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Antibacterial Activities of Persimmon Roots-derived Materials and 1,4-Naphthoquinone's Derivatives against Intestinal Bacteria

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Abstract The growth-inhibiting activities of persimmon roots-derived materials against intestinal bacteria were evaluated and compared with that of 1,4-naphthoquinone as a positive control. The active constituent isolated from persimmon roots was characterized as 5-hydroxy-2-methyl-1,4-naphthoquinone using various spectroscopic analyses. Treatment with 1,4-naphthoquinone at a dose of 1.0 mg/disc strongly inhibited the growth of 6 intestinal bacteria. Furthermore, when the structure-activity relationships of 1,4-naphthoquinone's derivatives were evaluated, 5-hydroxy-2-methyl-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone were found to strongly inhibit the growth of *Clostridium difficile*, *Clostridium perfringens*, and *Escherichia coli* without adversely affecting the growth of *Bifidobacterium adolescentis*, *Bifidobacterium longum*, and *Lactobacillus acidophilus*. Additionally, 2-hydroxy-1,4-naphthoquinone and 5-hydroxy-1,4-naphthoquinone strongly inhibited the growth of *C. difficile* and *C. perfringens*, but did not inhibit the growth of *E. coli*. Taken together, these results indicate that persimmon roots-derived materials and some of 1,4-naphthoquinone's derivatives could be useful preventive agents against diseases caused by harmful intestinal bacteria.

Keywords: persimmon, 5-hydroxy-2-methyl-1,4-napthoquinone, intestinal bacteria, growth-inhibiting, 1,4-naphthoquinone derivatives

Introduction

A wide variety of bacteria have adapted to live and grow in the human intestine. Specifically, the human intestine contains an ecologically complicated microbial community that is comprised of greater than 500 bacterial species, of which over 99% are anaerobic bacteria (1). These bacteria possess enzymatic and metabolic activities that have an important impact on human health and disease (1,2); therefore, it is possible that the global increase in allergic, immune, inflammatory, and neoplastic diseases that was observed during the second half of the 20th century may have occurred as a result of disruption of the delicate balance in the intestinal ecosystem (3). The Gram-negative bacteria, *Escherichia coli*, and the Gram-positive anerobes, *Clostridium difficile* and *Clostridium perfringens*, as well as other *Clostridium* species have been reported to be important causes of infection that are commonly found in the gut (4,5). These organisms biotransform various foods that have been ingested into harmful products such as aromatic steroids, indoles, *N*-nitroso compounds, and phenols, all of which cause sudden death, proinflammation, toxicity, and aging (6). Accordingly, antibiotics have been widely used for the purpose of prevention and disease therapy. However, their repeated use can bring about an increase in resistance and residual toxicity. Indeed, according to the World Health Organization (WHO), several antibiotic resistant strains of bacteria that have recently emerged in animals can be transmitted to humans, and this transmission occurs primarily via meat and other foods of animal origin or through direct contact with farm animals (7). In addition, many of these bacteria have the ability to spread genes that confer resistance to other bacteria via vertical gene transfer (8). Consequently, these resistant bacteria are able to cause serious diseases and therefore pose a major public health risk.

Studies designed to identify solutions to antibiotic resistance and residual toxicity have often focused on the isolation and characterization of novel antimicrobial compounds from a variety of sources, including medicinal plants (9,10). The fruits and leaves of the persimmon (*Diospyros kaki* L.) have long been used in Korean traditional medicines to treat cough, apoplexy, arteriosclerosis, diarrhea, and antibiotics (11). Due to their medicinal importance, extensive phytochemical studies have been conducted on more than 130 *Diospyros* species (12), the results of which have revealed the widespread presence of hydroxycarbon, terpene, naphthoquinone, and coumarin (13). In particular, 1,4-naphthoquinone pharmacophore and its derivatives, which are derived from persimmon roots, are known to induce acaricidal (14), antibacterial (15), antitermitic (16), and cytotoxic effects (17). However, few studies have been conducted to evaluate the effects of persimmon roots-derived materials on the growth of intestinal microorganisms in spite of its excellent pharmacological effects. Therefore, the active principle isolated from persimmon roots was characterized by various spectroscopic analyses and their growth-inhibiting activities against intestinal bacteria were then determined using the paper disc diffusion method. Additionally, the selective growth-inhibiting activities of commercially available derivatives against intestinal bacteria were investigated to evaluate the structure-activity relationships.

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Materials and Methods

Chemicals 2-Bromo-1,4-naphthoquinone, 2-(8-carboxyoctyl)-1,4-naphthoquinone, 2-chloro-3-morpholino-1,4-naphthoquinone, 2,3-dichloro-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, and 1,4-naphthoquinone were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals used in this study were of reagent grade.

Isolation and identification Persimmon (*Diospyros kaki* L.) roots, belonging to the family Ebenaceae, were purchased from a local market in Jeonju (Korea). The roots (10 kg) were ground in a blender, extracted twice with methanol (50 L) at room temperature for 2 days, and then filtered (No. 2; Advantec; Toyo Roshi, Tokyo, Japan). The combined filtrate was then concentrated *in vacuo* at 45°C to yield 35.16%. The extract (20 g) was then sequentially partitioned into *n*-hexane (3.86 g), chloroform (10.14 g), ethyl acetate (1.28 g), butanol (2.40 g), and water-soluble (2.32 g) portions for subsequent bioassay. The organic solvent portions were then concentrated to dryness at 45°C using a rotary vacuum evaporator (Eyela, N-N Series; Rikakikai, Tokyo, Japan), whereas the water portion was freeze-dried.

The chloroform (10 g) portion was sequentially chromatographed on a silica gel column (Merck 70-230 mesh; 620 g; 5.5 i.d.×68 cm, Rahway, NJ, USA), and then successively eluted with a stepwise gradient of *n*-hexane:chloroform (Fig. 1). The C2 fraction (1.95 g) of the 4 fractions (C1 to C4) eluted from the chloroform fraction showed a strong growth-inhibiting activity against *C. difficile*, *C. perfringens*, and *E. coli* at a concentration of 5.0 mg/disc. Therefore, the active C2 fraction was chromatographed on a silica gel column and then eluted with *n*-hexane:chloroform (4:6, v/v). The column fractions were then analyzed by thin layer chromatography (TLC, 0.25 mm, SIL G/UV 254; Macherey-Nagel, Düren, Germany; *n*-hexane:chloroform, 4:6, v/v), and fractions with similar TLC patterns were combined. The bioactive fraction was then rechromatographed on a silica gel column and successively eluted with *n*-hexane:chloroform (4:6, v/v).

To further separate the biologically active C21 fraction (975 mg), a Japanese analytical industry recycling preparative high performance liquid chromatography (HPLC, LC-908W-C60; Jai, Tokyo, Japan) was used, and the eluates were then examined for their biological activity. For HPLC, a JAI GEL GS-310 50 cm+GS-310 30 cm×2 column was used. *n*-Hexane:chloroform (4:6, v/v), which was used as the mobile phase, was applied at a flow rate 5.0 mL/min and detected at 264 nm. HPLC was repeated under these conditions 2 times consecutively. HPLC produced 4 fractions (C211 to C214) that were subsequently bioassayed at a concentration of 5.0 mg/disc. Due to the activity of fraction C212 (491 mg), it was further chromatographed on a JAI GEL W-253 50 cm+W-252 50 cm column under the same conditions described above. Finally, the potent active principle (C2122, 364 mg) was isolated.

The structure of the isolated fraction (C2122) was then determined by spectroscopic analysis. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were observed by dissolving the fraction in deuteriochloroform and then analyzing the solution using a JNM-LA 400F7 spectrometer (Jeol, Tokyo, Japan) at 400 and 100 MHz (TMS as an internal standard), respectively, to determine the chemical shifts (δ) in parts per million. Unambiguous ^{1}H-^{13}C NMR chemical shifts were obtained using DEPT spectrum as well as ^{1}H-^{13}H COSY spectrum. Ultra violet (UV) spectra were obtained in chloroform using a DR/4000 spectrometer (Hach, Tokyo, Japan).

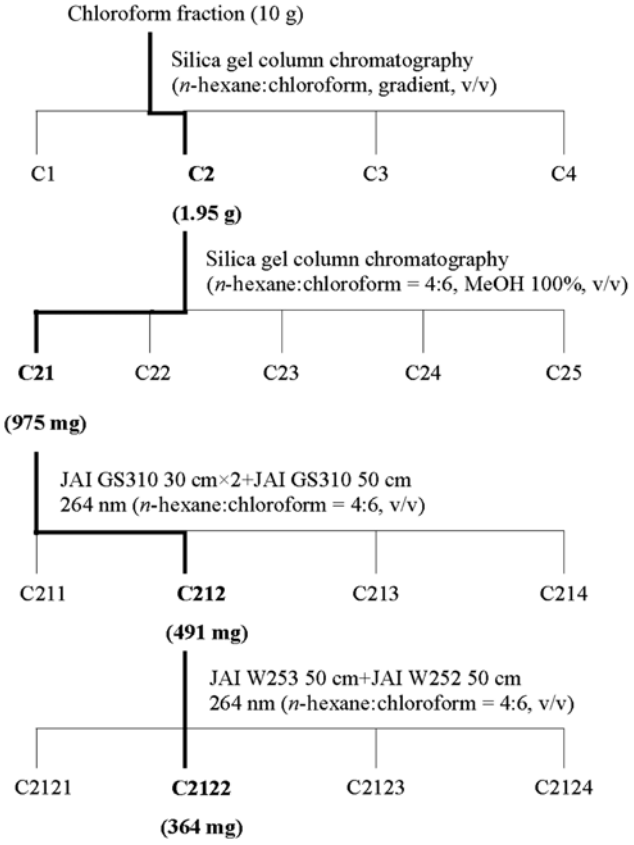

Fig. 1. Isolation of the growth-inhibiting component from the chloroform fraction.

Bacterial strains and culture conditions The intestinal bacteria used in this study were *Bifidobacterium adolescentis* ATCC 15073, *Bifidobacterium longum* ATCC 15707, *Clostridium difficile* ATCC 9689, *Clostridium perfringens* ATCC 13124, *Escherichia coli* ATCC 11775, and *Lactobacillus acidophilus* ATCC 4356. Stock cultures of these strains were routinely stored on an Eggerth-Gagnon (EG) liver extract-Field's slants at −80°C and subcultured on EG agar plates (Eiken Chemical, Tokyo, Japan) when required. The plates were incubated anaerobically at 37°C for 2 days in an anaerobic chamber (Hirayama, Tokyo, Japan) with an atmosphere comprised of 80% N_2, 15% CO_2, and 5% H_2. The bacteria were then grown in a brain heart infusion broth (pH 7.6) and de Man Rogosa and Sharpe broth (pH 5.7). All cultures were checked for contamination by plating at end of the growth cycle.

Growth-inhibiting assay To assay of the effect of the

test materials on the growth-inhibiting response of the test microorganisms used, 1 loopful of bacteria was suspended in 1 mL of sterilized physiological saline. An aliquot (0.1 mL) of the bacterial suspensions was then seeded on an EG agar plate. Each test compound was then dissolved in 0.1 mL of a methanol solution and applied to a paper disc using a Drummond glass microcapillary (8-mm diameter and 1-mm thickness; Advantec, Toyo Roshi). After evaporation of the solvents, the discs were placed on the agar surface that had been inoculated with the test bacteria. All plates were then incubated anaerobically at 37°C for 2 days. In addition, inoculated plates were treated with control discs that received 0.1 mL of methanol. All tests of growth inhibition were replicated 3 times and the inhibitory responses were classified as previously described (18): potent response, ++++, zone diameter >30 mm; strong response, +++, zone diameter 21-30 mm; moderate response, ++, zone diameter 16-20 mm; weak response, +, zone diameter 10-15 mm; and little or no response, -, zone diameter <10 mm.

Results and Discussion

Growth-inhibiting effect of fractions In routine screening, the methanol extract of persimmon roots exhibited a strong (+++) inhibition activity against *C. perfringens,* and a moderate (++) activity against *C. difficile* and *E. coli* at a concentration of 5 mg/disc, but showed no inhibitory response against *B. adolescentis, B. longum*, and *L. acidophilus* (Table 1). When the 5 fractions obtained from the methanol extract of persimmon roots were assayed at a dose of 5.0 mg/disc, significant differences were observed in their growth-inhibiting activities against intestinal bacteria (Table 1). In fractionation guided by the growth-inhibiting activity at a dose of 5.0 mg/disc, the chloroform fraction showed a strong (+++) activity against *C. perfringens,* and a moderate (++) activity against *C. difficile* and *E. coli*, but did not inhibit the growth of *B. adolescentis, B. longum*, and *L. acidophilus* (Table 1). Conversely, the butanol, ethyl acetate, hexane, and water fractions did not inhibit the growth of any of these 6 bacteria. The negative control that received 0.1 mL of methanol exhibited no inhibition against 6 intestinal bacteria tested.

Identification of the active principle Purification of the active constituent from the chloroform fraction was conducted by various types of silica gel chromatography and prep-HPLC. Structural determination of the active constituent was accomplished by conducting spectroscopic analysis using gas chromatography-mass spectrometry (GC-MS), electron impact (EI)-MS, ^{13}C NMR, ^{1}H NMR, ^{1}H-^{13}C COSY NMR and DEPT NMR and then comparing the results to those of authentic reference compounds. The active constituent was characterized as 5-hydroxy-2-methyl-1,4-naphthoquinone (Fig. 2b) based on the following evidence: 5-hydoxyl-2-methyl-1,4-naphthoquinone, ($C_{11}H_8O_3$, Mw: 188.18); EI-MS (70 eV) *m/z* (% relative intensity) M^+ 188 (100, base peak), 173 (21), 160 (19), 131 (27), 119 (12), 92 (18), 63 (10); ^{1}H-NMR ($CDCl_3$, 400 MHz); δ 7.518-7.543 (2H, t, J=10.0 Hz), 7.160-7.179 (1H, *d*, J=7.6 Hz), 6.723-6.726 (1H, d, J=1.2 Hz), 2.113-2.117 (3H, d, J=1.6 Hz); ^{13}C-NMR ($CDCl_3$, 100 MHz); δ 189.2, 183.7, 160.2, 148.6, 135.1, 134.4, 131.1, 123.1, 118.2, 114.1, 15.5. The ^{1}H-NMR and ^{13}C-NMR spectra of C2122 were found to be the same as those for 5-hydroxy-2-methyl-1,4-naphthoquinone derived from persimmon roots (14).

Growth-inhibiting activity of 1,4-naphtoquinone derivatives The growth-inhibiting activity of 5-hydroxy-2-methyl-1,4-naphthoquinone and 1,4-naphthoquinone's derivatives against *B. adolescentis, B. longum,* and *L. acidophilus* were evaluated using the paper disc diffusion method (Fig. 2, Table 2). Treatment with 2-(8-carboxyoctyl)-1,4-naphthoquinone, 2-chloro-3-morpholino-1,4-naphthoquinone, 2,3-dichloro-1,4-naphthoquinone, 5-hydroxy-2-methyl-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, and 2-methyl-1,4-naphthoquinone at a concentration of 5.0 mg/disc did not inhibit the growth of *B. adolescentis, B. longum,* or *L. acidophilus*. However, treatment with 2-bromo-1,4-naphthoquinone showed a weak growth-inhibiting activity at 2.0 mg/disc against *L. acidophilus*. Next, the effect of the positive control, 1,4-naphthoquinone, against beneficial intestinal bacteria was compared with that of its derivatives (Table 2). 1,4-Naphthoquinone exhibited a strong activity against *B. adolescentis, B. longum,* and *L. acidophilus* at a concentration of 1.0 mg/disc, and a concentration of 0.25 mg/disc moderately inhibited the growth of *B. adolescentis* and *L. acidophilus*. Furthermore, the growth-inhibiting effect of 1,4-naphthoquinone against *B. adolescentis, B. longum,* and *L. acidophilus* was more pronounced than that of its derivatives, which did not cause any adverse effects on the growth of *B. adolescentis, B. longum,* and *L. acidophilus* at a concentration of 0.25 mg/disc. The growth-

Table 1. Growth-inhibiting responses of various fractions obtained from the methanol extract of persimmon roots against intestinal bacteria

Compound[1)]	Bacteria strain[2)]					
	B. adolescentis	*B. longum*	*C. difficile*	*C. perfringens*	*E. coli*	*L. acidophilus*
Methanol fraction	-	-	++	+++	++	-
Hexane fraction	-	-	-	-	-	-
Chloroform fraction	-	-	++	+++	++	-
Ethyl acetate fraction	-	-	-	-	-	-
Butanol fraction	-	-	-	-	-	-
Water fraction	-	-	-	-	-	-

[1)]Inhibitory zone diameter >30 mm, ++++; 21-30 mm, +++; 16-20 mm, ++; 10-15 mm, +; and <10 mm, -.
[2)]Exposed to 5.0 mg/disc; -, cultured on EG agar at 37°C for 2 days in an atmosphere comprised of 80% N_2, 15% CO_2, and 5% H_2.

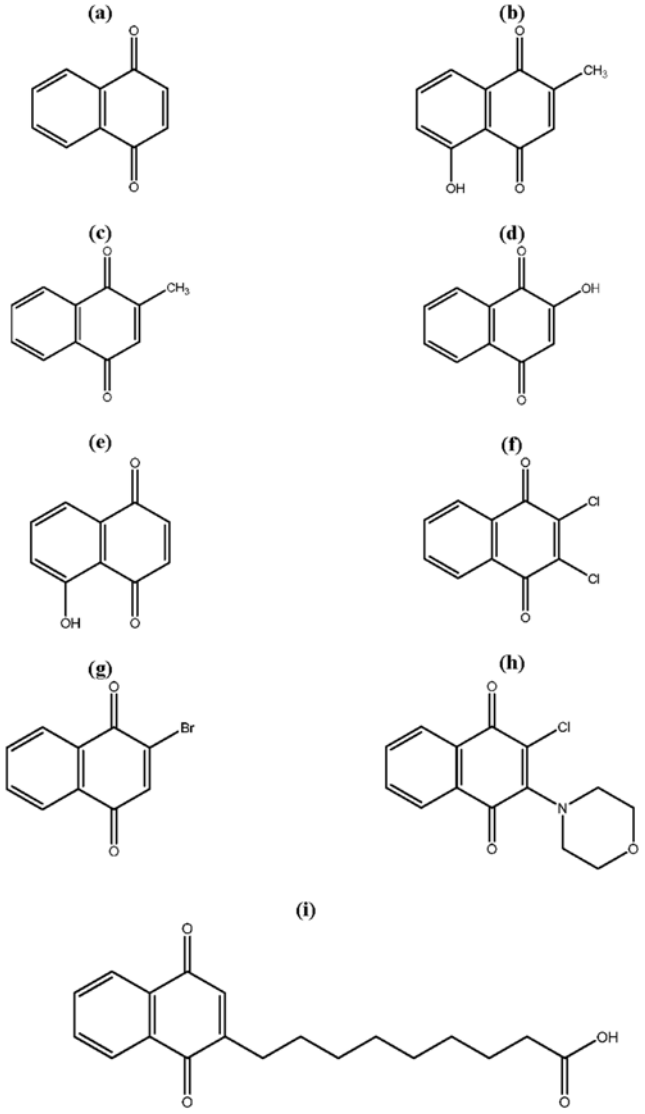

Fig. 2. Structures of 1,4-naphthoquinone and its derivatives. (a) 1,4-naphthoquinone, (b) 5-hydroxy-2-methyl-1,4-naphthoquinone, (c) 2-methyl-1,4-naphthoquinone, (d) 2-hydroxy-1,4-naphthoquinone, (e) 5-hydroxy-1,4-naphthoquinone, (f) 2,3-dichloro-1,4-naphthoquinone, (g) 2-bromo-1,4-naphthoquinone, (h) 2-chloro-3-morpholino-1,4-naphthoquinone, (i) 2-(8-carboxyoctyl)-1,4-naphthoquinone.

inhibiting activities of 1,4-naphthoquinone's derivatives against *C. difficile, C. perfringens*, and *E. coli* are shown in Table 2. 5-Hydroxy-2-methyl-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone strongly inhibited the growth of *C. difficile* at a concentration of 1.0 mg/disc, while a concentration of 5.0 mg/disc of 2-hydroxy-1,4-naphthoquinone and 5-hydroxy-1,4-naphthoquinone were required to inhibit the growth of *C. difficile*. When *C. perfringens* was evaluated, 5-hydroxy-1,4-naphthoquinone exhibited a strong-inhibiting activity at a concentration of 0.5 mg/disc, while 5-hydroxy-2-methyl-1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, and 2-hydroxy-1,4-naphthoquinone exerted a strong-inhibiting activity at a concentration of 1.0 mg/disc. When *E. coli* was evaluated, 5-hydroxy-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone showed a strong-inhibiting activity at a concentration of 1.0 and 2.0 mg/disc. However, 1,4-naphthoquinone strongly inhibited the growth of *C. perfringens* and *C. difficile* at a dose of 0.25 mg/disc.

1,4-Naphthoquinone's derivatives generally have various physiological effects, such as antibacterial, anticancer, anti-inflammatory, antifungal, and antiplatelet activities; therefore, they are often used in foods and medicine (19). In addition, 1,4-naphthoquinone's derivatives have been isolated and synthesized from various species of plants and analyzed (2,6,13,20-25). The results of these studies have shown that atovaquone (21), benanomicin (23), 2-hydroxynaphthoquinone (20), and nanaomycin A (24) have antibacterial and antifungal activities. These substances commonly contain 1,4-naphthoquinone, which indicates that the matrix of 1,4-naphthoquinone's nucleus is the primary compound involved in the activities of its derivatives. Specifically, the biological effects imparted by 1,4-naphthoquinone generally rely on its ability to accept 1 or 2 electrons to form radical anions or dianion species (26). However, the presence of electron-donating or -attracting substituents in 1,4-naphthoquinone modulates the generation of radical anions and the redox properties that are responsible for the catalytic cycle of various compounds, thereby resulting in the production of oxidative radicals such as hydrogen peroxide and superoxide, which damage the cells (27). In this study, we examined the growth-inhibiting activity of 5-hydroxy-2-methyl-1,4-naphthoquinone, which was isolated from the roots of persimmon, as well that of 1,4-naphthoquinone and its derivatives against intestinal bacteria.

While 1,4-naphthoquinone showed a strong growth-inhibiting activity against intestinal bacteria, among its derivatives, 5-hydroxy-2-methyl-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, and 2-methyl-1,4-naphthoquinone exhibited selective growth inhibition against the harmful Gram-positive bacteria, *C. difficile* and *C. perfingens*, but had no effect on *B. adolescentis*, *B. longum,* and *L. acidophilus*. Among 1,4-naphthoquinone's derivatives that inhibited the activity of intestinal bacteria, those that contained a methyl functional group (5-hydroxy-2-methyl-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone) inhibited the growth of Gram-negative *E. coli* as well as Gram-positive *C. difficile* and *C. perfingens*. Conversely, 2,3-dichloro-1,4-naphthoquinone, 2-bromo-1,4-naphthoquinone, 2-chloro-3-morpholino-1,4-naphthoquinone, and 2-(8-carboxyoctyl)-1,4-naphthoquinone showed weak or no growth-inhibiting activity.

The addition of various functional groups to 1,4-naphthoquinoe was found to decrease its growth-inhibiting activity or to induce selective growth-inhibiting activity. For example, the 1,4-naphthoquinone's derivatives that contain a hydroxyl functional group or methyl functional group were found to have no influence on beneficial intestinal bacteria, but to inhibit the growth of *Clostridium* spp. and *E. coli*, which can cause colitis, enteritis, and diarrhea. This finding is similar to the results of previous studies conducted by Park *et al.* (25) and Lim *et al.* (28), who reported that 1,4-naphthoquinone's derivatives that contain a hydroxyl or methyl functional group showed selective growth inhibition against harmful intestinal bacteria. However, the degree of the growth inhibition appears to differ based on the refining process or the purity

Table 2. Growth-inhibiting responses of the isolated compound and 1,4-naphthoquinone's derivatives against intestinal bacteria

Compound[1)]	Dose (mg/disc)	Bacteria strain[2)]					
		B. acidophilus	*B. longum*	*C. difficile*	*C. perfringens*	*E. coli*	*L. acidophollus*
5-Hydroxy-2-methyl-1,4-naphthoquinone	5.0	-	-	++++	++++	+++	-
	2.0	-	-	++++	++++	+++	-
	1.0	-	-	+++	+++	++	-
	0.5	-	-	++	++	+	-
	0.25	-	-	++	+	-	-
2-Methyl-1,4-naphthoquinone	5.0	-	-	++++	++++	++++	-
	2.0	-	-	++++	++++	+++	-
	1.0	-	-	+++	+++	+++	-
	0.5	-	-	++	++	++	-
	0.25	-	-	++	++	+	-
2-Hydroxy-1,4-naphthoquinone	5.0	-	-	+++	++++	-	-
	2.0	-	-	++	++++	-	-
	1.0	-	-	+	+++	-	-
	0.5	-	-	-	++	-	-
	0.25	-	-	-	++	-	-
5-Hydroxy-1,4-naphthoquinone	5.0	-	-	+++	++++	-	-
	2.0	-	-	++	++++	-	-
	1.0	-	-	++	+++	-	-
	0.5	-	-	-	+++	-	-
	0.25	-	-	-	++	-	-
2,3-Dichloro-1,4-naphthoquinone	5.0	-	-	++	+	-	-
	2.0	-	-	-	-	-	-
2-Bromo-1,4-naphthoquinone	5.0	-	-	-	-	-	+
	2.0	-	-	-	-	-	+
2-Chloro-3-morpholino-1,4-naphthoquinone	5.0	-	-	-	-	-	-
	2.0	-	-	-	-	-	-
2-(8-Carboxyoctyl)-1,4-naphthoquinone	5.0	-	-	-	-	-	-
	2.0	-	-	-	-	-	-
1,4-Naphthoquinone	5.0	+++	+++	++++	++++	+++	+++
	2.0	+++	+++	++++	++++	++	+++
	1.0	+++	+++	++++	++++	++	+++
	0.5	+++	++	+++	++++	++	++
	0.25	++	+	+++	++++	+	++

[1)]Each assay was conducted in triplicate.
[2)]Cultured on EG agar at 37°C for 2 days in an atmosphere of 80% N_2, 15% CO_2, and 5% H_2; -, inhibitory zone diameter >30 mm, ++++; 21-30 mm, +++; 16-20 mm, ++; 10-15 mm, +; and <10 mm, -.

of the compound. The results of this study clearly demonstrate that 5-hydroxy-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, and 2-methyl-1,4-naphthoquinone, which are 1,4-naphthoquinone's derivatives that contain a hydroxyl or metyl functional group, can be used to help maintain a balance of the microflora in the intestines due to their selective growth inhibition of harmful intestinal microflora.

We suggest that the derivatives of 5-hydroxy-2-methyl-1,4-naphthoquinone and 1,4-naphthoquinone play a role selective growth regulators of intestinal bacteria. Furthermore, the results of this study indicate that 5-hydroxy-2-methyl-1,4-naphthoquinone and 1,4-naphthoquinone's derivatives that show selective growth-inhibiting activity against intestinal bacteria can be added to the food and the feed of animals to stabilize the bacteria in their intestines. Finally, these results indicate that these compounds may be useful as food preservatives and medicines.

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