Selective Growth Inhibitor toward Human Intestinal Bacteria Derived from *Pulsatilla cernua* Root

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Among 21 medicinal plants, the growth-inhibiting activity of *Pulsatilla cernua* root-derived materials toward human intestinal bacteria was examined by using an impregnated paper disk method. The biologically active components of *P. cernua* roots were characterized as 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid by spectroscopic analysis. The activity was compared with that of six commercially available cinnamic acid derivatives *trans*-cinnamaldehyde, *trans*-cinnamic acid, cinnamyl alcohol, 2-methoxycinnamic acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid. The growth responses varied with each bacterial strain tested. Two isolated compounds revealed a potent inhibition against *Clostridium perfringens*, and moderate to weak activity against *Escherichia coli* was exhibited by 4-hydroxy-3-methoxycinnamic acid. Weak or no inhibitory activity was obtained against the bifidobacteria or *Lactobacillus acidophilus*. The inhibitory effect was much more pronounced in *C. perfringens* and *E. coli* as compared to *B. adolescentis*, *B. bifidum*, *B. fragilis*, *B. longum*, or *L. acidophilus*. Cinnamaldehyde exhibited a strong growth-inhibiting activity, but no inhibition was observed from treatments with *trans*-cinnamic acid, cinnamyl alcohol, 2-methoxycinnamic acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid. These results may be an indication of at least one of the pharmacological actions of *P. cernua* root.

Keywords: *Bifidobacteria; clostridia; 3,4-dihydroxycinnamic acid; 4-hydroxy-3-methoxycinnamic acid; intestinal bacteria; Pulsatilla cernua*

INTRODUCTION

Various microorganisms are resident in the human intestinal tract, which is known as a highly complex ecosystem with considerable species diversity. It has been well-established that the microbiota not only participates 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 (*1*, *2*). This biotransformation may influence drug efficacy, toxicity, carcinogenesis, and aging (*1*−*3*). 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 (*3*, *4*). The composition of the microbiota may also be influenced by factors such as diet and stress (*3*, *5*). The microbiota of cancer patients, patients with Alzheimer's disease, or elderly subjects is known to be mainly composed of clostridia and eubacteria with a few lactic acid-forming bacteria (*4*, *6*−*9*). Disturbance of the microbiota may cause a variety of diseases or abnormal physiological states.

Currently, 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 *Escherichia coli* since plants constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects (*10*−*13*). An earlier report confirmed that among medical plants, the methanol extract of *Pulsatilla cernua* roots revealed a potent growth-inhibiting activity toward *Streptococcus mutans* (*14*). This plant species not only is important as an herbicide but also is considered in East Asia to have medicinal properties, such as antiinflammatory, antimutagenic, antitumor, and antimicrobial activities (*13*−*16*). However, little work has been carried out on the effects of *P. cernua* (common name: Hulmi-flower) root-derived materials on growth of intestinal microorganisms as compared to other areas of intestinal microbiology despite its excellent pharmacological action.

In this investigation, we assessed the growth-inhibitory effects of 21 medicinal plants against human intestinal bacteria to develop new and safer types of antimicrobial agents. Among 21 medicinal plants, the active components of *P. cernua* roots to human intestinal bacteria were isolated and characterized by spectroscopic analysis. Additionally, the antimicrobial activities of commercially available cinnamic acid derivatives are also presented in relation to the results obtained.

MATERIALS AND METHODS

Chemicals. *trans*-Cinnamic acid, cinnamyl alcohol, and *trans*-cinnamaldehyde were obtained from Sigma (Sigma Chemical Co., St. Louis, MO). 2-Methoxycinnamic acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade.

Plants and Sample Preparation. Twenty-one plant samples consisting of fruit (2), leaf (3), root (12), seed (3), and stem (1) were collected from a market in Seoul, Republic of Korea. The plant materials were dried in the shade, finely powdered by using a blender and stirring with methanol for

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Table 1. Growth-Inhibitory Activity of Methanol Extracts of Medicinal Plants against Intestinal Bacteria

plant species	bacterial strain[a]				
	B. longum	*B. bifidum*	*B. adolescentis*	*C. perfringens*	*E. coli*
Clematis florida	++[b]	−	−	−	−
Codonopsis pilosula	−	−	−	−	−
Corydalis tutrschaninovii	−	−	++	++	−
Crataegus maximowiczii	−	−	−	−	−
Curcuma longa	−	−	−	++++	++
Epimedium koreanum	−	−	−	−	−
Equisetum hyemale	−	−	−	−	−
Eucommia ulmoides	−	−	−	+++	++
Euonymus japonica	−	−	−	−	−
Gastrodia elata	−	−	+	−	−
Imperata cylindrica	−	−	−	−	−
Liriope platyphylla	−	−	−	−	−
Lycium chinense	−	−	−	−	+
Pleuropterus multiflorus	−	−	−	−	−
Polygala tatarinowi	−	−	−	−	−
Pulsatilla cernua	−	−	+	++++	+++
Rheum undulatum	−	−	−	−	−
Schizandra chinensis	−	−	−	−	−
Schizandra nigra	−	−	−	++	++
Scrophularia buergeriana	−	−	−	−	−
Sinomenium acutum	−	−	++	+++	++

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

24 h at room temperature, extracted twice, and filtered (Toyo Filter Paper No. 2). The filtrate was concentrated in vacuo at 35 °C.

Bacterial Strains and Culture Conditions. The bacterial strains used in this study were as follows: *Bacteroides fragilis* ATCC 25289, *Bifidobacterium bifidum* ATCC 29521, *B. longum* ATCC 15707, *B. adolescentis* ATCC 15073, *Clostridium perfringens* ATCC 13124, *Lactobacillus acidophilus* KCTC 3145, and *Escherichia coli* ATCC 11775 isolated from human feces. Stock cultures of these 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 Co., Ltd, Tokyo, Japan). The plates were incubated anaerobically 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 Lab., Grass Lake, MI). The bacteria were then grown in EG broth (pH 6.8).

Isolation and Identification. The roots (4.1 kg) from *P. cernua* (family Ranunculaceae), which were purchased as a commercially available product, were dried in an oven at 60 °C for 3 days, finely powdered, extracted twice with methanol (10 L) at room temperature, and then filtered (Toyo Filter Paper No. 2, Japan). The combined filtrate was concentrated in vacuo at 35 °C to yield crude extract (9.2%, based on the dry weight of the root). The extract (20 g) was sequentially partitioned into hexane (2.9 g), chloroform (6.6 g), ethyl acetate (2.1 g), butanol (2.3 g), and water-soluble (6.1 g) portions for subsequent bioassay. The organic solvent portions were concentrated to dryness by rotary evaporation at 35 °C, and the water portion was freeze-dried.

The ethyl acetate portion (10 g) was chromatographed on a silica gel column (Merck 70-230 mesh, 500 g, 70 × 5.5 cm i.d.) and successively eluted with a stepwise gradient of ethyl acetate/methanol (0, 5, 10, 15, 20, and 25%). The active 5% fraction was chromatographed on a silica gel column and eluted with a stepwise gradient of chloroform/methanol (0, 10, 20, 30, 40, and 50%). The active 30% fraction was collected and analyzed by TLC (hexane/ethyl acetate, 3:1). Fractions with a similar TLC pattern were combined. The active fraction was chromatographed on a Sephadex LH-20 column (Pharmacia 25-100 mesh, 200 × 3.5 cm i.d.) and eluted with methanol/chloroform (4:1). For further separation of the biologically active substance(s), a Waters Delta Prep 4000 HPLC was used. The column was a 300 × 39 mm i.d. Bondapak C_{18} (Waters) that was eluted by using a stepwise gradient of methanol−water (30, 40, 50, 60, 70, 80, 90, and 100%) at a flow rate of 1 mL/min and UV detection at 254 nm. Finally, one potent active principle (**1**) was isolated from the 30% methanol/water fraction, and the other principle (**2**) potent active principle was isolated from the 60% methanol/water fraction.

Structural determination of the active isolates was based on spectroscopic analysis. ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded with a Bruker AM-500 spectrometer (Rheinspettem, Germany), and chemical shifts are given in parts per million. Ultraviolet spectra were obtained on a Waters 490 spectrometer (Massachusetts, USA), and mass spectra were obtained on a JEOL JMS-AX 302 WA spectrometer (Tokyo, Japan).

Microbiological Assay. For assaying the inhibitory effect of each test sample on the microorganisms, bacteria (0.1 mL) was suspended in 1 mL of sterile physiological saline. An aliquot (0.1 mL) of the bacterial suspensions was seeded on the EG agar. Samples of the extract dissolved in methanol were treated by using a Drummond glass microcapillary to 8 mm paper disks (Advantec, Toyo Roshi, Japan). After evaporation, the disks were placed on EG agar surface and incubated at 37 °C for 2 days in an anaerobic chamber. Control disks were only applied with methanol. All inhibition tests were carried out in triplicates. The growth responses of test samples were determined by making a comparison with those of the controls. The inhibitory responses were classified as previously described (*13*, *17*): inhibitory zone diameter >30 mm, ++++; 21−30 mm, +++; 16−20 mm, ++; 10−15 mm, +; and <10 mm, −.

RESULTS

Growth-inhibitory activities of the intestinal bacteria to the extracts of medicinal plants in East Asia to have some medicinal properties, such as a stomachic agent, an astringent agent, and a carminative agent (*10*), were assayed by the impregnated paper disk method (Table 1). Growth responses to the beneficial bacteria tested varied among plant species, plant parts, and bacterial strains at a concentration of 10 mg/disk. In tests with *B. longum*, which is dominant in the intestines of adults, *Clematis florida* extract moderately inhibited the growth of the bacteria (++), while the remaining samples showed no inhibitory responses. With *B. bifidum*, which is predominant in the intestines of infants, no inhibitory responses were obtained with all plant extracts tested.

Table 2. Growth-Inhibiting Responses of Human Intestinal Bacteria to Various Fractions Obtained from Methanol Extracts of *Pulsatilla cernua* Roots

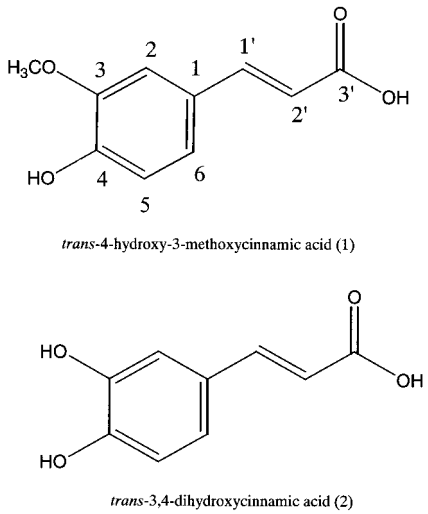
material[a]	bacterial strain[b]					
	B. longum	*B. bifidum*	*B. adolescentis*	*B. fragilis*	*C. perfringens*	*E. coli*
methanol extract	−[c]	−	+	+	++++	+++
hexane fraction	−	−	−	−	−	−
chloroform fraction	−	−	−	−	+	−
ethyl acetate fraction	−	−	+	+	++++	+++
butanol fraction	−	−	−	−	−	−
water fraction	−	−	−	−	−	−

[a] Exposed to 10 mg/disk. [b] 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, −.

Moderate growth-inhibitory responses (++) to *B. adolescentis*, also dominant in the intestines of adults, were determined with extracts of *Corydalis tutrschaninovii* and *Sinomenium acutum*, whereas weak activity (+) was obtained with the extracts of *Gastrodia elata* and *P. cernua*.

The growth inhibition to harmful bacteria also varied with plant species and plant parts. Extracts of *Curcuma longa*, *Eucommia ulmoides*, *P. cernua*, and *S. acutum* showed strong inhibitory activities against *C. perfringens*, and *C. turschaninvii* and *Schizandra nigra* showed a moderate inhibitory response. For *E. coli*, extracts of *P. cernua* revealed strong growth-inhibitory activity (+++), and moderate growth-inhibitory responses (++) were observed in the extracts of *C. longa*, *E. ulmoides*, and *S. acutum*. The remaining samples showed weak or no inhibitory responses against *E. coli*.

Because of the potent inhibitory responses of *P. cernua* extract against *C. perfringens* and *E. coli*, the methanol extract was sequentially partitioned into hexane, chloroform, ethyl acetate, butanol, and water-soluble fractions, and each fraction was evaluated at a concentration of 10 mg/disk (Table 2). A significant inhibitory effect against *C. perfringens* and *E. coli* was observed in the ethyl acetate fraction of *P. cernua*, whereas a weak inhibitory response showed against *B. adolescentis* and *B. fragilis*. No inhibitory response was produced from hexane, chloroform, butanol, and water fractions against human intestinal bacteria tested. Purification of the biologically active compounds from the ethyl acetate fraction was carried out by using silica gel column chromatography, Sephadex LH-20 column chromatography, and HPLC. Finally, two active principles were isolated. Structural determination of the isolates were made by spectroscopic analysis, including EI-MS and NMR and by direct comparison with authentic reference compounds, thus characterizing each as 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid, respectively (Figure 1). The compounds were identified based on the following evidence: 4-hydroxy-3-methoxycinnamic acid ($C_{10}H_{10}O_4$, MW, 194.2); EI-MS (70 eV) *m*/*z* (% relative intensity): M^+ 194 (95), 177 (100), 149 (45), 133 (65), 105 (30), 77 (53), 69 (35), 55 (40). ^{1}H NMR (CD_3OD, 400 MHz): 7.53 (1H, d, J = 15.6 Hz, H-1′), 7.15 (1H, d, J = 1.9 Hz, H-2), 7.04 (1H, dd, J = 8.2, 1.9 Hz, H-6), 6.80 (1H, d, J = 8.2 Hz, H-5), 6.33 (1H, d, J = 15.6 Hz, H-2′), 3.89 (3H, s, -OCH_3). ^{13}C NMR (CD_3OD, 100 MHz): 172.3, 150.1, 149.3, 145.5, 128.3, 123.7, 118.1, 116.5, 111.7, 56.5. 3,4-Dihydroxycinnamic acid ($C_9H_8O_4$, MW, 180.2); EI-MS (70 eV) *m*/*z* (% relative intensity): M^+ 180 (100), 163 (40), 134 (45), 117 (100), 89 (20), 57 (25). ^{1}H NMR (CD_3OD, 400 MHz): 7.39 (1H, d, J = 15.9 Hz, H-1′), 7.01 (1H, d, J = 1.8 Hz, H-2), 6.88 (1H, dd, J = 2.2, 8.1 Hz, H-6), 6.75 (1H, d, J = 8.3 Hz, H-5), 6.25 (1H, d, J = 15.9 Hz, H-2′). ^{13}C NMR (CD_3OD, 100 MHz): 173.87, 148.65, 146.70, 144.06, 128.74, 122.24, 119.74, 116.47, 114.88.



trans-4-hydroxy-3-methoxycinnamic acid (1)

trans-3,4-dihydroxycinnamic acid (2)

Figure 1. Structure of *trans*-4-hydroxy-3-methoxycinnamic acid and *trans*-3,4-dihydroxycinnamic acid.

The inhibitory activity of 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid at 5 and 10 mg/disk was compared to that of commercially available cinnamic acid derivatives (Table 3). Responses varied depending on the chemical and bacterial strain tested. The inhibitory activity of isolated compounds was much more pronounced in *C. perfringens* and *E. coli* as compared to *B. bifidum*, *B. longum*, *B. adolescentis*, and *L. acidophilus*. In the test using 10 mg/disk, 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid produced a very clear inhibitory effect on *C. perfringens* (++++), and 4-hydroxy-3-methoxycinnamic acid showed a clear inhibitory effect on *E. coli* (++++). Furthermore, at 5 mg/disk, the growth effect of *E. coli* was strongly inhibited by 4-hydroxy-3-methoxycinnamic acid. However, at the concentrations of 5 and 10 mg/disk, weak or no inhibition to beneficial bacteria such as bifidobacteria and lactobacilli was exhibited by 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid. In comparison with cinnamic acid derivatives, at the concentrations of 5 and 10 mg/disk, the growth of *B. bifidum* and *C. perfringens* was significantly inhibited (++++) by *trans*-cinnamaldehyde, and strong inhibitory activity (+++) against *B. longum* and *E. coli* was obtained. However, *trans*-cinnamic acid, cinnamyl alcohol, 2-methoxycinnamic acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid did not inhibit any intestinal bacteria tested.

Due to their potent growth-inhibiting activity toward the test bacteria, the growth-inhibiting effect of 4-hy-

Table 3. Growth-Inhibiting Responses of Intestinal Bacteria to Isolated Compounds and Cinnamic Acid Derivatives

		bacterial strain[a]					
plant species	dose (mg/disk)	*B. bifidum*	*B. longum*	*B. adolescentis*	*L. acidophilus*	*C. perfringens*	*E. coli*
4-hydroxy-3-methoxy-cinnamic acid	10	−[b]	−	−	−	++++	++++
	5	−	−	−	−	++++	+++
	2	−	−	−	−	+++	++
	1	−	−	−	−	+++	+
	0.5	−	−	−	−	+	−
	0.1	−	−	−	−	+	−
3,4-dihydroxycinnamic acid	10	−	−	+	−	++++	−
	5	−	−	+	−	++++	−
	2	−	−	+	−	++++	−
	1	−	−	−	−	+++	−
	0.5	−	−	−	−	++	−
	0.1	−	−	−	−	++	−
cinnamic acid	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
cinnamyl alcohol	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
cinnamaldehyde	10	++++	+++	−	++	++++	+++
	5	++++	++	−	+	++++	++
2-methoxycinnamic acid	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
3-methoxycinnamic acid	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
4-methoxycinnamic acid	10	−	−	−	−	−	−
	5	−	−	−	−	−	−

[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, −.

droxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid was determined at lower concentrations (0.1, 0.5, 1, and 2 mg/disk) (Table 3). Responses varied with the chemical and bacterial strains tested. In the test using 1 and 2 mg/disk, significant growth-inhibiting activities (>+++) against *C. perfringens* were exhibited by 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid. For *C. perfringens*, at 0.1 and 0.5 mg/disk, moderate (++) and weak (+) growth-inhibiting activities were produced by 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid, respectively. Furthermore, at 1 and 2 mg/disk, moderate and weak growth-inhibiting activities against *E. coli* were exhibited by 4-hydroxy-3-methoxycinnamic acid. In application of all concentrations tested, the growth of *B. bifidum*, *B. longum*, *B. adolescentis*, and *L. acidophilus* was not inhibited by 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid.

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 (*3*, *5*). Accordingly, alterations to the microbiota may cause abnormal physical conditions or diseases. In the present study, the growth-inhibitory responses of methanol extracts from 21 medicinal plants to 5 intestinal bacteria were investigated in vitro, and *P. cernua* (Ranunculaceae) root-derived materials showed most potent inhibitory activity toward two bacteria of intestinal bacteria tested. In this family, a great number of plant extracts have been investigated for their biological properties (*10*, *15*, *16*). Essential oils and constituents isolated from *P. cernua* have been extensively studied for pharmacological and herbicidal effects. The isolated components in previous investigations were identified as 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid (*15*, *16*, *18*).

Among the intestinal microorganisms, bifidobacteria are often considered to play important roles in metabolism, such as amino acid (*19*) and vitamin production (*20*), and to aid in the defense against infections (*3*) and are associated with longevity (*21*), antitumor activities (*22*), pathogen inhibition (*5*, *23*), improvement of lactose tolerance of milk products (*24*), and immunopotentiation (*25*, *26*). Bifidobacterial growth-promoting factors, usually called bifidus factors, have been extensively studied since György et al. (*27*) suggested their existence in human milk. Bifidus factors are classified into lacteal secretions, fructooligosaccharides, derivatives of lactose, xylooligosaccharides, and peptides (*1*, *28*). 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 (*1*, *2*).

It would therefore be desirable to both inhibit the formation of potential pathogens and/or increase the numbers of bifidobacteria in the human intestine. 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 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, based on the fact that many of 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 also to selectively inhibit various clostridia (*29*, *30*). In this study, the growth responses of *P. cernua* root-derived 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid varied according to the bacterial strain tested. Growth-inhibiting activity of 4-hydroxy-

3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid was more pronounced against *C. perfringens* and *E. coli*, as compared to the bifidobacteria and *L. acidophilus*. These results suggest that intake of *P. cernua* root-derived materials by human might induce the reduction of harmful bacteria such as *C. perfringens* and *E. coli*, while not causing any adverse effects toward growth of beneficial bacteria such as the bifidobacteria and *L. acidophilus* at higher doses. Inhibitory activity of two components isolated from *P. cernua* roots confirms their superiority and usefulness as bacteriocidal agents.

It has been reported that populations at risk for carcinoma of the intestine have higher levels of clostridia (*7*, *8*), suggesting that the organism may play a role in tumor formation by producing N-nitroso compounds or aromatic steroids, which are possibly carcinogenic (*1*, *2*). It may be of great interest to investigate relationships between growth-inhibiting action of 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid against clostridia and cancer. Epidemiological investigations have reported a negative relationship between death related to gastric cancer and frequent intake of green tea (*31*, *32*). Green tea components such as polyphenols may be effective by inhibiting the formation of carcinogens (*33*). 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 (*29*).

A structure−activity comparison of growth-inhibiting activity of the two isolated compounds and cinnamic acid derivatives against intestinal microorganisms has been made. In this study, the growth-inhibiting activity against *E. coli* was much more pronounced in 4-hydroxy-3-methoxycinnamic acid than in 3,4-dihydroxycinnamic acid. These results indicate that the methoxyl group seems to be essential for growth-inhibiting activity against *E. coli*. Ahn et al. (*30*) studied the structure−activity relationship between the six polyphenols derived from *Thea sinensis* leaves and growth inhibition against *C. perfringens* and *C. difficile*. The gallate moiety of polyphenols seemed to be required, but stereochemistry did not appear critical for the inhibitory activity. In the growth-inhibitory response of cinnamic acid derivatives, the growth-inhibiting activity against *C. perfringens* was much more pronounced in *trans*-cinnamaldehyde than other cinnamic acid derivatives. In this study, 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic acid have a modulatory function in the intestine, but *trans*-cinnamaldehyde is not a modulator because of potent inhibitory activities against harmful bacteria such as *C. perfringens* and *E. coli* and beneficial bacteria such as *B. bifidum*, *B. longum*, and *L. acidophilus***.** In previous investigations, cinnamic acid derivatives have antibacterial (*12*, *34*, *35*), antifungal (*36*, *37*), and antimutagenic properties (*38*).

In conclusion, our results indicate that two components isolated from *P. cernua* roots 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 4-hydroxy-3-methoxycinnamic acid and 3,4-dihydroxycinnamic against *C. perfringens* and *E. coli* may be an indication of at least one of the pharmacological actions of *P. cernua* roots. Further work is necessary to establish whether this activity is exerted in vivo after consumption of *P. cernua* roots by humans.

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