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RESEARCH NOTE

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Growth-inhibiting Effects of *Juniperus virginiana* Leaf-Extracted Components toward Human Intestinal Bacteria

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Abstract The growth responses of materials extracted from *Juniperus virginiana* leaves against *Bifidobacterium bifidum*, *B. longum*, *Clostridium perfringens*, *Escherichia coli*, *Lactobacillus acidophilus*, *L. casei*, and *Streptococcus mutans* were examined using impregnated paper disk agar diffusion. The biologically active constituent isolated from the *J. virginiana* extracts was characterized as α-cedrene using various spectroscopic analyses including IR, EI-MS, and NMR. The responses varied according to the dose, chemicals, and bacterial strain tested. Methanol extracts of *J. virginiana* leaves exhibited a strong and moderate inhibitory activity against *C. perfringens* and *E. coli* at 5 mg/disk, respectively. However, in tests conducted with *B. bifidum*, *B. longum*, *L. acidophilus*, *L. casei*, and *S. mutans*, the methanol extracts showed no or weak inhibitory response. At 2 mg/disk, a-cedrene strongly inhibited the growth of *C. perfringens* and moderately inhibited the growth of *E. coli* and *S. mutans*, without any adverse effects on the growth of four lactic acid-bacteria. Of the commercially available compounds originating from *J. virginiana* leaves, cedrol and α-pinene exhibited strong and moderate growth inhibition against *C. perfringens*, and α-copaene revealed moderate growth inhibition against *E. coli* at 1 mg/disk. Furthermore, cedrol exhibited moderate and weak growth inhibition against *S. mutans* at 2 and 1 mg/disk, respectively. However, little or no activity was observed for camphene, (+)-2-carene, *p*-cymene, limonene, linalool, and a-phellandrene against *B. bifidum*, *B. longum*, *C. perfringens*, *L. acidophilus*, *L. casei*, and *S. mutans* at 2 mg/disk. The observed inhibitory activity of the *J. virginiana* leaf-extracted materials against *C. perfringens*, *E. coli*, and *S. mutans* may be an indication of at least one of the pharmacological actions of the *J. virginiana* leaf.

Keywords: *Clostridium perfringens*, growth-inhibiting activity, *Juniperus virginiana*, lactic acid bacteria

Introduction

Intestinal microflora is classified, based on the effects on human health, into beneficial and harmful bacteria. In particular, the harmful bacteria such as *Clostridium*, *Escherichia coli*, *Proteus*, *Pseudomonas*, *Staphylococcus*, and *Veillonella* not only produce carcinogenic substances *de novo* but also change metabolites from dietary sources into tumor initiators or promoters (1). The infectious diseases caused by clostridia have a broad spectrum of clinical severity that ranges from mild outpatient illness to sudden death. Among clostridia, *C. perfringens* has been associated with sudden death, toxicity, and gastrointestinal disease in humans (1, 2). In contrast, bifidobacteria are often taken as useful indicators of human health under most environmental conditions, based on the fact that they play important roles in the metabolism such as amino acid and vitamin production, aid in the defense against infections, and are associated with longevity, pathogen inhibition, and immunopotentiation (3-6). Accordingly, it is desirable to both inhibit the growth of potential pathogens such as clostridia and increase the numbers of bifidobacteria in the human digestive system. Selective growth promoters for bifidobacteria or inhibitors for harmful bacteria are especially important for human health, because intake of these materials can normalize physiological functions that have been disturbed, thereby preventing and treating various diseases caused by pathogens in the gastrointestinal tract (7, 8).

In recent years, much attention has been focused on selective, plant-derived growth modulators in the intestine, based on the fact that most plant-extracted materials are relatively nontoxic to humans (9-12). For example, extracts from *Panax ginseng* and *Thea chinensis* have been shown to not only enhance the growth of bifidobacteria, but to selectively inhibit various clostridia (11, 12). Findings from our previous report confirmed that among 18 medicinal plants, the extract of *Juniperus virginiana* leaves revealed an antimicrobial activity against intestinal bacteria (13). This plant species is not only important as an insecticide, but is considered to possess some biological properties, such as an antibacterial and antioxidant activities (14). However, compared to other areas of intestinal microbiology, relatively little research has been conducted on the effects of *J. virginiana* leaf-extracted materials on the growth of human intestinal microorganisms. We assessed the growth-inhibiting effects of the constituents from *J. virginiana* leaves against human intestinal bacteria in order to develop new and safer types of growth inhibitors against harmful bacteria. In addition, the growth-inhibiting activities of the nine commercially available compounds identified in *J. virginiana* leaves (14, 15) were bioassayed against intestinal bacteria for the comparison.

Materials and Methods

Chemicals Camphene, (+)-2-carene, α-cedrene, cedrol, α-copaene, *p*-cymene, limonene, linalool, α-phellandrene, and α-pinene were provided by Fluka (Fluka Chemical

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Co., Milwaukee, WI, USA). All other chemicals were of reagent grade.

Bacteria strains and culture conditions The bacterial strains used in this study were *Bifidobacterium bifidum* ATCC 29521, *B. longum* ATCC 15707, *Clostridium perfringens* ATCC 13124, *Escherichia coli* ATCC 11775, *Lactobacillus acidophilus* KCTC 3145, *L. casei* ATCC 27216, and *Streptococcus mutans* KCTC 3289 isolated from human feces. Stock cultures of these strains were routinely stored on bacteria culture (Brain Heart Infusion broth (pH 7.6), deMan Rogosa Sharpe broth (pH 5.7), and 25% glycerol) at -80°C and subcultured on EG agar (Eiken Chemical, Tokyo, Japan) when required. The plates were incubated anaerobically at 37°C for 2 days in an anaerobic chamber with an atmosphere of 80% N_2, 15% CO_2, and 5% H_2. The bacteria were then grown in EG broth (pH 6.8).

Isolation and identification *J. virginiana* leaves (1.6 kg) were collected in June 2003 in Chonju (Korea), and dried at room temperature. A voucher specimen was authenticated by Prof. Sang-Hyun Lee at Chonbuk National University and deposited in the herbarium at the Department of Forestry, College of Agriculture, Chonbuk National University. The roots were ground in a blender, extracted twice with methanol (10 L) at room temperature for 2 days, and filtered (Toyo filter paper No. 2, Toyo Roshi, Tokyo, Japan). The combined filtrate was then concentrated *in vacuo* at 45°C using a rotary vacuum evaporator (EYELA autojack NAJ-100, Tokyo, Japan). The extract (322 g) was sequentially partitioned into hexane (49.3 g), chloroform (88.6 g), ethyl acetate (19.4 g), butanol (38.9 g), and water-soluble (125.8 g) portions. The organic solvent portions were concentrated to dryness by rotary evaporation at 45°C, and the water portion was freeze-dried. For isolation, 5 mg of each *J. procera* root-derived fraction in methanol was applied to paper disks (Advantec, 8 mm-diameter and 1-mm thickness, Toyo Roshi). The hexane (10 g × 5) portion was chromatographed on a silica gel column (Merck, 230-400 mesh, 600 g, 5.5 i.d. × 70 cm) and successively eluted with gradient steps of hexane: EtOAc (20:1 to 0:1, v/v) to give 14 fractions (F1-F14) (200 mL each). The bioactive F7 (2.9 g × 5) fraction was rechromatographed on a silica gel column and successively eluted with hexane:EtOAc (7:3, v/v). The column fractions were analysed by TLC and fractions with similar TLC patterns were pooled. In this step, six fractions (F71-F76) were obtained and bioassayed at 5 mg/disk as described below. The active F73 fraction (243 mg × 5) was purified by Prep HPLC (Spectra System P2000, Thermo Separation Products, Madison, WI, USA) to separate the biologically active constituent. The column was mPorasil (20 mm i.d. × 500 mm, Waters, Milford, MA, USA) using hexane: EtOAc (7:3) at a flow rate of 1.8 mL/min and detection was carried out at 260 nm. In this step, nine fractions (F731-F739) were obtained and bioassayed at 5 mg/disk. The active F734 fraction (45 mg × 5) was rechromatographed under the same conditions. Finally, an active compound (156 mg) with a retention time of 24.7 min was isolated. The structure of the active isolate was determined by spectroscopic analyses. ^1H and ^{13}C NMR spectra were recorded in deuterochloroform with a JNM-LA 400F7 spectrometer at 400 and 100 MHz, respectively. UV spectra were obtained in methanol with a Jasco V-550 spectrometer and EI-MS spectra on a JEOL GSX 400 spectrometer.

Growth-inhibiting assay To assay of the effect of the test materials on the growth-inhibiting response of the test microorganisms used, one loopful of bacteria was suspended in 1 mL of sterilized physiological saline. An aliquot (0.1 mL) of the bacterial suspensions was seeded on an EG agar. A sample in 100 μL of a methanol solution was applied using a Drummond glass microcapillary to a paper disk (Advantec 8 mm-diameter and 1-mm thickness). After evaporation of the solvents, the disks were placed on the agar surface inoculated with the test bacteria. All plates were incubated anaerobically at 37°C for 2 days. The control disks received 100 mL of methanol, which exhibited no adverse effect against the organisms used. All tests were performed in triplicate. The inhibitory responses were classified as previously described (16): 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 The growth-inhibiting activities of the five fractions obtained from methanol extracts of *J. virginiana* leaves were assayed toward human intestinal bacteria. In routine screening, the methanol extract exhibited a strong and moderate inhibiting activity against *C. perfringens* and *E. coli* at 5 mg/disk, respectively. However, in tests conducted with *B. bifidum*, *B. longum*, *L. acidophilus*, *L. casei*, and *S. mutans*, the methanol extracts showed no or weak inhibitory response (Table 1). In fractionation, guided by the growth-inhibiting activity at a dose of 5 mg/disk, the hexane fraction exhibited a strong and moderate inhibiting activity against *C. perfringens* and *E. coli*, respectively, but a weak inhibiting activity against *L. acidophilus*, *L. casei*, and *S. mutans* (Table 1). However, no or weak inhibitory activity was observed in the chloroform, ethyl acetate, butanol, and water fractions against the seven intestinal bacteria tested.

Isolation and identification The purification of the biologically active compound from the hexane fraction was performed by silica gel column chromatography and HPLC. Bioassay-guided fractionation of the *J. virginiana* extract afforded an active constituent identified by spectroscopic analyses, including IR, EI-MS, and NMR, and by direct comparison with an authentic reference compound. The active constituent was characterized as α-cedrene, and identified based on the following evidence: (-)-α-cedrene ($C_{15}H_{24}$, MW: 204.3); EI-MS (70 eV) m/z (% relative intensity): M^+ 204 (63), 189 (12), 175 (6), 161 (58), 147 (27), 136 (22), 120 (41), 105 (64), 93 (100), 69 (49), 55 (37), and 41 (70); ^1H-NMR (CD_3OD), δ 5.22 (1H, *d*, *J* = 1.2 Hz), 4.85 (1H, *s*), 2.20 (2H, *t*, *J* = 4.4 Hz), 2.16 (3H, *t*, *J* = 4.8 Hz), 1.85 (1H, *s*), 1.81 (2H, *s*), 1.74 (2H, *s*),

Table 1. Growth-inhibiting responses of *J. virginiana* leaf-derived materials against human intestinal bacteria

Material[1]	Bacterial strain[2]						
	B. bifidum	*B. longum*	*L. acidophilus*	*L. casei*	*C. perfringens*	*E. coli*	*S. mutans*
Methanol extract	+[3]	-	+	+	+++	++	+
Hexane fraction	-	-	+	+	+++	++	+
Chloroform fraction	+	-	-	-	+	-	-
Ethyl acetate fraction	-	-	-	-	-	-	-
Butanol fraction	-	-	+	-	+	+	-
Water fraction	-	+	-	-	-	-	+

[1]Exposed to 5 mg/disk.
[2]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.
[3]Inhibitory zone diameter >30 mm, ++++; 21-30 mm, +++; 16-20 mm, ++; 10-15 mm, +; and <10 mm, -.

1.71 (1H, *m*), 1.34 (2H, *m*), 1.03 (3H, *s*), 0.97 (3H, *s*), and 0.84 (3H, *d*, *J* = 7.2 Hz); ^{13}C-NMR (CD_3OD); 141.4, 120.2, 60.4, 56.2, 55.0, 49.8, 42.9, 41.7, 39.9, 37.1, 28.2, 26.0, 25.7, 25.0, and 15.8.

Growth-inhibiting activity of α-cedrene The growth-inhibiting activity of α-cedrene against the six intestinal bacteria was examined using the impregnated paper disk method (Table 2). The responses varied according to the dose and the bacterial strain tested. In the test with *C. perfringens*, α-cedrene exhibited strong growth inhibition (>21 mm) at 2 and 1 mg/disk and moderate growth inhibition (>16 mm) at 0.5 and 0.25 mg/disk. Furthermore, this isolate revealed moderate activity against *E. coli* and *S. mutans* at 2 and 1 mg/disk and weak activity against *E. coli* at 0.5 mg/disk. However, α-cedrene exhibited little or no inhibition toward *B. bifidum*, *L. acidophilus*, and *L. casei* at 2 mg/disk (Table 2). The results of the current study indicated that the growth-inhibiting activity of α-cedrene was more pronounced in *C. perfringens*, *E. coli*,

Table 2. Growth-inhibiting responses of isolated compounds and *J. virginiana* leaf-derived components against human intestinal bacteria

Compound	Dose (mg/disk)	Bacterial strain[1]					
		B. bifidum	*L. acidophilus*	*L. casei*	*C. perfringens*	*E. coli*	*S. mutans*
α-Cedrene	2.0	-[2]	-	-	++++	++	++
	1.0	-	-	-	+++	++	++
	0.5	-	-	-	++	+	-
	0.25	-	-	-	++	-	-
	0.1	-	-	-	+	-	-
Camphene	2.0	-	-	-	-	-	-
	1.0	-	-	-	-	-	-
(+)-2-Carene	2.0	-	-	-	-	-	-
	1.0	-	-	-	-	-	-
Cedrol	2.0	-	-	-	+++	+	++
	1.0	-	-	-	++	-	+
	0.5	-	-	-	+	-	-
α-Copaene	2.0	-	-	-	-	++	-
	1.0	-	-	-	-	++	-
	0.5	-	-	-	-	+	-
p-Cymene	2.0	-	-	-	-	-	-
	1.0	-	-	-	-	-	-
Limonene	2.0	-	-	-	-	-	-
	1.0	-	-	-	-	-	-
Linalool	2.0	-	-	-	-	-	-
	1.0	-	-	-	-	-	-
α-Phellandrene	2.0	-	-	-	-	-	-
	2.0	-	-	-	-	-	-
α-Pinene	2.0	-	-	-	+++	+	-
	1.0	-	-	-	++	-	-

[1]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.
[2]Inhibitory zone diameter > 30 mm, ++++; 21-30 mm, +++; 16-20 mm, ++; 10-15 mm, +; and <10 mm, -.

and *S. mutans*, as compared to bifidobacteria and lactobacilli. The isolate did not adversely affect the growth of the bifidobacteria and lactobacilli used. These results for the growth inhibitory activity of a-cedrene confirm its superiority and usefulness as a bactericidal agent.

The growth-inhibiting activity of the nine commercially available compounds (camphene, (+)-2-carene, cedrol, α-copaene, *p*-cymene, limonene, linalool, α-phellandrene, α-pinene) identified in *J. virginiana* leaves (14, 15) was examined against the six intestinal bacteria (Table 2). Of the nine compounds, cedrol and α-pinene exhibited strong and moderate growth inhibition against *C. perfringens*, and α-copaene revealed moderate growth inhibition against *E. coli* at 2 and 1 mg/disk. Furthermore, cedrol exhibited moderate and weak growth inhibition against *S. mutans* at 2 and 1 mg/disk, respectively. However, little or no activity was observed for camphene, (+)-2-carene, *p*-cymene, limonene, linalool, and a-phellandrene against *B. bifidum*, *B. longum*, *C. perfringens*, *L. acidophilus*, *L. casei*, and *S. mutans* at 2 and 1 mg/disk. The results of the current study indicated that the growth-inhibiting activity of *J. virginiana* extracts against *C. perfringens*, *E. coli*, and *S. mutans* can be mostly attributed to α-cedrene and cedrol.

In conclusion, the current results indicate that the materials extracted from *J. virginiana* leaves have growth-inhibiting effects *in vitro* against specific bacteria from the human intestine. The observed inhibitory action of the *J. virginiana* components toward *C. perfringens*, *E. coli*, and *S. mutans* may indicate at least one of the pharmacological actions of the *J. virginiana* leaf. Furthermore, the LD_{50} level of α-cedrene was safely administered to rats (LD_{50}: 5,000 mg/kg) by NTP (The National Toxicology 2002 Program, as approved by the U.S. Public Health Service) (17). In this regard, further research is necessary to establish whether this activity is still exerted *in vivo* after human consumption of *J. virginiana* leaves.

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