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Growth-Inhibiting Effects of *Cinnamomum cassia* Bark-Derived Materials on Human Intestinal Bacteria

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The growth-inhibiting activity of *Cinnamomum cassia* (Blume) bark-derived materials toward five intestinal bacteria was examined using an impregnated paper disk method and compared with that of tetracycline and chloramphenicol, as well as four commercially available compounds (cinnamyl alcohol, *trans*-cinnamic acid, eugenol, and salicylaldehyde). The biologically active component of *C. cassia* bark was characterized as cinnamaldehyde by spectral analysis. The growth responses varied with each bacterial strain tested. In a test using 1 and 0.5 mg/disks, cinnamaldehyde revealed potent inhibition against *Clostridium perfringens* and *Bacteroides fragilis*. At 1 and 0.5 mg/disk, growth of *Bifidobacterium bifidum* was significantly inhibited, whereas weak or no inhibitory activity was obtained against *Bifidobacterium longum* or *Lactobacillus acidophilus*. The inhibitory effect was much more pronounced in *Cl. perfringens*, *B. fragilis*, and *Bi. bifidum*, compared to *Bi. longum* or *L. acidophilus*. Salicylaldehyde exhibited moderate growth-inhibiting activity, but little or no inhibition was observed from treatments with cinnamyl alcohol, *trans*-cinnamic acid, and eugenol. In contrast, tetracycline and chloramphenicol significantly inhibited growth of all test bacteria as low as 0.01 mg/disk. These results may be an indication of at least one of the pharmacological actions of *C. cassia* bark.

Keywords: *Cinnamomum cassia; intestinal bacteria; bifidobacteria; clostridia; cinnamaldehyde*

INTRODUCTION

Various microorganisms are resident in the human intestinal tract as a highly complex ecosystem with considerable species diversity. It has been well acknowledged that the microbiota not only participate in normal physiological functions but may also contribute to the genesis of various disease states by biotransforming a variety of ingested or endogenously formed compounds to potentially harmful agents such as *N*-nitroso compounds (Modler et al., 1990; Hughes and Hoover, 1991). This biotransformation may influence drug efficacy, toxicity, carcinogenesis, and aging. Gastrointestinal ecological investigations have indicated that there are some differences in the intestinal bacterial composition between patients and healthy control subjects as well as between young and elderly subjects (Mitsuoka, 1982; Hentges, 1983). The composition of the microbiota may also be influenced by factors such as diet and stress (Hentges, 1983; Rasic, 1983). The microbiota of cancer patients, patients with Alzheimer's disease, or elderly subjects is known to be mainly composed of clostridia and eubacteria with few lactic acid forming bacteria (Finegold et al., 1975; Mastromarino et al., 1978; Mitsuoka, 1982; Fujisawa et al., 1992). 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, which promote the growth of bifidobacteria or growth inhibitors against harmful bacteria such as clostridia, eubacteria, and *Escherichia coli* because plants consitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects. We have already reported that among 78 oriental medicinal plant species, methanol extract of *Cinnamomum cassia* bark revealed potent growth-inhibiting activity toward *Clostridium perfringens* (Ahn et al., 1994; Oh et al., 1996). This plant species not only is important as a spice but in East Asia is considered to have some medicinal properties, such as a stomachic agent, an astringent agent, and a carminative agent (Namba, 1986). It is rich in essential oils and tannins (Morimoto et al., 1986; Namba, 1986; Buckingham, 1992). However, relatively little work has been carried out on the effects of *C. cassia* bark-derived materials on growth of intestinal microorganisms compared to other areas of intestinal microbiology in spite of its excellent pharmacological action.

In the laboratory study described herein, we assessed the growth-inhibiting responses of five human intestinal bacteria to *C. cassia* bark-derived materials, the antibiotics tetracycline and chloramphenicol, and four commercial components of *C. cassia*. The antitumor action of plant-derived extracts or phytochemicals is also

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discussed in connection with the results obtained from the intestinal bacteria examined.

MATERIALS AND METHODS

Chemicals. Tetracycline, chloramphenicol, *trans*-cinnamic acid, eugenol, and salicylaldehyde were purchased from Sigma (St. Louis, MO). Cinnamyl alcohol was supplied by Tokyo Kasei (Tokyo, Japan).

Bacterial Strains and Culture Conditions. The bacterial strains used in this study were as follows: *Bifidobacterium longum* ATCC 15707, *Bifidobacterium bifidum* ATCC 29521, *Lactobacillus acidophilus* JCM 1028, *Clostridium perfringens* ATCC 13124, and *Bacteroides fragilis* isolated from human feces. Stock cultures of these five strains were routinely stored on Eggerth−Gagnon liver extract−Fieldes slant 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). The bacteria were then grown in EG broth (pH 6.8).

Isolation and Identification. The bark from *C. cassia* (3.6 kg) purchased as a commercially available product was dried in an oven at 60 °C for 2 days, finely powdered, extracted twice with methanol (10 L) at room temperature, and filtered (Toyo filter paper No. 2). The combined filtrate was concentrated *in vacuo* at 35 °C to yield ~10.4% (based on the weight of the bark). The extract (20 g) was sequentially partitioned into hexane (3.9 g), chloroform (4.5 g), ethyl acetate (1.9 g), and water-soluble (9.7 g) portions for subsequent bioassay with *Cl. perfringens* and *B. fragilis*. The organic solvent portions were concentrated to dryness by rotary evaporation at 35 °C, and the water portion was freeze-dried. For isolation, 5 mg of each *C. cassia* bark-derived fraction in methanol was applied to paper disks (Advantec 8-mm diameter and 1-mm thickness, Toyo Roshi).

The hexane portion (10 g) was chromatographed on a silica gel column (Merck 70−230 mesh, 500 g, 5.5 i.d. × 70 cm) and successively eluted with a stepwise gradient of hexane/ethyl acetate (0, 10, 30, 50, 80, and 100%). The active 50% fraction (4.1 g) was chromatographed on a silica gel column and eluted with hexane/ethyl acetate (2:1). Twenty-five column fractions were collected and analyzed by TLC (hexane/ethyl acetate, 3:1). Fractions with similar TLC patterns were combined. The active fraction (2.4 g) was chromatographed on a silica gel column and eluted with hexane/ethyl acetate (8:2). For further separation of the bioactive substance(s), a preparative HPLC (Waters Delta Prep 4000) was used. The column was a 29 i.d. × 300 mm Bondapak C_{18} (Waters) using methanol/water (3:7) at a flow rate of 10 mL/min and detection at 260 nm. Finally, a potent active principle (0.1 g) was isolated.

Structural determination of the active isolate was based on spectral analysis. ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker AM-500 spectrometer, and chemical shifts are given in parts per million. 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.

Microbiological Assay. For assay of effects of *C. cassia* bark-derived materials on the growth-inhibiting responses of the test microorganisms, 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 (5, 1, 0.5, 0.1, and 0.01 mg) in methanol solution was applied by syringe (0.1 mL) 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 for 2 days at 37 °C. Control disks received methanol. All tests on growth inhibition were repeated in triplicate.

The growth-inhibiting responses of the most active isolate toward the five bacterial strains were examined and compared with those of the antibiotics tetracycline and chloramphenicol and four commercial compounds (cinnamyl alcohol, *trans*-cinnamic acid, eugenol, and salicylaldehyde), which are components of the *C. cassia* bark (Namba, 1986; Buckingham, 1992). Tests were repeated three times. The inhibitory responses were classified as previously described (Ahn et al., 1994): highly strong 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.

Table 1. Growth-Inhibiting Activity of *C. cassia* Bark-Derived Materials toward *Cl. perfringens* and *B. fragilis*

material[a]	bacterial strain[b]	
	C. perfringens	*B. fragilis*
MeOH extract	+++[c]	+++
hexane fraction	+++	+++
chloroform fraction	−	−
ethyl acetate fraction	−	−
water fraction	−	−

[a] Exposed to 5 mg/disk. [b] 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. [c] Inhibitory zone diameter >30 mm, ++++; 21−30 mm, +++; 16−20 mm, ++; 10−15 mm, +; and <10 mm, −.

Table 2. Growth-Inhibiting Responses of Intestinal Bacteria to *C. cassia* Bark-Derived Compounds

bacterial strain[a]	compound[b]				
	CA[c]	CD	CL	EN	SA
Bi. longum	+[d]	−	−	−	++
L. acidophilus	−	−	−	−	++
C. perfringens	+++	−	−	−	++
B. fragilis	++++	−	−	−	+++

[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 1 mg/disk. [c] CA, *trans*-cinnamaldahyde; CD, *trans*-cinnamic acid; CL, cinnamyl alcohol; EN, eugenol; SA, salicylaldahyde [d] Inhibitory zone diameter >30 mm, ++++; 21−30 mm, +++; 16−20 mm, ++; 10−15 mm, +; and <10 mm, −.

RESULTS

Fractions obtained from methanol extracts of *C. cassia* bark were assayed according to the impregnated paper disk method (Table 1). The hexane fraction showed strong growth-inhibiting activity (+++) toward *Cl. perfringens* and *B. fragilis*. Purification of the biologically active compound(s) from the fraction was done by using silica gel column chromatography and HPLC, and the isolates were bioassayed. One active isolate showed inhibitory activity. Structural determination of the isolate was made by spectral techniques, and it was characterized as *trans*-cinnamaldehyde. The compound was identified on the basis of the following evidence: C_9H_8O (MW, 132); EI-MS (70 eV), *m*/*z* (% rel intensity) M^+ 132 (3), 103 (2), 74 (83), 59 (100), 58 (75); IR (neat) max (cm^{-1}): 2920, 1680, 1630, 1130; ^{1}H NMR (CD_3OD, 400 MHz) δ 6.60 (dd, J = 8 and 18 Hz), 7.35 (d, J = 18 Hz), 7.1−7.7 (m), 9.52 (d, J = 8 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ 195.6, 154.4, 135.0, 132.1, 129.9 129.7, 129.5, 129.0, 128.9.

The growth-inhibiting activities of cinnamaldehyde and other components of this plant species toward intestinal bacteria when treated with 1 mg/disk were determined (Table 2). Responses varied with the chemical and bacterial strain tested. Cinnamaldehyde and salicylaldehyde showed strong and moderate growth-inhibiting activity, respectively. Little or no activity was observed for cinnamyl alcohol, cinnamic acid, and eugenol.

Table 3. Growth-Inhibiting Responses of Intestinal Bacteria to Antibiotics and *C. cassia* Bark-Derived Cinnamaldehyde

		dose (mg/disk)				
compound	bacterial strain[a]	0.01	0.1	0.5	1.0	5.0
cinnamaldehyde	*Bi. longum*	−[b]	−	−	+	++
	Bi. bifidum	−	−	+	+++	++++
	L. acidophilus	−	−	−	−	+
	C. perfringens	−	−	++	+++	+++
	B. fragilis	−	−	+++	++++	++++
tetracycline	*Bi. longum*	+++	++++	++++	nd[c]	
	Bi. bifidum	+	+++	++++	nd	
	L. acidophilus	++	++++	+++	nd	
	C. perfringens	+++	++++	++++	nd	
	B. fragilis	+++	++++	++++	nd	
chloramphenicol	*Bi. longum*	−	+	++++	nd	
	Bi. bifidum	−	++++	++++	nd	
	L. acidophilus	+++	++++	++++	nd	
	C. perfringens	+++	++++	++++	nd	
	B. fragilis	+++	++++	++++	nd	

[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 >30 mm, ++++; 21−30 mm, +++; 16−20 mm, ++; 10−15 mm, +; and <10 mm, −. [c] Not determined.

Due to its potent growth-inhibiting activity toward test bacteria, cinnamaldehyde was compared with tetracycline and chloramphenicol (Table 3). The inhibitory activity was much more pronounced in *C. perfringens*, *B. fragilis*, and *Bi. bifidum*, as compared to *Bi. longum* and *L. acidophilus*. In the test using 5 mg/disk, cinnamaldehyde produced a very clear inhibitory effect on *Cl. perfringens* (+++), *B. fragilis* (++++), and *B. bifidum* (++++). For *Bi. longum* and *L. acidophilus*, moderate (++) and weak (+) growth-inhibiting activities were produced by cinnamaldehyde, respectively. At 1 mg/disk, growth of *Cl. perfringens*, *B. fragilis*, and *Bi. bifidum* was significantly inhibited, whereas weak or no inhibitory activity was obtained against *Bi. longum* and *L. acidophilus*. Application of 0.5 mg/disk did not adversely affect the growth of the bifidobacteria or *L. acidophilus*. Growth of *Cl. perfringens* and *B. fragilis* was greatly inhibited.

Antibiotics are known to cause disturbance of intestinal microbiota, which results in various diseases or abnormal physiogical states. Therefore, the effect of antibiotics on intestinal bacterial growth was determined (Table 3). Tetracycline and chloramphenicol significantly inhibited growth of all bacteria with the exception of *Bi. longum* at 0.01 mg/disk. The growth-inhibiting effect of tetracycline toward *B. longum* was more pronounced than that of chloramphenicol, indicating that this organism may be more tolerant to the these antibiotics, as compared to the other bacteria. Cinnamaldehyde did not cause any adverse effects against the bacteria at 0.1 mg/disk.

DISCUSSION

The intestinal microbiota in healthy people remains relatively constant but is known to be significantly influenced by physical, biological, chemical, environmental, or host factors (Hentges, 1983; Rasic, 1983). Accordingly, alterations to the microbiota may cause abnormal physical conditions or diseases. In our study, *C. cassia* bark-derived materials showed growth-inhibiting activities toward five intestinal bacteria. Antibacterial properties of spices, essential oils, and their constituents have been also reported (Ismaiel and Pierson, 1990; Moleyar and Marasimham, 1992; Kim et al., 1995).

Among the intestinal microorganisms, bifidobacteria are often considered to play important roles in metabolism such as amino acid (Matteuzzi et al., 1978) and vitamin production (Rasic and Kurmann, 1983), aid in defense against infections (Hentges, 1983), association with longevity (Mitsuoka and Hayakawa, 1973), antitumor activities (Tsuyuki et al., 1991), pathogen inhibition (Bullen and Willis, 1971; Rasic, 1983), improvement of lactose tolerance of milk products (Savaiano and Levitt, 1987), and immunopotentiation (Pereyra and Lemonnier, 1993; Perdigon et al., 1995). Bifidobacterial growth-promoting factors, usually called bifidus factors, have been extensively studied since György et al. (1954) suggested their existence in human milk. Bifidus factors are classified into lacteal secretions, fructooligosaccharides, derivatives of lactose, xylooligosaccharides, and peptides (Modler et al., 1990; Ibrahim and Bezkorovainy, 1994). Clostridia are possible causative agents of a variety of human diseases such as sudden death, toxicity, mutagenesis, carcinogenesis, or aging 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 (Modler et al., 1990; Hughes and Hoover, 1991).

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 which result in the prevention and treatment of various diseases caused by pathogens in the gastrointestinal tract. In recent years, much concern has been focused on selective plant-derived growth modulators in the intestine, on the basis of the fact that many medicinal plant-derived materials are relatively nontoxic to human. For example, extracts from ginseng (*Panax ginseng* C. A. Meyer) and green tea (*Thea chinensis* L.) have been shown not only to enhance the growth of bifidobacteria but to selectively inhibit various clostridia (Ahn et al., 1990a,b, 1991). In our study, the growth responses of *C. cassia* bark-derived cinnamaldehyde varied according to bacterial strain tested. Growth-inhibiting activity was more pronounced in *Cl. perfringens* and *Bi. fragilis*, as compared to the bifidobacteria and *L. acidophilus*. Growth of *B. longum* was mildly affected at higher doses (5 mg/disk), whereas potent growth-inhibiting activity

toward *Bi. bifidum* was obtained. These results suggest that intake of *C. cassia* bark-derived materials by an infant should be limited since *Bi. bifidum* is more common to the intestinal tract of infants. Although cinnamaldehyde did not cause any adverse effects toward growth of all the test bacteria at 0.1 mg/disk, tetracycline and chloramphenicol significantly inhibited the growth of all test bacteria, indicating an alteration of intestinal microbiota that could result in an abnormal physiological state. The inhibitory effect of tetracycline toward *Bi. longum* was more pronounced than that of chloramphenicol, although Rasic and Kurmann (1983) reported that bifidobacteria have moderate tolerance to penicillin and tetracycline but are susceptible to chloramphenicol. Investigations have shown that cinnamaldehyde has antibacterial (Moleyar and Narasimham, 1992; Bowles and Miller, 1993), antifungal (Thompson, 1989; Vaughn and Spencer, 1994) and antimutagenic properties (Kakinuma et al., 1984).

It has been reported that populations at risk for carcinoma of the intestine have higher rates of carriage of clostridia (Finegold et al., 1975; Mastromarino et al., 1978), suggesting that the organism may play a role in tumor formation by production of *N*-nitroso compounds or aromatic steroids, which are possibly carcinogenic (Modler et al., 1990; Hughes and Hoover, 1991). It may be of great interest to investigate relationships of growth-inhibiting action of cinnamaldehyde against clostridia. Epidemiological investigations of gastric cancer have reported a negative relationship between death related to gastric cancer and frequent intake of green tea (Oguni et al., 1983; Tajima and Tominaga, 1985). Green tea components such as polyphenols may be effective in inhibiting the formation of carcinogens (Kuwata et al., 1988). More recent *in vivo* investigations using human volunteers have shown that intake of ginseng extract favorably affected the fecal microbiota and biochemical aspects of feces (Ahn et al., 1990b).

In conclusion, our results indicate that *C. cassia* bark-derived materials have growth-inhibiting effects in vitro against specific bacteria from the human intestine. On the basis of our limited data and some earlier findings, the inhibitory action of cinnamaldehyde toward *Cl. perfringens* may be an indication of at least one of the pharmacological actions of *C. cassia* bark, although many aspects of its therapeutic effects are thought to be due to tannins (Morimoto et al., 1986). Further work is necessary to establish whether this activity is exerted in vivo after consumption of *C. cassia* bark by humans.

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