In Vitro Growth-inhibiting Effects of Leaf Extracts from *Pinus* Species on Human Intestinal Bacteria

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Methanol extracts of leaves from 15 *Pinus* species belonging to the family Pinaceae were tested for their *in vitro* growth-inhibiting activities against 10 bacteria commonly found in the gastrointestinal tracts of human, using impregnated paper disk methods. The inhibitory activities varied with both bacterial strain and *Pinus* species used. At a concentration of 10 mg/disk, a clear growth inhibition was produced from the extracts of *Pinus armandii, P. banksiana, P. bungeana, P. densiflora, P. rigida*, and *P. thunbergii* against *Clostridium perfringens*, whereas all *Pinus* samples revealed weak or little growth-inhibiting activity against *Escherichia coli*, *Bacteroides fragilis*, and *Staphylococcus aureus*. At 5 mg/disk, the extracts of *P. banksiana* and *P. thunbergii* exhibited potent growth inhibition toward *C. perfringens*. All the extracts except the one from *P. densiflora* did not adversely affect growth of *Bifidobacterium adolescentis*, *B. longum*, *B. bifidum*, *B. breve*, *B. animalis*, and *Lactobacillus casei*. The growth-inhibiting activity was more pronounced in *C. perfringens*, as compared to the lactic acid-producing bacteria. These results may be an indication of at least one of the pharmacological activities of these *Pinus* species.

Key words: Pinus *species*, Pinaceae, *intestinal bacteria, growth inhibition.*

In relation to human health, in recent years, much concern has been focused on plant-drived bifidus factors and growth inhibitors against harmful bacteria such as clostridia and *Escherichia coli* because plant materials constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects and have excellent pharmacological actions. In East Asia, *Pinus* species have long been considered to have natural medicinal properties, being rich in terpenoids.[1,2] We already reported that the extracts of *Pinus densiflora* leaves and shoots had potent growth-inhibiting activity against *Clostridium perfringens*.[3] However, the effects of Pinus species on the growth of bacteria commonly found in the gastrointestinal tracts of human have not been fully investigated despite excellent pharmacological activities of *P. densiflora*.

In the laboratory study described herein, we assessed the growth-inhibiting and promoting activities of leaf extracts from 15 *Pinus* species against 4 harmful and 6 lactic acid-producing bacteria.

Materials and Methods

Bacterial strains and culture conditions. The bacterial strains used in this study were as follows: *Bifidobacterium adolescentis* ATCC 15703, *B. longum* ATCC 15707, *B. bifidum* ATCC 29521, *B. breve* ATCC 15700, *B. animalis* ATCC 25527, *Lactobacillus casei* ATCC 27216, *Clostridium perfringens* ATCC 13124, *Bacteroides fragilis* ATCC 25289, *Staphylococcus aureus* ATCC 12600, and *Escherichia coli* ATCC 11775. Stock cultures of these 10 strains were routinely stored on Eggerth-Gagnon liver extract-Fieldes slants at -60°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 5% H_2, 15% CO_2, and 80% N_2 in an anaerobic chamber (Hirayama, Japan). The bacteria were then grown in BHI broth (pH 7.6). All cultures were checked for contamination at the end of the growth cycle.

Plant materials and sample preparation. Leaves of 15 *Pinus* species used in this study were collected from the

Table 1. List of *Pinus* species tested.

Scientific name	Yield (%)[a]
Pinus armandii var. *amamiana*	11.0
Pinus banksiana	10.6
Pinus bungeana	16.4
Pinus densiflora	10.0
Pinus densiflora for. *erecta*	20.2
Pinus densiflora for. *multicaulis*	7.2
Pinus densiflora for. *pendula*	12.0
Pinus griffithii	10.5
Pinus koraiensis	8.5
Pinus parviflora	9.2
Pinus rigida	13.4
Pinus strobus	8.2
Pinus sylvestris	12.4
Pinus taeda	6.8
Pinus thunbergii	15.0

[a](Dried weight of methanol extract/dried weight of sample) 100

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Table 2. Growth-inhibiting responses of harmful intestinal bacteria to leaf extracts of *Pinus* species.

Pinus species	Bacterial Strain[a]								
	C. perfringens ATCC 13124			*S. aureus* ATCC 12600			*B. fragilis* ATCC 25289		*E.coli* ATCC 11775
	10[b]	5	2.5	10	5	2.5	10	5	10
P. armandii var. *amamiana*	++[c]	+	-	+	-	-	+	-	-
P. banksiana	++	++	+	-	-	-	-	-	-
P. bungeana	++	+	-	+	+	-	-	-	-
P. densiflora	++	+	+	-	-	-	-	-	-
P. densiflora for. *erecta*	-	-	-	-	-	-	-	-	-
P. densiflora for. *multicaulis*	+	+	-	-	-	-	-	-	-
P. densiflora for. *pendula*	+	+	+	-	-	-	-	-	-
P. griffithii	+	+	-	+	-	-	-	-	-
P. koraiensis	+	+	-	+	-	-	-	-	-
P. parviflora	+	+	+	-	-	-	-	-	-
P. rigida	++	+	-	-	-	-	-	-	-
P. strobus	+	+	-	-	-	-	-	-	-
P. sylvestris	+	-	+	-	-	-	-	-	-
P. taeda	+	+	+	+	-	-	-	-	-
P. thunbergii	++	++	+	-	-	-	-	-	-

[a]They were cultured on Eggerth-Gagnon agar at 37°C for 2 days in an atmosphere of 5% H_2, 15% CO_2, and 80% N_2.
[b]Unit, /disk (8 mm-diameter and 1-mm thickness).
[c]Inhibitory zone diameter >20 mm, +++, strong response; 16~20 mm, ++, moderate response; 10~15 mm, +, weak response; and <10 mm, -, no response.

Table 3. Growth-inhibiting responses of lactic acid-producing bacteria to leaf extracts of *Pinus* species.

Pinus species	Bacterial Strain[a]					
	B. adolescentis ATCC 15703	*B. animalis* ATCC 25527	*B. bifidum* ATCC 15696	*B. breve* ATCC 15700	*B. longum* ATCC 15707	*L. casei* ATCC 27216
P. armandii var. *amamiana*	-[b]	-	-	-	-	-
P. banksiana	-	-	-	-	-	-
P. bungeana	-	-	-	-	-	-
P. densiflora	+	+	-	-	+	-
P. densiflora for. *erecta*	-	-	-	-	-	-
P. densiflora for. *multicaulis*	-	-	-	-	-	-
P. densiflora for. *pendula*	-	-	-	-	-	-
P. griffithii	+	-	-	-	-	-
P. koraiensis	-	-	-	-	-	-
P. parviflora	-	-	-	-	-	-
P. rigida	-	-	-	-	-	-
P. strobus	-	-	-	-	-	-
P. sylvestris	-	-	-	-	-	-
P. taeda	-	-	-	-	-	-

[a]They were cultured on Eggerth-Gagnon agar at 37°C for 2 days in an atmosphere of 5% H_2, 15% CO_2, 80% N_2.
[b]Exposed to 10 mg/disk (8 mm-diameter and 1-mm thickness). Inhibitory zone diameter >20 mm, +++, strong response; 16~20 mm, ++, moderate response; 10~15 mm, +, weak response; and <10 mm, -, no response.

Forestry Research Institute (Seoul, Korea) on mid-July to late August 1998 and are listed in Table 1. These dried samples were finely powdered using a blender, extracted twice with methanol (100 g/300 ml) at room temperature for 2 days and filtered (Toyo filter No. 2). The combined filtrate was concentrated *in vacuo* at 45°C with rotary evaporator. The yield of each plant extract is shown in Table 1.

Growth-inhibiting assay. For assay of effects of the *Pinus* extracts on the growth-inhibiting responses of the microorganisms used, one loopful of bacteria was suspened in 1 ml of sterile physiological saline. An aliquot (0.2 ml) of the bacterial suspensions was seeded on EG agar. Each sample (2.5, 5, and 10 mg) 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 triplicates.

The growth-inhibiting responses of test samples toward the 10 bacterial strains were examined. The inhibitory responses were classified as previously described.[4,5]

Results and Discussion

The inhibitory activities of the *Pinus* leaf extracts toward harmful bacteria used are given in Table 2. Responses were plant species- and bacterial strain-dependent. In tests with the bacteria at 10 mg/disk, a clear growth inhibition was produced from the extracts of *Pinus armandii*, *P. banksiana*, *P. bungeana*, *P. densiflora*, *P. rigida*, and *P. thunbergii* against *Clostridium perfringens*, whereas all *Pinus* samples revealed weak or little growth-inhibiting activity against *E. coli*, *B. fragilis*, and *S. aureus*. At 5 mg/disk, the extracts of *P. banksiana* and *P. thunbergii* exhibited potent growth inhibition toward *C. perfringens*.

The effects of test samples on growth inhibition of lactic acid-producing bacteria used were investigated (Table 3). All samples did not adversely affect growth of *B. adolescentis*, *B. longum*, *B. bifidum*, *B. breve*, *B. animalis*, and *L. casei*.

In our study, the growth-inhibiting activity was plant species- and bacterial strain-dependent. In particular, the potent growth-inhibiting activities of *P. banksiana* and *P. thunbergii* extracts confirms their superiority and usefulness as selective growth inhibitors against *C. perfringens*. Additionally, they did not affect the growth of *B. adolescentis* and *B. longum*, dominant in intestine of adults, and *B. bifidum* and *B. breve*, dominant in intestine of infants. These results suggest that intake of these plants might be very helpful, regardless of age.

In conclusion, our results indicate that *Pinus* species have growth-inhibiting effects *in vitro* against specific bacteria from the human intestine. Based upon our limited data and some earlier findings, development of functional foods using *Pinus* plants described herein is expected to alter the growth and composition of the microbial community and modulate the genesis of potentially harmful products due to their mild growth-inhibiting activity *in vitro* against specific intestinal bacteria. Further work is necessary to establish whether this activity is exerted *in vivo* after the consumption of functional foods by humans.

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