Growth-Inhibiting Effects of *Coptis japonica* Root-Derived Isoquinoline Alkaloids on Human Intestinal Bacteria

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The growth-inhibiting activity of *Coptis japonica* (Makino) root-derived materials toward eight human intestinal bacteria was examined using an impregnated paper disk method and compared to that of four commercially available isoquinoline alkaloids [berberine sulfate (BS), berberine iodide (BI), palmatine chloride (PC), and palmatine sulfate(PS)], as well as that of *Thea sinensis* leaf-derived epigallocatechin gallate (EGCG). The biologically active constituents of the *Coptis* extract were characterized as the isoquinoline alkaloids berberine chloride (BC), palmatine iodide (PI), and coptisine chloride (CC) by spectral analysis. The growth responses varied with both chemical and bacterial strain used. In a test using 500 μg/disk, BC and PI produced a clear inhibitory effect against *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Clostridium perfringens*, and *Clostridium paraputrificum*, whereas weak or no inhibition was observed in *Bifidobacterium adolescentis*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Escherichia coli*. At 1000 μg/ disk, CC revealed weak or no growth inhibition toward all test bacteria, whereas EGCG exhibited weak growth inhibition against only *C. perfringens* and *C. paraputrificum*. Among various isoquinoline alkaloids, BC exhibited more potent inhibitory activity toward *C. perfringens* than BI and BS, whereas the inhibitory effect was more pronounced in PI compared to PC and PS. The *Coptis* root-derived materials did not promote growth of *B. longum* and *C. perfringens*.

Keywords: *Coptis japonica; Coptidis rhizoma; Thea sinensis; growth inhibition; intestinal bacteria; isoquinoline alkaloid*

INTRODUCTION

Methanol extracts of *Coptis japonica* roots (*Coptidis rhizoma*) have significant growth-inhibiting activities toward *Clostridium perfringens* (Ahn et al., 1994). In East Asia, *Coptis japonica* has long been considered to have natural medicinal properties, being rich in alkaloids and phenolics (Yahara et al., 1985; Namba, 1986; Kawaguchi et al., 1989).

In humans, intestinal microorganisms 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 agents (Hentges, 1983; Modler et al., 1990; Mitsuoka, 1992). Such biotransformations may influence drug efficacy, toxicity, carcinogenesis, and aging. Gastrointestinal ecological investigations have indicated that there are some differences in intestinal bacteria between patients and healthy control subjects, between young and elderly subjects, as well as between breast- and bottle-fed infants (Finegold et al., 1975; Modler et al., 1990; Mitsuoka, 1992). The microbiota of cancer patients or patients with Alzheimer's disease is known to be mainly composed of clostridia and eubacteria, with few lactic acid-producing bacteria (Finegold et al., 1975; Fujisawa et al., 1992). It has also been reported that bottle-fed infants harbor fewer bifidobacteria but higher levels of enterobacteria, streptococci, and anaerobes other than bifidobacteria than breast-fed infants (Modler et al., 1990). Accordingly, disturbance of the microbiota may cause a variety of diseases or abnormal physiological states.

In relation to human health, much current concern has been focused on plant-derived bifidus factors and growth inhibitors against harmful bacteria such as clostridia and *Escherichia coli* because plant materials constitute a rich source of biologically active chemicals and many of them are largely free from harmful adverse effects. For example, extracts from ginseng (*Panax ginseng* C. A. Meyer) and green tea (*Thea chinensis* L.) have been shown to not only enhance the growth of bifidobacteria but also selectively inhibit various clostridia (Ahn et al., 1990a,c, 1991). However, little work has been carried out on the effects of oriental medicinal plant-derived materials on the growth of intestinal microorganisms despite their excellent pharmacological actions (Namba, 1986).

In the laboratory study described herein, we assessed the growth-inhibiting responses of eight human intestinal bacteria to *C. japonica* root-derived isoquinoline alkaloids [berberine chloride (BC), palmatine iodide (PI), and coptisine chloride (CC)], their four commercially available isoquinoline derivatives [berberine sulfate (BS), berberine iodide (BI), palmatine chloride (PC), and palmatine sulfate (PS)] and green tea leaf-derived epigallocatechin gallate (EGCG), which was found to be

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a selective growth inhibitor against several clostridia (Ahn et al., 1991).

MATERIALS AND METHODS

Chemicals. BI, BS, PS, and tetracyclin were purchased from Sigma (St. Louis, MO). PC was obtained from Wako (Osaka). EGCG was obtained from green tea leaves as previously described (Ahn et al., 1991). All other chemicals were of reagent grade.

Bacterial Strains and Culture Conditions. Eight bacterial strains were used in this study: *Bifidobacterium longum* ATCC 15707, *Bifidobacterium adolescentis* ATCC 15073, *Bifidobacterium bifidum* ATCC 29521, *Lactobacillus acidophilus* JCM 1028, *Lactobacillus casei* ATCC 14916, *Clostridium perfringens* ATCC 13124, *Clostridium paraputrificum* ATCC 25780, and *Escherichia coli* ATCC 11775. Stock cultures of these four strains were routinely stored on Eggerth−Gagnon liver extract−Fieldes slants at −80 °C, and when required were subcultured on Eggerth−Gagnon (EG) agar (Eiken Chemical, Japan). The plates were incubated at 37 °C for 2 days in an atmosphere of 80% N_2, 15% CO_2, and 5% H_2 in an anaerobic chamber (Coy Laboratory, Ann Arbor, MI). The bacteria were then grown in EG broth (pH 6.8).

Isolation and Identification. The *C. japonica* (Makino) roots (4.5 kg) purchased as a commercially available product were dried in an oven at 50 °C for 2 days, finely powdered, extracted twice with methanol (20 L) at room temperature and filtered (Toyo No. 2 filter paper). The combined filtrate was concentrated in vacuo at 40 °C to give a yield of ∼21% (based on the weight of the root). The extract (945 g) was sequentially partitioned into hexane (70.9 g), chloroform (37.8 g), ethyl acetate (14.2 g), butanol (165.4 g), and water-soluble (656.7 g) portions for subsequent bioassay with *C. perfringens*. The organic solvent portions were concentrated to dryness by rotary evaporator at 40 °C, and the water portion was freeze-dried. For isolation, 1000 μg of each *C. japonica* root-derived fraction in solvents was applied to paper disks (Advantec, 8-mm diameter and 1-mm thickness, Toyo Roshi).

The chloroform fraction (12 g) was chromatographed on a silica gel column (Merck 70−230 mesh, 600 g, 5.5 i.d. × 70 cm) and successively eluted with a stepwise gradient of chloroform/methanol (0, 1, 2.5, 5, 10, 30, and 100%). The active 10% fraction (1.75 g) was chromatographed on a silica gel column and eluted with chloroform/methanol (10:1). Column fractions were analyzed by TLC (chloroform/methanol, 10:1), and fractions with similar TLC patterns were combined. For further separation of the biologically active substances, a preparatory HPLC (Waters Delta Prep 4000) was used. The column was a Bondapak C_{18} (2.9 i.d. × 300 mm, Waters) using methanol/water (3:2) at a flow rate of 10 mL/min and detection at 254 nm. The chemical substances in the active second peak (20 mg) among three peaks showed two main yellow spots on TLC, which were developed by benzene/ethyl acetate/*n*-propanol/methanol/ethylamine (8:4:2:1:1). The two spots were orange-red when reacted with the Dragendorff reagent, suggesting that the chemical substances were alkaloids. The active fraction was chromatographed on a silica gel column using the previous solvent system to give fractions I and II. The active compounds were isolated according to the method of Kawaguchi et al. (1989). Fractions I (100 mg) and II (120 mg) were dissolved in water, and precipitates were obtained from the aqueous solution by adding 1 N HCl to pH 5.0 and a 7.5% HI solution to pH 4.0, respectively. The precipitates were collected by filtration, washed with water, and dried under reduced pressure over P_2O_5. The yield from fractions I and II was 50.2 mg (compound **A**) and 44.6 mg (compound **B**), respectively. R_f values of compounds **A** and **B** were 0.65 and 0.40 in benzene/ethyl acetate/*n*-propanol/methanol/ethylamine (8:4:2:1:1), respectively.

The butanol fraction (12 g) was chromatographed on a silica gel column, as mentioned earlier, and eluted with benzene/ethyl acetate/*n*-propanol/methanol/ethylamine (8:4:2:1:1). The active fraction (2 g) was chromatographed on a silica gel column and eluted with butanol/acetone/water (3:1:1). Column fractions were analyzed by TLC (butanol/acetone/water, 3:1:1), and fractions with similar TLC patterns were combined. The chemical substances in the active fraction among three fractions showed a main yellow spot on TLC, which was developed by benzene/ethyl acetate/*n*-propanol/methanol/ethylamine (8:4:2:1:1). The spot was orange-red when reacted with the Dragendorff reagent, suggesting that the chemical substance was an alkaloid. For further separation of the biologically active substance(s), a preparatory HPLC was used, as mentioned above. Finally, an active principle (96 mg) was isolated. The R_f value of the isolate was 0.96 in the previous solvent system.

Structural determination of the active isolates was made by spectral analysis. ^1H and ^{13}C NMR spectra were recorded with a Bruker AM-500 spectrometer. UV spectra were obtained on a Waters 490 spectrometer, IR spectra on a Bio-Rad FT-80 spectrophotometer, and mass spectra on a JEOL JMS-DX 30 spectrometer.

Growth-Inhibiting Assay. For assay of effects of test materials on the growth-inhibiting responses of the microorganisms used, one loopful of bacteria was suspended in 1 mL of sterile physiological saline. An aliquot (0.1 mL) of the bacterial suspensions was seeded on EG agar. A sample (1000, 500, 250, 125, and 62.5 μg) in 100 μL of methanol solution was applied by syringe to a paper disk (Advantec, 8-mm diameter and 1-mm thickness). After evaporation of solvents, the paper disks were placed on the agar surface inoculated with test bacteria. All plates were incubated anaerobically at 37 °C for 2 days. Control disks received 100 μL of methanol, which exhibited no adverse effect against the organisms used. All tests on growth inhibition were repeated in triplicate.

The growth-inhibiting responses of the active isolates toward the eight bacterial strains were examined and compared with those of green tea leaf-derived EGCG and four commercial compounds (BI, BS, PC, and PS) which are derivatives of constituents of the *Coptis* root. The inhibitory response were classified as previously described (Ahn et al., 1994): strong response, +++, zone diameter >20 mm; moderate response, ++, zone diameter 15−20 mm; weak response, +, zone diameter 10−15 mm; and no response, −, zone diameter <10 mm.

Growth-Promoting Assay. The growth-promoting responses of test materials to the microorganisms used were spectrophotometrically determined, as previously described (Lee and Ahn, 1997). In the experiments for growth-promoting factors derived from non-carbon and carbon sources, György (1954) broth (pH 6.8) as modified by Yoshioka et al. (1968) and modified RCM broth, respectively, were used. One percent of each culture was inoculated in the test media, and 0.1% of each filter-sterilized test material was added to the media in a final volume of 10 mL. Solutions of the test materials were prepared using methanol as a solvent. The methanol concentration in the solutions was 2%, which was found to be without adverse effect on the bacteria used. Samples from test and control solutions were assayed according to the membrane filter procedure (Ahn et al., 1990a). The media were anaerobically incubated at 37 °C for 2 days, and the bacterial growth was measured at 600 nm.

Growth-promoting responses were expressed as growth increase rate (GIR = A_{600} sample/A_{600} reference). The responses were classified as previously described (Lee and Ahn, 1997); strong response, +++, GIR > 2.0; moderate response, ++, 2.0 > GIR > 1.6; weak response, +, 1.5 > GIR > 1.0; and little or no response, −, GIR < 1.0. Each assay was replicated three times.

RESULTS

Identification. Fractions obtained from methanol extracts of the *Coptis* roots were assayed according to the impregnated paper disk method (Table 1). At 1000 μg/disk, chloroform and butanol fractions showed strong and moderate growth-inhibiting activity toward *C.*

Table 1. Growth Responses of *C. japonica* Root-Derived Materials to *C. perfringens* and *B. longum*[a]

material	inhibition[b]		promotion[c]	
	C. perfringens ATCC 13124	*B. longum* ATCC 15707	*C. perfringens* ATCC 13124	*B. longum* ATCC 15707
methanol extract	++	++	−[d]	−
hexane fraction	−	−	−	−
chloroform fraction	+++	++	−	−
ethyl acetate fraction	−	−	−	−
butanol fraction	+++	++	−	−
water fraction	−	−	−	−

[a] They were cultured on Eggerth−Gagnon agar at 37 °C for 2 days in an atmosphere of 80% N_2, 15% CO_2, and 5% H_2. [b] Exposed to 1000 μg/disk (8-mm diameter and 1-mm thickness). Inhibitory zone diameter >20 mm, +++; 16−20 mm, ++; 10−15 mm, +; and <10 mm, −. [c] For determination of bacterial growth, two kinds of media were used: modified György broth, as modified by Yoshioka et al. (1968), as carbon-containing medium and modified RCM broth (Ahn et al., 1994) as a carbon-free medium. [d] Applied at 0.1% of test materials. Each test material exhibited no growth-promoting activity to *B. longum* and *C. perfringens* on both modified György and RCM media.

Table 2. Growth-Inhibiting Responses of Lactic Acid-Producing Bacteria to *C. japonica* Root-Derived Isoquinoline Alkaloids and Green Tea Leaf-Derived Epigallocatechin Gallate (EGCG)

compound	dose μg/disk	bacterial strain[a]				
		B. bifidum ATCC 29521	*B. longum* ATCC 15707	*B. adolescentis* ATCC 15073	*L. acidophilus* JCM 1028	*L. casei* ATCC 14916
berberine chloride	1000	+++[b]	+++	+	+	−
	500	++	++	−	−	−
	250	+	+	−	−	−
	125	+	+	−	−	−
	62.5	−	−	−	−	−
palmatine iodide	1000	++	++	+	−	−
	500	++	++	−	−	−
	250	+	+	−	−	−
	125	+	+	−	−	−
	62.5	−	−	−	−	−
coptisine chloride	1000	+	+	−	−	−
	500	+	+	−	−	−
	250	+	+	−	−	−
	125	−	−	−	−	−
	62.5	−	−	−	−	−
EGCG	1000	−	−	−	−	−
	500	−	−	−	−	−

[a] They were cultured on Eggerth−Gagnon agar at 37 °C for 2 days in an atmosphere of 80% N_2, 15% CO_2, and 5% H_2. [b] Inhibitory zone diameter >20 mm, +++; 16−20 mm, ++; 10−15 mm, +; and <10 mm, −.

perfringens, respectively. Purification of the biologically active compounds from the fractions was done by silica gel column chromatography and HPLC, and the isolates were bioassayed. Two and one active principles were isolated from the chloroform and butanol fractions, respectively. The isolates were identified as isoquinoline alkaloids by color reaction with the Dragendorff reagent.

Structural determination of the isolates was made by spectroscopic methods including MS and NMR and by direct comparison with authentic reference compounds, and they were characterized as the isoquinoline alkaloids BC, PI, and CC.

Growth-Promoting Activity. The effects of the *Coptis* root-derived materials on the bacterial growth promotion are given in Table 1. For determination of bacterial growth, two kinds of media were used: modified György broth as carbon-containing medium and modified RCM broth as carbon-free medium. At a concentration of 0.1%, test materials from the *Coptis* roots exhibited little or no growth-promoting activity to *B. longum* and *C. perfringens* on both modified György and RCM media.

Growth-Inhibiting Activity. The inhibitory activities of BC, PI, and CC toward lactic acid-producing bacteria used were compared to that of green tea leaf-derived EGCG (Table 2). Responses varied with the chemical and bacterial strain tested. At 500 and 1000 μg/disk, BC and PI produced a clear inhibitory effect against *B. longum* and *B. bifidum*, whereas weak or no inhibition toward *B. adolescentis*, *L. acidophilus*, and *L. casei* was obtained; however, CC revealed weak or no growth inhibition toward bifidobacteria and lactobacilli used when exposed to 1000 μg/disk. Unlike the *Coptis* roots, green tea leaf-derived EGCG exhibited no growth-inhibiting effect against bifidobacteria and lactobacilli tested.

Table 3 shows growth-inhibiting responses of harmful intestinal bacteria to test compounds. Responses were chemical and bacterial strain-dependent. At 500 and 1000 μg/disk, BC and PI exhibited a potent growth-inhibiting effect against *C. perfringens* and *C. paraputrificum*, whereas weak inhibition was obtained in application of 1000 μg/disk of CC; however, at 1000 μg/disk, the three isoquinoline alkaloids isolated did not cause any adverse effects against *E. coli*. In a test with 1000 μg/disk of EGCG, this compound revealed weak activity toward *C. perfringens* and *C. paraputrificum*.

The growth-inhibiting effect of various isoquinoline alkaloids against *C. perfringens* is shown in Table 4. BC exhibited more potent inhibitory activity than BI and BS; however, the growth-inhibiting activity of PI was more potent than that of PC and PS.

Table 3. Growth-Inhibiting Responses of Harmful Intestinal Bacteria to *C. japonica* Root-Derived Isoquinoline Alkaloids and Green Tea Leaf-Derived Epigallocatechin Gallate (EGCG)

		bacterial strain[a]		
compound	dose μg/disk	*C. perfringens* ATCC 13124	*C. paraputrificum* ATCC 25780	*E. coli* ATCC 11775
berberine chloride	1000	+++[b]	++	−
	500	++	++	−
	250	+	+	−
	125	+	+	−
	62.5	+	−	−
palmatine iodide	1000	++	++	−
	500	++	++	−
	250	+	+	−
	125	+	+	−
	62.5	+	−	−
coptisine chloride	1000	++	++	−
	500	+	+	−
	250	+	+	−
	125	+	−	−
	62.5	+	−	−
EGCG	1000	+	+	−
	500	−	−	−

[a] They were cultured on Eggerth−Gagnon agar at 37 °C for 2 days in an atmosphere of 80% N_2, 15% CO_2, and 5% H_2. [b] Inhibitory zone diameter >20 mm, +++; 16−20 mm, ++; 10−15 mm, +; and <10 mm, −.

Table 4. Growth-Inhibiting Effect of Various Isoquinoline Alkaloids against *C. perfringens* ATCC 13124

	isoquinoline compound[a]					
concn, μg/disk	BC	BI	BS	PC	PI	PS
1000	+++[b]	++	++	++	++	++
500	++	+	+	+	++	+
250	+	+	+	+	+	+
125	+	+	+	+	+	+
62.5	+	+	−	+	+	+

[a] BC, berberine chloride; BI, berberine iodide; BS, berberine sulfate; PC, palmatine chloride; and PL, palmatine iodide. [b] Inhibitory zone diameter >20 mm, +++; 16−20 mm, ++; 10−15 mm, +; and <10 mm, −.

DISCUSSION

Microorganisms resident in the human intestinal tract as a highly complex ecosystem with considerable species diversity are greatly influenced by physical, biological, chemical, environmental, or host factors (Modler et al., 1990; Hughes and Hoover, 1991; Mitsuoka, 1992; Hoover, 1993). Alterations to the microbiota may cause abnormal physical conditions or diseases. In our study, the growth-inhibiting effect of the *Coptis* root extract toward *C. perfringens* was investigated. Its active components were identified as isoquinoline alkaloids berberine, palmatine, and coptisine, although the responses varied with chemical and bacterial strain tested; however, the *Coptis* root extract revealed no growth-promoting effect against *C. perfringens* and *B. longum*.

Among the intestinal microorganisms, bifidobacteria are often taken as useful indicators of human health under most environmental conditions, on the basis of the facts that they play important roles in maintaining optimum human health (Modler et al., 1990; Hughes and Hoover, 1991; Mitsuoka, 1992; Hoover, 1993). Bifidobacteria growth-promoting factors, usually called bifidus factors, have therefore been extensively studied since György et al. (1954) suggested their existence in human milk. On the contrary, infectious diseases caused by clostridia have a broad spectrum of clinical severity that ranges from mild out-patient illness to sudden death. Among the clostridia, *C. perfringens* has been associated with sudden death, toxicity, and gastrointestinal disease in humans by biotransforming a variety of ingested or endogenously formed compounds to harmful agents such as *N*-nitroso compounds or aromatic steroids within the gastrointestinal tract (Bokkenheuser and Winter, 1983; Goldman, 1983).

It would therefore be desirable to both inhibit the growth of potential pathogens and/or increase the numbers of bifidobacteria in the human gut. Selective growth promoters for bifidobacteria or inhibitors for harmful bacteria are especially important for human health, because intake of these materials may normalize disturbed physiological functions that result in the prevention of diseases caused by pathogens in the gastrointestinal tract. Recent in vivo investigations using human volunteers have shown that intake of ginseng extract or green tea extract favorably affected the fecal microbiota and biochemical aspects of feces (Ahn et al., 1990b; Okubo et al., 1992), suggesting an indication of at least one of their pharmacological actions. In our study, the growth-inhibiting activity of the *Coptis* isoquinoline alkaloids was much more pronounced in *C. perfringens*, *C. paraputrificum*, *B. longum*, and *B. bifidum* as compared to *B. adolescentis*, *L. acidophilus*, *L. casei*, and *E. coli*. These results suggest that intake of the *Coptis* root-derived materials should be limited; however, methanol extracts of the *Coptis* roots inhibited *C. perfringens* and *B. longum* (Table 1) but not *B. adolescentis* (Ahn et al., 1994). It has been also reported that the *Coptis* root-derived materials including isoquinoline alkaloids have antibacterial (Subbaiah and Amin, 1967), antitumor (Sethi, 1983; Kumazawa et al., 1984), antidiarrhea (Sharda, 1970), and antiinflammatory effects (Otsuka et al., 1981).

In conclusion, our results indicate that *C. japonica* root-derived materials have growth-inhibiting effects in vitro against specific bacteria of the human intestine. On the basis of our limited data and some earlier findings, the inhibitory action of the *Coptis* root-derived isoquinoline alkaloids toward *C. perfringens* and *C. paraputrificum* may be an indication of at least one of the pharmacological actions of this plant species. Fur-

ther work is necessary to establish whether this activity is exerted in vivo after consumption of the *Coptis* roots by humans.

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