JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Selective Growth-Inhibiting Effects of Compounds Identified in *Tabebuia impetiginosa* Inner Bark on Human Intestinal Bacteria

BYEOUNG-SOO PARK,† JUN-RAN KIM,† SUNG-EUN LEE,† KYOUNG SOON KIM,‡ GARY R. TAKEOKA,§ YOUNG-JOON AHN,† AND JEONG-HAN KIM*,†

School of Agricultural Biotechnology, Seoul National University, San 56-1, Shillim-dong, Gwanak-gu, Seoul 151-742, Korea, College of Pharmacy, Seoul National University, Seoul 151-742, Korea, and Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, California 94710

The growth-inhibiting activity of anthraquinone-2-carboxylic acid and lapachol identified in the inner bark of taheebo, *Tabebuia impetiginosa*, toward 10 human intestinal bacteria was evaluated by using a paper disk diffusion bioassay and compared to those of seven lapachol congeners (1,4-naphthoquinone, naphthazarin, menadione, lawsone, plumbagin, juglone, and dichlone) as well as two commercially available antibiotics, chloramphenicol and tetracycline. Anthraquinone-2-carboxylic acid exhibited very strong growth inhibition of *Clostridium paraputrificum* at 1 μg/disk while 100 μg/disk of lapachol was needed for moderate growth inhibition of the same organism. These two isolates exhibited weak inhibition of *Clostridium perfringens* and *Escherichia coli* at 100 μg/disk while no adverse effects were observed on the growth of *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, and *Lactobacillus casei* at 1000 μg/disk. Structure−activity relationships indicate that a methyl group in the C-2 position of 1,4-naphthoquinone derivatives might play an important role in antibacterial activity.

KEYWORDS: Natural antibacterial agent; taheebo; intestinal bacteria; anthraquinone-2-carboxylic acid; lapachol; 1,4-naphthoquinones; structure−activity relationship

INTRODUCTION

In humans, various microorganisms are resident in the human intestinal tract, which is known as a highly complex ecosystem with considerable species diversity. These microbiota participate in normal physiological functions and also contribute to the genesis of various disease states by biotransforming a variety of ingested or endogenously formed compounds to potentially harmful metabolites such as *N*-nitroso compounds or aromatic steroids (*1*−*4*). This biotransformation may influence drug efficacy, toxicity, carcinogenesis, and aging (*1*, *5*, *6*). Intestinal microbiota have numerous effects on the immune system and host health. The maintenance of particular lactic acid-producing bacteria in the intestine, such as *Bifidobacterium* spp. and *Lactobacillus* spp., has beneficial effects in the host, although the mechanisms involved are not fully understood. To date, efforts to improve intestinal microflora have resulted in the development of probiotics, prebiotics, and synbiotics (*7*).

Gastrointestinal ecological investigations have indicated that there are age- and disease-associated differences in the intestinal bacterial composition. The normal gastrointestinal microbiota is found to be predominantly composed of Gram-negative rods belonging to the genus *Bacteroides*. The other main groups are Gram-positive rods and cocci, many of which are lactic acid bacteria and bifidobacteria (*8*). In contrast, the microbiota of cancer patients or elderly subjects are known to have a higher proportion of species from the genus *Clostridium* with few lactic acid-producing bacteria (*1*, *4*, *6*, *9*, *10*). Disturbance of the microbiota may cause a variety of diseases or abnormal physiological states. Growth-promoting agents of lactic acid-producing bacteria and growth-inhibiting agents against harmful bacteria such as *Clostridium* and *Escherichia coli* would be expected to alter the growth and composition of the microbiota and modulate the genesis of potentially harmful agents, thus maintaining optimal human health. The most common and significant cause of disturbances in the normal intestinal microbiota is the administration of antimicrobial agents. This disturbance can cause bacterial overgrowth and emergence of resistant microorganisms, which may lead to serious infections and also encourage transfer of resistance factors among bacteria (*11*).

In relation to human health, much concern has been focused on plant-derived bifidus factors that promote the growth of bifidobacteria or growth inhibitors against harmful bacteria such as clostridia, eubacteria, and *E. coli*. Plants are potential sources of growth modulators of intestinal bacteria because they

* To whom correspondence should be addressed. Tel: +82-2-880-4644. Fax: +82-2-873-4415. E-mail: kjh2404@snu.ac.k.
† School of Agricultural Biotechnology, Seoul National University.
‡ College of Pharmacy, Seoul National University.
§ U.S. Department of Agriculture.

10.1021/jf0486038 CCC: $30.25
Published on Web 01/29/2005

constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects (*12*). *Tabebuia* spp. (Bignoniaceae) are native to tropical rain forests throughout Central and South America. The herbal products obtained from the bark of tabebuia trees are called "taheebo", "lapacho", "pau d'arco", and "ipe roxo" (*13*). Taheebo has astringent, anti-inflammatory, antibacterial, antifungal, diuretic, and laxative properties (*14*). Key constituents identified in taheebo include naphthoquinones, furanonaphthoquinones, anthraquinones, benzoic acid derivatives, benzaldehyde derivatives, iridoids, coumarins, and flavonoids (*15*). Very little work has been done on the growth-inhibiting activity of *Tabebuia impetiginosa* inner bark compounds toward human intestinal bacteria, despite its wide-ranging pharmacological actions.

This paper describes a laboratory study aimed at isolating antibacterial constituents from the inner bark of *T. impetiginosa* active against four harmful intestinal bacteria. The growth-inhibiting activity of *T. impetiginosa* inner bark-derived compounds was compared with those of the widely used antibiotics, chloramphenicol and tetracycline. Additionally, structure−antibacterial activity relationships of test compounds were also investigated.

MATERIALS AND METHODS

Chemicals. Anthraquinone-2-carboxylic acid, dichlone, juglone, lapachol, lawsone, menadione, 1,4-naphthoquinone, naphthazarin, and plumbagin were purchased from Aldrich (Milwaukee, WI). *N*,*N*-Dimethylformamide was obtained from EM Science (Gibbstown, NJ). Chloramphenicol and tetracycline were supplied by Sigma (St. Louis, MO). All other chemicals were of reagent grade.

Bacterial Strains and Culture Conditions. The intestinal bacteria used in this study were *Bifidobacterium adolescentis* ATCC 15076, *Bifidobacterium infantis* ATCC 25962, *Bifidobacterium bifidum* ATCC 29521, *Bifidobacterium longum* ATCC 15707, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus casei* ATCC 393, *Clostridium difficile* ATCC 9689, *Clostridium paraputrificum* ATCC 25780, *Clostridium perfringens* ATCC 13124, and *E. coli* ATCC 11775. Stock cultures of these 10 strains were routinely stored on Eggerth−Gagnon (EG) liver extract−Fieldes slants at −60 °C and when required were subcultured on EG agar (Eiken Chemical, Tokyo, Japan). The plates were incubated at 37 °C for 2 days in an atmosphere of 5% H_2, 15% CO_2, and 80% N_2 in an anaerobic chamber (Hirayama, Tokyo, Japan), except for the plates of *E. coli*, which were incubated at 37 °C for 2 days under aerobic conditions. The bacteria were then grown in brain heart infusion broth (pH 7.6). All cultures were checked for contamination by plating at the end of the growth cycle.

Isolation and Identification. The air-dried inner bark (3.0 kg) of *T. impetiginosa* Mart. ex DC was purchased from Frontier Natural Products Co-op (Norway, IA). The material was extracted two times with 25 L of methanol at room temperature for 2 days and filtered. The resultant extract was pooled and concentrated under reduced pressure at 40 °C to yield ca.12% (based on the initial weight of the dried inner bark). The methanol extract (150 g) was sequentially partitioned into hexane (29.7 g), chloroform (13.8 g), ethyl acetete (19.2 g), butanol (40.2 g), and water (47.1 g) portions for subsequent bioassay. The organic solvent portions were concentrated to dryness by rotary evaporation at 40 °C while the water portion was freeze-dried.

The bioactive chloroform portion (10 g) was chromatographed on a silica gel column (70−230 mesh, 500 g, 5.5 cm × 70 cm; Merck, Darmstadt, Germany) and successively eluted with a stepwise gradient of chloroform/methanol (100/0, 90/10, 80/20, 70/30, 60/40, 50/50, and 0/100 by volume). Column fractions were monitored by thin-layer chromatography (TLC) on silica gel plates (Kieselgel 60 F_{254}, 0.20 mm thickness, Merck) with hexane/ethyl acetate (6:4). Fractions with similar streaking patterns on the TLC plates were pooled. Spots were detected by spraying with 30% H_2SO_4 followed by heating on a hot plate. The bioactive fraction (1.5 g) was rechromatographed on a silica gel column, using a stepwise gradient of chloroform/methanol (95/5, 90/10, 80/20, 70/30, and 0/100 by volume). For further separation of the constituents from the active 95/5 fraction (600 mg), a preparative high-performance liquid chromatograph (HPLC; Spectra System P2000, Thermo Separation Products, San Jose, CA) was used. The column was a 250 mm × 4.6 mm i.d. Cosmosil 5C_{18}-MS (Nacalai Tesque, Kyoto, Japan). A linear gradient of methanol−acetonitrile−0.1% H_3PO_3 (25:20:55 by volume; isocratic for 5 min) to methanol−acetonotrile−0.1% H_3PO_3 (25:45:20 v/v) in 20 min at a flow rate of 1.3 mL/min was employed. Column effluent was monitored at 254 nm. Finally, two active principles, **1** (0.75 mg) and **2** (1.25 mg), were isolated at the retention times of 16.5 and 20.1 min, respectively.

Structural determination of the active isolates was made by spectroscopic analyses. ^1H and ^{13}C NMR spectra were recorded with a JNM-LA 400 F7 spectrometer (JEOL, Tokyo, Japan), and chemical shifts were given in ppm. COSY, HMQC, and HMBC spectra were acquired using the standard JEOL software. Fourier transform infrared (FT-IR) spectra were obtained on a VECTOR 22 spectrometer (Bruker, Frankfurt, Germany). The HPLC system (model HP1100) from Hewlett-Packard (Agilent, Waldbronn, Germany) consisted of a binary pump (model G1312A) and an autosampler (model G1313A). The analytical HPLC column (XterraC18; average particle size, 3.5 μm, 150 mm × 2.1 mm i.d.) was purchased from Waters (Milford, MA). The mobile phase was a mixture of acetonitrile/water (95:5 by volume) with a flow rate of 0.3 mL/min at room temperature. The mobile phase was filtered through a 0.45 μm Whatman nylon membrane filter (Maidstone, United Kingdom) and degassed under vacuum before use. Mass spectrometric detection was performed using an ion-trap mass spectrometer (Finnigan LCQ, San Jose, CA) equipped with an electrospray source. The ESI source was operated under the following conditions: heated capillary temperature, 200 °C; the nitrogen sheath gas, 70 psi; and the auxiliary gas, 10 units.

Growth-Inhibiting Assay. For the assay of the growth-inhibiting effects of *T. impetiginosa* inner bark-derived materials on test microorganisms, one loopful of bacteria was suspended in 1 mL of sterile physiological saline. An aliquot (0.1 mL) of the bacterial suspension was seeded on EG agar. A sample (10, 1, and 0.5 mg) in 0.1 mL of methanol was applied by Drummond microcapillary to a paper disk (ADVANTEC, 8 mm diameter and 1 mm thickness; Toyo Roshi, Japan). After evaporation of solvent, the disks were placed on the agar surface inoculated with test bacteria. All plates were incubated under the same conditions mentioned above. The widely used antibiotics, chloramphenicol and tetracycline, served as standards for comparison in inhibition tests. Control disks received 0.1 mL of methanol. All tests of growth inhibition were replicated three times.

The inhibitory responses were classified as previously described (*16*): very strong response, ++++, zone diameter >25 mm; strong response, +++, zone diameter 21−25 mm; moderate response, ++, zone diameter 16−20 mm; weak response, +, zone diameter 10−15 mm; and no response, −, zone diameter <10 mm.

RESULTS

Growth-Inhibiting Activity of *T. impetiginosa* Inner Bark-Derived Materials. Fractions obtained from the methanol extract of *T. impetiginosa* inner bark were bioassayed with five lactic acid-producing bacteria according to the paper disk diffusion (**Table 1**). Significant differences were observed in the growth-inhibiting activity toward the bacteria tested. At a dose of 10 mg/disk, the chloroform fraction showed moderate (++) and weak (+) inhibitory activity toward *B. longum* and *L. acidophilus*, respectively. The hexane fraction exhibited weak growth-inhibiting activity toward *B. longum*, *L. acidophilus*, and *L. casei*, whereas no inhibitory activity was observed with the ethyl acetate, butanol, and water fractions.

The growth-inhibiting activity of *Tabebuia* inner bark-derived fractions toward harmful bacteria is shown in **Table 2**. At 10 mg/disk, the chloroform fraction exhibited strong growth-inhibiting effects (+++) on *C. paraputrificum* and *C. perfringens* with moderate effects on the growth of *E. coli*. The other

Table 1. Growth-Inhibiting Responses of Various Fractions Obtained from the Methanol Extract of Taheebo Toward Lactic Acid-Producing Bacteria Using the Paper Disk Diffusion Bioassay

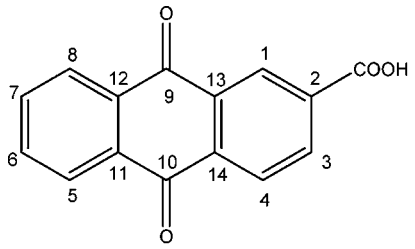
	bacterial strain[a]				
material	*B. adolescentis* ATCC 15076	*B. infantis* ATCC 25962	*B. longum* ATCC 15707	*L. acidophilus* ATCC 4356	*L. casei* ATCC 393
methanol extract	–[b]	–	+	+	+
hexane fraction	–	–	+	+	+
chloroform fraction	–	–	++	+	–
ethyl acetate fraction	–	–	–	–	–
butanol fraction	–	–	–	–	–
water fraction	–	–	–	–	–

[a] Exposed to 10 mg/disk. [b] Inhibitory zone diameter >25 mm, ++++; 21–25 mm, +++; 16–20 mm, ++; 10–15 mm, +; and <10 mm, –.

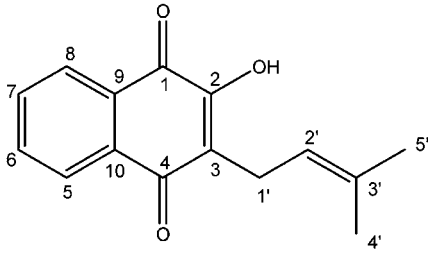
Table 2. Growth-Inhibiting Responses of Various Fractions Obtained from the Methanol Extract of Taheebo toward Harmful Intestinal Bacteria Using the Paper Disk Diffusion Bioassay

	bacterial strain[a]			
material	*C. difficile* ATCC 9689	*C. paraputrificum* ATCC 25780	*C. perfringens* ATCC 13124	*E. coli* ATCC 11775
methanol extract	–[b]	+++	+++	++
hexane fraction	–	++	+	–
chloroform fraction	–	+++	+++	++
ethyl acetate fraction	–	++	+	+
butanol fraction	–	++	–	–
water fraction	–	++	–	–

[a] Exposed to 10 mg/disk. [b] For explanation, see **Table 1**.



(1)



(2)

Figure 1. Structures of anthraquinone-2-caboxylic acid and lapachol, antibacterial constituents from the dried inner bark of *T. impetiginosa*.

four fractions were moderately effective against *C. paraputrificum* but ineffective against *C. difficile*, *C. perfringens*, and *E. coli*.

Identification of Active Principles. Paper disk diffusion bioassay-guided fractionation of *Tabebuia* inner bark extract afforded two active principles identified by spectroscopic analyses, including MS and NMR. The active principles were characterized as anthraquinone-2-carboxylic acid (**1**) and 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol) (**2**) (**Figure 1**). These two compounds were identified on the basis of the following evidence. Anthraquinone-2-carboxylic acid (**1**): ($C_{15}H_8O_4$; MW, 252.2). FT-IR: ν_{max} (Nujol): 1699 (C═O), 1678 (C═O) cm^{-1}. FAB-MS: *m*/*z* 253 $[M + H]^+$. LC-ESI-MS: *m*/*z* 253 $[M + H]^+$. EI-MS (rel int): *m*/*z* 252 $[M]^+$ (100), 224 (36), 207 (33), 151 (35). ^1H NMR (400 MHz, DMSO *d*-6): 8.58 (1H, *d*, $J = 1.6$ Hz, H-1), 8.33 (1H, *dd*, $J = 1.6$, 8.0, H-3), 8.20 (1H, *d*, $J = 8.0$, H-4), 8.16 (2H, *m*, H-5,8), 7.92 (2H, *m*, H-6,7). ^{13}C NMR (100 MHz, DMSO *d*-6): 182.0, 181.9, 165.9, 135.7, 135.5, 134.7, 134.4, 133.1, 132.9, 127.3, 127.5, 126.8, 126.7, 126.6, 123.9. Lapachol (**2**): ($C_{15}H_{14}O_3$; MW, 242.7). FT-IR: ν_{max} (Nujol): 3350 (OH), 1660 (C═O), 1630 (aromatic ring) cm^{-1}. FAB-MS: *m*/*z* 243 $[M + H]^+$. LC-ESI-MS: *m*/*z* 243 $[M + H]^+$. EI-MS (rel int): *m*/*z* 242 $[M]^+$ (37), 227 (100), 199 (35), 181 (32), 152 (37), 128 (35), 105 (35). ^1H NMR (400 MHz, DMSO *d*-6): 1.61 (3H, *s*, CH_3-'), 1.70 (3H, *s*, CH_3-), 3.15 (2H, *d*, $J = 6.7$, $-CH_2-$), 5.10 (1H, *m*, $-CH=$), 7.7 (2H, *m*, Ar−H), 7.9 (2H, *m*, Ar−H). ^{13}C NMR (100 MHz, DMSO *d*-6): 184.2, 181.1, 155.0, 134.5, 133.1, 131.9, 131.6, 129.9, 125.6, 125.1, 122.8, 120.6, 25.4, 22.0, 17.7.

Growth-Inhibiting Activity of Test Compounds. The inhibitory activity of anthraquinone-2-carboxylic acid (**1**) and lapachol (**2**) toward various lactic acid-producing bacteria were compared to those of chloramphenicol and tetracycline (**Table 3**). These compounds did not cause growth inhibition of *B. adolescentis*, *B. bifidum*, *B. infantis*, *L. acidophilus*, and *L. casei* at 1000 μg/disk. *B. longum* was the most sensitive lactic acid-producing bacteria tested as both taheebo compounds caused weak inhibition at 1000 μg/disk. Tetracycline and chloramphenicol caused strong to moderate growth inhibition of the two lactobacilli at doses as low as 10 μg/disk. *B. longum* was strongly inhibited by tetracycline at 10 μg/disk while *B. adolescentis* was strongly inhibited by tetracycline and chloramphenicol at 200 μg/disk.

The growth-inhibiting effect in the paper disk diffusion bioassay of the test compounds on three harmful intestinal bacteria is shown in **Table 4**. Anthraquinone-2-carboxylic acid very strongly inhibited the growth of *C. paraputrificum* at 1 μg/disk while 200 μg/disk of lapachol was needed to strongly inhibit the same organism. Lapachol at 1000 μg/disk showed moderate and weak inhibition of *C. perfringens* and *E. coli*, respectively, while anthraquinone-2-carboxylic acid at 200 μg/disk caused strong to moderate growth inhibition of these two bacteria. Tetracycline at 0.1 μg/disk was highly effective on both clostridia, while chloramphenicol at 10 μg/disk strongly inhibited the growth of the two clostridia and *E. coli*.

Structure−Activity Relationships. The growth-inhibiting activity of seven 1,4-naphthoquinone derivatives (**Figure 2**) was compared to that of lapachol (**Table 5**). Potencies varied according to test compound and bacterial strain. Of the eight test compounds, the most toxic compounds toward *C. paraputrificum* and *C. perfringens* were menadione and plumbagin followed by 1,4-naphthoqunone, lawsone, naphthazarin, juglone, and lapachol. Dichlone was least active. Against *E. coli*,

Table 3. Growth-Inhibiting Activity of Taheebo Isolates and Antibiotics toward Lactic Acid-Producing Bacteria Using the Paper Disk Diffusion Bioassay

		bacterial strain					
compound	dose (μg/disk)	*B. adolescentis* ATCC 15073	*B. bifidum* ATCC 29521	*B. infantis* ATCC 15697	*B. longum* ATCC 15707	*L. acidophilus* JCM 1028	*L. casei* ATCC 14916
anthraquinone-2-carboxylic acid (**1**)	1000	–[a]	–	–	+	–	–
	200	–	–	–	+	–	–
	100	–	–	–	+	–	–
	10	–	–	–	+	–	–
	1	–	–	–	–	–	–
	0.1	–	–	–	–	–	–
lapachol (**2**)	1000	–	–	–	+	–	–
	200	–	–	–	+	–	–
	100	–	–	–	+	–	–
	10	–	–	–	+	–	–
	1	–	–	–	–	–	–
	0.1	–	–	–	–	–	–
tetracycline	1000	++++	–	–	++++	++++	++++
	200	+++	–	–	++++	++++	++++
	100	++	–	–	++++	++++	+++
	10	+	–	–	+++	++	++
	1	+	–	–	++	+	+
	0.1	–	–	–	+	–	–
chloramphenicol	1000	++++	–	–	–	++++	++++
	200	+++	–	–	–	++++	++++
	100	+	–	–	–	++++	++++
	10	–	–	–	–	+++	+++
	1	–	–	–	–	+	+
	0.1	–	–	–	–	–	–

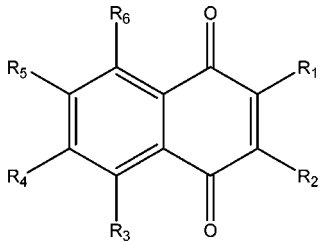
[a] For explanation, see **Table 1**.

Table 4. Growth-Inhibiting Activity of Taheebo Isolates toward Harmful Intestinal Bacteria Using the Paper Disk Diffusion Bioassay

		bacterial strain		
compound	dose (μg/disk)	*C. paraputrificum* ATCC 25780	*C. perfringens* ATCC 13124	*E. coli* ATCC 11775
anthraquinone-2-carboxylic acid (**1**)	1000	++++[a]	++++	+++
	200	++++	+++	++
	100	++++	+	+
	10	++++	–	+
	1	++++	–	–
	0.1	+	–	–
lapachol (**2**)	1000	++++	++	+
	200	+++	+	+
	100	++	+	+
	10	+	+	–
	1	–	–	–
	0.1	–	–	–
tetracycline	1000	++++	++++	++++
	200	++++	++++	++++
	100	++++	++++	++++
	10	+++	+++	+++
	1	+++	+++	+
	0.1	+++	+++	–
chloramphenicol	1000	++++	++++	++++
	200	++++	++++	++++
	100	+++	+++	+++
	10	+++	+++	+++
	1	+	+	–
	0.1	–	–	–

[a] For explanation, see **Table 1**.

plumbagin was most effective. Menadione was also highly effective. Lawsone was moderastely active. Weak or no inhibitory activity was observed with 1,4-naphthoqunone, naphthazarin, juglone, lapachol, and dichlone.



$R_1=R_2=R_3=R_4=R_5=R_6=H$ (1,4-naphthoquinone)
$R_1=R_2=R_4=R_5=H$; $R_3=R_6=OH$ (naphthazarin)
$R_2=R_3=R_4=R_5=R_6=H$; $R_1=CH_3$ (menadione)
$R_2=R_3=R_4=R_5=R_6=H$; $R_1=OH$ (lawsone)
$R_2=R_4=R_5=R_6=H$; $R_1=CH_3$; $R_3=OH$ (plumbagin)
$R_1=OH$; $R_2=CH_2CH=C(CH_3)_2$ (lapachol)
$R_1=R_2=R_4=R_5=R_6=H$; $R_3=OH$ (juglone)
$R_3=R_4=R_5=R_6=H$; $R_1=R_2=Cl$ (dichlone)

Figure 2. Structures of 1,4-naphthoquinone derivatives.

DISCUSSION

The intestinal microbiota in healthy subjects remain relatively constant but are known to be greatly influenced by physical, biological, chemical, environmental, or host factors (*1*, *2*, *4*, *6*). Accordingly, alterations to the microbiota may cause abnormal physical conditions or diseases. Infectious diseases caused by clostridia have a broad spectrum of clinical severity that ranges from mild outpatient illness to sudden death. Among the clostridia, *C. perfringens* has been associated with sudden death, toxicity, and gastrointestinal disease in man (*17*, *18*). On the contrary, bifidobacteria are often taken as useful indicators of human health under most environmental conditions because they play important roles in metabolism such as amino acid and vitamin production (*19*), aid in the defense against infections (*6*), are associated with longevity (*20*), exhibit antitumor activities (*21*), pathogen inhibition (*22*, *23*), and immuno-

Table 5. Growth-Inhibiting Activity of Naphthoquinone Derivatives toward Several Human Intestinal Bacteria Using the Paper Disk Diffusion Bioassay

		bacterial strain		
compounds	dose (μg/disk)	*C. paraputrificum* ATCC 25780	*C. perfringens* ATCC 13124	*E. coli* ATCC 11775
1,4-naphthoquinone	1000	++++[a]	++++	+
	200	++++	++++	+
	100	++++	+++	+
	10	++++	++	−
	1	+++	+	−
	0.1	+	−	−
naphthazarin	1000	++++	+++	−
	200	++++	++	−
	100	++++	++	−
	10	+++	+	−
	1	+	+	−
	0.1	−	−	−
menadione	1000	++++	++++	++++
	200	++++	++++	+++
	100	++++	++++	+++
	10	++++	++++	++
	1	++++	+++	+
	0.1	++++	−	+
lawsone	1000	++++	++++	++
	200	++++	++++	++
	100	++++	+	+
	10	+++	+	+
	1	+++	−	+
	0.1	−	−	−
plumbagin	1000	++++	++++	++++
	200	++++	++++	++++
	100	++++	++++	++++
	10	++++	++++	+++
	1	++++	+++	++
	0.1	+++	+	−
juglone	1000	++++	+	+
	200	++++	+	+
	100	++++	−	−
	10	+++	−	−
	1	−	−	−
	0.1	−	−	−
dichlone	1000	++	+	+
	200	++	−	+
	100	+	−	+
	10	+	−	−
	1	−	−	−
	0.1	−	−	−

[a] For explanation, see **Table 1**.

potentiation (*24*, *25*). Accordingly, it would be desirable to both inhibit the growth of potential pathogens such as clostridia and increase the number of bifidobacteria in the human gut. Selective growth promoters for bifidobacteria or inhibitors of harmful bacteria are especially important for human health because intake of these materials may normalize disturbed physiological functions, resulting in the prevention and treatment of various diseases caused by pathogens in the gastrointestinal tract. Recently, attention has been focused on the inhibitory roles of natural compounds in suppressing the carcinogenic and mutagenic effects of clostridia. Plants are potential alternatives to synthetic antibacterial agents because they exhibit selective growth inhibition effects on *Clostridium* spp. with no inhibitory effects on the other human intestinal bacteria and may be applied to humans in the same way as other conventional antibiotics (*26*, *27*). Extracts from ginseng (*Panax ginseng*, C. A. Meyer) and green tea (*Thea sinensis* L.) have been shown not only to enhance the growth of bifidobacteria but also to selectively inhibit various clostridia (*28*, *29*). Additionally, plants and/or their constituents are reported to be highly effective against drug resistant organisms. Furanonaphthoquinone, an analogue of lapachol, isolated from the bark of *T. impetiginosa* was found to be highly effective against a methicillin resistant *Staphylococcus aureus* strain as compared to methicillin sensitive *S. aureus*. Lapachol possesses potent growth-inhibiting activity against *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Bacillus*, and *Clostridium.* It has also been shown to have an activity similar to amphotericin B against *C. albicans*, *C. tropicalis*, and *C. neoformans.* The presumed antifungal activity of lapachol is believed to be due to its interaction with the cellular membrane (*30*−*32*).

In the present study, taheebo extract exhibited growth inhibition of *C. paraputrificum* and *C. perfringens*. The growth-inhibiting constituents of *T. impetiginosa* inner bark were identified as anthraquinone 2-carboxylic acid and lapachol with species selective activity. These compounds had potent growth-inhibiting activities against *C. paraputrificum* and *C. perfringens*, whereas no growth inhibition was observed against five lactic acid-producing bacteria (*B. adolescentis*, *B. bifidum*, *B. infantis*, *L. acidophilus*, and *L. casei*). However, growth of *B. longum* was slightly inhibited. This is the first report on the growth-inhibiting activity of anthraquinone 2-carboxylic acid and lapachol against *C. paraputrificum* and *C. perfringens*. Lapachol is the major naphthoquinone derivative found in the heartwood of several plants species belonging to Bignoniaceae and Verbenaceae. Considerable recent attention has been focused on lapachol and its analogues, because these compounds have potent antimalarial (*33*), antibiotic (*34*), and antitumor activity (*34*, *35*). Orally administered lapachol had a single dose LD_{50} of 0.487 and 0.792 g/kg in male and female mice, respectively, and greater than 2.4 g/kg in albino rats (*36*). Human studies with lapachol showed that intestinal absorption was considerably less than that determined for rats (*37*).

Structure−growth inhibitory activity relationships of natural antibacterial compounds have been well-studied. Ahn et al. (*29*) reported a clear structure−activity relationship between the six polyphenols derived from *T. sinensis* leaves and growth inhibition against *C. perfringens* and *C. difficile*. The gallate moiety of polyphenols seemed to be required, but their stereochemistries did not appear to be critical for the inhibitory activity. In *Pinus densiflora* leaf compounds, (1*R*)-(+)-pinene was found to have much more pronounced growth inhibitory activity than (1*S*)-(−)-, (1*R*)-(+)-, and (1*S*)-(−)-pinenes against *C. perfringens* (*38*). In the present study, menadione was about 100 times more active against the test bacteria than 1,4-naphthoquinone without a methyl group at C-2. Similarly, juglone without a methyl group at C-2 had much less activity than plumbagin with one. The introduction of Cl group at C-2 (dichlone) or 3-methyl-2-butenyl side chain at C-3 (lapachol) significantly reduced the growth inhibitory activity toward the test bacteria. These results indicate that a methyl group at the C-2 position appeared to be an important role for growth inhibition.

In conclusion, the inhibitory action of anthraquinone-2-carboxylic acid and lapachol toward harmful bacteria combined with almost no growth effects on lactic acid-producing bacteria may be an indication of at least one of the pharmacological actions of taheebo. As naturally occurring growth-inhibiting agents, anthraquinone-2-carboxylic acid and lapachol could be useful as new preventive agents against various diseases caused by harmful intestinal bacteria such as clostridia. Further work is necessary to establish whether this activity is exerted in vivo after consumption of taheebo by humans.

LITERATURE CITED

(1) Modler, H. W.; McKellar, R. C.; Yaguchi, M. Bifidobacteria and bifidogenic factors. *Can. Inst. Food Sci. Technol. J.* **1990**, *23*, 29−41.

(2) Hoover, D. G. Bifidobacteria: Activity and potential benefits. *Food Technol.* **1993**, *47*, 120−124.

(3) Rood, J. I.; McClane, B. A.; Songer, J. G.; Tithall, R. W. *The Clostridia Molecular Biology and Pathogenesis*; Academic Press: San Diego/London, 1997.

(4) Mituoka, T. A. *Color Atlas of Anaerobic Bacteria*; Shobansha: Tokyo, Japan, 1984.

(5) Hughes, D. B.; Hoover, D. G. Bifidobacteria: Their potential for use in American dairy products. *Food Technol*. **1991**, *45*, 74−83.

(6) Hentges, D. J. Role of the intestinal microflora in host defense against infection. In *Human Intestinal Microflora in Health and Disease*; Hentges, D. J., Ed; Academic Press: New York, 1983; pp 311−331.

(7) Tuomola, E.; Crittenden, R.; Playne, M.; Isolauri, E.; Salminen, S. Quality assurance criteria for probiotic bacteria. *Am. J. Clin. Nutr*. **2001**, *73*, 393S−398S. Supplement.

(8) Šušković, J.; Kos, B.; Goreta, J.; Matošić, S. Role of lactic acid bacteria and bifidobacteria in synbiotic effect. *Food Technol. Biotechnol*. **2001**, *39*, 227−235.

(9) Mitsuoka, T. The human gastrointestinal tract. In *The Lactic Acid Bacteria*; Wood, B. J. B., Ed.; Elsevier Applied Science: London, 1992; Vol. 1, pp 69−114.

(10) Moore, W. E. C.; Moore, L. H. Intestinal flora of populations that have a high risk of colon cancer. *Appl. Environ. Microbiol*. **1995**, *61*, 3202−3207.

(11) Nord, C. E. The effect of antimicrobial agents on the ecology of the human intestinal microflora. *Vet. Microbiol*. **1993**, *35*, 193−197.

(12) Wink, M. Production and application of phytochemicals from an agricultural perspective. In *Phytochemistry and Agriculture*; van Beek, T. A., Breteler, H., Eds.; Oxford: Clarendon, 1993; Vol. 34, pp 171−213.

(13) Jones, K. *Pau d'arco: Immune Power from the Rain Forest*; Healing Arts Press: Rochester, VT, 1995; pp 54−58.

(14) Hashimoto, G. *Illustrated Cyclopedia of Brazilian Medical Plants*; Aboc-sha: Kamakura, 1996; p 158.

(15) Mills, S.; Bone, K. *Principles and Practice of Phytotherapy: Modern Herbal Medicine*; Churchill Livingstone: London; 2000; pp 499−506.

(16) Cho, S. H.; Na, Y. E.; Ahn, Y. J. Growth-inhibiting effects of seco-tanapartholides identified in *Artemisia princeps* var. *orientalis* whole plant on human intestinal bacteria. *J. Appl. Microbiol*. **2003**, *95*, 7−12.

(17) Bokkenheuser, V. D.; Winter, J. Biotransformation of steroids. In *Human Intestinal Microflora in Health and Disease*; Hentges, D. J., Ed.; Academic Press: New York, 1983; pp 215−239.

(18) Goldman, P. Biochemical pharmacology and toxicology involving the intestinal flora. In *Human Intestinal Microflora in Health and Disease*; Hentges, D. J., Ed.; Academic Press: New York, 1983; pp 241−263.

(19) Rasic, J. L.; Kurmann, J. A. *Bifidobacteria and Their Role*; Birkhauser Verlag: Boston, MA, 1983.

(20) Mitsuoka, T.; Hayakawa, K. Die faekalflora bei menschen. I. Mitteilung: Die zusammensetzung der faekalflora der verschiedenen altersgruppen. *Zentralbl. Bakteriol., Mikrobiol. Hyg. Abt. I* **1973**, *A223*, 333−342.

(21) Tsuyuki, S.; Yamazaki, S.; Akashiba, H.; Kamimura, H.; Sekine, K.; Toida, T.; Saito, M.; Kawashima, T.; Ueda, K. Tumor-suppressive effect of a cell wall preparation, WPG, from *Bifidobacterium infantis* in germfree and flora-bearing mice. *Bifidobact. Microflora* **1991**, *10*, 43−52.

(22) Bullen, C. L.; Willis, A. T. Resistance of the breast-fed infant to gastroenteritis. *Br. Med. J*. **1971**, *3*, 338−343.

(23) Rasic, J. L. The role of dairy foods containing bifido- and acidophilus-bacteria in nutrition and health. *N. Eur. Dairy J*. **1983**, *48*, 80−88.

(24) Perdigon, G.; Alvarez, S.; Rachid, M.; Aguero, G.; Gobbato, N. Immune system stimulation by probiotics. *J. Dairy Sci*. **1995**, *78*, 1597−1606.

(25) Pereyra, B. S.; Lemonnier, D. Induction of human cytokines by bacteria used in dairy foods. *Nutr. Res*. **1993**, *13*, 1127−1140.

(26) Lee, H. S.; Beon, M. S.; Kim, M. K. Selective growth inhibitor toward human intestinal bacteria derived from *Pulsatilla cernua* root. *J. Agric. Food Chem.* **2002**, *49*, 4656−4661.

(27) Lee, H. S.; Kim, M. J. Selective responses of three Ginkgo biloba leaf-derived constituents on human intestinal bacteria. *J. Agric. Food Chem.* **2002**, *50*, 1840−1844.

(28) Ahn, Y. J.; Kim, M.; Kawamura, T.; Yamamoto, T.; Fujisawa, T.; Mitsuoka, T. Effects of *Panax ginseng* extract on growth responses of human intestinal bacteria and bacterial metabolism. *Korean J. Ginseng Sci.* **1990**, *4*, 253−264.

(29) Ahn, Y. J.; Kawamura, T.; Kim, M.; Yamamoto, T.; Mistuoka, T. Tea polyphenols: Selective growth inhibitors of *Clostridium* sp. *Agric. Biol. Chem*. **1991**, *55*, 1425−1426.

(30) Nagata, K.; Hirai, K.; Koyama, J.; Wada, Y.; Tamura, T. Antimicrobial activity of novel furanonaphthoquinone analogues. *Antimicrob. Agents Chemother*. **1998**, *42*, 700−702.

(31) Guiraud, P.; Steiman, R.; Campos-Takaki, G. M.; Seigle-Murandi, F.; Simeon de Buochberg, M.; Comparison of antibacterial and antifungal activities of lapachol and B-lapachol. *Planta Med*. **1994**, *60*, 373−374.

(32) Portillo, A.; Vila, R.; Freixa, B.; Adzet, T.; Cañigueral, S. Antifungal activity of Paraguayan plants used in traditional medicine. *J. Ethnopharmacol*. **2001**, *76*, 93−98.

(33) Fieser, L. F.; Berliner, E.; Bondhus, F. J.; Chang, F. C.; Dauben, W. G.; Ettlinger, M. G.; Fawaz, G.; Fields, M.; Fieser, M.; Heidelberger, C.; Heymann, H.; Seligman, A. M.; Vaughan, W. R.; Wilson, A. G.; Wilson, E.; Wu, M. I.; Leffler, M. T.; Hamlin, K. E.; Hathaway, R. J.; Matson, E. J.; Moore, E. E.; Moore, M. B.; Rapala, R. T.; Zaugg, H. E. Naphthoquinone antimalarials. General survey. *J. Am. Chem. Soc*. **1948**, *70*, 3151−3155.

(34) Goncalves de Lima, O.; de B. Coelho, J. S.; d'Albuquerque, I. L.; Francisco de Mello, J.; Martins, D. G.; Lacerda, A. L.; De Moraes e Souza, M. A. Antimicrobial compounds from higher plants. XXXV. Antimicrobial and antitumor activity of lawsone (2-hydroxy-1,4-naphthoquinone) compared with that of lapachol, 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone. *Rev. Inst. Antibiot*. (*Recife*) **1971**, *11*, 21−26.

(35) Rao, R. V.; McBride, R. J.; Oleson, J. J. Recognition and evaluation of lapachol as an antitumor agent. *Cancer Res*. **1968**, *28*, 1952−1954.

(36) Morrison, R. K.; Brown, D. E.; Oleson, J. J.; Cooney, D. A. Oral toxicity studies with lapachol. *Toxicol. Appl. Pharmacol*. **1970**, *17*, 1−11.

(37) Block, J. B.; Serpick, A. A.; Miller, W.; Wiernic, P. H. Early clinical studies with lapachol (NSC-11905). *Cancer Chemother. Rep*. [2] **1974**, *4*, 27−28.

(38) Jeon, H. J.; Lee, K. S.; Ahn, Y. J. Growth-inhibiting effects of constituents of *Pinus densiflora* leaves on human intestinal bacteria. *Food Sci. Biotechnol.* **2001**, *10*, 403−407.

Received for review August 23, 2004. Revised manuscript received November 18, 2004. Accepted December 4, 2004. This work was supported by the Brain Korea 21.

JF0486038