PHENOLIC COMPOUNDS IN *ERYTHRINA* X *BIDWILLII* AND THEIR ACTIVITY AGAINST ORAL MICROBIAL ORGANISMS

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Abstract --- The bioassay-directed fractionation of an acetone extract of the root bark of *Erythrina* x *bidwillii* which shows a significant antibacterial activity against oral bacteria led to isolate three new phenolic compounds (bidwillols A and B, and bidwillon C) and a known pterocarpene (erycristagallin), the structures of which were characterized by spectral and physical properties. Among them, erycristagallin showed potent microbial activity against *Streptococcus mutans*, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*.

In our previous study[1] oriented to exploration of antimicrobial agent in leguminous plants, two phenolic compounds (a pterocarpan and an isoflavanone) with a moderate activity were isolated from an acetone extract of *Erythrina* x *bidwillii*. Although the crude extract showed a higher activities against such microorganisms, the active principle(s) has not necessarily been characterized yet. In the present paper, other bioactive compounds and their structures elucidation are described.

The dried root bark of *E.* x *bidwillii* was extracted with acetone. The acetone solution was concentrated *in vacuo* to give an extract which was subjected to silica gel column chromatography (si-cc) eluted with *n*-hexane-acetone (3 : 1), and divided into seven fractions in order of polarity. Each fraction was monitored by microbial activity

against eleven microorganisms (Table 1). In the fractions 2-5, bioactive compounds against *Streptococcus mutans*, *Lactobacillus casei*, *L. fermentum*, *Prevotella intermedia* and *Staphylococcus aureus* were contained.

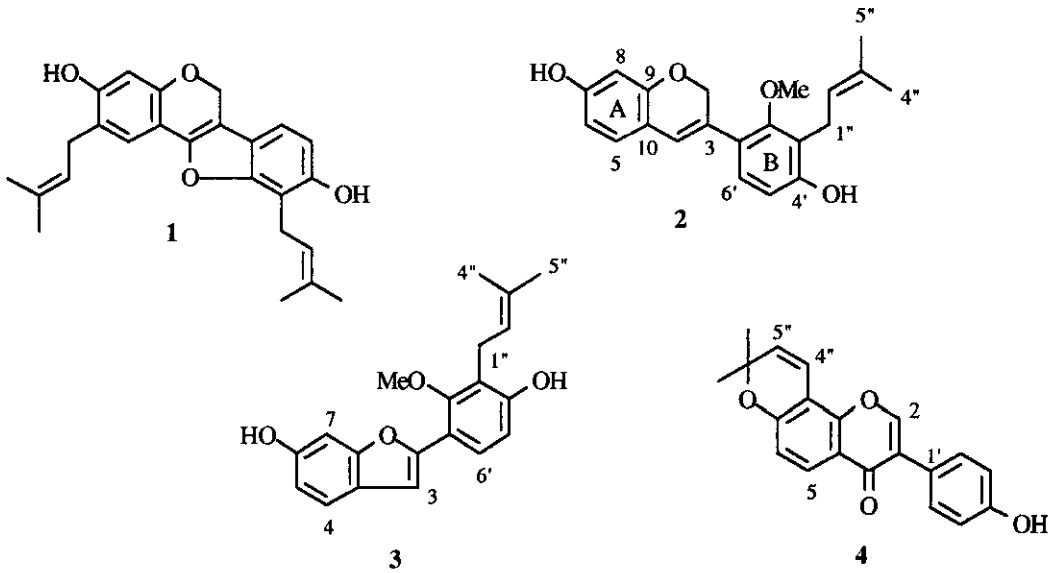
Table 1 In vitro activity of each fraction of the acetone extract against oral microorganisms

	microorganisms (MIC, μg/ml)										
	1	2	3	4	5	6	7	8	9	10	11
Fr. 1	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
Fr. 2	12.5	25	12.5	25	>100	6.3	>100	>100	>100	50	>100
Fr. 3	12.5	12.5	12.5	12.5	>100	25	>100	>100	>100	25	>100
Fr. 4	6.3	6.3	3.2	6.3	>100	6.3	>100	100	>100	6.3	>100
Fr. 5	6.3	12.5	12.5	12.5	>100	6.3	>100	100	>100	6.3	>100
Fr. 6	50	>100	50	100	>100	6.3	>100	100	100	100	>100
Fr. 7	>100	>100	>100	>100	>100	50	>100	>100	>100	>100	>100

1: *Lactobacillus casei* ATCC 7469, 2: *L. fermentum* YIT 1182, 3: *Streptococcus mutans* OZ 1, 4: *S. mutans* Ingbritt, 5: *Fusobacterium nucleatum* k1, 6: *Prevotella intermedia* ATCC 26551, 7: *Porphyromonas gingivalis* ATCC 33277, 8: *Actinobacillus actinomycetemcomitans* ATCC 29552,9: *Actinomyces naeslundii* ATCC 10953, 10: *Staphylococcus aureus* NIHJ 209P, 11: *Escherichia coli* K12.

These fractions were further subjected to si-cc, preparative thin layer chromatography and recrystallization to give four flavonoid compounds (**1**-**4**). Among them, compound (**1**) was identified as a known pterocarpan erycristagallin by comparison of spectroscopic data with those in a previous report.[2]

Compound (**2**), $C_{21}H_{22}O_4$, obtained as a yellow oil, showed negative in the Gibbs reaction. The uv absorption maxima at 218, 242sh, 301sh and 322 nm suggested that **1** was an isoflav-3-ene.[3] The presence of a γ,γ-dimethylallyl [δ 1.66, 1.78 (3H each, s, Me), 3.37 (2H, d, *J*= 7 Hz, CH_2), 5.26 (1H, t, *J*= 7 Hz, CH)], a



methoxyl (δ 3.65), and two hydroxyl groups (δ 8.44 and 8.47) was suggested in the ^1H-nmr spectrum. In an aromatic region, three protons in an ABX spin system [δ 6.34 (1H, d, *J*= 2 Hz), 6.42 (1H, dd, *J*= 8, 2 Hz) and 6.97 (1H, d, *J*= 8 Hz)], and a pair of *ortho*-coupled one-proton doublets were also observed. Further, the spectrum exhibited A_2X-type proton signals at δ 4.94 (2H, d, *J*= 2 Hz) and 6.58 (1H, br s) characteristically assigned to H-2 and H-4 of the isoflav-3-ene.[4] In the ^{13}C-nmr spectrum, the methoxyl carbon signal at δ 61.0 indicated that the methoxyl group is surrounded with substituents at both *ortho*-positions,[5] and the both *ortho*-positions of the γ,γ-dimethylallyl group were also occupied with *O*-functions because a methylene carbon of it was observed at δ 23.6.[6] The nOes were observed between the methoxyl group and H-2, H-4, and the methoxyl group and the methylene protons of the γ,γ-dimethylallyl group, respectively, which indicated that the methoxyl group was substituted at C-2', the γ,γ-dimethylallyl group was at C-3', and then one of the hydroxyl groups was at C-4'. Another hydroxyl group was located at C-7 because of ABX-type signals of ring-A protons. Consequently, the structure of **2** was concluded to be 3'-(γ,γ-dimethylallyl)-7,4'-dihydroxy-2'-methoxyisoflav-3-ene (bidwillol A).

Compound (**3**) obtained as a yellow oil showed negative in Gibbs reaction. The HRms data supported the molecular formula of **3** to be $C_{20}H_{20}O_4$ (M^+ 324.1336, calcd 324.1361). The uv absorption maxima at 248sh, 317 and 331 nm closely resembled those of a 2-arylbenzofuran derivative.[7] The ^1H-nmr spectrum revealed the presence of a γ,γ-dimethylallyl [δ 1.67, 1.81 (3H each, s, Me), 3.44 (2H, d, *J*= 7 Hz, CH_2) and 5.30 (1H, m, CH)], a methoxyl [δ 3.74 (3H, s)] and two hydroxyl groups [δ 8.43, 8.69 (1H each, s)] in addition to five resonances (three protons in an ABX and two protons in *ortho*-coupled one-proton doublets) in the aromatic region. Furthermore, the spectrum showed a broad singlet proton signal at δ 7.11 assignable to H-3 of a 2-arylbenzofuran.[8] By the same reasons of the ^{13}C-nmr data mentioned above, a partial structure of a B-ring moiety was identical to that of **2**. The other hydroxyl group was substituted at C-7 due to an ABX-type signal of A-ring protons. Therefore, the structure of **3** was determined to be 3'-(γ,γ-dimethylallyl)-6,4'-dihydroxy-2'-methoxy-2-arylbenzofuran (bidwillol B).

Compound (**4**) obtained as a colorless amorphous powder showed negative in Gibbs reaction. The hrms data showed the molecular formula to be $C_{20}H_{16}O_4$ (M^+ 320.1070, calcd 320.1048). In the ^1H-nmr spectrum, a typical singlet (δ 8.37) assigned to H-2 of an isoflavone skeleton was observed. The ^1H-nmr spectrum also exhibited the presence of a dimethylpyran ring [δ 1.46 (6H, s), 5.95 (1H, d, *J*= 10 Hz), 6.80 (1H, d, *J*= 10 Hz)] and four protons in an A_2B_2 spin system due to a 4'-oxygenated isoflavone, as well as a hydroxyl group

and a pair of *ortho*-coupled one-proton doublets. A carbonyl carbon signal appeared at δ 175.2 in higher field of the ^{13}C-nmr spectrum indicated that **4** is a 5-deoxygenated type. The structure of **4** was, then, characterized as 4'-hydroxy-[6",6"-dimethylpyrano(2",3":7,8)]isoflavone (bidwillon C).

The minimum inhibitory concentrations (MIC) of **1-4** against oral bacteria were estimated according to our previous method,[1] and the results are shown in Table 2. The potent activities were found to be less than those of the known antibiotics bekanamycin, but erycristagallin (**1**) showed remarkable activity against *Streptococcus mutans*, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (each MIC is 6.3 μg/ml). The others (**2-4**) had a relatively weak activity.

Table 2 Activity of the compounds (**1-4**) isolated from *E.* x *bidwillii* against oral microorganisms

	microorganisms[a] (MIC, μg/ml)										
	1	2	3	4	5	6	7	8	9	10	11
1	50	>50	6.3	>50	>50	>50	6.3	6.3	>50	>50	>50
2	>50	>50	25	>50	>50	>50	>50	>50	25	>50	>50
3	>15	>15	15	>15	>15	>15	>15	>15	>15	>15	>15
4	>50	>50	25	>50	>50	50	50	>50	>50	>50	>50
bkm[b]	0.8	0.8	0.8	0.8	25	0.8	0.8	0.8	0.8	3.2	50

a) Although microorganisms are listed in Table 1, strain number about 2, 5 and 7 is different; 2: *L. fermentum* YIT 0082, 5: *F. nucleatum* K3, and 7: *P. gingivalis* ATCC 33275.
b) bekanamycin.

EXPERIMENTAL

Plant Material. The roots of *Erythrina* x *bidwillii* were collected at Okinawa in summer 1990. The voucher specimens are deposited in the Herbarium of Gifu Pharmaceutical University.

Extraction and isolation. The air-dried root bark (1 kg) was extracted with acetone (2 l x 3) at 80°C for 6 h. After evaporating the solvent, the crude extract (140 g) was dissolved in acetone and chromatographed on si-cc (1.2 kg) eluting with *n*-hexane-acetone (3 : 1) to give seven fractions (each 2.5 l). The fr. 4 was further chromatographed on si-cc eluting with C6H6-EtOAc (5 : 1) to give **1** (280 mg), **2** (5 mg) and **4** (7 mg). From the fr. 3, **3** (5 mg) was isolated after purification by preparative tlc (C6H6-EtOAc = 5 : 1).

Compound 1 (erycristagallin). Eims *m/z* (%): 390 [M$^+$] (100), 334 (42), 279 (47); uv λ (MeOH, nm) (log ε): 214 (4.19), 244 (3.91), 251sh (3.91), 288sh (3.52), 321sh (4.19), 338 (4.14), 355 (4.09); ^{1}H-nmr (acetone-d_6) δ: 1.68, 1.75, 1.77, 1.92 (3H each, s, Me), 3.30 (2H, d, *J*= 7 Hz, H-1'), 3.63 (2H, d, *J*= 7 Hz, H-1"),

5.38 (2H, m, H-2', 2"), 5.49 (2H, s, H-6), 6.46 (1H, s, H-4), 6.83 (1H, d, *J*= 8 Hz, H-8), 7.10 (1H, d, *J*= 8 Hz, H-7), 7.21 (1H, s, H-1), 8.34, 8.57 (1H each, br s, OH).

Compound 2 (bidwillol A). Hrms *m/z*: 338.1515 (calcd for $C_{21}H_{22}O_4$: 338.1518); eims *m/z* (%): 338 [M^+] (100), 282 (51), 267 (49), 147 (26); uv λ (MeOH, nm): 218, 242sh, 301sh, 322; ^{1}H-nmr (acetone-d_6) δ: 1.66, 1.78 (3H each, s, Me), 3.37 (2H, d, *J*= 7 Hz, H-1"), 3.65 (3H, s, OMe), 4.94 (2H, d, *J*= 2 Hz, H-2), 5.26 (1H, t, *J*= 7 Hz, H-2"), 6.34 (1H, d, *J*= 2 Hz, H-8), 6.42 (1H, dd, *J*= 8, 2 Hz, H-6), 6.58 (1H, br s, H-4), 6.68 (1H, d, *J*= 8 Hz, H-5'), 6.97 (1H, d, *J*= 8 Hz, H-5), 7.03 (1H, d, *J*= 8 Hz, H-6'), 8.44, 8.47 (1H each, s, OH); ^{13}C-nmr (acetone-d_6) δ: 68.7 (C-2), 124.4 (C-3), 127.6 (C-4), 129.5 (C-5), 109.4 (C-6), 158.5 (C-7), 103.5 (C-8), 155.7 (C-9), 117.0 (C-10), 124.3 (C-1'), 157.0 (C-2'), 121.8 (C-3'), 159.1 (C-4'), 112.0 (C-5'), 128.5 (C-6'), 23.6 (C-1"), 122.8 (C-2"), 131.2 (C-3"), 18.0 (C-4"), 25.9 (C-5"), 61.0 (OMe).

Compound 3 (bidwillol B). Hrms *m/z* 324.1336 (calcd for $C_{20}H_{20}O_4$: 324.1361); eims *m/z* (%): 324 [M^+] (100), 268 (42), 253 (53), 133 (15), 89 (29); uv λ (MeOH, nm): 284sh, 317, 331; ^{1}H-nmr (acetone-d_6) δ: 1.67, 1.81 (3H each, s, Me), 3.44 (2H, d, *J*= 7 Hz, H-1"), 3.74 (3H, s, OMe), 5.30 (1H, m, H-2"), 6.79 (1H, d, *J* = 8 Hz, H-5'), 6.80 (1H, dd, *J*= 8, 2 Hz, H-5), 6.97 (1H, d, *J*= 2 Hz, H-7), 7.11 (1H, br s, H-3), 7.40 (1H, d, *J*= 8 Hz, H-4), 7.62 (1H, d, *J*= 8 Hz, H-6'), 8.43, 8.69 (1H each, s, OH); ^{13}C-nmr (acetone-d_6) δ: 152.5 (C-2), 98.2 (C-3), 123.5 (C-4), 112.8 (C-5), 157.5 (C-6), 104.0 (C-7), 156.3 (C-8), 116.9 (C-9), 124.1 (C-1'), 155.8 (C-2'), 121.6 (C-3'), 157.4 (C-4'), 112.3 C-5'), 125.8 (C-6'), 23.7 (C-1"), 123.3 (C-2"), 131.4 (C-3"), 18.0 (C-4"), 25.9 (C-5"), 60.6 (OMe).

Compound 4 (bidwillon C). Hrms *m/z* 320.1070 (calcd for $C_{20}H_{16}O_4$: 320.1048); eims *m/z* (%): 320 [M^+] (22), 306 (21), 305 (100), 187 (15), 152 (16); uv λ (MeOH, nm) (log ε): 212 (4.24), 257 (4.47), 323 (3.70); ^{1}H-nmr (DMSO-d_6) δ: 1.46 (6H, s, Me x 2), 5.95 (1H, d, *J*= 10 Hz, H-5"), 6.80 (1H, br d, *J*= 10 Hz, H-4"), 6.81 (2H, d, *J*= 9 Hz, H-3', 5'), 6.93 (1H, br d, *J*= 9 Hz, H-6), 7.39 (2H, d, *J*= 9 Hz, H-2', 6'), 7.89 (1H, d, *J*= 9 Hz, H-5), 8.37 (1H, s, H-2), 9.54 (1H, br s, OH); ^{13}C-nmr (DMSO-d_6) δ: 152.1 (C-2), 122.7 (C-3), 175.2 (C-4), 126.5 (C-5), 115.4 (C-6), 157.7 (C-7), 109.4 (C-8), 153.3 (C-9), 118.3 (C-10), 124.1 (C-1'), 130.6 (C-2',6'), 115.5 (C-3',5'), 157.0 (C-4'), 114.6 (C-4"), 131.8 (C-5"), 78.3 (C-6"), 28.1 (C-7", 8").

Growth of Bacteria. Facultative anaerobes (*Lactobacillus*, *Streptococcus*, *Staphylococcus* and *Escherichia*) were cultivated in Müller-Hinton broth (Difco) containing 0.5% yeast extract and 0.5% glucose in the air at 37°C for 18-48 h. *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Actinobacillus* and *Actinomyces* were cultivated in Müller-Hinton broth containing 0.5% yeast extract, 0.5% glucose, hemin (5 μg/ml) and menadione (1 mg/ml) in an

anaerobic glove box (model AZ: Hirasawa, Tokyo) in the atmosphere of 80% N_2, 10% H_2 and 10% CO_2 at 37°C for 3-5 day.

Sensitivity test. MICs were determined by the agar dilution method. Inocula were prepared by dilution of 18 h - 15 day broth (10^8 - 10^9 cells/ml) with buffered saline to $1x10^6$ colony forming units/ml. Plates containing 5% (v/v) defibrinated horse blood culture medium (*vide supra*) were inoculated with a loop (i.d. 1 mm). The inoculated plates were incubated at 37°C for 3-5 day in the air or an anaerobic glove box. The MIC was defined as the lowest concentration of antimicrobial agent in the agar medium resulting in complete inhibition of visible growth.

REFERENCES

1. M. Iinuma, T. Tanaka, M. Mizuno, H. Yamamoto, Y. Kobayashi, and S. Yonemori, *Chem. Pharm. Bull.*, 1992, **40**, 2749.
2. L.A. Mitscher, J.A. Ward, S. Drake, and G.S. Rao, *Heterocycles*, 1984, **22**, 1673.
3. R.B. Bradbury and D.E. White, *J. Chem. Soc.*, 1953, 871.
4. T. Miyase, A. Ueno, T. Noro, and S. Fukushima, *Chem. Pharm. Bull.* , 1980, **28**, 1172.
5. K.S. Dhami and J.B. Stothers, *Can. J. Chem.*, 1966, **44**, 2855.
6. T. Fukai and T. Nomura, *Heterocycles*, 1989, **29**, 2379.
7. N.W. Preston, K. Chamberlain, and R.A. Skipp, *Phytochemistry*, 1975, **14**, 1843.
8. M. Komatsu, I. Yokoe, and Y. Shirataki, *Chem. Pharm. Bull.*, 1978, **26**, 1274.

Received, 1st March, 1994