: 520 nm, FLUOstar OPTIMA, BMG Labtechnologies).

3.2.3. WST-8 Assay. After 20, 44, or 68 h incubation of U937 cells (5×10^3 cells/100 μL/well) with test compounds in RPMI1640 [FCS (+)] in 96-well microplates, 10 μL of WST-8 solution (Cell Counting Kit-8™) was added to each well. After a further 4 h in culture, the optical density of the water-soluble formazan produced by the cells was measured with a microplate reader (Model 550, Bio-Rad) at 450 nm (reference: 655 nm). Camptothecin was used as a reference compound. Inhibition (%) was calculated by the following formula and IC_{50} value was determined graphically.

$$\text{Inhibition } (\%) = (A - B)/A \times 100$$

where A and B indicate optical density or fluorescence intensity of vehicle and test compound-treated groups ($n = 4$).

3.3. Gelatin zymography

HT1080 cells (2×10^5 cells/400 μL/well) were seeded onto 24-well multiplates. Test compound solution was added to each well. After 24 h incubation, the medium was removed and MEM [FCS (−)] without test compound was added. After further culture for 24 h, proMMPs secreted in the FCS free medium were analyzed by a gelatin zymography. Briefly, the medium was centrifuged and 20 μL of the supernatant was applied to a SDS–10% polyacrylamide gel electrophoresis containing 0.1% gelatin. After washing twice with 2.5% Triton X-100 for 15 min, the gel was then incubated in a Tris-buffer (50 mM Tris–

HCl, 200 mM NaCl, and 10 mM $CaCl_2$, pH7.4) for 24 h at 37 °C. The lytic bands, indicating the presence of proMMPs, were visualized by Coomassie brilliant blue staining (Quick CBB, Wako Pure Chemical Industries).

3.4. Differentiation-inducing activity in HL-60

HL-60 cell differentiation was measured by NBT reduction test. Briefly, HL-60 cells (3.0×10^6 cells/15 mL/100 mm i.d. dish) were incubated for 72 h with test compound. Living HL-60 cells (1.0×10^6 cells) were washed twice with RPMI1640 [FCS (+)] and incubated for 30 min at 37 °C in 1 mL RPMI1640 [FCS (+)] containing 0.1% (w/v) NBT and 100 ng/mL PMA. After the incubation, cells were washed with PBS, and NBT-positive cells were counted under a microscope. 1,25-Dihydroxyvitamin D_3 was used as a reference compound.

3.5. Statistical analysis

For statistical analysis, one-way analysis of variance followed by Dunnett's test (Tables 1–4) or Kruskal-Wallis test followed by Steel's test (Table 5) was used. Probability (p) values less than 0.05 were considered significant.

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