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Growth Responses of Seven Intestinal Bacteria Against *Phellodendron amurense* Root-Derived Materials

KIM, MIN-JEONG, SANG-HYUN LEE, JANG-HEE CHO, MOO-KEY KIM, AND HOI-SEON LEE*

Research Center for Industrial Development of Biofood Materials and Institute of Agricultural Science and Technology, College of Agriculture, Chonbuk National University, Chonju 561-756, Korea

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Abstract The growth responses of *Phellodendron amurense* root-derived materials against seven intestinal bacteria were examined, using an impregnated paper disk agar diffusion method and spectrometric method under O_2-free condition. The biologically active constituent of the *P. amurense* root extract was characterized as berberine chloride ($C_{20}H_{18}NO_4Cl$) using various spectroscopic analyses. The growth responses varied depending on the bacterial strain, chemicals, and dose tested. At 1 mg/disk, berberine chloride strongly inhibited the growth of *Clostridium perfringens*, and moderately inhibited the growth of *Escherichia coli* and *Streptococcus mutans* without any adverse effects on the growth of three lactic acid-bacteria (*Bifidobacterium bifidum*, *B. longum*, and *Lactobacillus acidophilus*). The structure-activity relationship revealed that berberine chloride exhibited more growth-inhibiting activity against *C. perfringens*, *E. coli*, and *S. mutans* than berberine iodide and berberine sulfate. These results, therefore, indicate that the growth-inhibiting activity of the three berberines was much more pronounced as chloridated analogue than iodided and sulphated analogues. As for the morphological effect caused by 1 mg/disk of berberine chloride, most strains of *C. perfringens* were damaged and killed, indicating that berberine chloride showed a strong inhibition against *C. perfringens*. As naturally occurring growth-inhibiting agents, the *P. amurense* root-derived materials described could be useful as a preventive agent against diseases caused by harmful intestinal bacteria such as clostridia.

Key words: Intestinal bacteria, growth inhibition, morphological activity, *Phellodendron amurense*, berberine

Various microorganisms are resident in the human intestinal tract in a highly complex ecosystem with considerable species diversity [12, 13, 29]. It is well known that they not only participate in the normal physiological functions, but also contribute significantly to the genesis of various diseases by biotransforming a variety of ingested or endogenously formed compounds into useful or harmful derivatives [28, 29]. Such biotransformations can influence drug efficacy, toxicity, carcinogenesis, and aging [12, 27]. Gastrointestinal ecological investigations have indicated that there are some differences in the intestinal bacteria between patients and healthy subjects and between younger and elderly subjects. The gastrointestinal microbiota of cancer patients or Alzheimer's patients is composed of high concentrations of clostridia and eubacteria, with few lactic acid-producing bacteria [8]. In contrast, normal gastrointestinal microbiota is found to be predominantly composed of lactic acid bacteria, which seem to play a large role in both metabolism and host defense against infection, aging, and immunopotentiation [12, 27]. It has also been reported that elderly subjects harbor fewer bifidobacteria and more clostridia than younger subjects. Accordingly, the disturbance of the microbiota appears to cause a variety of diseases or abnormal physiological states [8, 10, 23].

Although the relationship between the microbial community structure and the health of the host remains to be clearly elucidated, recent attention 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*, since plants constitute a rich source of bioactive chemicals and many of them are largely free of harmful adverse effects [3, 6, 14–15, 17–20]. However, relatively little work has been carried out on the effects of *Phellodendron amurense* root-derived materials on the growth of intestinal microorganisms, despite their excellent pharmacological activity [7, 22, 30].

In the present study, the active component of *P. amurense* roots was isolated and characterized by spectroscopic analyses to develop new and safer agents to modulate

*Corresponding author
Phone: 82-63-270-2544; Fax: 82-63-270-2550;
E-mail: hoiseon@moak.chonbuk.ac.kr

intestinal bacteria. In addition, the antibacterial activities of berberine chlorides and structurally-related components, such as berberine iodide and berberine sulfate, against five intestinal bacteria and the morphological changes of *C. perfringens* induced by berberine chloride were also examined [16].

MATERIALS AND METHODS

Chemicals

Adenine, alanine, amino benzoic acid, asparagine, bacto-casamino acid, bacto-tryptose, bacto-yeast extract, berberine, biotin, L-cysteine, L-cysteine hydrochloride, lactose, folic acid, guanine, nicotinic acid, proteose peptone, pyridoxine, riboflavin, sodium chloride, tryptophan, uracil, and xanthine were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, U.S.A.) and Aldrich Chemical Co. (Milwaukee, WI, 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* ATCC 15707, *B. breve* ATCC 15700, *Clostridium perfringens* ATCC 13124, *Escherichia coli* ATCC 11775, *Lactobacillus acidophilus* KCTC 3145, and *Streptococcus mutans* KCTC 3289 isolated from human feces [21]. Stock cultures of these strains were routinely stored on an Eggerth-Gagnon (EG) liver extract-Fields slant at -80°C and subcultured on an EG agar (Eiken chemical, 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 a BHI broth (pH 7.6) and MRS broth.

Isolation and Identification

The *P. amurense* roots (2 kg), belonging to the family Rutaceae, were purchased from a local market in Seoul, Republic of Korea. 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, Japan). The combined filtrate was then concentrated *in vacuo* at 45°C using a rotary vacuum evaporator (EYELA autojack NAJ-100, Japan). The extract (240 g) was sequentially partitioned into hexane (29.3 g), chloroform (28 g), ethyl acetate (1.3 g), butanol (80.4 g), and water-soluble (101.0 g) portions. The organic solvent fractions were concentrated to dryness by rotary evaporation at 45°C, and the water fraction was freeze-dried. For isolation, 10 mg of each *P. amurense* root-derived methanol fraction was applied to paper disks (Advantec, 8 mm-diameter and 1-mm thickness, Toyo Roshi). The butanol (10 g) fraction was chromatographed on a silica gel column (Merck 70-230 mash, 600 g, 5.5 i.d.×70 cm) and successively eluted with a gradient of chloroform/butanol/methanol (2:1:1, 1:1:1, 1:1:2, 100% methanol, v/v) and benzene/ethyl acetate/*n*-propylalcohol/methanol/ethyl amine (8:4:2:1:1, v/v). The bioactive fraction was rechromatographed on a silica gel column, and successively eluted with chloroform/butanol/methanol (1:1:1, v/v). The active fractions were analyzed by TLC and fractions with similar TLC patterns were pooled. The chemical substance in the active fraction exhibited a yellow spot on TLC, which was developed by benzene/ethyl acetate/*n*-propanol/methanol/ethylamine (8:4:2:1:1, v/v). The spot became orange-red, when reacted with the Dragendorff reagent, suggesting that the chemical substance was alkaloid. For further separation of the biologically active substance, a Japanese Analytical Industry Recycling Preparative HPLC (LC-908W) was used, and eluates were examined for their biological activity. The column was a JAI GEL GS-310 Column using MeOH/MeCN (1:1, v/v) at a flow rate of 5 ml/min and detected at 220 nm. Finally, a potent active principle (15 mg) was isolated. The R_f value of the isolate was 0.65 in the above solvent system. The structure of the active isolate was determined by spectroscopic analyses: The ^{1}H- and ^{13}C-NMR spectra were recorded in deuterochloroform using a JNM-LA 400F7 spectrometer at 600 and 100 MHz, respectively. The UV spectra were obtained in methanol using an HP 8452A diode array spectrometer and the EI-MS spectra obtained using a JEOL JMS AX505 spectrometer.

Microbiological Assay

Growth-Inhibiting Assay. To assay the effect of the test material on the growth-inhibiting response of microorganisms, 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 µl of methanol, which exhibited no adverse effect on the organisms used. All tests were performed in triplicate. The inhibitory responses were classified as previously described [13-19]: 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.

Growth-Promoting Assay. The growth-promoting responses of the *P. amurense* root-derived materials toward human intestinal microorganisms were spectrophotometrically determined. In the experiment for growth-promoting factors derived from noncarbon and carbon sources, a György

Table 1. Growth-inhibiting responses of *P. amurense* root-derived materials against human intestinal bacteria.

Bacterial strain[b]	Material[a]						
	B. longum	*B. bifidum*	*B. breve*	*L. acidophilus*	*C. perfringens*	*E. coli*	*S. mutans*
Methanol extract	-[c]	+	-	+	++++	++	+
Hexane fraction	-	-	-	-	-	-	-
Chloroform fraction	-	-	-	-	-	-	-
Ethyl acetate fraction	-	-	-	-	-	-	-
Butanol fraction	-	+	-	+	++++	+++	+
Water fraction	-	-	-	-	-	-	-

[a]Exposed to 5 mg/disk.

[b]Cultured on Eggerth-Gagnon agar at 37°C for 2 days in 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, -.

(1954) broth modified by Yoshioka (1968) and modified RCM broth media, were used, respectively [11, 34]. One per cent of each culture was inoculated into the test media, and 0.1% of each filter-sterilized material was added to the media in a final volume of 10 ml. Methanol was used as the solvent to dissolve the test materials. The methanol concentration in the solutions was 2%, which was found to have no adverse effect on the bacteria. The cultures were anaerobically incubated at 37°C for 2 days, and the bacterial growth was spectrophotometrically measured at 600 nm. Tubes without bacteria and a sample were used as a reference. The growth-promoting response was expressed as the Growth Increase Rate (GIR) as follows; GIR= A_{600} sample/A_{600} reference. GIR was classified as follows; strong response +++, GIR>2.0; moderate response ++, 2.0>GIR>1.6; weak response +, 1.5>GIR>1.0; and little or no response -, GIR<1.0.

Scanning Electron Microscopy

Morphological changes of *C. perfringens* induced by the active constituent of the *P. amurense* roots were observed with a scanning electron microscope (SEM). The strains were prepared by cutting the agar, fixing for a minimum 4 h in 2.5% (v/v) glutaraldehyde, and then fixing in 1% (wt/v) osmium tetroxide for 1 h. The agar blocks were dehydrated through a graded ethanol series (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 were dried on a grid with a critical-point drier using liquid CO_2, then coated using a gold-coater for 5 min. The coated samples were observed under a JSM-5600LV with an accelerating voltage of 10 kV.

Results and Discussion

The growth-inhibitory activities of human intestinal bacteria toward the fractions obtained from methanol extracts of *P. amurense* roots were assayed using the impregnated paper disk method. In routine screening, the methanol extracts at 5 mg/disk exhibited a potent and moderate-inhibiting activity against *Clostridium perfringens* and *Escherichia coli*, respectively. However, in tests conducted with *Bifidobacterium bifidum*, *B. breve*, *B. longum*, and *Lactobacillus acidophilus*, the methanol extracts showed no or weak inhibitory response (Table 1). To determine the growth-promoting effect on intestinal bacteria, the methanol extracts of *P. amurense* roots were tested on *B. bifidum*, *B. breve*, and *C. perfringens* using two kinds of media: a modified György broth as the carbon-containing medium and modified RCM broth as the carbon-free medium. At 0.1% concentration, the methanol extracts showed little and no growth-promoting activity towards *B. bifidum*, *B. breve*, and *C. perfringens* on both the modified György broth and RCM media (data not shown). The butanol fraction at a dose of 5 mg/disk exhibited a potent and strong growth-inhibitory activity on *C. perfringens* and *E. coli*, respectively (Table 1). However, no activity was observed in the hexane, chloroform, and ethyl acetate fractions. The purification of the biologically active compound from the butanol fraction was performed by silica gel column chromatography and HPLC. The isolated compound was identified as an isoquinoline alkaloid based on the color reaction with the Dragendorff reagent. Bioassay-guided fractionation of the *P. amurense* extract resulted in an active constituent, which was identified by spectroscopic analyses, including IR, EI-MS, and NMR, and also by direct comparison with an authentic reference compound (Figs. 1, 2, 3, and 4). The active constituent was determined as berberine chloride ($C_{20}H_{18}NO_4Cl$).

The growth-inhibitory activity of berberine chloride, berberine iodide, and berberine sulfate against five intestinal bacteria was examined using the impregnated paper disk method (Table 2). The responses varied depending on the chemical, dose, and bacterial strain tested. In the test with *C. perfringens*, berberine chloride exhibited strong growth inhibition at 2, 1, and 0.5 mg/disk, and moderate growth inhibition at 0.2 mg/disk. Furthermore, this isolate revealed moderate activity against *E. coli* and *Streptococcus*

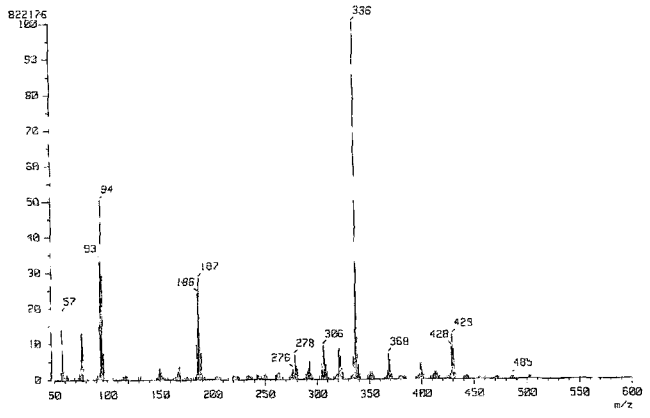

Fig. 1. Mass spectra of berberine chloride isolated from *Phellodendron amurense* roots.

mutans at 2 and 1 mg/disk, and weak activity against *E. coli* and *S. mutans* at 0.1 mg/disk. However, berberine chloride exhibited little or no inhibition of *B. bifidum* and *L. acidophilus* at 2 mg/disk (Table 2). As regards the structure-activity relationships against *C. perfringens*, berberine iodide and berberine sulfate revealed strong and moderate growth-inhibitory activity at 2 and 1 mg/disk, respectively, yet no growth-inhibitory activity toward *B. bifidum*, *E. coli*, and *L. acidophilus* at various concentrations. Berberine iodide and berberine sulfate exhibited weak inhibition against *S. mutans* at 2 and 1 mg/disk. In intestinal microorganisms, the structure-activity relationships between the six polyphenols isolated from *T. chinensis* have been studied for their growth-inhibitory activity against *C. perfringens* and *C. difficile*: the gallate moiety of polyphenols seems to be required, yet their stereochemistries do not appear critical for the inhibitory activity [4]. In the

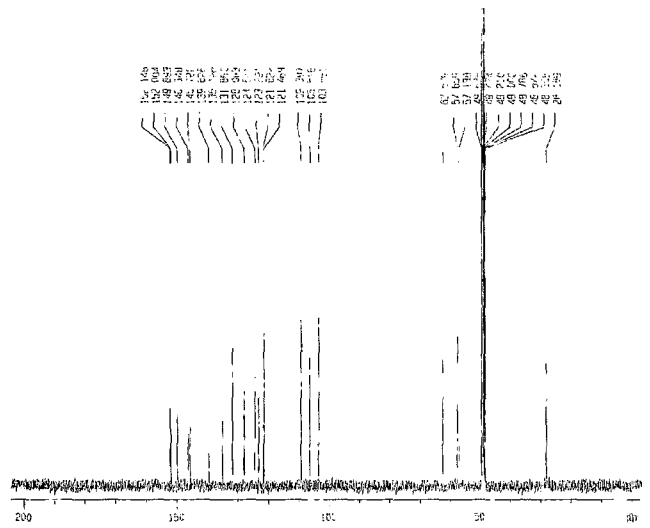

Fig. 2. ^{13}C-NMR spectra of berberine chloride isolated from *Phellodendron amurense* roots.

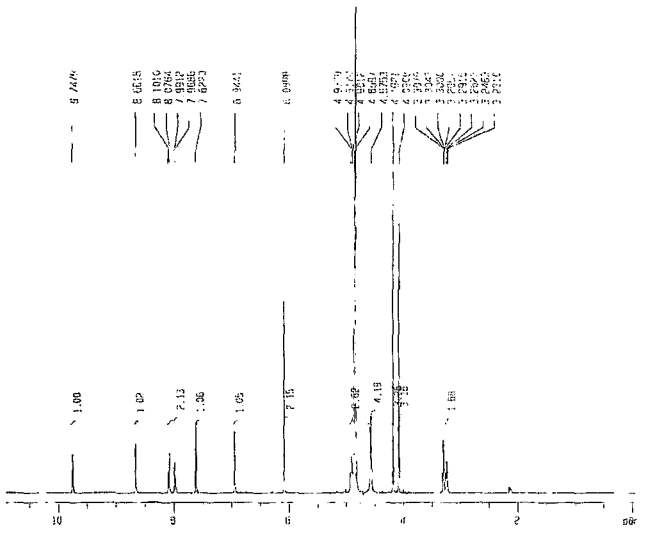

Fig. 3. ^{1}H-NMR spectra of berberine chloride isolated from *Phellodendron amurense* roots.

current study, the results indicated that the growth-inhibiting activity of three berberines against *C. perfringens*, *E. coli*, and *S. mutans* was much more pronounced in a chloridated analogue than iodided and sulfated analogues.

The infectious diseases caused by clostridia have a broad spectrum of clinical severity, ranging from mild outpatient illness to sudden death. Among clostridia, *C. perfringens* has been associated with sudden death, toxicity, and gastrointestinal disease in humans [4, 5, 9]. In contrast, bifidobacteria are often regarded as useful indicators of human health under most environmental conditions, based on the fact that they play important roles in metabolism such as amino acid [26] and vitamin production [33], aid in the defense against infections [12], and are associated with longevity [26], antitumor activities [9, 25], pathogen inhibition [33], and immunopotentiation [31, 32]. Accordingly, it is desirable either to inhibit the growth of potential pathogens such as clostridia and/or increase the numbers of bifidobacteria in the human

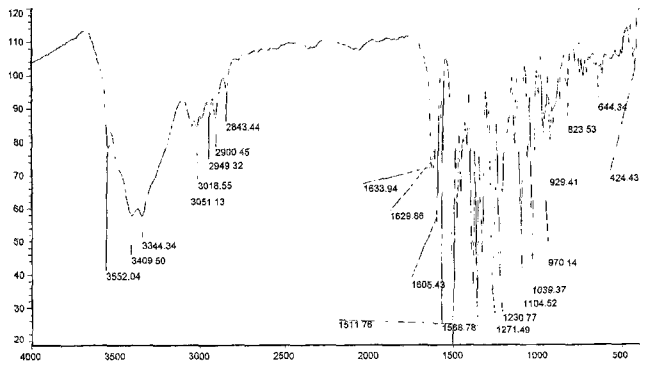

Fig. 4. IR spectrum of berberine chloride isolated from *Phellodendron amurense* roots.

Table 2. Growth-inhibiting responses of various isoquinone alkaloids against human intestinal bacteria.

Compound	Dose (mg/disk)	Bacterial strain[a]				
		B. bifidum	*L. acidophilus*	*C. perfringens*	*E. coli*	*S. mutans*
Berberine chloride	2.0	-[b]	+	++++	++	++
	1.0	-	-	++++	++	++
	0.5	-	-	+++	+	+
	0.2	-	-	++	-	+
	0.1	-	-	+	-	-
Berberine iodide	2.0	-	-	+++	-	+
	1.0	-	-	++	-	+
	0.5	-	-	+	-	-
	0.2	-	-	+	-	-
	0.1	-	-	-	-	-
Berberine sulfate	2.0	-	-	+++	-	+
	1.0	-	-	++	-	+
	0.5	-	-	+	-	-
	0.2	-	-	+	-	-
	0.1	-	-	-	-	-

[a]Bacteria were cultured on Eggerth-Gagnon agar at 37°C for 2 days in 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, -.

digestive system. Selective growth promoters for bifidobacteria or inhibitors for harmful bacteria are especially important for human health, because intake of these materials may alleviate disturbed physiological functions, resulting in the prevention and treatment of various diseases caused by pathogens in the gastrointestinal tract. In recent years, much attention has been focused on selective plant-derived growth modulators in the intestine, based on the fact that most 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 microbial study, the growth inhibitory constituent of *P. amurense* roots was identified as berberine chloride with a species selectivity. At a dose of 1 mg/disk, berberine chloride strongly inhibited the growth of *C. perfringens* and exhibited moderate inhibitory activity against *E. coli* and *S. mutans*, without any adverse effects on the growth of bifidobacteria and lactobacilli.

Because of the potent growth-inhibitory activity of berberine chloride against *C. perfringens*, the morphological change of *C. perfringens* in the presence of berberine chloride was compared by SEM (Fig. 5). In the control with no added berberine chloride, the strains of *C. perfringens* exhibited no morphological change (Fig. 5A). However, the most obvious effect caused by the 1 mg/disk of berberine chloride is shown in Fig. 5B, where most of *C. perfringens* were damaged and extensively destroyed, although *C. perfringens* were still present. As such, these results indicate that the strong activity of berberine chloride was morphologically exhibited against *C. perfringens*.

In conclusion, the current results indicate that *P. amurense* root-derived materials have growth-inhibitory effects *in vitro* on specific bacteria from human intestine. On the basis of this data, the inhibitory action of the *P.*

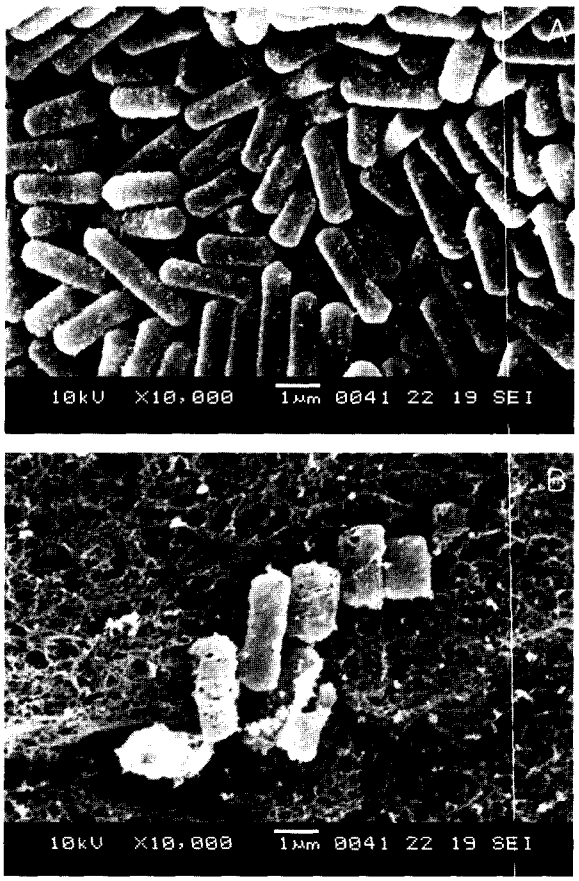

Fig. 5. Morphological effect of *Clostridium perfringens* with no addition (A) and addition (1 mg/disk (B)) of berberine chloride.

amurense-derived isoquinoline alkaloid toward *C. perfringens*, *E. coli*, and *S. mutans* may indicate at least one of the pharmacological actions of *P. amurense* root. In this regard, further work is necessary to establish whether this activity is still active *in vivo* after the consumption of *P. amurense* roots by humans.

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