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Antibacterial Activity of *Pinus densiflora* Leaf-Derived Components Toward Human Intestinal Bacteria

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Abstract The growth-inhibiting effects of *Pinus densiflora* leaf-derived materials on nine human intestinal bacteria were investigated using the impregnated paper disk method, and their activities were compared with those of 13 commercially available terpenes. The biologically active constituent of the extract of *P. densiflora* leaf was characterized as the monoterpene (1*R*)-(+)-α-pinene by various spectroscopic analyses. Responses varied according to bacterial strain, chemicals, and dose. At 10 mg/disk, limonene and (1*R*)-(+)-α-pinene strongly inhibited the growth of *Clostridium perfringens*, *Staphylococcus aureus*, and *Escherichia coli*, without adverse effects on the growth of five lactic acid-bacteria (*Bifidobacterium adolescentis*, *B. bifidum*, *B. longum*, *Lactobacillus acidophilus*, and *L. casei*). Little or no inhibition against seven bacteria was observed with anethole, borneol, camphor, caryophyllene, 1,8-cineole, estragole, linalool, and α-terpineol. Structure-activity relationship revealed that (1*R*)-(+)-α-pinene had more growth-inhibiting activity against *C. perfringens* than (1*R*)-(+)-β-, (1*S*)-(−)-α-, and (1*S*)-(−)-β-pinenes. Furthermore, the growth-inhibition against *L. casei* was much more pronounced in (1*R*)-(+)-β- and (1*S*)-(−)-β-pinenes than (1*R*)-(+)-α- and (1*S*)-(−)-α-pinenes. These results indicate that the (+)-α form seems to be required against *C. perfringens* and β form against *L. casei* for growth-inhibiting activity. Morphologically, most strains of *C. perfringens* were damaged and disappeared at 5 and 2 mg/disk of (1*R*)-(+)-α-pinene. Morphological study revealed that (1*R*)-(+)-α-pinene had more growth-inhibiting activity against *C. perfringens* than (1*R*)-(+)-β-, (1*S*)-(−)-α-, and (1*S*)-(−)-β-pinenes. As naturally occurring growth-inhibiting agents, the *Pinus* leaf-derived materials described above could be useful preventive agents against diseases caused by harmful intestinal bacteria such as clostridia.

Key words: Intestinal bacteria, growth inhibition, morphological activity, *Pinus densiflora*, α-pinene

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Various microorganisms are resident in the human intestinal tract in a highly complex ecosystem with considerable species diversity [12, 26]. They not only participate in the normal physiological functions, but also contribute significantly to the genesis of various disease states, by biotransforming a variety of ingested or endogenously formed compounds to useful or harmful derivatives [12, 26]. Accordingly, these biotransformations may influence drug efficacy, toxicity, carcinogenesis, and aging [11, 23, 24]. Differences in the intestinal bacteria between patients and healthy subjects and between younger and elderly subjects have been observed. A normal gastrointestinal microbiota is found to be predominantly composed of lactic acid bacteria which seem to play a large role not only in metabolism, but also host defense against infection, aging, and immunopotentiation [11, 24]. In contrast, the microbiota of cancer patients is composed of high concentrations of clostridia and eubacteria with few lactic acid bacteria. It has also been reported that elderly subjects harbour fewer bifidobacteria and more clostridia than the younger subjects. Thus, any disturbance of the microbiota may cause a variety of diseases [8, 10, 21].

In relation to human health, many studies have been carried out how to promote beneficial bacteria by intake of milk products incorporated with bifidobacteria [7, 31]. However, the intake of the bifidobacteria supplement does not significantly change the intestinal microbiota, because ingested bifidobacteria do not become easily fixed in the digestive system [28]. Much concern has, therefore, been focused on plant-derived bifidus factors and plant-derived growth inhibitors against harmful bacteria such as *Clostridium perfringens* and *Escherichia coli*, because plants constitute a rich source of bioactive chemicals and many of them do not have largely harmful adverse effects [3, 14–19]. In this regard, methanolic extract of leaves from *Pinus densiflora* has potent growth-inhibiting activity against *C. perfringens* [6, 13].

In the present study, the active components of *P. densiflora* leaves were isolated and characterized by

spectroscopic analyses in order to develop new and safer agents of intestinal bacteria modulators. Additionally, structure-activity relationship between four pinenes, and morphological changes of *C. perfringens* induced by the active constituents, were investigated.

MATERIALS AND METHODS

Chemicals

Anethole, borneol, camphor, caryophyllene, 1,8-cineole, estragole, glutaraldehyde, limonene, linalool, osmium tetroxide, and α-terpineol were purchased from Tokyo Kasei (Tokyo, Japan). (1*R*)-(+)-α-, (1*S*)-(−)-β-, (1*R*)-(+)-β-, and (1*S*)-(−)-β-pinenes were supplied by both Aldrich (Milwaukee, WI, U.S.A.) and Sigma (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade.

Bacterial Strains and Culture Conditions

The bacterial strains used in this study were: *Bifidobacterium bifidum* ATCC 29521, *B. longum* ATCC15707, *B. adolescentis* ATCC15073, *Clostridium perfringens* ATCC13124, *Escherichia coli* ATCC11775, *Lactobacillus acidophilus* KCTC3145, *L. casei* ATCC7469, and *Staphylococcus aureus* ATCC12600 isolated from human feces. Stock cultures of these strains were routinely stored on Eggerth-Gagnon (EG) liver extract-Fielde's slant at −80°C, and were subcultured on EG agar (Eiken Chemical Co., Ltd., Tokyo, Japan) when required. 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, U.S.A.). The bacteria were then grown in BHI broth (pH 7.6).

Isolation and Identification

P. densiflora leaves (5 kg), belonging to the family Pinaceae, were collected in July 2000 at the College of Agriculture, Chonbuk National University, Chonju, Korea. They were ground in a blender, extracted twice with methanol (10 liter) at room temperature for 2 days, and filtered. The combined filtrate was concentrated under vacuum at 45°C to yield ~9.6%. The extract (20 g) was sequentially partitioned into hexane (5.4 g), chloroform (2.6 g), ethyl acetate (1.2 g), butanol (2.9 g), and water-soluble (7.9 g) portions. The organic solvent-soluble fractions were concentrated to dryness by rotary evaporation at 45°C, while the water-soluble fraction was freeze-dried. For isolation, 10 mg of each *P. densiflora* leaf-derived fraction in methanol was applied to paper disks (Advantec, 8-mm diameter and 1-mm thickness, Toyo Roshi) as mentioned below. The hexane (10 g) portion was chromatographed on a silica gel column (Merck 70–230 mash, 700 g, 5.5 i.d.× 80 cm), and successively eluted with chloroform/methanol (10:1, v/v). Column fractions were analyzed by TLC (Silica gel G), and fractions with a similar TLC pattern were pooled. The bioactive fraction (4.1 g) was successively rechromatographed on a silica gel column, using hexane/ethyl acetate (95:5, v/v). For further separation of the biologically active substance, a Waters Delta Prep 4000 HPLC was used. The column [300 mm×39 i.d Bondclone 10 Silica (Phenomenex)] was eluted with hexane at a flow rate of 2 ml/min, and eluate was measured at 254 nm. Finally, a potent active principle (15 mg) was isolated. Structural determination of the active isolate was made by spectroscopic analyses. ^{1}H- and ^{13}C-NMR spectra were recorded in deuterochloroform with a JNM-LA 400F7 spectrometer at 600 and 100 MHz, respectively. UV spectra were obtained in methanol with a HP 8452A diode array spectrometer and EI-MS spectra on a JEOL JMS AX505 spectrometer.

Microbiological Assay

For assay of the *Pinus* leaf-derived materials on the growth-inhibitory responses of the test microorganisms, one loopful of bacteria was suspended in 1 ml of sterilized physiological saline. An aliquot (200 μl) of the bacterial suspensions was seeded on EG agar. A sample dissolved in methanol (100 μl) was applied by syringe to a paper disk (Advantec 8-mm diameter and 1-mm thickness). After evaporation of solvents, the 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 methanol (100 μl). All tests were performed in triplicate. The inhibitory responses were classified, as described previously [3, 14–19]: strong response, +++, zone diameter >20 mm; moderate response, ++, zone diameter 16–20 mm; weak response, +, zone diameter 10–15 mm; and little or no response, −, zone diameter <10 mm.

Scanning Electron Microscopy

The morphological changes of *C. perfringens* induced by the active constituents were observed with a scanning electron microscope (SEM). Strains were prepared by cutting the agar, fixed for a minimum of 4 h in 2.5% (v/v) glutaraldehyde, and then fixed in 1% (wt/v) osmium tetroxide for 1 h. The agar blocks were dehydrated through a graded series of ethanol (50, 70, 90, and 100%; each level was applied twice for 20 min each time) and ethanol:amyl acetate (3:1, 1:1, 1:3, 100% amyl acetate twice for 30 min). The agar blocks on grid were dried with a critical-point drier using liquid CO_2 and coated with gold-coater for 5 min. The coated samples were observed under JSM-5600LV with accelerating voltage of 10 kV.

RESULTS AND DISCUSSION

In routine screening, it was observed that methanol extracts of the *Pinus* leaves showed significant inhibition against

Table 1. Growth-inhibiting activities of *P. densiflora* leaf-derived materials against human intestinal bacteria.

Materials[a]	Bacterial strains[b]					
	B. longum	*B. bifidum*	*B. adolescentis*	*L. casei*	*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]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 >20 mm, +++; 16–20 mm, ++; 10–15 mm, +; and <10 mm, -.

human intestinal bacteria (Table 1). Fractionation revealed that the hexane fraction from methanol extracts, at a dose of 10 mg/disk, showed the strongest growth-inhibiting activity against *L. casei*, *C. perfringens*, and *E. coli*, while showing no adverse effects on the growth of *B. adolescentis*, *B. bifidum*, and *B. longum*, and that the chloroform fraction exhibited weak to moderate inhibitory activity against *B. longum*, *L. casei*, and *C. perfringens* (Table 1). However, no activity was present in the butanol, ethyl acetate, and water fractions. Purification of the biologically active constituents from the hexane fraction was done by silica gel column chromatography and HPLC. Bioassay-guided fractionation afforded an active constituent identified by spectroscopic analyses such as EI-MS and NMR, and also by direct comparison with the authentic reference compound (Figs. 1, 2, 3). The active constituent was identified as monoterpene (1*R*)-(+)-α-pinene.

The growth-inhibiting activity of the isolate and other nine constituents of the *Pinus* leaves [20] against six intestinal bacteria were examined by the impregnated paper disk method (Table 2). Responses varied depending on the chemical, dose, and bacterial strain tested. In a test with *C. perfringens*, (1*R*)-(+)-α-pinene produced strong and moderate growth inhibition at 5 and 2 mg/disk, respectively. Furthermore, this isolate revealed moderate activity against *E. coli* at a dose of 10 mg/disk. However, no growth inhibition against *B. bifidum*, *B. longum*, *L. casei*, and *S. aureus* was observed with (1*R*)-(+)-α-pinene. At 10 mg/disk, limonene showed moderate inhibitory activities against *E. coli* and *S. aureus*. At 10 and 5 mg/disk, no growth inhibition against the six human bacteria was obtained with anethole, borneol, camphor, caryophyllene, 1,8-cineole, estragole, linalool, and α-terpineol.

Infectious diseases caused by clostridia have a broad spectrum of clinical severity that ranges from mild outpatient illness to sudden death. Among the clostridia, *C. perfringens* has been associated with sudden death, toxicity, and gastrointestinal disease in man [4, 9]. In contrast, bifidobacteria are often taken as useful indicators of human health under most environmental conditions, because they play important roles in metabolism such as amino acid [22] and vitamin production [33], aid in the defense against infections [11], association with longevity [25], antitumor activities [34], pathogen inhibition [5, 32], and immunopotentiation [29, 30]. Accordingly, it would be desirable to inhibit the growth of potential pathogens like clostridia and/or increase the

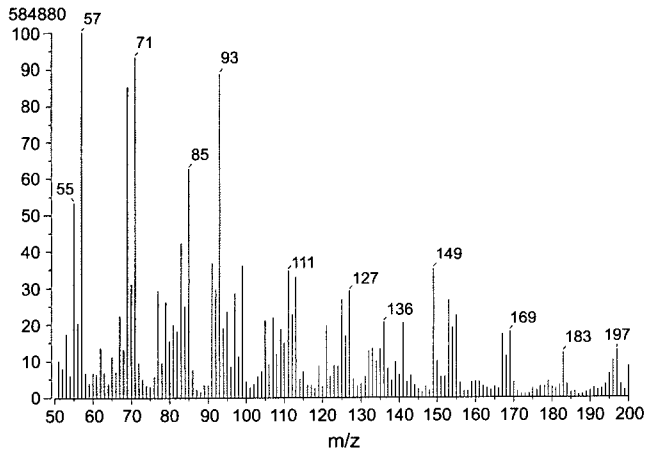

Fig. 1. Mass spectra of (1*R*)-(+)-α-pinene isolated from *Pinus densiflora* leaves.

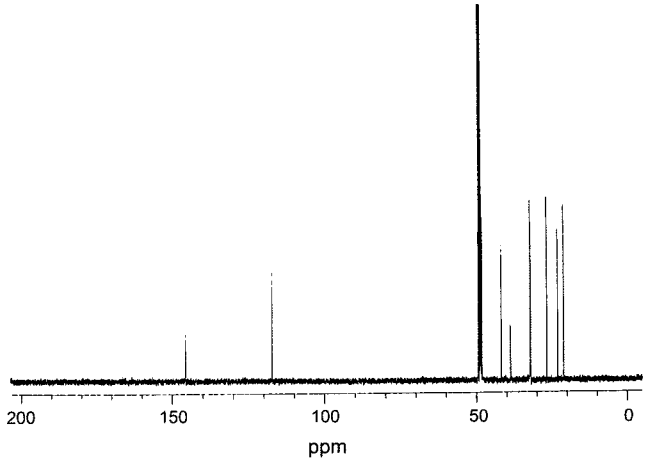

Fig. 2. ^{13}C-NMR spectra of (1*R*)-(+)-α-pinene isolated from *Pinus densiflora* leaves.

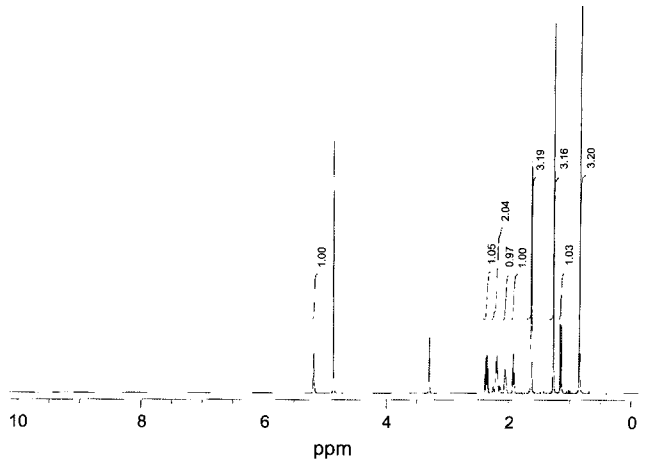

Fig. 3. ^{1}H-NMR spectra of (1*R*)-(+)-α-pinene isolated from *Pinus densiflora* leaves.

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 may normalize disturbed physiological functions, and consequently 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 plant-derived materials are relatively nontoxic to humans. For example, extracts from *Panax ginseng* and *Thea chinensis* have been shown to not only enhance the growth of bifidobacteria, but selectively inhibit various clostridia [1, 2]. In the present study, the growth inhibitory constituents of the *Pinus* leaves were identified as the monoterpene (1*R*)-(+)-α-pinene which was species specific. At a dose of 10 mg/disk, (1*R*)-(+)-α-pinene and limonene strongly inhibited the growth of *C. perfringens*, *S. aureus*, and *E. coli*, respectively, without adverse effects on the growth of three bifidobacteria and two lactobacilli.

Structure-activity relationships between four pinenes and growth inhibition against six intestinal bacteria was determined (Table 3). At 10 and 5 mg/disk, (1*R*)-(+)-α-pinene revealed strong growth-inhibiting activity against *C. perfringens*, and moderate and weak growth-inhibiting activity against *E. coli*, respectively. (1*S*)-(−)-α-, (1*R*)-(+)-β-, and (1*S*)-(−)-β-pinenes at 10 mg/disk did not inhibit growth of *C. perfringens*, *E. coli*, *B. adolescentis*, *B. bifidum*, and *L. acidophilus*. However, (1*R*)-(+)-β- and (1*S*)-(−)-β-pinenes at 5 mg/disk showed moderate growth-inhibiting activity against *L. casei*. In intestinal microorganisms, the structure-activity relationships between the six polyphenols

Table 2. Growth-inhibiting responses of *P. densiflora* leaf-derived monoterpenes against human intestinal bacteria.

Compounds	Dose (mg/disk)	Bacterial strains[a]					
		C. perfringens	*B. longum*	*B. bifidum*	*L. casei*	*S. aureus*	*E. coli*
(1*R*)-(+)-α-Pinene	10	++++[b]	−	−	−	−	++
	5	+++	−	−	−	−	+
	2.5	++	−	−	−	−	−
	1	+	−	−	−	−	−
Limonene	10	−	−	−	−	++	++
	5	−	−	−	−	+	+
	1	−	−	−	−	−	+
Anethole	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
Borneol	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
Camphor	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
Caryophyllene	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
1,8-Cineole	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
Estragole	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
Linalool	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
α-Terpineol	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, −.

Table 3. Structure-activity relationship between four pinenes and growth inhibition against six human intestinal bacteria.

Strains[a]	(1*R*)-(+)-α-Pinene		(1*S*)-(−)-α-Pinene		(1*R*)-(+)-β-Pinene		(1*S*)-(−)-β-Pinene	
	10[b]	5	10	5	10	5	10	5
B. adolescentis	−[c]	−	−	−	−	−	−	−
B. bifidum	−	−	−	−	−	−	−	−
L. acidophilus	−	−	−	−	−	−	−	−
L. casei	−	−	−	−	+++	++	++	++
C. perfringens	++++	+++	−	−	−	−	−	−
E. coli	++	+	−	−	−	−	−	−

[ab]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]mg/disk.
[c]Inhibitory zone diameter >30 mm, ++++; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; and <10 mm, −.

isolated from *T. chinensis* have been studied in the growth-inhibiting activity against *C. perfringens* and *C. difficile*: the gallate moiety of polyphenols seems to be required, but their stereochemistries do not appear critical for the inhibitory activity [32]. In this study, the growth-inhibition against *C. perfringens* was much more pronounced with (1*R*)-(+)-α-pinene than (1*S*)-(−)-α-, (1*R*)-(+)-β-, and (1*S*)-(−)-β-pinenes. Furthermore, the growth-inhibition against *L. casei* was much more pronounced in (1*R*)-(+)-β- and (1*S*)-(−)-β-pinenes than in (1*R*)-(+)-α- and (1*S*)-(−)-α-pinenes. These results indicate that (+)-α form against *C. perfringens* and β form against *L. casei* appear to be required for growth-inhibiting activity.

Because of the potent growth-inhibiting activity of (1*R*)-(+)-α-pinene against *C. perfringens*, the morphological change of *C. perfringens* by the addition of (1*R*)-(+)-α-pinene was compared by SEM (Fig. 4). In the control with no addition of (1*R*)-(+)-α-pinene, the strains of *C. perfringens* appeared not to have changed morphologically (Fig. 4A). However, as shown in Fig. 4B, 5 mg/disk of (1*R*)-(+)-α-pinene caused the most obvious effect. Most strains of *C. perfringens* were damaged and then extensively disappeared by the addition of 5 mg/disk of (1*R*)-(+)-α-pinene, although the strains of *C. perfringens* were still present (Fig. 4B). Furthermore, in this study, 15% strains of *C. perfringens* were morphologically changed by the addition of 2 mg/disk of (1*R*)-(+)-α-pinene. Although strains of *C. perfringens* were much less destroyed at 5 mg/disk than at 2 mg/disk of (1*R*)-(+)-α-pinene, most of strains were still destroyed and disappeared. However, the morphological structure of *C. perfringens* was not changed by the addition of (1*S*)-(−)-α-, (1*R*)-(+)-β-, and (1*S*)-(−)-β-pinenes at 5 mg/disk (data not shown). These results indicate that strong activity of (1*R*)-(+)-α-pinene against *C. perfringens* was also morphologically manifested.

In conclusion, the results indicate that *P. densiflora* leaf-derived materials have growth-inhibiting effects *in vitro* against specific bacteria from the human intestine. Based on the data, the inhibitory action of limonene and (1*R*)-(+)-α-pinene against *S. aureus*, *C. perfringens*, and

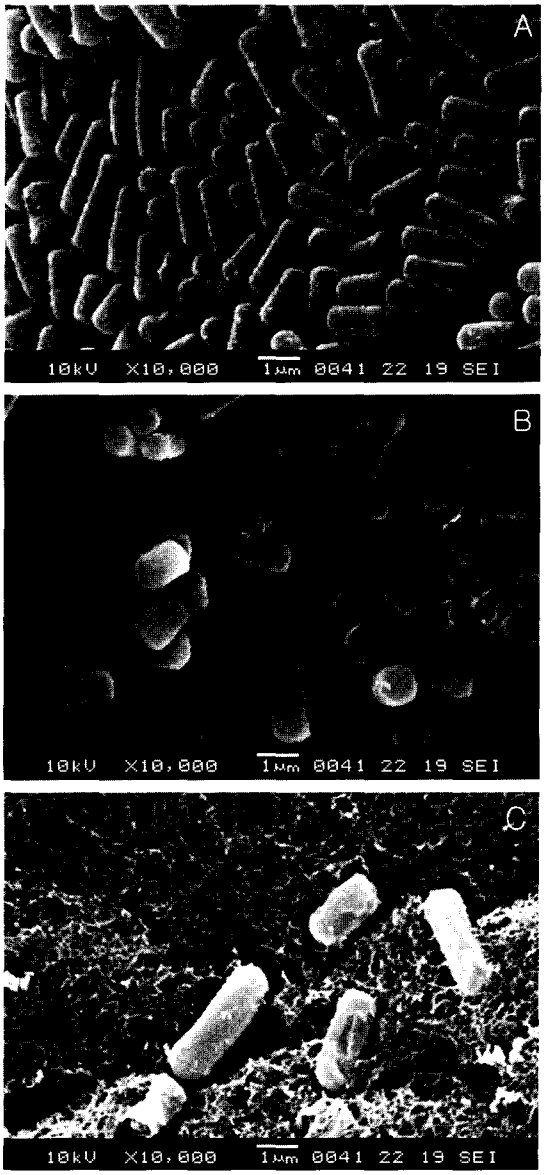

Fig. 4. Morphological effect of *Clostridium perfringens* with no addition (A) and the addition (5 mg/disk (B) and 2 mg/disk (C)) of (1*R*)-(+)-α-pinene.

E. coli may indicate at least one of the pharmacological actions of *P. densiflora* leaves [33]. Furthermore, LD_{50} of α-pinene for the safety in rats (LD_{50}: 3,700 mg/kg) was given by NTP (The National Toxicology 2002 Program was approved by U.S. Public Health Service) [27]. Therefore, further work is warrented to establish whether this activity could occur in humans after consumption of the *Pinus* leaves.

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