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Studies on the Antibacterial Constituents of Baenongtang

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Abstract - The water extracts of 83 oriental herbal medicines (Hanbang) which have been clinically used to treat bacterial infections in Korea were screened for *in vitro* antibacterial activity by the paper disc assay method. Two Gram positive bacteria, *Staphylococcus aureus* SG 511, *Bacillus subtilis* ATCC 6633, and two Gram negative bacteria, *Escherichia coli* 055, *Pseudomonas aeruginosa* 9027 were used as test organisms. Among the extracts tested, MeOH extract of Baenongtang showed remarkably potent antibacterial activity. Activity-guided chromatographic fractionations of the CH_2Cl_2 extract of Baenongtang afforded seven antibacterial compounds.

Key words - Oriental herbal medicine (Hanbang), antibacterial activity, *Staphylococcus aureus* SG 511, *Bacillus subtilis* ATCC 6633, *Escherichia coli* 055, *Pseudomonas aeruginosa* 9027, paper disc assay method, Baenongtang.

Introduction

By taking advantage of long history and experience in the usuage of herbal medicines, development of new drugs derived from herbal medicines may avoid side effects or toxicities that synthetic drugs might have. Owing to recent progress in high-throughput screening, the biological activities of unexplored natural products and traditional medicines can be tested very rapidly. Therefore, the role of natural products in drug discovery becomes more and more important, and the natural products can be considered as a gold mine for new drug development. In order to develop new antibacterial agents from oriental herbal medicines, water extracts of 83 of oriental herbal medicines, most of which are commonly used to treat bacterial infections were screened for *in vitro* antibacterial activity by the paper disc assay method (Kahn *et al.*, 1980, Acar and Goldstein 1991). Among them, water extract of Baenongtang showed remarkably potent antibacterial activity. Activity-guided chromatographic fractionations of the CH_2Cl_2 extract of Baenongtang afforded seven antibacterial compounds. This paper describes isolation of active ingredients of Baenongtang and their antibacterial activities.

Experimental

General - Melting points were determined on a Thomas-Hoover capillary melting point apparatus (uncorrected). UV spectra were taken by a Pharmacia Biotech

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Ultrospec 2000 UV/Visible Spectrometer. IR spectra were recorded on a Midac High Resolution FT-IR Spectrometer using potassium bromide pellets. ^{1}H-NMR spectra were recorded on a Varian Unity 300 (300 MHz) spectrometer using TMS as internal standard. ^{13}C-NMR spectra were recorded on a Varian Unity 300 (75.5 MHz) spectrometer. EIMS spectra were determined on a Finnigan MAT 95S. HPLC was performed by Waters pump (model 501) with UV detector (λ 254 nm, Waters model 441) using a LiChrosorb RP-18 (4 mm i.d.×250 mm, Merck) column. TLC and column chromatography were carried out on precoated Silica gel F_{254} plates (Merck, art. 5715), RP-18 F_{254s} plates (Merck, art. 15423), Silica gel 60 (Merck, 230-400 mesh), LiChroprep RP-18 (Merck, 40-63 μm), and Sephadex LH-20 (Sigma).

Oriental Herbal Medicines – Oriental herbal medicines were purchased at Kyoungdong market in Korea and authenticated from the division of herbology at Kyung Hee University. The constitutional drugs of Baenongtang were Platycodi Radix 1,850 g, Glycyrrhizae Radix 1,110 g, Zingiberis Rhizoma 555 g, and Zizyphi inermis Fructus 1,480 g (Ge Hong, 1978).

Extraction and Isolation – Baenongtang (6.2 kg) were extracted with MeOH twice at room temperature. The MeOH extract was evaporated to dryness (1.2 kg) under reduced pressure and suspended in distilled water. The water suspension was partitioned with CH_2Cl_2, EtOAc, and BuOH consecu- tively. A portion of the CH_2Cl_2 extract (20.0 g) which showed most strong antibacterial activity was subjected to column chromato- graphy over Sephadex LH-20 eluting with MeOH. Fracions were combined based on their TLC pattern to yield six fractions designated as BD1-BD6. Fractions BD4 (4.9 g) and BD5 (0.78 g) showed potent antibact- erial activity in our assay system. Active fraction BD5 was further chromatographed on a silica gel column eluting with a hexane-EtOAc-MeOH (10:5:1), hexane-EtOAc-MeOH (10:6:1.3), and hexane-EtOAc-MeOH (10:10:4) to afford thirteen fractions designated as BD5a-BD 5m. Fractions BD5f (80.6 mg), BD5 g (109.0 mg), and BD5h (64.1 mg) were further chromatographed by prep. RP-18 TLC with 70% CH_3CN, and Sephadex LH-20 column with MeOH to give compounds **1** (16.2 mg), **2** (8.5 mg), **3** (6.9 mg), **4** (10.5 mg), and **5** (8.1 mg), respectively. Also, active fraction BD4 was chromatographed on a silica gel column eluting with a hexane-EtOAc-MeOH (10:5:1), hexane-EtOAc-MeOH (10:6:1.3), and hexane-EtOAc-MeOH (10:10:4) to afford six fractions designated as BD4a-BD4h. Fractions BD4c (64.3 mg) and BD4f (29.7 mg) were purified by prep. RP-18 TLC with 65% CH $_3$CN and Sephadex LH-20 column with MeOH to give compounds **6** (17.0 mg) and **7** (18.5 mg).

Licocumarone (**1**): mp 183-5°C; UV λ_{max} (MeOH) 320, 334 nm; λ_{max} (MeOH+$AlCl_3$) 320, 334 nm; λ_{max} (MeOH+NaOMe) 293, 339 nm; IR ν_{max}^{KBr} 3511, 3375, 2965, 2925, 1620, 1508, 1474, 1319, 1217, 1171 cm^{-1}; MS *m/z* 340 [M$^+$]; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.64 (3H, s, H-4"), 1.76 (3H, s, H-5"), 3.34 (2H, d, *J*=6.9 Hz, H-1"), 3.97 (3H, s, OMe), 5.20 (1H, br t, H-2"), 6.38 (1H, dd, *J*=9.0, 1.8 Hz, H-5'), 6.40 (1H, d, *J*=1.8 Hz, H-3'), 6.64 (1H, s, H-7), 7.18 (1H, s, H-3), 7.60 (1H, d, *J*=8.3 Hz, H-6'); ^{13}C NMR (CD_3OD, 75.5 MHz); see Table 1.

Glycycoumarin (**2**): mp 244-6°C; UV λ_{max} (MeOH) 251 (sh), 352 nm; λ_{max} (MeOH+$AlCl_3$) 286 (sh), 351 nm; λ_{max} (MeOH+NaOMe) 269, 406 nm; IR ν_{max}^{KBr}; 3380, 2963, 2923, 1687, 1606, 1514, 1458, 1371, 1224, 1228, 1169, 1100 cm^{-1}; MS *m/z* 368 [M$^+$]; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.67 (3H, s, H-4"), 1.78 (3H, s, H-5"), 3.35 (2H, d, *J*=6.6 Hz, H-1"), 3.82 (3H, s, OMe), 5.21 (1H, t, *J*=6.7 Hz, H-2"), 6.35 (1H, dd, *J*=8.3, 2.4 Hz, H-5'), 6.38 (1H, d, *J*=2.5 Hz, H-3'), 6.57 (1H, s, H-8), 7.13 (1H, d, *J*=8.2 Hz, H-6'), 7.95 (1H, s, H-

Table 1. ^{13}C NMR data of compounds (**1-7**)

No \ Cpds	1	2	3	4	5	6	7
1					127.9		
2	113.5	161.7	154.6	146.2	131.8	81.0	154.4
3	102.3	120.5	129.7	135.5	116.9	44.9	123.2
4	153.6	137.8	182.2	175.8	161.9	193.5	182.3
5	114.7	158.5	159.7	162.2	116.9	129.9	160.5
6	155.2	114.1	100.4	110.8	131.8	111.7	113.1
7	91.9	159.9	163.8	159.0	145.6	166.8	163.7
8	157.7	97.6	94.9	92.2	118.4	103.8	93.9
9	114.7	155.7	166.7	154.7	193.5	165.6	157.5
10		119.2	106.1	102.8		114.9	106.0
1'	109.9	106.7	123.1	122.4	114.7	131.3	124.0
2'	150.5	153.4	114.8	129.1	166.5	128.9	122.2
3'	100.3	102.6	144.6	114.8	103.9	116.3	145.8
4'	152.9	155.9	145.8	159.0	167.5	158.9	144.6
5'	106.6	106.5	125.1	114.8	109.2	116.3	129.7
6'	126.5	131.3	122.2	129.1	133.4	128.9	114.9
1"	22.1	22.2	29.3	20.7			22.3
2"	123.8	122.4	123.9	122.1			123.5
3"	129.3	130.8	132.9	130.5			132.0
4"	16.4	16.5	17.9	16.4			17.9
5"	24.4	24.4	25.9	24.4			25.9
1"'							29.3
2"'							124.9
3"'							132.9
4"'							17.9
5"'							25.9
OMe	59.1	62.2					

4); ^{13}C NMR(CD_3OD, 75.5 MHz); see Table 1.

Glycyrrhisoflavone (**3**): mp 185-7°C; UV λ_{max} (MeOH) 261 nm; λ_{max} (MeOH+$AlCl_3$) 266, 371, 429 nm; λ_{max} (MeOH+NaOMe) 269, 327, 400 nm; IR ν_{max}^{KBr}; 3398, 2968, 2920, 1652, 1621, 1575, 1506, 1438, 1366, 1280, 1177 cm^{-1}; MS *m/z* 354 [M^+]; ^1H NMR (CD_3 OD, 300 MHz) δ 1.73 (6H, s, H-4", H-5"), 3.33 (2H, d, *J*=7.7 Hz, H-1"), 5.35 (1H, t, *J*=7.2 Hz, H-2"), 6.19 (1H, s, H-6), 6.30 (1H, s, H-8), 6.71 (1H, s, H-2'), 6.87 (1H, s, H-6'), 7.97 (1H, s, H-2); ^{13}C NMR (CD_3OD, 75.5 MHz); see Table 1.

Licoflavonol (**4**): mp 185-7°C (decomp.); UV λ_{max} (MeOH) 254 (sh), 268, 300, 366 nm; λ_{max} (MeOH+$AlCl_3$) 231, 270, 364, 429 nm; λ_{max} (MeOH+NaOMe) 278, 324, 418 nm; IR ν_{max}^{KBr}; 3398, 2965, 2923, 1648, 1608, 1561, 1485, 1366, 1316, 1265, 1179 cm^{-1}; MS *m/z* 354 [M^+]; ^1H NMR (CD_3OD, 300 MHz) δ 1.65 (3H, s, H-4"), 1.78 (3H, s, H-5"), 3.33 (2H, d, *J*=6.9 Hz, H-1"), 5.24 (1H, t, *J*=7.2 Hz, H-2"), 6.40 (1H, s, H-8), 6.89 (2H, d, *J*=9.0 Hz, H-3', H-5'), 8.05 (2H, d, *J*=9.0 Hz, H-2', H-6'); ^{13}C NMR (CD_3OD, 75.5 MHz); see Table 1.

Isoliquiritigenin (**5**): mp 180-2°C; UV λ_{max} (MeOH) 240 (sh), 370 nm; λ_{max} (MeOH+$AlCl_3$) 237, 329, 424 nm; λ_{max} (MeOH+NaOMe) 246 (sh), 430 nm; IR ν_{max}^{KBr} 3390, 1627, 1606, 1543, 1513, 1370, 1290, 1228, 1165, 1144 cm^{-1}; MS *m/z* 256 [M^+]; ^1H NMR (CD_3OD, 300 MHz) δ 6.27 (1H, d, *J*=2.4 Hz, H-3'), 6.40 (1H, dd, *J*=8.7, 2.4 Hz, H-5'), 6.84 (2H, d, *J*= 8.6 Hz, H-3, H-5), 7.58 (1H, d, *J*=15.1 Hz, H-8), 7.60 (2H, d, *J*=8.6 Hz, H-2, H-6), 7.78 (1H, d, *J*=15.4 Hz, H-7), 7.97 (1H, d, *J*=9.0 Hz, H-6'); ^{13}C NMR (CD_3OD, 75.5 MHz); see Table 1.

Liquiritigenin (**6**): mp 208-10°C; UV λ_{max} (MeOH) 230, 274, 309 nm; λ_{max} (MeOH+$AlCl_3$) 230, 274, 309, 356 nm; λ_{max} (MeOH+NaOMe) 248, 334 nm; IR ν_{max}^{KBr} 3416, 1658, 1602, 1518, 1464, 1332, 1252, 1158, 1124 cm^{-1}; MS *m/z*

256 [M$^+$]; ^{1}H NMR (CD_3OD, 300 MHz) δ 2.68 (1H, dd, J=16.9, 2.9 Hz, H-3a), 3.04 (1H, dd, J=16.9, 13.1 Hz, H-3b), 5.36 (1H, dd, J=13.1, 2.9 Hz, H-2), 6.34 (1H, d, J=2.2 Hz, H-8), 6.49 (1H, dd, J=8.7, 2.2 Hz, H-6), 6.80 (2H, d, J= 8.6 Hz, H-3', H-5'), 7.31 (2H, d, J=8.5 Hz, H-2', H-6'), 7.72 (1H, d, J=8.7 Hz, H-5); ^{13}C NMR(CD_3OD, 75.5 MHz); see Table 1.

Isoangustone A (**7**): mp 191-3°C; UV λ_{max} (MeOH) 267 nm; λ_{max} (MeOH+$AlCl_3$) 270 nm; λ_{max} (MeOH+NaOMe) 274, 337, 552 nm; IR ν_{max}^{KBr} 3418, 2914, 1648, 1621, 1576, 1461, 1436, 1302, 1227, 1186 cm^{-1}; MS m/z 422 [M$^+$]; ^{1}H NMR (CD_3OD, 300 MHz) δ 1.65 (3H, s, H-4"), 1.72 (6H, s, H-4"', H-5"), 1.77 (3H, s, H-5"'), 3.30 (4H, m, H-1", H-1"'), 5.22 (1H, t, J=7.1 Hz, H-2"'), 5.33 (1H, t, J=7.2 Hz, H-2"), 6.36 (1H, s, H-8), 6.69 (1H, d, J=1.9 Hz, H-2'), 6.86 (1H, d, J=1.9 Hz, H-6'), 7.95 (1H, s, H-2); ^{13}C NMR (CD_3OD, 75.5 MHz); see Table 1.

Test Organisms – Two Gram positive bacteria, *Staphylococcus aureus* SG 511, *Bacillus subtilis* ATCC 6633 and two Gram negative bacteria, *Escherichia coli* 055, *Pseudomonas aeruginosa* 9027 were used as test organisms.

Antibacterial activity – The antibacterial activity was carried out according to a procedure described previously (Bae *et al.* 1998).

Results and Discussion

The antibacterial activity of 83 of oriental herbal medicines, most of which are commonly used to treat bacterial infections in Korea were screened by the paper disc assay method. Among them, Baenongtang

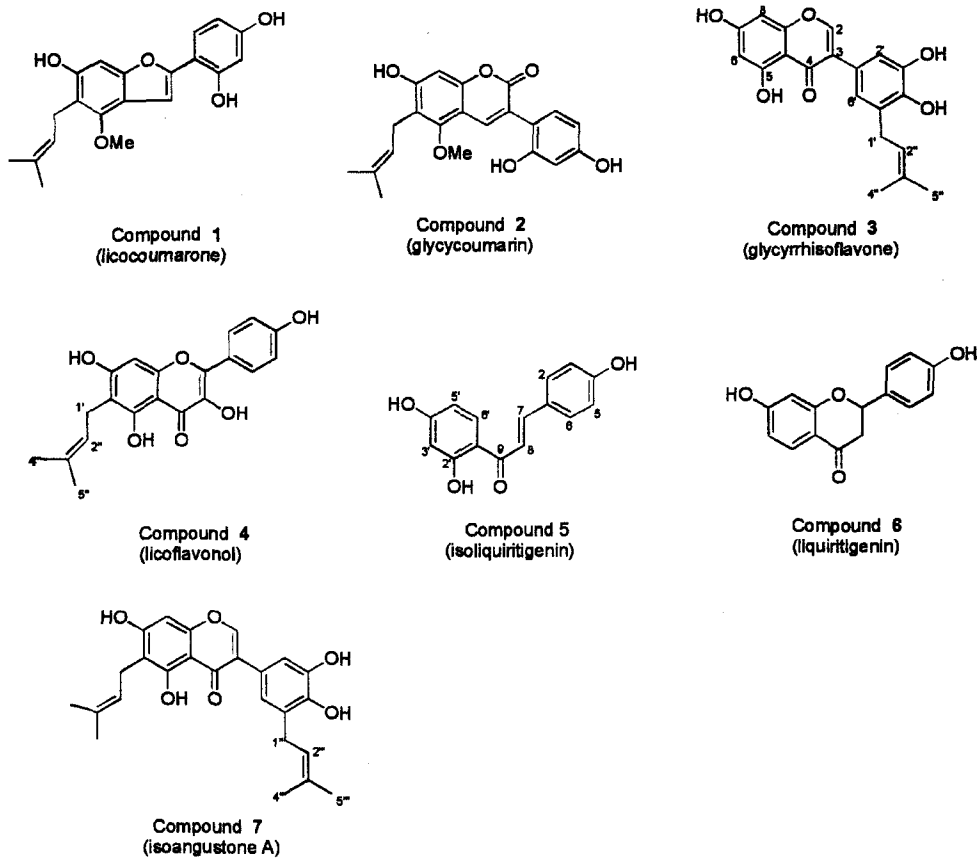

Fig. 1. Structures of isolated compounds **1-7** from Baenongtang.

showed potent antibacterial activity. In order to identify the antibacterial constituents, and the interactions of their constituents of Baenongtang, fractionation and activity-guided purification procedures were repeatedly carried out by silica gel, prep. RP-18 TLC, and Sephadex-LH 20 column chromatography to afford seven known antibacterial compounds (Fig. 1).

Compound **1**, mp 183-5°C was recognized as one of the coumarin derivatives from the strong fluorescene at UV 365 nm. The UV spectrum exhibited absorption maxima at 320 and 340 nm that are characteristic absorption bands of a 2-arylbenzofuran (Dewick, 1982). In the IR spectrum, a hydroxyl peak at 3375 cm^{-1} and aromatic ring peaks at 1620, 1508 cm^{-1} were apparent. The EIMS spectrum showed a molecular ion peak at m/z 340. The ^{1}H-NMR spectrum showed peaks at δ 1.64 (3H, s), 3.34 (2H, d, J =6 Hz), and 5.21 (1H, br. s) due to the isoprenyl group and peaks at δ 6.38 (1H, dd, J=9.0, 1.8 Hz, H-5'), 6.40 (1H, d, J=1.8 Hz, H-3'), and 7.60 (1H, d, J=8.3 Hz, H-6') due to 3H ABX system of the B ring in compound **1**. Peaks at δ 6.64 (1H, s) and 7.18 (1H, s) were assignable to H-3 of A ring and H-7 of C ring, respectively. In addition, the -OCH_3 peak at C-4 was observed at δ 3.97. When these ^{1}H-NMR and ^{13}C-NMR data (see Table 1) were compared with those reported in the literature, compound **1** was identified as licocoumarone, which have already been reported from Glycyrrhizae Radix (Demizu *et al.*, 1988).

The other six compounds **2-7** were identified as glycycoumarin, glycyrrhisoflavone, licoflavonol, isoliquiritigenin, liquiritigenin, isoangustone A, respectively, comparision with published UV, MS, ^{1}H-NMR and ^{13}C-NMR data (Hatano *et al.*, 1988, Saitoh *et al.*, 1976, Jensen *et al.*, 1977, Yahara *et al.*, 1984, Nakanishi *et al.*, 1985,

Table 2. Antibacterial Activity of Compounds **1-7** from Baenongtang

Antibacterial activity (mg/ml)[a]		compound						
		1	**2**	**3**	**4**	**5**	**6**	**7**
S. aureus SG 511	100	++++	++++	++++	+++	+++	+++	++
	50	++++	++++	++++	+++	+++	+++	++
	25	++++	++++	++++	+++	++	++	++
	12.5	++++	++++	++++	++	++	-	+
	6.25	++++	+++	+++	-	-	-	-
	3.125	+++	+++	++	-	-	-	-
	1.563	++	++	++	-	-	-	-
	0.781	++	++	+	-	-	-	-
B. subtilis ATCC 6633	100	++++	++++	++++	++	+++	+++	++
	50	++++	++++	++++	++	++	++	++
	25	++++	++++	++++	++	++	++	++
	12.5	++++	++++	+++	++	+	+	+
	6.25	+++	+++	++	-	-	-	-
	3.125	+++	++	++	-	-	-	-
	1.563	++	++	+	-	-	-	-
	0.781	++	-	-	-	-	-	-
E. coli 055	100	+++	++	+	-	-	++	-
	50	+++	++	+	-	-	+	-
	25	++	++	+	-	-	-	-
	12.5	+	+	+	-	-	-	-
P. aeruginosa 9027	100	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-
	12.5	-	-	-	-	-	-	-

[a]The antibacterial activity is represented as described previously (Bae *et al.*, 1998)

Kiuchi *et al.*, 1990). Antibacterial activity of isolates from Baenongtang was evaluated by the paper disc assay method. Table 2 summarizes the results of antibacterial activities of these compounds. All of the isolated compounds from Baenongtang showed potent antibacterial activity against tested Gram positive bacteria. Moreover, compounds **1, 2, 3** and **5** showed antibacterial activity against Gram negative bacteria, *E. coli* 055. Among these compounds, antibacterial activities of licocoumarone (**1**), glycycoumarin (**2**) (Demizu *et al.*, 1988), and isoliquilitigenin (**5**) (Eiji *et al.*, 1993) have already been reported, while, the antibacterial activities of compounds **3, 4, 6** and **7** have not yet been reported previously. Based on the foregoing results, compounds **1-7** considerd as major antibacterial constituents of Baenongtang. Further purification studies for the active EtOAc soluble fraction of Baenongtang are under study.

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