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Antimicrobial Activities of Hydroxybiphenyl Derivatives (I)

Antibacterial Activities and HPLC Determination of Magnolol and Honokiol

Kihwan Bae, Beongtae Yoo, Myungkoo Lee, and Wonjun Seo

College of Pharmacy, Chungnam National University, Taejeon 300-31, Korea

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Abstract □ It was revealed that magnolol and honokiol isolated from the stem bark of *Magnolia obovata*, had potent antibacterial activity against *Bacillus anthracis*. A quantitative analytical method of magnolol and honokiol by HPLC has been established, and the amounts of the two components in the dried stem bark of *M. obovata* were 1.94% and 0.44%, respectively.

Keywords □ Magnolol, Honokiol, HPLC method, *Magnolia obovata*, *Bacillus anthracis*, antibacterial activity.

Magnolol and honokiol are naturally occurring hydroxybiphenyl compounds(Fig. 1). Magnolol was extracted from the stem bark of *Magnolia obovata* and its structure was elucidated by Sugii.[1] Honokiol was investigated by Fujita *et al.*[2] The two diallylbiphenols were reported to have potent antibacterial activities against most fo Gram positive bacteria[3-10] including a carcinogenic bacterium, *Streptococcus mutans*.[3-7]

We studied the antibacterial activities of magnolol and honokiol against *Bacillus anthracis* ATCC 11949, 14186 and 14578 by paper disk method, minimal inhibitory concentration and bactericidal time. Furthermore, a quantitative analytical method of magnolol and honokiol from the methanol extract of the stem bark of *M. obovata* was developed by using of HPLC.

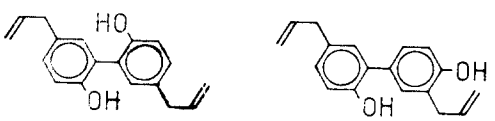

Fig. 1: Structures of magnolol and honokiol.

EXPERIMENTAL METHODS

Isolation of magnolol and honokiol

The pulverized stem bark of *M. obovata* (1kg) was refluxed with benzene. The benzene solution was evaporated to a small volume *in vacuo*. The benzene solution was extracted 3 times with 300 ml of 0.2N sodium hydroxide. The resulting extract was acidified with 6 N hydrochloric acid to pH 3, and extracted 3 times with 300ml of ether. The ethereal solution was evaporated to dryness (20g) *in vacuo*. The residue was dissolved in 30 ml of benzene and applied to a column of silica gel (6×40cm). The column was eluted with benzene, benzene-ethylacetate (9:1, v/v) and benzene-ethylacetate (8:2, v/v). The fractions containing mainly magnolol were pooled

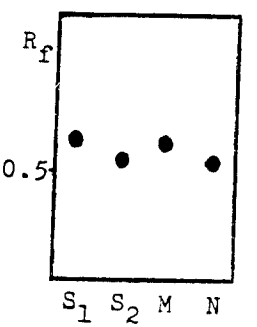

Fig. 2: TLC of magnolol and honokiol isolated from the stem bark of *Magnolia obovata*. Plate: Kieselgel 60G, Solvent: $CHCl_3$-MeOH (9:1), Detection: I_2 vapor, S: Standards (1: Magnolol, 2: Honokiol), M: Magnolol, H: Honokiol.

and concentrated *in vacuo* to give crystalline magnolol (yield, 5.0g). Magnolol was then recrystallized from benzene, mp 102°C. The fractions eluted with the latter solvent (benzene-ethylacetate, 8:2) were pooled and evaporated to dryness *in vacuo*. The residue was dissolved in 10ml of benzene and applied on a column of polyamide (3×40cm). The column was then eluted with benzene to give additional magnolol (1.0 g) and with benzene-ethylacetate to give honokiol (1.5 g). The fractions containing honokiol was crystallized from benzene-petroleum ether (1:1, v/v), mp 85~87°C. They were identified by direct comparision with authentic samples on TLC. For thin layer chromatography, kieselgel 60G (Merck) plates were used and developed by $CHCl_3$-MeOH (9:1, v/v). The spots were detected by exposing to iodine vapor. The Rfs of magnolol and honokiol were 0.60 and 0.56, respectively (Fig. 2).

Other Chemicals

Brain Heart Infusion (BHI) was the product of Difco Lab., and berberine hydrochloride was that of Wako Pure Chemical Industries, Ltd.

Determination of Antibacterial Activity

Prior to testing, the strains of *B. anthracis* were cultured in liquid BHI broth at 37°C overnight, and subcultured again 6 hours. The turbidity of bacterial cell suspensions was adjusted with the same sterile broth to 0.07 OD unit at 550 nm and then used for the tests. For the paper disk method, 0.6ml of the bacterial cell suspension was poured uniformly into the plates made of BHI as medium. Five paper disks containing 5, 10, 20, and 40μg of the components and one control were carefully placed on the seeded plates. The culture was carried out at 37°C for 24hours. Antibacterial activity was measured as inhibitory zones around paper disks. At least, three replicate plates were examined for an antibacterial activity of the components.

Determination of Minimal Inhibitory Concentration (MIC)

Test components (1 mg) were dissolved in a minimum volume of ethanol or water and prepared for two-fold step dilution series of the solution. The solution (0.1 ml) was then added to the inoculated BHI broth (4.9 ml) which had about 0.01 OD unit at 550 nm. The MIC was determined by judging visually the bacterial growth in the series of test tubes.

Minimal Time for Bactericidal Action

The precultured bacterial suspension (0.1 ml) which had about 0.07 OD unit at 550 nm was mixed with BHI broth (4.9 ml) containing antibacterial compound (100 μg/ml). The suspension (0.1 ml) was transferred to BHI medium (5 ml) at an indicated time. The bacterial growth was cultured for 48 hours and measured turbidimetrically at 550 nm.

Quantitative Analysis of Magnolol and Honokiol by HPLC

Methanol extract of dried stem bark of *M. obovata* was applied to HPLC. The HPLC was equipped with the Pondapak C18 and the mobile phase of methanol and water (4:1, v/v) was used for the quantitative analysis of magnolol and honokiol. Methanol extract (1.0g) was concentrated and diluted to 100ml with methanol. Methanol solution (10.0 μl) was injected for each chromatographic analysis. The method in quantitative analysis was followed by the standard addition method.[11] The authentic magnolol or honokiol (0.765 mg) was added to the methanol solution (5.0 ml), and the mixture was injected for the analysis.

RESULTS AND DISCUSSION

Antibacterial Activity

The comparison of antibacterial activities of magnolol, honokiol, o,o-biphenol, berberine hyd-

Table I: Antibacterial Activity of Magnolol, Honokiol, Berberine Hydrochloride and Erythromycin against *Bacillus anthracis* ATCC 11949, ATCC 14186 and ATCC 14578

Compounds	μg/disk	Diameter of inhibitory zone (mm)		
		11949	14186	14578
Magnolol	5	17.1	13.1	11.9
	10	18.2	16.7	15.7
	20	20.4	18.1	17.1
	40	21.3	20.2	19.1
Honokiol	5	12.2	9.4	10.5
	10	16.8	12.3	12.3
	20	20.0	14.9	14.1
	40	24.4	17.1	15.9
Berberine Hydrochloride	5	—	—	—
	10	—	—	—
	20	8.9	—	—
	40	11.2	—	—
Erythromycin	5	28.9	20.1	15.1
	10	31.2	22.4	17.8
	20	32.6	24.1	20.0
	40	36.4	26.4	23.0

Average values were calculated from three observations, and each inhibitory zone around the paper disk (6 mm in diameter, 1.5mm in thickness) was measured in 4 different directions.

rochloride and erythromycin by the paper disk method were shown in Table I. The control blank showed no antibacterial activity. Both magnolol and honokiol were potent antibacterial agents against all the strains of *B. Anthracis*. The inhibitory zones of magnolol and honokiol were greater in diameter than that of a typical antibacterial alkaloid, berberine hydrochloride, under comparable conditions. The diameter of inhibitory zones was a linear function of logarithmic concentration in a range of 5 μg to 40 μg/disk (Table 1).

Minimal Inhibitory Concentrations

The minimal inhibitory concentrations of the antibacterial constituents determined by the broth dilution method were shown in Table II. Both magnolol and honokiol completely inhibited the bacterial growth at 6.25 μg/ml against all strains of *B. anthracis*. On the other hand, berberine hydrochloride and erythromycin inhibited the growth at 200 and 0.78 μg/ml, respectively.

Table II: Minimal Inhibitory Concentration Values

Compounds	MIC(μg/ml)[1]		
	Bacillus anthracis		
	11949	14186	14578
Magnolol	6.25	6.25	6.25
Honokiol	6.25	6.25	6.25
Berberine Hydrochloride	200.00	200.00	200.00
Erythromycin	0.78	0.78	0.78

1) MIC values were determined by two-fold dilution method

Minimal Time for Bactericidal Action

All the strains of *B. anthracis* were almost sterilized in 2～5 minutes by exposing to magnolol or honokiol, and the compounds had

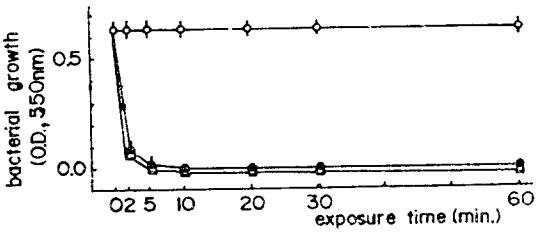

Fig. 3: Bacterial cells were exposed to 100μg/ml of magnolol and honokiol. Bacterial cells were exposed to 110μg/ml of magnolol and honokiol. ○ : Control, ● : Magnolol, □ : Honokiol.

a same trend of killing rate (Fig. 3). These findings showed that both magnolol and honokiol hade a rapid bactericidal mode of action.

Quantitative Analysis of Magnolol and Honokiol by HPLC

A standard chromatogram of the methanol extract of the stem bark of *M. obovata*, and the operational condition of HPLC were shown in Fig. 4. The peaks of magnolol and honokiol were completely resolved under the condition. The standard addition method was employed for the analysis of magnolol and honokiol, and the

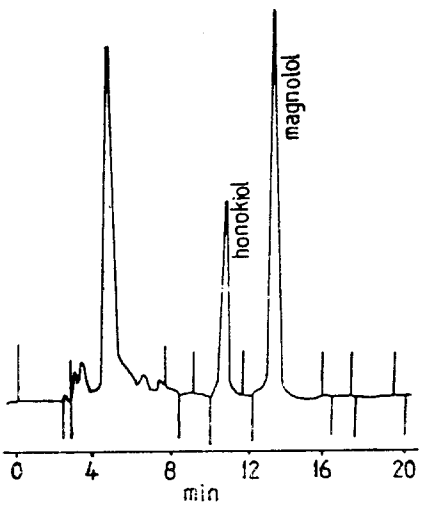

Fig. 4: HPLC chromatogram of methanol extract of stem bark of *Magnolia obovata*. Analytical conditions: column, μBondaPak C_{18}, 30cm × 7.8mm; eluent, Methanol- Water (4:1, v/v); flow rate, 2.0ml/min; detector, UV (254nm).

Table III: Amounts of Magnolol and Honokiol

	Average of peak area[1] ± S.D.[2]		Amounts[3] (%)
	in standard	after addition	
Magnolol	6679.67±242.69	10609.83±95.17	1.94
Honokiol	2697.54± 97.43	7429.17±36.23	0.44

1) The values were averages of three measurements
2) Standard deviation
3) % to dried stem bark of *Magnolia obovata*

analyzed amounts in the stem bark of *M. obovata* were 1.94% and 0.44% respectively, as shown in Table III. The HPLC condition used in this study may be useful and convenient for the quantitative analysis of magnolol and honokiol from the view of simple operation as compared with other methods.[9,12] This method can be applied to the studies of the distributions. variations and evidence of magnolol and honokiol in plant organs of *M. obovata*, *M. officinalis*, *M. grandifolia*, *M. virgininan* and other allied plants.

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