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Anti-tumor-initiating effects of phenolic compounds isolated from the bark of *Picea jezoensis* var. *jezoensis*

Shun-ichi Wada [a,*], Yumiko Yasui [a], Harukuni Tokuda [b], Reiko Tanaka [a]

[a] Laboratory of Medicinal Chemistry, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-1094, Japan
[b] Department of Biochemistry, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-0841, Japan

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ABSTRACT

We have previously reported the isolation of nine phenolic compounds including three new flavonostilbenes, jezonocinols A, B, and C, from the MeOH extract of the bark of *Picea jezoensis* var. *jezoensis*. Further investigation of the MeOH extract led to the isolation of three new stilbene-type compounds and one new 1,4-benzodioxane-type compound, together with seven known phenolic compounds. These compounds were tested for their inhibitory effects on the activation of (±)-(*E*)-methyl-2-[(*E*)-hydroxy-imino]-5-nitro-6-methoxy-3-hexemide (NOR 1), a nitric oxide (NO) donor, as a primary screening test for anti-tumor initiators. All compounds tested exhibited potent inhibitory effects on NOR 1 activation. Furthermore, jezonocinol B, the most potent inhibitor of NOR 1 activation, showed remarkable anti-tumor-initiating activity in the in vivo two-stage mouse skin carcinogenesis test using peroxynitrite ($ONOO^-$; PN) as the initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as the promoter.

1. Introduction

We have been searching for biologically active constituents from the leaves and bark of coniferous trees that have been treated as waste in the forestry industry. As part of this undertaking, we have been studying the constituents of the bark of *Picea jezoensis* var. *jezoensis* (Japanese name: *Ezomatsu*), and have isolated several triterpenoids including 13α,14α-epoxy-3β-methoxyserratan-21β-ol (PJJ-34) from the $CHCl_3$ extract,[1–3] and nine phenolic compounds, namely, three flavonostilbenes named jezonocinols A (**12**), B (**13**), and C (**14**), 3,4-dihydroxybenzaldehyde (**15**), 4-hydroxycinnamic acid (**16**), dihydrodehydrodiconiferyl alcohol (**17**), isolariciresinol (**18**), protocatechuic acid (**19**), and dihydroquercetin (**20**), from the MeOH extract (Fig. 1).[4] In our previous publication of their biological activities, we have reported that PJJ-34 can inhibit carcinogenesis in both cancer initiation and promotion periods in the in vivo two-stage mouse skin carcinogenesis tset,[5,6] and the phenolic compounds have potent radical scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion radicals.[4] Our continuing studies of phenolic compounds in the MeOH extract have led to the isolation of three new stilbene-type compounds and one new 1,4-benzodioxane-type compound (**1**–**4**), together with seven known phenolic compounds, 12-hydroxydehydroabietic acid (**5**),[7] (+)-lariciresinol (**6**),[8] quercetin (**7**),[9] piceatanol (**8**),[10] *trans*-scirpusin A (**9**), *trans*-scirpusin B (**10**),[11] and cassigarol E (**11**)[12] (Fig. 1). Recently, a stilbene-type natural compound, resveratrol (3,4,3′-trihydroxystilbene), which is produced in grapes and wines, was found to have cancer chemopreventive potential and to be a potent inhibitor of the three stages of carcinogenesis: initiation, promotion, and progression.[13] Since several stilbenoids that are structurally similar to resveratrol could be isolated from *P. jezoensis* var. *jezoensis*, the anti-tumor-initiating activities of these stilbenoids and other phenolic compounds were investigated in this study. All 20 isolated phenolic compounds were tested for their inhibitory effects on the activation of (±)-(*E*)-methyl-2-[(*E*)-hydroxy-imino]-5-nitro-6-methoxy-3-hexemide (NOR 1), a nitric oxide (NO) donor, as a primary screening test for anti-tumor-initiating activity,[14] since it has been confirmed that the overproduction of NO or NO radicals induces mutagenesis in genes and strongly initiates multistage carcinogenesis.[15] Furthermore, jezonocinol B, the most potent inhibitor of NOR 1 activation, was examined in an in vivo two-stage mouse skin carcinogenesis test using peroxynitrite ($ONOO^-$; PN) as the initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as the promoter.[15] We describe herein the structure elucidation of phenolic compounds isolated from the MeOH extract of the bark of *P. jezoensis* var. *jezoensis*, and the evaluation of their anti-tumor-initiating activities.

2. Results and discussion

We have previously reported about the preliminary fractionation of the MeOH extract of *P. jezoensis* var. *jezoensis* on silica

* Corresponding author. Tel.: +81 72 690 1088; fax: +81 72 690 1005.
E-mail address: wada@gly.oups.ac.jp (S. Wada).

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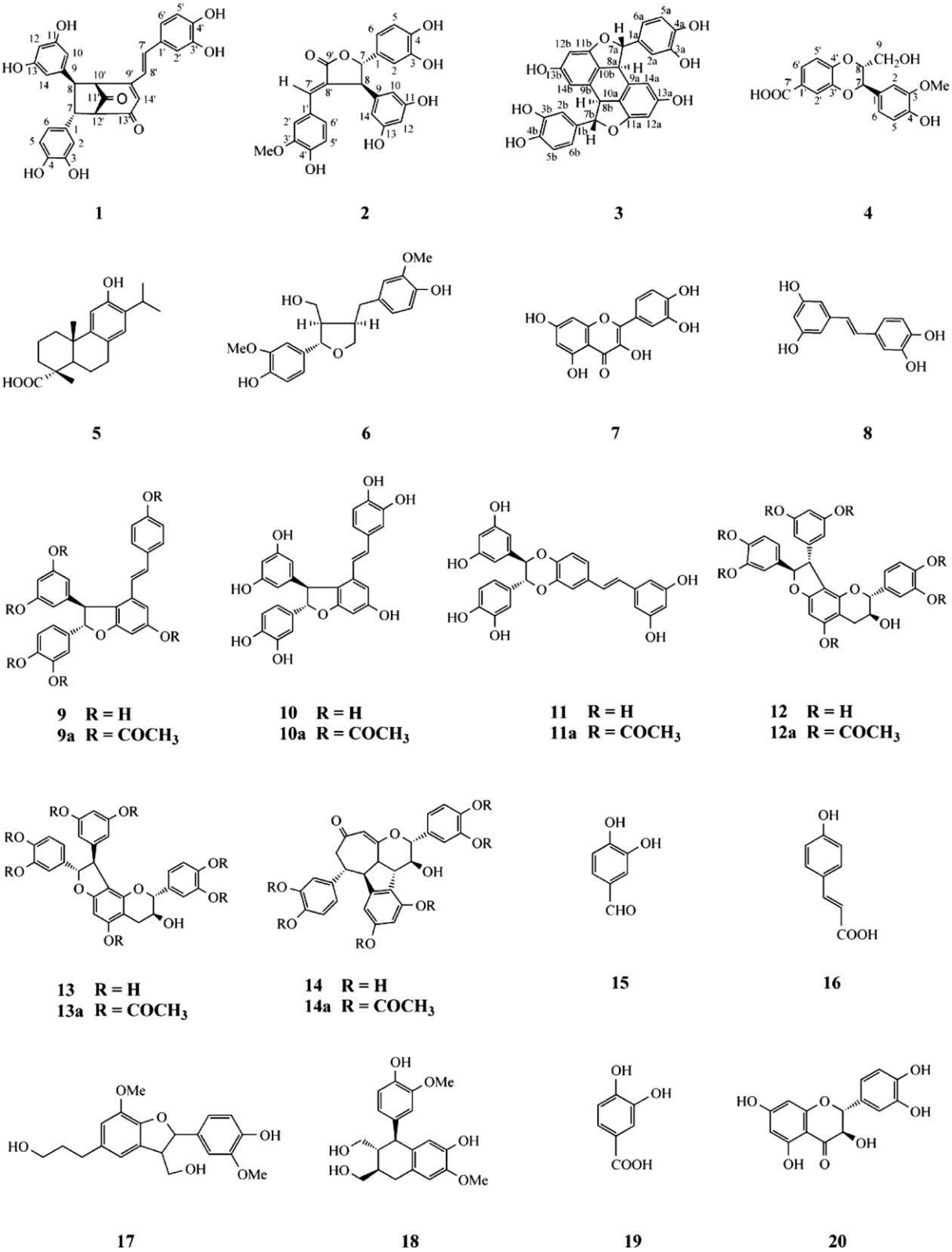

Figure 1. Structures of phenolic compounds isolated from the bark of *Picea jezoensis* var. *jezoensis*.

gel.[4] The fractions containing phenolic compounds were selected for further purification by a combination of medium pressure liquid chromatography (MPLC), Sephadex LH-20 gel permeation chromatography, preparative TLC, and HPLC to obtain new compounds, jezonodione (**1**), jezonolide (**2**), jezonofol (**3**), and 3-*O*-methyl isoamericanoic acid A (**4**) together with seven known phenolic compounds **5**–**11**.

Jezonodione (**1**) (brown amorphous powder, $[\alpha]_D$ = +33.2 (*c* 0.47, MeOH), $C_{28}H_{22}O_8$, high-resolution secondary ion MS (HRSIMS) *m/z*: 487.1389 $[M+H]^+$) showed absorption maxima at 277 (log ε 3.9) and

Table 1
^{1}H and ^{13}C NMR chemical shifts and ^{1}H–^{1}H COSY, HMBC, and NOESY correlation data of jezonodione (**1**) in acetone-d_6

Position	δ_H[a]	δ_C		^{1}H–^{1}H COSY	HMBC (H to C)	NOESY
1		130.8	s			
2	6.73 d (2.0)	121.0	d		1, 3, 4, 7	7, 8
3		145.8	s			
4		145.2	s			
5	6.71 d (8.3)	116.0	d	6	1, 3, 4	6
6	6.59 dd (8.3, 2.0)	116.5	d	5	2, 4, 7	5, 7, 8
7	3.71 dd (7.0, 6.5)	52.2	d	8, 12′	1, 2, 6, 8, 9, 12′, 13′	2, 6, 10, 14, 12′
8	3.39 d (7.0)	54.6	d	7, 10′	1, 7, 9, 10, 14, 9′, 10′, 11′	2, 6, 10, 14
9		147.1	s			
10, 14	6.19 d (2.4)	105.8	d		8, 11, 12, 13	7, 8, 10′
11, 13		159.9	s			
12	6.25 t (2.4)	102.3	d		10, 14	
1′		129.1	s			
2′	7.19 d (2.0)	115.1	d		3′, 4′, 6′, 7′	7′
3′		148.4	s			
4′		146.4	s			
5′	6.85 d (8.0)	116.5	d	6′	1′, 3′, 4′	6′
6′	7.05 dd (8.0, 2.0)	122.2	d	5′	2′, 3′	5′, 7′
7′	7.21 d (16.5)	139.1	d	8′	2′, 6′, 8′, 9′	2′, 6′, 10′
8′	7.09 d (16.5)	123.3	d	7′	1′, 7′, 9′, 10′, 14′	14′
9′		161.4	s			
10′	3.85 br s	56.4	d	8, 12′, 14′	7, 9, 8′, 9′, 12′, 14′	10, 14, 7′
11′		204.2	s			
12′	3.64 ddd (6.5, 1.4, 1.4)	73.3	d	7, 10′, 14′	11′, 13′	7
13′		194.9	s			
14′	6.25 dd (1.4, 1.4)	126.7	d	10′, 12′	8′, 12′, 10′	8′

[a] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (J/Hz).

386 (4.0) nm indicative of a highly conjugated chromophore in the UV spectrum. The IR spectrum of **1** showed absorption bands at 3304 br, 1750, 1602, 1558, and 1518 cm^{-1}, indicative of the presence of hydroxyl, carbonyl, aromatic ring, and olefin functions. The ^{1}H NMR spectrum showed signals assignable to four methines [δ_H 3.39 (1H, d, J = 7.0 Hz, H-8), 3.64 (1H, ddd, J = 6.5, 1.4, 1.4 Hz, H-12′), 3.71 (1H, dd, J = 7.0, 6.5 Hz, H-7), 3.85 (1H, br s, H-10′)], one olefin proton [δ_H 6.25 (1H, dd, J = 1.4, 1.4 Hz, H-14′)], *trans*-olefinic protons [δ_H 7.09, 7.21 (1H each, both d, J = 16.5 Hz, H-7′, 8′)], and nine aromatic protons including those of one *meta*-coupled A_2B-type dihydroxybenzene [δ_H 6.19 (2H, d, J = 2.4 Hz, H-10, 14), 6.25 (1H, t, J = 2.4 Hz, H-12)] and two *ortho*- and *meta*-coupled ABC-type dihydroxybenzenes [δ_H 6.59 (1H, dd, J = 8.3, 2.0 Hz, H-6), 6.71 (1H, d, J = 8.3 Hz, H-5), 6.73 (1H, d, J = 2.0 Hz, H-2); 6.85 (1H, d, J = 8.0 Hz, H-5′), 7.05 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 7.19 (1H, d, J = 2.0 Hz, H-2′)] (Table 1). The ^{13}C NMR spectrum supported the above assignments and two additional signals assignable to the carbonyl group were noted [δ_C 194.9 (s, C-13′), 204.2 (s, C-11′)] (Table 1). The bicyclo[3.2.1]oct-3-ene-2,8-dione structure in **1** was determined from ^{1}H–^{1}H COSY and HMBC experiments (Fig. 2 and Table 1). The ^{1}H–^{1}H COSY spectrum of **1** exhibited a set of mutually connected methine protons as shown in Figure 2a (C-12′–C-7–C-8–C-10′). Analysis of the HMBC spectrum of **1** gave the following cross peaks, H-12′/C-11′,-13′, H-10′/C-9′,-11′, and H-14′/C-12′ (Fig. 2a and Table 1). The substitution pattern of the bicyclo ring was also elucidated by HMBC experiment, which showed long-range correlations, H-7/C-2,-6, H-8/C-10,-14, H-8′/C-10′,-14′, and H-8′/C-9′,-10′,-14′. These data revealed linkages of C-1/C-7, C-8/C-9, and C-8′/C-9′. From these data, **1** was suspected to be composed of two piceatannol units and to be formed by the oxidative coupling of these units, similar to stilbene dimers, *trans*-scirpusin A (**9**), *trans*-scirpusin B (**10**), and cassigarol E (**11**). The relative stereostructure of C-7, C-8, C-10′, and C-12′ in the bicyclo ring moiety was characterized by NOESY experiment on **1**, in which NOE correlations were observed for H-7/H-10,-14,-12′, H-8/H-2,-6, H-10′/H-10,-14,-7′, and H-8′/H-14′ (Fig. 2b). Therefore, the structure of **1** was confirmed as shown in Figure 1.

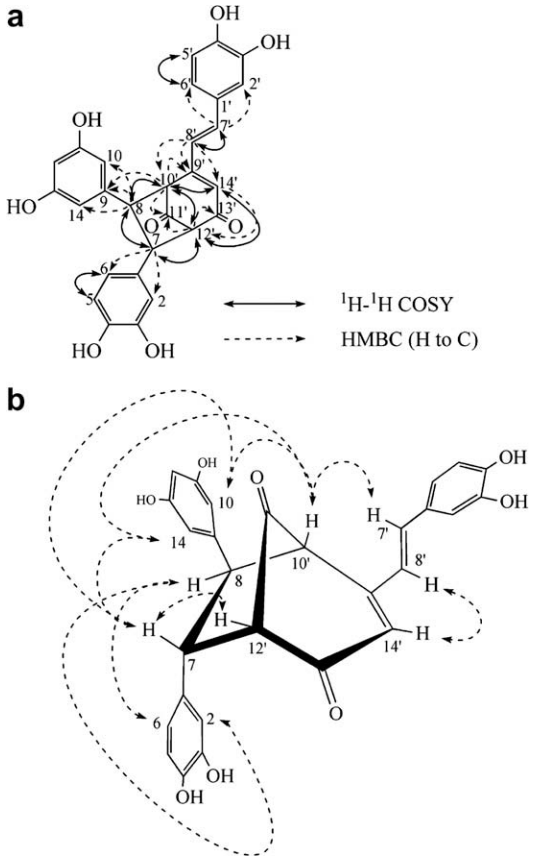

Figure 2. ^{1}H–^{1}H COSY and HMBC correlations (a) and relevant NOESY correlations (b) for jezonodione (**1**).

Jezonolide (**2**) (brown amorphous powder, $[\alpha]_D$ = ±0 (*c* 0.29, MeOH), $C_{24}H_{20}O_8$) showed absorption bands at 3377 br, 1718, 1601, and 1516 cm^{-1} due to hydroxyl, γ-lactone carbonyl, aromatic ring, and olefin functions in the IR spectrum, while its UV

Table 2
^{1}H and ^{13}C NMR chemical shifts and ^{1}H–^{1}H COSY, HMBC, and NOESY correlation data of jezonolide (**2**) in acetone-d_6

Position	δ_H[a]	δ_C		^{1}H–^{1}H COSY	HMBC (H to C)	NOESY
1		133.5	s			
2	6.82 d (2.5)	113.5	d		1	7, 8
3		146.4	s			
4		146.3	s			
5	6.83 d (8.3)	116.3	d	6	3, 4, 6	6
6	6.70 dd (8.3, 2.5)	118.2	d	5		5, 7, 8
7	5.21 d (2.8)	87.1	d	8	1, 2, 6, 9, 8′, 9′	2, 6, 10, 14
8	4.43 dd (2.8, 2.5)	53.9	d	7, 7′	1, 7, 10, 14, 7′, 8′	2, 6, 10, 14, 2′, 6′
9		144.6	s			
10, 14	6.34 d (2.4)	106.2	d	12	8, 11, 12, 13	7, 8, 2′, 6′, OMe
11, 13		160.3	s			
12	6.28 t (2.4)	102.6	d	10, 14	11, 13	
1′		126.7	s			
2′	7.01 d (2.4)	113.8	d		3′, 4′, 6′, 7′	8, 10, 14, OMe
3′		148.4	s			
4′		149.9	s			
5′	6.81 d (8.4)	115.9	d	6′	4′, 6′	6’
6′	7.09 dd (8.4, 2.4)	127.4	d	5′		8, 10, 14, 5′
7′	7.68 d (2.5)	140.0	d	8	8′, 9’	
8′		124.2	s			
9′		172.8	s			
OMe	3.63 s	56.2	q		3′	10, 14, 2′

[a] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (*J*/Hz).

spectrum showed absorption maxima at 287 (log ε 3.7) and 337 (3.8) nm due to a stilbene chromophore. The ^{1}H and ^{13}C NMR spectra showed signals assignable to one ethylene [δ_H 4.43 (1H, dd, *J* = 2.8, 2.5 Hz, H-8), 5.21 (1H, d, *J* = 2.8 Hz, H-7)], one methoxy proton [δ_H 3.63 (3H, s)], one olefin proton [δ_H 7.68 (1H, d, *J* = 2.5 Hz, H-7′)], nine aromatic protons including those of one *meta*-coupled A_2B-type dihydroxybenzene [δ_H 6.28 (1H, t, *J* = 2.4 Hz, H-12), 6.34 (2H, d, *J* = 2.4 Hz, H-10,14)] and two *ortho*- and *meta*-coupled ABC-type dihydroxybenzenes [δ_H 6.70 (1H, dd, *J* = 8.3, 2.5 Hz, H-6), 6.82 (1H, d, *J* = 2.5 Hz, H-2), 6.83 (1H, d, *J* = 8.3 Hz, H-5); 6.81 (1H, d, *J* = 8.4 Hz, H-5′), 7.01 (1H, d, *J* = 2.4 Hz, H-2′), 7.09 (1H, dd, *J* = 8.4, 2.4 Hz, H-6′)], and one γ-lactone carbonyl carbon [δ_C 172.8 (s, C-9′)] (Table 2). The planar structure of **2** was established on the basis of 2D NMR experiments (Fig. 3a). Analysis of the HMBC spectrum of **2** gave the following cross peaks, H-7/C-2,-6,-9′, H-8/C-10,-14,-7′,-8′ H-2′/C-7′, MeO-3′/C-3′, and H-7′/C-8′,-9′. Furthermore, the relative stereostructure of C-7 and C-8 and the geometry of C-7′ were deduced on the basis of NOESY experiment (Fig. 3b and Table 2), in which NOE correlations were observed for H-7/H-10,-14, H-8/H-2,-6, and H-2′,-6′/H-10,14. Therefore, the structure of **2** was determined as shown in Figure 1, and could be formed by the oxidative coupling of piceatanol and ferulic acid.

Jezonofol (**3**) and 3-*O*-methyl isoamericanoic acid A (**4**) are a stilbene dimer and a 1,4-benzodioxane-type compound, respectively, as shown in Figure 1, and are analogues of betulifol A from

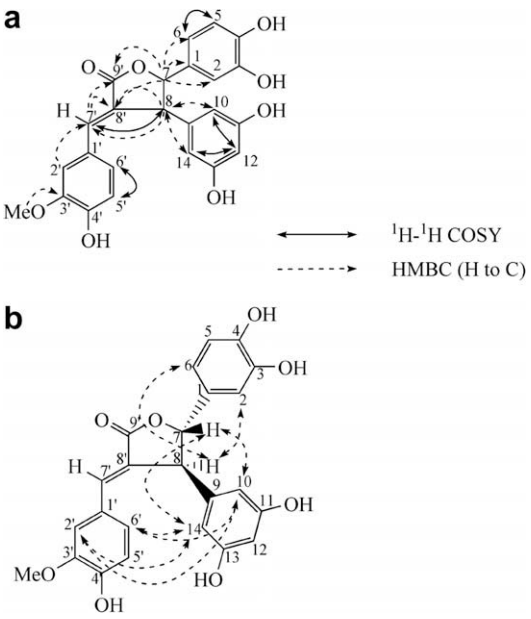

Figure 3. ^{1}H–^{1}H COSY and HMBC correlations (a) and relevant NOESY correlations (b) for jezonolide (**2**).

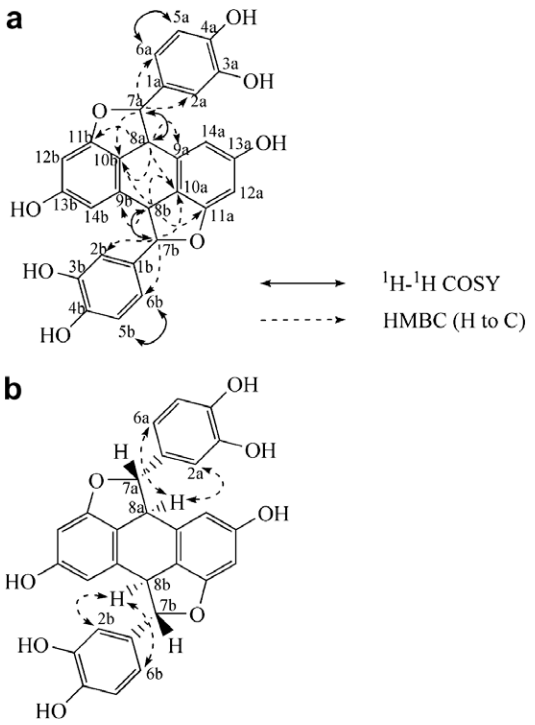

Figure 4. ^{1}H–^{1}H COSY and HMBC correlations (a) and relevant NOESY correlations (b) for jezonofol (**3**).

Table 3
^{1}H and ^{13}C NMR chemical shifts and ^{1}H–^{1}H COSY, HMBC, and NOESY correlation data of jezonofol (**3**) in acetone-d_6

Position	δ_H[a]	δ_C		^{1}H–^{1}H COSY	HMBC (H to C)	NOESY
1a (1b)		132.0	s			
2a (2b)	7.24 d (2.2)	115.6	d		4a (4b), 6a (6b), 7a (7b)	7a (7b), 8a (8b)
3a (3b)		146.7	s			
4a (4b)		146.3	s			
5a (5b)	6.96 d (8.2)	116.2	d	6a (6b)	1a (1b), 3a (3b)	6a (6b)
6a (6b)	7.09 dd (8.2, 2.2)	120.3	d	5a (5b)	2a (2b), 4a (4b), 7a (7b)	5a (5b), 7a (7b), 8a (8b)
7a (7b)	5.44 d (11.0)	92.9	d	8a (8b)	2a (2b), 6a (6b), 8a (8b), 9a (9b), 10b (10a)	2a (2b), 6a (6b)
8a (8b)	4.47 d (11.0)	48.5	d	7a (7b)	1a (1b), 9a (9b), 10a (10b), 10b (10a), 11b (11a)	2a (2b), 6a (6b)
9a (9b)		136.9	s			
10a (10b)		122.0	s			
11a (11b)		159.9	s			
12a (12b)	6.19 d (2.0)	96.7	d		10a (10b), 11a (11b), 14a (14b)	
13a (13b)		159.5	s			
14a (14b)	6.25 d (2.0)	104.0	d		8a (8b), 10a (10b), 12a (12b)	

[a] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (J/Hz).

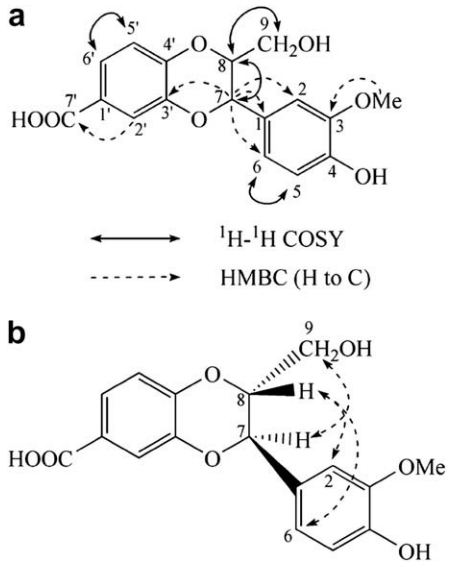

Figure 5. ^{1}H–^{1}H COSY and HMBC correlations (a) and relevant NOESY correlations (b) for 3-*O*-methyl isoamericanoic acid A (**4**).

Vitis betulifolia[16] and isoamericanoic acid A methyl ester from *Phytolacca americana*.[17] The detailed structural elucidations of **3** and **4** were carried out by comparison of their NMR data with the respective reported data, and by the analyses of their 2D NMR data as shown in Figure 4 and Table 3, and Figure 5 and Table 4, respectively.

As a primary screening test for anti-tumor initiating activity, compounds **1**–**20** were evaluated for their inhibitory effects on the activation of NOR 1, a nitric oxide (NO) donor.[14] The inhibitory effect on NOR 1 activation is expressed by the inhibitory ratio (IR), which represents the percentage transformation of Chang liver cells with NOR 1 alone divided by the percentage transformation with NOR 1 and the test compound that inhibits the activation of NO. Table 5 shows the IR values of 26 phenolic compounds and two reference compounds, curcumin and quercetin. All the test compounds showed high IR values ranging from 1.7 to 2.9. The IR values of the test compounds that have less than two phenolic hydroxyl groups in the molecule, such as **4**–**6** and **15**–**19**, were low ($R \leqslant 2.0$), while those of stilbene dimers, flavonostilbenes and flavonoids, such as **1**–**3**, **7**, **9**–**14**, and **20**, were high ($R \geqslant 2.0$). Especially, test compounds possessing the flavonoid skeleton exhibited the strongest inhibitory effects (**7**, **12**–**14**, and **20**). Furthermore, acetylation of the phenolic hydroxyl group (**9a**, **10a**, **11a**, **12a**, **13a**, and **14a**) suppressed the inhibitory effects on NOR 1 activation. These data indicated that

Table 4
^{1}H and ^{13}C NMR chemical shifts and ^{1}H–^{1}H COSY, HMBC, and NOESY correlation data of 3-*O*-methyl isoamericanoic acid A (**4**) in acetone-d_6

Position	δ_H[a]	δ_C		^{1}H–^{1}H COSY	HMBC (H to C)	NOESY
1		128.8	s			
2	7.15 d (2.0)	111.9	d		1, 3, 6, 7	7, 8, OMe
3		148.5	s			
4		148.1	s			
5	6.89 d (8.5)	115.8	d	6	1, 2, 3, 7	6
6	6.99 d (8.5, 2.0)	121.6	d	5	2, 4, 7	5, 7, 8
7	5.03 d (8.3)	77.1	d	8	1, 2, 5, 6, 8, 9, 3′	2, 6, 9
8	4.22 ddd (8.3, 4.2, 2.5)	80.0	d	7, 9	7	2, 6, 9
9	3.78 dd (12.7, 2.5)	61.7	t	8	7, 8, 4′	7, 8
	3.55 dd (12.7, 4.2)					
1′		124.4	s			
2′	7.56 d (2.2)	119.3	d		1′, 3′, 4′, 7′	6′
3′		144.6	s			
4′		148.9	s			
5′	7.01 d (8.4)	117.6	d	6′	3′, 4′, 6′, 7′	
6′	7.59 dd (8.4, 2.2)	124.2	d	5′	5′	2′
7′		167.1	s			

[a] ^{1}H chemical shift values (δ ppm from $SiMe_4$) followed by multiplicity and then the coupling constants (J/Hz).

Table 5
Inhibitory ratios (IRs) of phenolic compounds isolated from the bark of *Picea jezoensis* var. *jezoensis* and reference compounds

Test compound[a]	Inhibitory ratio (IR)[b]
Positive control NOR1	1.0
Curcumin	2.1
Quercetin	2.9
1	2.2
2	2.1
3	2.3
4	1.7
5	2.0
6	2.0
7	2.9
8	2.0
9	2.0
9a	1.8
10	2.2
10a	1.9
11	2.1
11a	1.8
12	2.7
12a	1.9
13	2.7
13a	1.9
14	2.3
14a	1.8
15	1.8
16	1.9
17	1.8
18	2.0
19	1.8
20	2.5

[a] All compounds were tested at 350 nmol.
[b] IR = percentage transformation of cells (NOR 1 alone)/percentage transformation of cells (NOR 1 + test compound).

the phenolic hydroxyl group and the flavonoid skeleton are critical for the inhibition of NOR 1 activation.

Among the 20 test compounds, jezonocinols (**12** and **13**), which possess the flavonostilbene skeleton, exhibited inhibitory effects (IR = 2.7) that were almost equivalent to that of quercetin and were stronger than that of curcumin. From this result, jezonocinol B (**13**) was selected for the in vivo two-stage mouse skin carcinogenesis test. In in vivo study, we have applied a tumor model using a single dose of PN as an initiator and promoted the tumors with TPA.[15,18,19] As shown in Figure 6a, the percentage of papilloma bearers in the control group (PN and TPA only) increased from week 7 to reach 100% at week 11, whereas the group treated with **13** by oral administration in drinking water (0.0025%), along with the initiator and the promoter, showed a two-week delay in tumor promotion, and a reduction in the percentage of papilloma bearing mice to 27% at week 11. Moreover, upon treatment with **13**, the average number of papillomas per mouse at week 20 was reduced from 7.1 for the control group to 3.1 for the test group (Fig. 6b). The oral administration of **13** had a significant inhibitory effect on the PN-induced and TPA-promoted mouse skin carcinogenesis. These data suggest that jezinocinol B (**13**) may intercept and neutralize potent chemical carinogens, such as reactive oxygen species (ROS; superoxide, and peroxy and hydroxyl radicals) and NO donors.

In this study, we showed that phenolic compounds isolated from the bark of *P. jezoensis* var. *jezoensis* have anti-tumor-initiating activity and that PJJ-34 is a cancer chemopreventive agent.[5,6] Considering these results, *P. jezoensis* var. *jezoensis* may be a vital natural source of cancer chemopreventive agents.

3. Experimental

3.1. General

Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-1000 digital polarimeter. IR spectra were run on a JASCO FT/IR-680 Plus spectrophotometer, and UV spectra on a HITACHI U-2000 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Varian INOVA 500 spectrometer with standard pulse sequences, operating 500 and 125 MHz, respectively. Acetone-d_6 and $CDCl_3$ were used as the solvent and TMS as the internal standard. EIMS and SIMS data were recorded on a Hitachi 4000H double-focusing mass spectrometer, using Ce^{+} as primary ion for SIMS. Column chromatography was carried out over silica gel (70–230 mesh, Merck) and MPLC was carried out with silica gel (230–400 mesh, Merck). HPLC was performed on a Waters Delta 600. Fractions obtained from column chromatography were monitored by TLC (Silica Gel 60 F254, Merck). Preparative TLC was carried out on Merck silica gel F254 plates (20 × 20 cm, 0.5 mm thick). Acetylation (ca. 1 mg sample) was carried out as usual (Ac_2O/pyridine, each 1 mL).

3.2. Isolation procedure

The chopped stem bark (18 kg) of *P. jezoensis* var. *jezoensis* was extracted with successively with $CHCl_3$ and MeOH. It has been previously reported that preliminary separation with silica gel column chromatography of the MeOH extract (325 g) gave 8 fractions (I–VIII) and that rechromatography with silica gel column chromatography of fractions II and III afforded four (II-A–II-D) and eleven (III-A–III-K) fractions, respectively.[4]

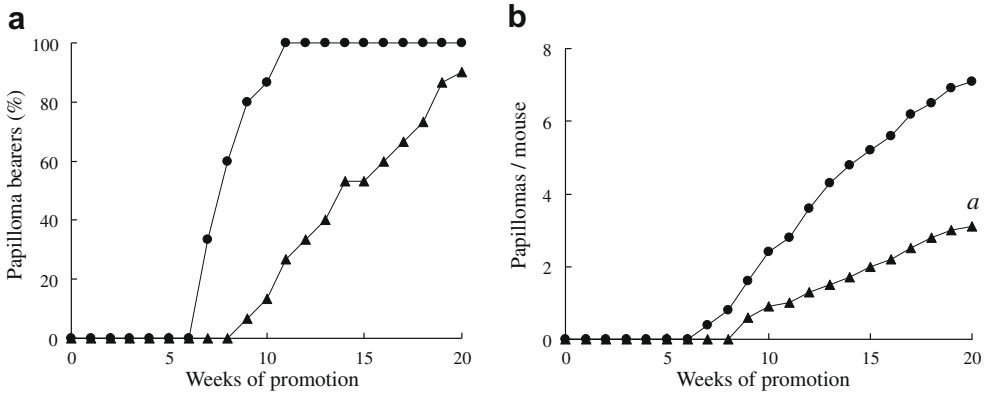

Figure 6. Inhibitory effect of jezonocinol B (**13**) on mouse skin carcinogenesis, initiated by peroxynitrite (PN) and promoted by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). (a) Percentage of mice bearing papillomas; and (b) average number of papillomas per mouse. ●: Positive control; PN (390 nmol) + TPA (1.7 nmol). ▲: PN (390 nmol) + 0.0025% jezonocinol B (**13**) in drinking water for 2 weeks + TPA (1.7 nmol). [a] Statistically different from the positive control ($P < 0.05$).

Further fractionation of II-A (1.2 g) by a combination of MPLC ($CHCl_3$–MeOH, 10:1), Sephadex LH-20 gel permeation chromatography (MeOH–$CHCl_3$, 1:1) and preparative TLC (*n*-hexane–AcOEt, 8:2) gave 3-*O*-methyl isoamericanoic acid A (**4**) (2.5 mg), 12-hydroxydehydroabietic acid (**5**) (6.4 mg) and (+)-lariciresinol (**6**) (8.8 mg). Frs. III-C (4.4 g), III-D (5.6 g) and III-E (4.4 g) were, respectively separated by Sephadex LH-20 (MeOH), followed by HPLC [column TSKgel ODS-80Ts (TOSOH, 21.5 mm × 300 mm); solv. MeOH–H_2O (40:60); flow rate 6 mL/min; detection UV 280 nm] to afford jezonolide (**2**) (4.2 mg), quercetin (**7**) (5.0 mg) and piceatanol (**8**) (6.2 mg) from fr. III-C, jezonofol (**3**) (2.4 mg), *trans*-scirpusin A (**9**) (71.2 mg) and cassigarol E (**11**) (12.2 mg) from fr. III-D, and jezonodione (**1**) (7.8 mg) and *trans*-scirpusin B (**10**) (30.3 mg) from fr. III-E.

3.2.1. Jezonodione (1)

Brown amorphous powder; SIMS *m/z* (rel. int): 487 (7), 391 (5), 299 (10), 223 (11), 207 (60), 115 (100); HRSIMS: $C_{28}H_{23}O_8$ (*m/z* 487.1389 $[M+H]^+$: calcd 487.1391); $[\alpha]_D$ = +33.2 (*c* 0.47, MeOH); UV (EtOH) λ_{max} (log ε): 208.5 (4.46), 277.0 (3.87), 385.5 (3.96) nm; IR ν_{max} (KBr): 3304 br, 1750, 1602, 1558, 1518, 1448, 1360, 1278, 1159, 1115, 1009, 963 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1.

3.2.2. Jezonolide (2)

Brown amorphous powder; SIMS *m/z* (rel. int): 437 (5), 391 (2), 299 (4), 223 (3), 207 (52), 115 (100); HRSIMS: $C_{24}H_{21}O_8$ (*m/z* 437.1232 $[M+H]^+$: calcd 437.1235); $[\alpha]_D$ = ±0 (*c* 0.29, MeOH); UV (EtOH) λ_{max} (log ε): 212.5 (4.13), 286.5 (3.70), 336.5 (3.82) nm; IR ν_{max} (KBr): 3377 br (OH), 1718, 1601, 1516, 1456, 1384, 1288, 1163, 1009, 819 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 2.

3.2.3. Jezonofol (3)

Brown amorphous powder; $[\alpha]_D$ = ±0 (*c* 0.28, MeOH); UV (EtOH) λ_{max} (log ε): 235.0 (3.99), 284.0 (3.90) nm; IR ν_{max} (KBr): 3367 br (OH), 1592, 1522, 1375, 1281, 1164, 980, 824 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 3. The molecular-related ion in the MS spectra of **3** could not be observed, however, that of the hexaacetates (**3a**) could be clearly showed in the EIMS. The molecular formula of **3** was determined from the analyses of the EIMS and HREIMS of **3a**.

3.2.4. Jezonofoyl hexaacetates (3a)

Pale yellow amorphous powder; EIMS: *m/z* 736 (20), 694 (62), 652 (100), 610 (54), 568 (31), 526 (15),484, 388 (10), 346 (10), 328 (9); HREIMS: $C_{40}H_{32}O_{14}$ (*m/z* 736.1782 $[M]^+$, calcd 736.1790), ^1H NMR (300 MHz, $CDCl_3$): δ_H 7.56 (2H, dd, *J* = 8.4, 2.4 Hz), 7.55 (2H, d, *J* = 2.4 Hz), 7.33 (2H, d, *J* = 8.4 Hz), 6.51 (4H, s), 5.73 (2H, d, *J* = 9.8 Hz), 4.62 (2H, d, *J* = 9.8 Hz), 2.324 (6H, s), 2.318 (6H, s), 2.29 (6H, s).

3.2.5. 3-*O*-Methyl isoamericanoic acid A (4)

Colorless amorphous powder; EIMS *m/z* (rel. int): 332 (89), 314 (12), 180 (37), 137 (100), 124 (53), 91 (13), 31 (24); HREIMS: $C_{17}H_{16}O_7$ (*m/z* 332.0901 $[M+Na]^+$: calcd 327.0895); $[\alpha]_D$ = ±0 (*c* 0.33, MeOH); UV (EtOH) λ_{max} (log ε): 229.5 (3.89), 255.5 (3.76), 285.5 (3.62) nm; IR ν_{max} (KBr): 3419 br (OH), 1700 (C=O), 1611, 1520, 1452, 1384, 1274, 1033, 898, 770 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 4.

3.3. In vitro NOR 1 test

Chang liver cells (normal human hepato cells; 5×10^5/ml), derived from human liver in MEM Eagle medium, were cultured 3 days before NOR 1 was added into culture dish and incubated for 1 h under CO_2 incubator as control. For screening assay, test samples to culture dish were added before 1 min of NOR 1 treatment. Transformed cells were observed under light-microscopy (×100). All observed cells count for more than 250. The inhibitory ratio (IR) was then calculated using the following equation: IR = percentage transformation of cells (NOR1 alone)/percentage transformation of cells (NOR1 + test compound). All samples were carried out the three times experiments per one sample and showed a average of numbers into Table 5.

3.4. In vivo two-stage mouse-skin-carcinogenesis assay

Mouse studies were approved by the Committee of Animal Experimental Center for Kyoto Prefectural University of Medicine throughout all treatments. Specific pathogen-free female ICR mice (6 weeks old) were obtained from Japan SLC Inc., Shizuoka, Japan, and the animals were housed, five per polycarbonate cage, in a temperature-controlled room at 24 °C and given food and water and libitum throughout the experiment. The protocol involved treatment of three groups of mice, each comprising five animals. One week prior to initiation, the test group was administrated 0.0025 wt % of jezonocinol B (**13**) in drinking water, whereas the control group received regular drinking water. All treated mice were taken in total 2.6 mg (0.187 mg/day) by free oral drinking during two weeks and after one week of initiating treatment, they were freely oral drinking of normal water to end of experiment. As initiation, the dry-shaved dorsal skin of the test mice was topically treated with PN (390 nmol) in NaOH (1 mM, 0.1 ml). One week after initiation, the oral feeding of **13** to the test group was stopped, and replaced with regular drinking water. Then, the animals in both the test and control groups were topically treated with TPA (1.7 nmol) for 20 weeks. The incidence of papillomas was examined weekly over a period of 20 weeks. Student's *t*-test was used for all statistical analysis.

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