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Antibacterial and Antifungal Activities of a Naphthoquinone Derivative Isolated from the Fruits of *Catalpa ovata* G. D_{ON}

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Abstract An antimicrobial compound was isolated from the MeOH extract of *Catalpa ovata* G. D_{ON} fruits, and its structure was identified as 4,9-dihydroxy-2,2-dimethyl-3,4-dihydronaphtho[2,3-*b*]pyran-5,10-dione (HMNP). The antimicrobial activity of the HMNP was determined by measuring the dose-response inhibiton of microbial growth in liquid cultures and then compared with that of lapachol, a well known antimicrobial 1,4-naphthoquinone. The antimicrobial activity of the HMNP was more effective than that of lapachol over a wide range of test organisms. Gram-positive bacteria, yeast, and fungi (IC_{50} 20–75 μM) were found to be more sensitive to the HMNP than Gram-negative bacteria (IC_{50} >100 μM). The HMNP also inhibited germination of spores of many fungi. The morphological deformation of the fungal spores was induced by the treatment of HMNP, as illustrated by Scanning Electron Microscopy (SEM).

Key words: *Catalpa ovata*, antimicrobial activity, naphthoquinone, 4,9-dihydroxy-2,2-dimethyl-3,4-dihydronaphtho[2,3-*b*]pyran-5,10-dione

A number of chemicals used for the control of saprophytic and pathogenic microorganisms have serious side effects on human health. Furthermore, with the emergence of drug resistant species, there is a need to develop novel antimicrobial compounds. Therefore, much attention has been focused on the discovery of new antimicrobial compounds from natural sources. Several studies on the identification of these antimicrobial substances have been attempted with plants. Generally, plants synthesize various antimicrobial compounds as a self-defense system against microbial infections [7]. It is well known that spices such as garlic, onion, oregano, thyme, and cinnamon have an inhibitory effect on the growth of microorganisms [11, 15, 20, 24, 29, 30], and several antimicrobial compounds including organic acids, phenolic acids, flavonoids, and quinones have also been isolated from plants [2, 4, 9, 28, 31]. However, only a limited number of studies on the antimicrobial compounds of traditional plants in Korea have been reported. We have been investigating antimicrobial activities in Korean traditional plants to develop more effective and safer antimicrobial agents, and isolated a number of antimicrobial compounds such as pulsaquinone and several phenolic acids [5, 6, 16, 18, 21].

Catalpa ovata G. D_{ON} (Bignoniaceae) is a deciduous tall tree that grows naturally in Korea, and its fruits have been used as a diuretic for chronic nephritis, and the root and stem bark have been utilized as a folk medicine for treating fever, jaundice, and edema which are caused by nephritis. Several phenolic acids, flavone glycosides, and monoterpene glycosides from the stem bark and leaves of *Catalpa ovata* have been reported [14, 19, 23]. Recent studies on antimutagenic or antitumor activity have resulted in isolation of several active compounds including naphthoquinone derivatives and iridoid [8, 22]. However, few studies on antimicrobial activity of the *Catalpa ovata* have yet been reported. In this paper, we isolated and identified an antimicrobial compound from the MeOH extract of *Catalpa ovata* fruits and examined its activity against several microorganisms.

The fruits of *Catalpa ovata* were collected in October at Chonnam, Korea. The fruits were air-dried at 9.90±0.07% moisture and then powdered. The dried fruits (5,934 g) of *Catalpa ovata* were extracted with methanol (MeOH, 97 l) and antimicrobial activity was determined by paper disc method [30], employing 16 kinds of microorganisms (eight

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Gram-positive bacteria, three Gram-negative bacteria, two yeast, and three fungi). Thus, the paper disc (8 mm, Whatman, Maidstone, England) loaded with each sample was placed on the agar plate which was seeded with each test microorganism. The plates with bacteria and yeast were incubated at 30°C or 37°C for 24 h and at 24°C or 30°C for 48 h with fungi, and the diameter (mm) of inhibition zone was measured to determine the antimicrobial activity. Benzoic acid (Hayashi Pure Chemical Industry, Osaka, Japan) was used as a positive control. The MeOH extract from fruits of *Catalpa ovata* showed strong antibacterial and antifungal activities against Gram-positive bacteria, Gram-negative bacteria, and fungi, although weak activity was observed against yeast (Table 1). The MeOH concentrates (670.9 g, 5,831 g dry wt. eq.) were partitioned using ethyl acetate (EtOAc) and 0.2 M glycine buffer (pH 3.0). The organic phase was washed with 0.2 M phosphate buffer (pH 8.0) to give the EtOAc-soluble neutral fraction. The aqueous layer (pH 8.0) was adjusted to pH 3.0 with 1.0 M HCl and extracted with EtOAc to obtain the EtOAc-soluble acidic fraction [18, 21]. The antimicrobial activity was found in both fractions (Table 1). Since several aromatic compounds, known as EtOAc-soluble acidic antimicrobial compounds such as vanillic acid, ferulic acid, *p*-hydroxybenzoic acid, and *p*-hydroxycinnamic acid, have been isolated from wood extracts of *C. ovata* [14, 16], EtOAc-soluble neutral fraction was selected for further investigation. As shown in Fig. 1, the EtOAc-soluble neutral fraction (68.2 g) was successively purified with a silica gel (Kieselgel 60, Merck, 70–230 mesh, Darmstadt, Germany), adsorption column chromatography, Sephadex LH-20 (25–100 mesh, Pharmacia Fine Chemicals, Uppsala, Sweden) column chromatography, and octadecylsilane (ODS, YMC-GEL, 70–230 mesh, YMC Co, Kyoto, Japan) column chromatography. The bioassay for the antimicrobial active substances during purification was performed with *Staphylococcus aureus*. The final purification by HPLC (Model 510 solvent delivery system with 486 Tunable Absorbance Detector, Waters, Milford, MA, U.S.A.) with an ODS column (Senshu pak, 0.8×25 cm, Tokyo, Japan; elution with 60% MeOH for 1.5 ml/min) resulted in an analytically pure antimicrobial compound (t_R 21.7 min, 76.6 mg, yellow needle crystal, 13.9 μg/g dry weight of *Catalpa ovata* fruits).

The molecular formula, $C_{15}H_{14}O_5$, of the isolated compound was determined by applying a high resolution EI-MS analysis [*m/z* 274.0837 (−0.4 mmu for $C_{15}H_{14}O_5$)]. The ^{13}C-NMR spectrum [100 MHz, DMSO-d_6; δ 25.6 (C-11), 28.4 (C-12), 38.9 (C-3), 56.5 (C-4), 78.5 (C-2), 113.9 (C-9a), 117.6 (C-6), 120.9 (C-4a), 122.7 (C-8), 131.6 (C-5a), 136.6 (C-7), 152.8 (C-10a), 159.8 (C-9), 180.0 (C-5), 183.8 (C-10)] showed fifteen signals that included two carbonyl

Table 1. The antimicrobial activity of MeOH extract and solvent fractionated fractions from fruits of *Catalpa ovata*.

Microorganism	Inhibition (Clear zone, mm)[a]			
	MeOH extract[b]	EtOAc-soluble acidic fr.[b]	EtOAc-soluble neutral fr.[b]	Benzoic acid (0.5 mg)
Gram-positive bacteria				
Staphylococcus aureus KCTC 1928	22.7±0.47	13.7±0.47	18.7±0.47	13.5±0.41
Staphylococcus epidermidis KCTC 1917	17.5±0.41	9.5±0.41	-[c]	10.5±0.41
Bacillus subtilis KCTC 1021	23.5±0.41	15.7±0.47	17.7±0.47	14.5±0.41
Micrococcus luteus KCTC 3523	17.7±0.47	13.7±0.47	13.7±0.47	13.7±0.47
Enterococcus faecalis KCTC 3195	12.5±0.41	-	-	-
Lactobacillus brevis KCTC 3102	11.7±0.47	-	12.5±0.41	-
Leuconostoc mesenteroides KCTC 3100	17.7±0.47	17.7±0.47	-	8.7±0.24
Pediococcus damnosus KCTC 1628	16.5±0.41	11.5±0.41	10.7±0.47	11.7±0.47
Gram-negative bacteria				
Escherichia coli KCTC 2593	18.5±0.41	10.7±0.47	-	10.3±0.47
Pseudomonas aeruginosa KCTC 2513	16.7±0.47	9.7±0.47	8.7±0.47	10.7±0.47
Salmonella typhi ATCC 19214	11.7±0.47	9.7±0.47	8.7±0.47	10.7±0.47
Yeast				
Saccharomyces cerevisiae KCTC 7904	9.5±0.47	8.7±0.47	8.7±0.47	9.7±0.47
Candida albicans KCTC 7965	9.5±0.47	8.7±0.47	8.7±0.47	11.7±0.47
Fungi				
Aspergillus flavus ATCC 15517	16.5±0.47	11.5±0.47	10.5±0.47	14.7±0.47
Aspergillus parasiticus ATCC 22789	14.7±0.47	10.7±0.47	10.5±0.47	12.5±0.47
Penicillium citrinum KCTC 6927	20.7±0.41	12.7±0.47	11.7±0.47	15.5±0.47

[a]Each value is the mean±S.D. (n=3).

[b]Extract of 1 g eq. of dry weight per 8 mm paper disc.

[c]No inhibition.

Fruits of *C. ovata*
5934 g (dry wt.)
├— extract with MeOH (97 l)
MeOH extract
(670.9 g, 5831 g dry wt. eq.)
├— solvent fractionation
EtOAc-soluble neutral fr.
(68.2 g, 5788 g dry wt. eq.)
├— silica gel adsorption chromatography ($CHCl_3$-MeOH, 100:0, 90:10, 80:20, 70:30, 60:40, v/v)
10% MeOH fr.
(48.7 g, 5748 g dry wt. eq.)
├— silica gel adsorption chromatography (Hexane:EtOAc:MeOH, 8:6:1, 6:8:1, 4:10:1, 2:12:1, v/v)
H:E:M 8:6:1, 6:8:1 fr.
(29.4 g, 5670 g dry wt. eq.)
├— silica gel adsorption chromatography (Hexane:EtOAc:MeOH, 10:4:1, 8:6:1, 6:8:1, 4:10:1, v/v)
H:E:M 10:4:1, 8:6:1 fr.
(20.8 g, 5630 g dry wt. eq.)
├— silica gel adsorption chromatography (Hexane:EtOAc:MeOH, 14:1:1, 12:3:1, 10:5:1, 8:7:1, v/v)
H:E:M 14:1:1 fr.
(9.7 g, 5600 g dry wt. eq.)
├— silica gel adsorption chromatography (Hexane:EtOAc, 10:4, 9:5, 8:6, 7:7, 6:8, v/v)
H:E 10:4, 9:5 fr.
(3.8 g, 5570 g dry wt. eq.)
├— Sephadex LH-20 chromatography (MeOH:$CHCl_3$, 4:1, v/v)
Ve/Vt 0.74 - 1.04 fr.
(554.0 mg, 5550 g dry wt. eq.)
├— ODS column chromatography (MeOH:H_2O, 60:40, 70:30, 80:20, 90:10, 100:0, v/v)
80% MeOH fr.
(253.1 mg, 5530 g dry wt. eq.)
├— HPLC (ODS column, 60% MeOH)
Active compound
(76.6 mg, 5530 g dry wt. eq.)

Fig. 1. The isolation procedure of antimicrobial compound from the fruits of *Catalpa ovata*.

carbons [183.8 (C-10) and 180.0 (C-5)] and eight sp^2-carbons (δ 159.8-113.9). In the ^{1}H-NMR spectrum [400 MHz, DMSO-d_6; δ 1.43 (3H, s, H-12), 1.45 (3H, s, H-11), 1.89 (1H, dd, *J*=4.8 and 14.5 Hz, H-3a), 2.00 (1H, dd, *J*= 4.8 and 14.5 Hz, H-3b), 4.76 (1H, dt, *J*=4.0 and 4.8 Hz, H-4), 5.21 (1H, d, *J*=4.0 Hz, 4-OH), 7.29 (1H, dd, *J*=1.2 and 8.2 Hz, H-8), 7.53 (1H, dd, *J*=1.2 and 7.5 Hz, H-6), 7.74 (1H, dd, *J*=7.5 and 8.2 Hz, H-7), 11.59 (1H, s, 9-OH)], three aromatic protons (δ 7.29, 7.54, and 7.74) showing ABC-type coupling pattern and a phenolic hydroxyl proton (δ 11.59, 1H, s, 9-OH) were observed. The position of the hydroxyl group was assigned to be at C-9 with a low field signal (δ 159.8) of C-9 and with a cross peak from H-7 to C-9 in HMBC spectrum (Fig. 2). These data suggested that active compound contained a naphthoquinone moiety.

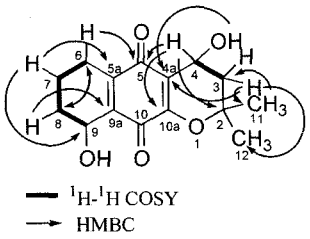

Fig. 2. Important connectivities observed in HMNP by ^{1}H-^{1}H COSY and HMBC.

The remaining partial structure and its connection with naphthoquinone moiety was determined by comprehensive ^{1}H- and ^{13}C-NMR measurements including ^{1}H-^{1}H COSY (Fig. 2), HSQC, and HMBC (Fig. 2) analyses. The ^{1}H-NMR data revealed the presence of a geminal-dimethyl group (δ 1.43 and 1.45), a set of AB type methylene protons (δ 1.89 and 2.00, 1 H each, dd, *J*=4.8 and 14.5, H-3a and -3b, respectively), and a proton coupling with methylene protons on a hydroxy bearing carbon atom at δ 4.76 (1 H, dt, *J*=4.0 and 4.8 Hz, H-4) in addition to a doublet alcoholic hydroxyl proton (δ 5.21, 1 H, *J*=4.0, 4-OH). Also, the presence of cross peaks from H-4 to C-5 and C-10a, and from H-3 and 4-OH to C-4a in the HMBC experiment indicated that the remaining five sp^3-carbons (δ 78.5, 56.5, 38.9, 28.4, and 25.6) could be assigned to a dimethyl-hydroxy-pyran structure combined with naphthoquinone moiety. The positions of dimethyl groups and a hydroxyl group on the pyran ring were determined to be at C-2 and C-4, respectively, from the coupling constants and the cross peaks between dimethyl protons (H-11 and 12) to C-3 and H-4 to C-2 in the HMBC spectrum (Fig. 2).

Table 2. The antimicrobial activity (IC_{50}) of HMNP against various microorganisms.

Microorganism	IC_{50} (M)[a]	
	HMNP	Lapachol
Gram-positive bacteria		
Staphylococcus aureus KCTC 1928	20±0.6	36±1.8
Micrococcus luteus KCTC 3523	50±0.6	81±1.4
Bacillus subtilis KCTC 1021	43±0.8	73±2.1
Gram-negative bacteria		
Escherichia coli KCTC 2593	356±3.2	>500
Pseudomonas aeruginosa KCTC 2513	117±1.6	>500
Yeast		
Saccharomyces cerevisiae KCTC 7904	56±1.5	>500
Candida albicans KCTC 7965	73±1.1	>500
Fungi		
Aspergillus flavus ATCC 15517	33±1.7	77±1.9
Aspergillus parasiticus ATCC 22789	21±0.8	56±1.6
Penicillium citrinum KCTC 6927	71±1.5	>500

[a]IC_{50} was shown as the mean±S.D. (n=3); Each value was determined from a dose-response curve.

From these data, the isolated compound was identified as 4,9-dihydroxy-2,2-dimethyl-3,4-dihydronaphtho[2,3-*b*] pyran-5,10-dione (HMNP).

Although several naphthoquinones have been isolated from the plants of the Bignoniaceae family [1, 2, 9, 25] and HMNP has been shown to be an antitumor compound [8], the antimicrobial activity of HMNP has never been reported. Therefore, its antimicrobial activities were determined by the growth inhibition rate in a liquid culture using a microplate reader assay [3, 5] and compared with those of lapachol. Lapachol, 1,4-naphthoquinone, is well known for its antimicrobial activity as well as for its mode of action against bacteria, yeast, and fungi, [2, 10] (Table 2).

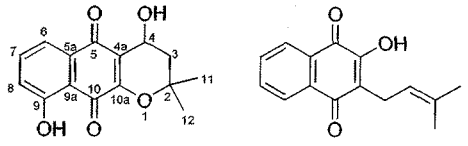

Fig. 3. The structures of HMNP and lapachol.

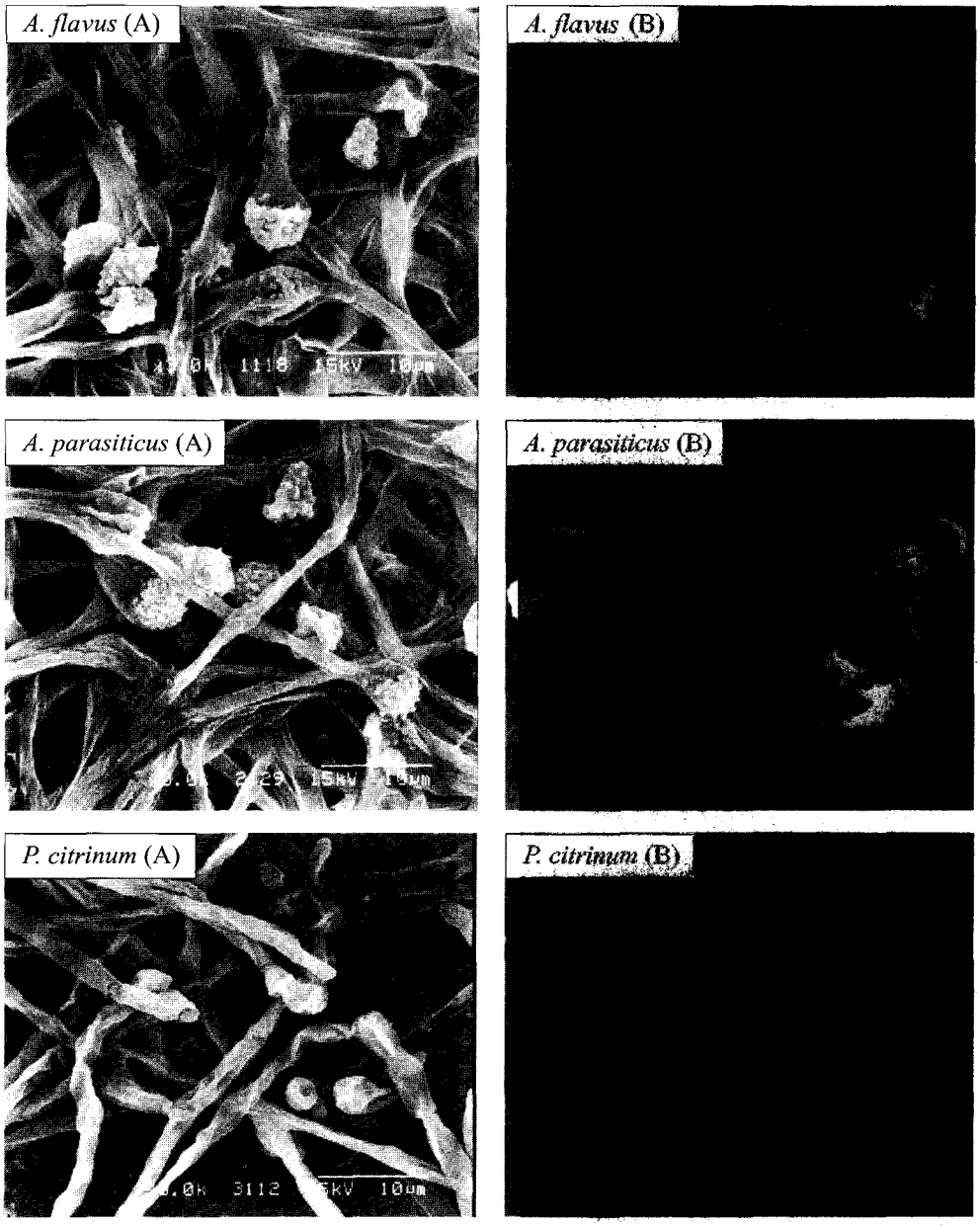

Fig. 4. The Scanning Electron Micrographs (SEM, ×3,000) of fungi spores nontreated (A) and treated (B) with HMNP (250 μM).
(A) is normally germinated spores after incubating for 24 h. (B) is deformed spores after incubating with HMNP for 24 h.

To determine the growth inhibition of HMNP, the inocula of liquid cultures of each test microorganism were prepared by the conventional method to a final microbial concentrations of approximately 10^5 cfu/ml (for bacteria and yeast) or 10^5 spores/ml (for fungi). Each prepared culture (20 μl) was inoculated onto a well and diluted with 180 μl of respective growth media (BHI broth for *Enterococcus faecalis* KCTC 3195, *Lactobacilli* MRS broth for *Lactobacilli*, YM broth for yeast, potato dextrose broth for fungi, and nutrient broth for the rest bacteria), and HMNP or lapachol (Sigma Chemical Co., St. Louis, MO, U.S.A.) was then added into each well at various concentrations (10, 25, 50, 100, 250, and 500 μM). Growth inhibition of each test organism was measured at $OD_{630\,nm}$ with an EL-800 microspectrophotometry (Bio-Tek instruments, VT, U.S.A.) after incubation for 17 h for bacteria and yeast, and 24 h with shaking (130 rpm) for fungi at their respective optimum temperatures. The growth inhibition is expressed as the concentration of each substance required to inhibit 50% growth (IC_{50} value), determined by a dose-response curve (percentage growth inhibition *vs.* concentration) [5].

The HMNP displayed strong activities against various strains such as Gram-positive bacteria, yeast, and fungi. The IC_{50} values against Gram-positive bacteria, fungi, and yeast were in the range of 20 to 75 μM. The HMNP showed relatively weak activities against Gram-negative bacteria (*E. coli* and *P. aeruginosa,* IC_{50} >115 μM and >352 μM, respectively). On the other hand, lapachol showed negligible activities (IC_{50} >500 μM) against Gram-negative bacteria (*E. coli* and *P. aeruginosa*), yeast (*S. cerevisiae* and *C. albicans*), and a fungus (*P. citrinum*), whereas it showed fairly strong activities against Gram-positive bacteria (*S. aureus*, *M. luteus*, and *B. subtilis*) and two kinds of fungi (*A. flavus* and *A. parasiticus*) (Table 2). This is in good agreement with a report of a narrow antimicrobial spectrum of lapachol [10]. In conclusion, these results show that the HMNP has more effective antimicrobial activity and shows a wider antimicrobial spectrum than lapachol.

The antimicrobial activities of naphthoquinones are known to be due to inhibition of electron transport [27] or uncoupling of oxidative phosphorylation in the respiratory chain [10, 13]. Quinones also act as electron acceptors to stimulate the superoxide production [12]. The mechanism of antimicrobial activity of lapachol has been suggested to be due to its activity on uncoupling oxidative phosphorylation [10, 13]. However, the mode of action of the HMNP has not yet been investigated and will be the subject for future research.

To further confirm the growth inhibitory effect of the HMNP against fungi such as *A. flavus*, *A. parasiticus*, and *P. citrinum*, morphological changes of spores were examined by SEM after treatment with the HMNP (Fig. 4). The fungal spore suspensions (nontreated or treated with 250 μM concentration of HMNP) were incubated for 24 h and filtered through a membrane filter (pore size 0.22 μm, Millipore, Ireland). The spores on the membrane filter were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h, rinsed with the same buffer, and dehydrated with a series of graded ethanol at room temperature. After freeze-drying, the spores were sputter-coated with gold and its shape was observed under a scanning electron microscope (S-2400, HITACHI, Tokyo, Japan) [26]. Most of the spores treated with the HMNP showed irregular and wrinkled form, indicating morphological deformation. The germination of spores was also inhibited by the HMNP, whereas the nontreated spores showed normal germination. To the best of our knowledge, this is the first study ever made on the HMNP as an antimicrobial substance, and also the first report on the isolation of the HMNP from fruits of *Catalpa ovata*. It may provide a strong support for the discovery of new antimicrobial compound from natural sources, and also to design effective antimicrobial naphthoquinone derivatives.

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