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Inhibitory Effects of Quinizarin Isolated from *Cassia tora* Seeds Against Human Intestinal Bacteria and Aflatoxin B_1 Biotransformation

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Abstract The growth-inhibitory activity of *Cassia tora* seed-derived materials against seven intestinal bacteria was examined *in vitro*, and compared with that of anthraquinone, anthraflavine, anthrarufin, and 1-hydroxyanthraquinone. The active constituent of *C. tora* seeds was characterized as quinizarin, using various spectroscopic analyses. The growth responses varied depending on the compound, dose, and bacterial strain tested. At 1 mg/disk, quinizarin exhibited a strong inhibition of *Clostridium perfringens* and moderate inhibition of *Staphylococcus aureus* without any adverse effects on the growth of *Bifidobacterium adolescentis*, *B. bifidum*, *B. longum*, and *Lactobacillus casei*. Furthermore, the isolate at 0.1 mg/disk showed moderate and no activity against *C. perfringens* and *S. aureus*. The structure-activity relationship revealed that anthrarufin, anthraflavine, and quinizarin moderately inhibited the growth of *S. aureus*. However, anthraquinone and 1-hydroxyanthraquinone did not inhibit the human intestinal bacteria tested. As for the morphological effect of 1 mg/disk quinizarin, most strains of *C. perfringens* were damaged and disappeared, indicating that the strong activity of quinizarin was morphologically exhibited against *C. perfringens*. The inhibitory effect on aflatoxin B_1 biotransformation by anthraquinones revealed that anthrarufin (IC_{50}, 11.49 μM), anthraflavine (IC_{50}, 26.94 μM), and quinizarin (IC_{50}, 4.12 μM) were potent inhibitors of aflatoxin B_1-8,9-epoxide formation. However, anthraquinone and 1-hydroxyanthraquinone did not inhibit the mouse liver microsomal sample to convert aflatoxin B_1 to aflatoxin B_1-8,9-epoxide. These results indicate that the two hydroxyl groups on A ring of anthraquinones may be essential for inhibiting the formation of aflatoxin B_1-8,9-epoxide. Accordingly, as naturally occurring inhibitory agents, the *C. tora* seed-derived materials described could be useful as a preventive agent against diseases caused by harmful intestinal bacteria, such as clostridia, and as an inhibitory agent for the mouse liver microsomal conversion of aflatoxin B_1 to aflatoxin B_1-8,9-epoxide.

Key words: Anthraquinone, aflatoxin B_1, aflatoxin B_1-8,9-epoxide, *Cassia tora*, intestinal bacteria

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Approximately 400 kinds of microorganisms are resident in the human intestinal tract in a highly complex ecosystem with considerable species diversity [13, 14, 28]. These microorganisms 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 into useful or harmful derivatives [13, 14, 28]. Accordingly, these biotransformations can influence drug efficacy, toxicity, carcinogenesis, and aging [10, 12, 13, 25]. Differences in the intestinal bacteria between patients and healthy subjects and between younger and elderly subjects have already been observed. A normal gastrointestinal microbiota is found to be predominantly composed of lactic acid bacteria, which seem to play a significant role in metabolism and host defense against infection, aging, and immunopotentiation [10, 12, 13, 25]. In contrast, the microbiota of cancer patients is composed of high concentration of clostridia and eubacteria with few lactic acid bacteria. Harmful microflora, such as *Escherichia coil*, *Pseudomonas*, *Staphylococcus*, and *Clostridium* not only produce carcinogenic substances *de novo*, but also change metabolites from dietary sources into tumor initiators or promoters. It has also been reported that elderly subjects harbor fewer bifidobacteria and more clostridia than younger subjects. Thus, any disturbance of the microbiota can cause a variety of diseases and abnormal physiological states [10, 12, 25].

In relation to human health, many studies have already been conducted on methods to promote beneficial bacteria based on the intake of milk products including bifidobacteria

[8, 33]. However, the intake of a supplement including bifidobacteria does not result in any significant changes in the intestinal microbiota, as ingested bifidobacteria do not become easily colonized in the digestive system [29]. Therefore, much concern has been focused on plant-derived bifidus factors and plant-derived growth inhibitors against harmful bacteria, such as clostridia, eubacteria, and *E. coli*, because plants constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects [6, 15, 18-20]. The current authors already reported and confirmed that among 25 leguminous seeds, the methanol extract of *Cassia tora* seeds exhibits a potent growth-inhibitory activity against *C. perfringens* [17, 23]. This plant species is not only important as an antifungicide, but is also considered to possess some medicinal properties, such as antiseptic, diuretic, diarrheal, antioxidant, and antimutagen [7, 21, 39, 40]. However, in spite of these pharmacological activities, relatively little work has been carried out on the inhibitory effects of *C. tora* seed-derived materials on the growth of human intestinal bacteria and formation of the 8,9-epoxide derivative or aflatoxicol from aflatoxin B_1.

Aflatoxins are biologically active, secondary metabolites produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus* [9, 22]. The toxic and carcinogenic effects of aflatoxins are intimately linked with their biotransformation to the corresponding 8,9-epoxides. The main enzymes involved in the biotransformation of aflatoxin B_1 in animals, birds, and fishes are cytochromes P450 from the liver [1, 30]. An alternative biotransformation of aflatoxin B_1 into aflatoxicol is mediated by a reductase, and the product can be reconverted into aflatoxin B_1 by a dehydrogenase. Aflatoxin B_1 reductase and aflatoxicol dehydrogenase have not been well studied, yet this mechanism is considered to be very important, as aflatoxicol may play a role as a reservoir of aflatoxin B_1 in some organisms. The biotransformation of aflatoxin may be influenced by dietary phytochemicals that modulate various biological activities [24]. For example, natural compounds may suppress the formation of aflatoxin epoxides or prevent the dehydrogenation of aflatoxicol retroactively into aflatoxin B_1. Thus, the second objective of the current study was to investigate the effects of five anthraquinones on aflatoxin B_1-8,9-epoxide or aflatoxicol formation from aflatoxin B_1 and study the structural features governing the interaction between anthraquinones and cytochromes P450 or reductases. The result is expected to provide a fundamental basis for further investigation of the effects of specific dietary compounds in mitigating the toxification or promoting the detoxification of aflatoxins.

Therefore, the growth response of the constituents from *C. tora* seeds toward human intestinal bacteria was assessed to develop new and safer types of growth agents for the intestine. In addition, the inhibitory activity of the *C. tora* seeds constituents in suppressing carcinogenic and mutagenic effects of aflatoxins was also considered in relation to the results.

MATERIALS AND METHODS

Chemicals and Reagents

Anthraflavine, anthraquinone, anthrarufin, *m*-chloroperbenzoic acid, dithiothreitol, 1-hydroxyanthraquinone, and quinizarin were provided by Fluka Chemical Corp. (Milwaukee, WI, U.S.A.). Phenylmethanesulfonyl fluoride (PMSF) and reduced glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis. MO, U.S.A.), and aflatoxins B_1, B_2, G_1, and G_2 were from Sigma Chemical Co. and used without further purification. The aflatoxin B_1-8,9-epoxide was synthesized as previously described [16]. Aflatoxin epoxide-glutathione conjugate was biosynthesized according to the protocols of Raney *et al.* [34]. All purchased chemicals were of the highest grade commercially available. Mouse liver and fresh chicken livers were obtained from Dong Nam Laboratory Animal Research Center Co. (Chonju, Chonbuk, South Korea).

Bacterial Strains and Culture Conditions

The bacterial strains used in the current study were: *Bifidobacterium adolescentis* ATCC 15073, *B. bifidum* ATCC 29521, *B. longum* ATCC 15707, *Clostridium perfringens* ATCC 13124, *Escherichia coli* ATCC 11775, *Lactobacillus casei* ATCC 7469, and *Staphylococcus aureus* ATCC 12600 isolated from human feces. Stock cultures of these strains were routinely stored on an Eggerth-Gagnon (EG) liver extract-Fieldes slant at -80°C and, when required, subcultured on an EG agar (Eiken Chemical Co., Ltd., Tokyo, Japan) [27]. 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 an EG broth (pH 6.8).

Isolation and Identification

C. tora seeds (5.1 kg), belonging to the family Leguminosae, were ground in a blender, extracted twice with methanol (10 l) at room temperature for 2 days, and filtered. The combined filtrate was then concentrated under a vacuum at 45°C to yield ~13.1%. Next, the extract (20 g) was sequentially partitioned into hexane (1.9 g), chloroform (4.1 g), ethyl acetate (2.3 g), butanol (3.0 g), and water-soluble (8.7 g) portions. The organic solvent fractions were concentrated to dryness by rotary evaporation at 45°C, while the water fraction was freeze-dried. For isolation, 5 mg of each *C. tora* seed-derived fractions in acetone was applied to paper disks (Advantec, 8 mm-diameter and 1-mm thickness, Toyo Roshi) as mentioned below.

The chloroform (15 g) extract was chromatographed on a silica gel column (Merck 70-230 mash, 900 g, 6.0 i.d.×

90 cm), and successively eluted with a stepwise gradient of chloroform/methanol (0, 10, 20, 30, 40, and 50%). The active 10% fraction (5.3 g) was chromatographed on a silica gel column and eluted with chloroform/methanol (25:1). The column fractions were analyzed by TLC (chloroform/methanol, 20:1), and the fractions with similar TLC patterns combined. The bioactive fraction (2.1 g) was chromatographed on Sephadex LH-20 column (Pharmacia, 800×49 mm, U.S.A.) using chloroform/acetone/methanol (50:2:2). This operation was repeated three times. The active fraction (409 mg) was chromatographed on Polyclar AT column (Touzart and Matignon, 100 g, U.S.A.) packed with chloroform/acetone (50:2) and eluted with an increasing ratio of methanol (1, 2, 5, and 10%). The active fraction (112 mg) was finally purified successively on Sephadex LH-20 column (Pharamacia, U.S.A.) and cellulose (Merck), eluted with chloroform/methanol (3:7), to yield 29.5 mg of the active compound. The structure of the active isolate was determined by spectral analysis. The ^{1}H- and ^{13}C-NMR spectra were recorded using a Bruker AM-500 spectrometer. The UV spectra were obtained by Waters 490 spectrometer, the IR spectra by Biorad FT-80 spectrophotometer, and the mass spectra by JEOL JMS-DX 30 spectrometer.

Growth-Inhibitory Assay

To assay the inhibitory effect of each test sample on the microorganisms, one loopful of bacteria was suspended in 1 ml of sterile physiological saline. Aliquots (0.1 ml) of the bacterial suspensions were then seeded on an EG agar, and samples of the extract dissolved in acetone were applied to paper disks (Advantec, 8 mm, Toyo Roshi, Japan), using a Drummond glass microcapillary. After evaporation, the disks were placed on the EG agar surface and incubated at 37°C for 2 days in an anaerobic chamber. The control disks were treated only with methanol. All the inhibition tests were performed in triplicate. The growth-inhibiting responses of the test samples were determined by comparing with the control. The inhibitory responses were classified as previously described: inhibitory zone diameter >30 mm, ++++; 21-30 mm, +++; 16-20 mm, ++; 10-15 mm, +; and <10 mm, - [3, 19].

Scanning Electron Microscopy

Morphological changes of *C. perfringens* induced by the active *C. tora* seeds constituents were observed using scanning electron microscopy (SEM). The strains were prepared by cutting the agar, then fixing the blocks for a minimum 4 h in 2.5% (v/v) glutaraldehyde and 1 h in 1% (wt/v) osmium tetroxide. Next, 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 then dried on a grid with a critical-point drier using liquid CO_2 and coated with a gold-coater for 5 min. Finally, the coated samples were observed under JSM-5600LV with an accelerated voltage of 10 kV.

Preparation of Animal Liver Homogenates

All preparations were carried out at 4°C. One gram of mouse or chicken liver was homogenized in a glass homogenizer with 15 ml of 100 mM phosphate buffer, pH 7.4, containing 0.4 mM PMSF, 0.1 mM DTT, and 1 mM EDTA. The resultant homogenates were then filtered through four layers of cheesecloth, and centrifuged at 12,100 ×g at 4°C for 20 min using an Eppendorf centrifuge 5417R. The supernatants were saved as crude enzyme extracts, which were then transferred to 15 ml polycarbonate ultracentrifuge tubes and centrifuged at 100,000 ×g at 4°C for 1 h, including the acceleration time, in a Beckman L8-M ultracentrifuge using a Ti 70 rotor. The supernatant was designated as cytosolic fraction. The microsomal pellet was rinsed twice with 4 ml of 200 mM phosphate buffer (pH 7.4), containing 1 mM EDTA, and fully resuspended by a glass homogenizer in 5 ml.

Inhibition of Aflatoxin B_1-8,9-Epoxide Production from Aflatoxin B_1 by Mouse Liver Microsomal Proteins Using Isolated Compound

To detect aflatoxin B_1-8,9-epoxide, mouse cytosolic glutathione *S*-transferase (GST) was employed to conjugate GSH to any aflatoxin epoxide produced by the mouse liver preparations. In a typical experiment, 10 µl of 1,000 ppm aflatoxin B_1 solution (in DMSO) was added to 0.6 ml of a reaction mixture consisting of 92 mM sodium phosphate buffer, pH 7.4, 0.5 mM GSH, 0.5 mM NADPH, and the mouse liver cytosolic fraction (2.0 mg of protein/ml). The mouse liver microsomal fractions (1.5 mg of protein/ml) were then added to the reaction mixture. After preincubation for 10 min at 37°C, NADPH was added to the reaction mixture to initiate the reaction. After 3 h of incubation, the reaction was stopped by the addition of 1 ml of ice-cold methanol containing aflatoxin G_1 (10 µM) as the internal standard. This reaction mixture was then centrifuged at 12,100 ×g for 10 min at room temperature. The supernatant was analyzed by reversed-phase Supelcosil LC-18 column (250×4.6 mm) equipped with a fluorescence detector. The mobile phase was a mixture of water/acetonitrile/methanol (60:20:20).

Inhibition of Aflatoxin B_1 Metabolism into Aflatoxicol by Chicken Liver Cytosol Using Naturally Occurring Compounds

The metabolism of aflatoxin B_1 into aflatoxicol was studied using an incubation mixture (250 µl final volume) consisting of 92 mM sodium phosphate buffer (pH 7.4), 0.5 mM NADPH, and 2 mg/ml protein of the chicken liver cytosol

preparation. After a preincubation period of 10 min at 37°C, aflatoxin B_1 (10 μl of a 1,000 ppm solution in DMSO) was added to the reaction mixture as a substrate. After 1 h of incubation, the reactions were stopped by the addition of 1 ml of ice-cold methanol containing aflatoxin G_1 (10 μM) as the internal standard. This mixture was then centrifuged at 12,100 ×g for 10 min at room temperature. The supernatant was analyzed by a reversed-phase Supelcosil LC-18 column (250×4.6 mm) equipped with a fluorescence detector. The mobile phase was a mixture of water/acetonitrile/methanol (60:20:20). The ID_{50} (expressed as micromolar) was the concentration at which a 50% inhibition of aflatoxin B_1-8,9-epoxide or aflatoxicol formation was reached as calculated from the dose-response curve.

RESULTS AND DISCUSSION

In routine screening, it was observed that the methanol extracts from *C. tora* seeds significantly inhibited the growth of human intestinal bacteria (Table 1). In fractionation, guided by the growth-inhibiting activity at a dose of 5 mg/disk, the chloroform fraction from the methanol extracts showed the strongest growth-inhibitory activity (++++) against *C. perfringens* and *E. coli* without any adverse effects on the growth of *B. adolescentis*, *B. bifidum*, *B. longum*, and *L. casei*, while the hexane fraction exhibited a moderate inhibitory activity (++) against *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 chloroform fraction was then performed by silica gel column, Polyclar AT column, Sephadex LH-20 column, and cellulose chromatographies. The bioassay-guided fractionation of the *C. tora* seed extract yielded an active constituent identified by various spectroscopic analyses and by direct comparison with authentic reference compounds (Figs. 1, 2, 3). This active constituent was identified as quinizarin, and identified based on the following evidence: quinizarin ($C_{14}H_8O_4$, MW, 240); EI-MS (70 eV) *m/z* (% relative intensity): M^+ 240 (100), 239 (91), 212 (20), 183 (25), 155 (9), 128 (27), 102 (20), 77 (13), 51(12); ^{1}H-NMR (CD_3COCD_3, 500 MHz); δ 8.4 (*m*, 4H), 8.0 (*d*, 2H, *J*=8 Hz), 7.4 (*m*, 4H); ^{13}C-NMR (CD_3OD, 100 MHz): 188.1, 158.6, 158.2, 135.8, 134.3, 130.3, 130.2, 130.1, 127.7, 113.7.

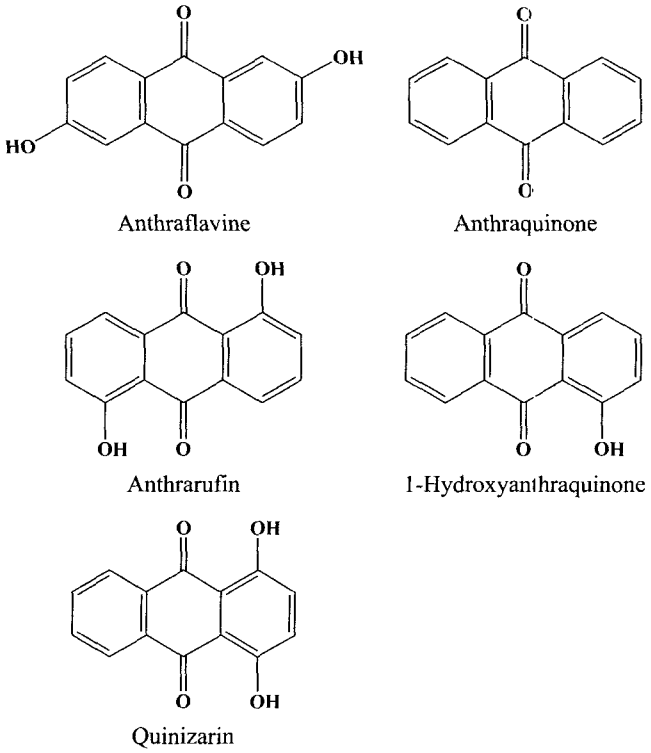

Fig. 1. Structures of quinizarin isolated from *C. tora* seeds and anthraquinones.

The growth-inhibitory activity of the isolate against six intestinal bacteria was examined using the impregnated paper disk method (Table 2). The responses varied according to the compound, dose, and bacterial strain tested. In a test with *C. perfringens*, quinizarin produced strong growth inhibition at 5, 2, 1, and 0.5 mg/disk, and moderate (++)

Table 1. Growth-inhibiting effect of various fractions obtained from methanol extract of *Cassia tora* seeds against human intestinal bacteria.

Material[a]	Bacterial strain[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	-	-	-	-	-	-
H_2O 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, -.

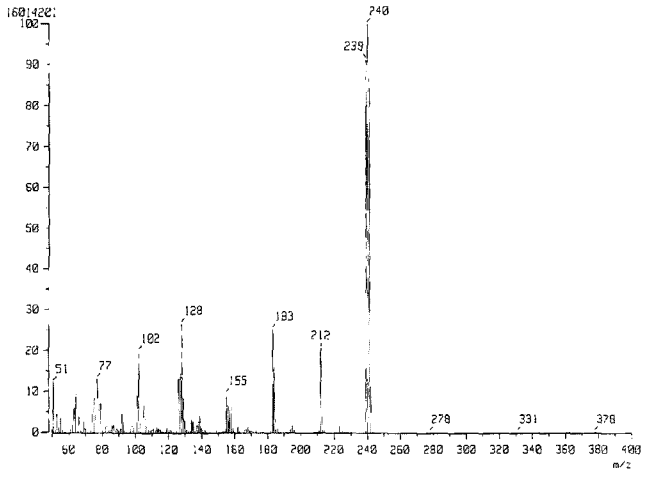

Fig. 2. Mass spectra of quinizarin isolated from *C. tora* seeds.

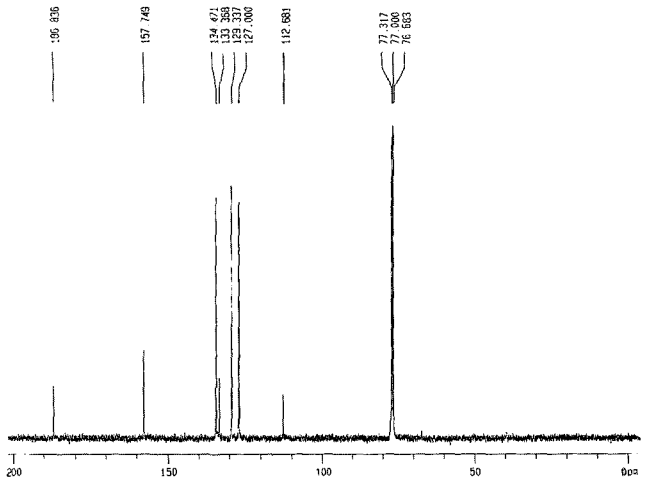

Fig. 3. ^{13}C-NMR spectra of quinizarin isolated from *C. tora* seeds.

growth inhibition at 0.1 mg/disk. Furthermore, the isolate at 5 and 2 mg/disk revealed strong activity against *S. aureus*, moderate activity at 1 mg/disk, and weak (+) activity at 0.5 mg/disk. However, no growth inhibition of *B. adolecentis*, *B. bifidum*, and *L. casei* was observed with quinizarin.

The infectious diseases caused by clostridia have a broad spectrum of clinical severity, ranging from mild outpatient illness to sudden death. Among the strains of clostridia, *C. perfringens* has been associated with sudden death, toxicity, and gastrointestinal disease in human [4, 11]. In contrast, bifidobacteria are often taken as useful indicators of human health under most environmental conditions, based on the fact that they play an important role in metabolism, such as amino acid [26] and vitamin production [36], aid in the defense against infections [13], and are associated with longevity [27], antitumor activities [38], pathogen inhibition [5, 35], and immunopotentiation [31, 32]. Accordingly, it is desirable to either inhibit the growth of potential pathogens, such as clostridia, and/or increase the numbers of bifidobacteria in the human digestive system. Selective growth promoters of bifidobacteria or inhibitors for harmful bacteria are especially important for human health, because the intake of these materials can normalize disturbed physiological functions, resulting 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 plant-derived materials are relatively nontoxic to humans. For example, extracts from *Cassia obtusifolia* and *Pulsatilla cernua* have been shown to not only enhance the growth of bifidobacteria,

Table 2. Growth-inhibiting effect of *Cassia tora* seed-derived compound and anthraquinones against human intestinal bacteria.

Compound	Dose (mg/disk)	Bacterial strain[a]					
		B. adolecentis	*B. bifidum*	*L. casei*	*C. perfringens*	*E. coli*	*S. aureus*
Quinizarin	5.0	-[b]	-	-	++++	-	+++
	2.0	-	-	-	++++	-	++
	1.0	-	-	-	++++	-	++
	0.5	-	-	-	+++	-	+
	0.1	-	-	-	++	-	-
Anthrarufin	0.5	-	-	-	-	-	++
	2.0	-	-	-	-	-	+
Anthraflavine	0.5	-	-	-	-	-	++
	2.0	-	-	-	-	-	+
Anthraquinone	0.5	-	-	-	-	-	-
	2.0	-	-	-	-	-	-
1-Hydroxyanthraquinone	0.5	-	-	-	-	-	-
	2.0	-	-	-	-	-	-

[a]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, -.

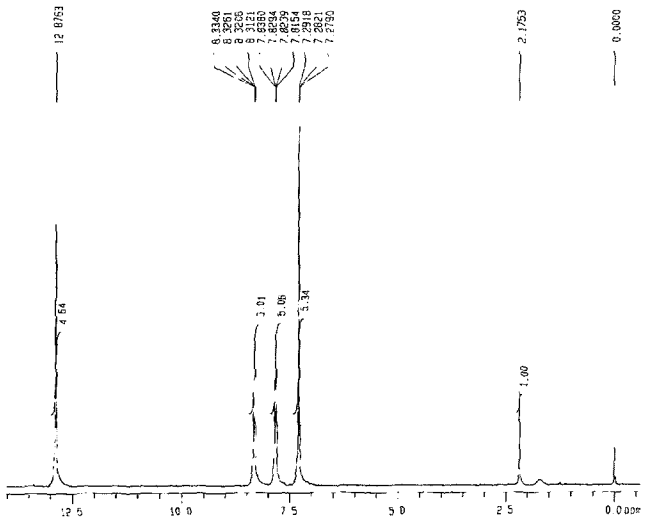

Fig. 4. ^{1}H-NMR spectra of quinizarin isolated from *C. tora* seeds.

but selectively inhibit various clostridia [3, 20]. Accordingly, in the present study, we identified the selective growth inhibitory constituent of *C. tora* seeds as quinizarin. At 5, 2, and 1 mg/disk, quinizarin strongly inhibited the growth of *C. perfringens* and *S. aureus* without any adverse effects on the growth of three bifidobacteria and one lactobacilli.

The activity of structurally-related anthraquinones, including anthrarufin, anthraflavine, and 1-hydroxyanthraquinone, against six intestinal bacteria was examined using the impregnated paper disk method (Table 2). In the structure-activity relationship against *S. aureus*, anthrarufin and anthraflavine revealed a moderate and weak growth-inhibitory activity at 5 and 2 mg/disk, respectively, yet did not inhibit the growth of *B. adolecentis*, *B. bifidum*, *C. perfringens*, *E. coli*, and *L. casei* at the concentrations tested. On the other hand, anthraquinone and anthrone did not inhibit the growth of *B. adolecentis*, *B. bifidum*, *C. perfringens*, *E. coli*, *L. casei*, and *S. aureus* at 5 and 2 mg/disk, respectively. Among intestinal microorganisms, the structure-activity relationships between the six polyphenols isolated from *T. chinensis* have already been studied on their growth-inhibiting activity against *C. perfringens* and *C. difficile*, where the gallate moiety of polyphenols seemed to be required, yet their stereochemistries did not appear to be critical for the inhibitory activity [4]. In the current study, the results indicated that two hydroxyl groups of anthraquinones appeared to be required for growth-inhibiting activity of *S. aureus*. However, it is not clear which factor of anthraquinones inhibited the growth of *C. perfringens*. Therefore, further work is still necessary to identify the factor.

Because of the potent growth-inhibitory activity of quinizarin against *C. perfringens*, the morphological changes of *C. perfringens* induced by quinizarin were also examined by SEM (Fig. 5). In the control with no addition of quinizarin, the strains of *C. perfringens* appeared normal with no change of morphology (Fig. 5A). However, the most obvious effect caused by 1 mg/disk of quinizarin is shown in Fig. 5C: Most strains of *C. perfringens* were damaged and then extensively disappeared at 1 mg/disk of quinizarin (Fig. 5C). Furthermore, the strains of *C. perfringens* were morphologically examined based on a comparison between

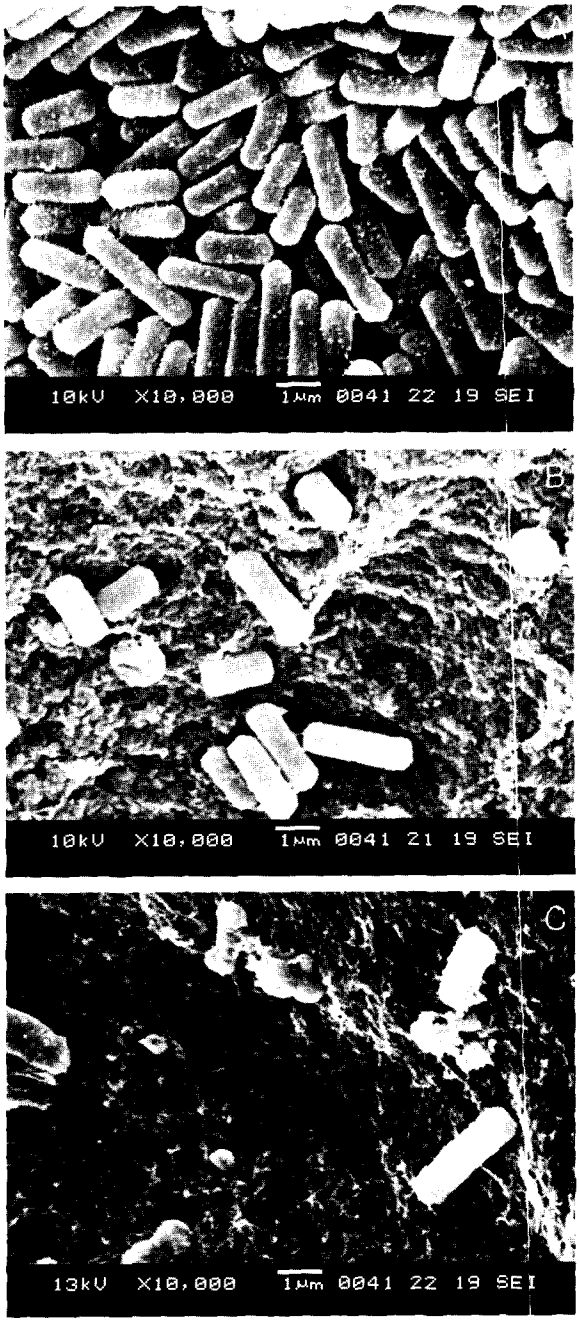

Fig. 5. Morphological change in *Clostridium perfringens* without addition (A) and with addition (0.5 mg/disk (B) and 1 mg/disk (C)) of quinizarin.

Table 3. IC_{50} values for inhibition of biotransformation of aflatoxin B_1 into aflatoxin B_1-8,9-epoxide by *Cassia tora* seed-derived compound and anthraquinones using mouse liver microsomal cytochrome P450.

Compound	IC_{50} (ppm)	IC_{50} (μM)
Quinizarin	0.99	4.12
Anthrarufin	2.75	11.49
Anthraflavine	5.86	26.94
Anthraquinone	>500	-
1-Hydroxyanthraquinone	>500	-

the control and the addition of 0.5 mg/disk of quinizarin (Figs. 5A and B). In the current study, only 15% of the *C. perfringens* were observed after the addition of 0.5 mg/disk of quinizarin. After the addition of 1 mg/disk of quinizarin, even fewer strains of *C. perfringens* remained as most of the strains were destroyed and disappeared. Therefore, these results indicate that the strong activity of quinizarin was morphologically exhibited against *C. perfringens*.

In an additional study on the effect of anthraquinones on the biotransformation of aflatoxin B_1, it was observed that anthrarufin (IC_{50}, 11.49 μM), anthraflavine (IC_{50}, 26.94 μM), and quinizarin (IC_{50}, 4.12 μM) were potent inhibitors of aflatoxin B_1-8,9-epoxide formation (Table 3). However, the addition of anthraquinone and 1-hydroxyanthraquinone did not inhibit the mouse liver microsomal conversion of aflatoxin B_1 into aflatoxin B_1-8,9-epoxide, a metabolically activated mutagenic product. Anthraquinones are tricyclic compounds found in higher plants and fungi, and have been widely used as colorants in foods, cosmetics, hair dyes, textiles, and as phytotherapeutic drugs [37]. Anthraquinones are known as mutagens and the genotoxicity of these compounds are related to their planar structures that are capable of reacting with DNA [41]. Quinizarin potentially inhibits cytochrome P450 1A1/2 ethoxyresorufin-O-deetylase activity [2]. Similarly, quinizarin strongly inhibits aflatoxin B_1-8,9-epoxide formation by mouse liver microsomes. Thus, the two hydroxyl groups in the A ring of anthraquinones may be essential for inhibiting the formation of aflatoxin B_1-8,9-epoxide. Thus, further studies are needed to elucidate the relationship between naturally occurring compounds in diet and the risk of cancer induced by aflatoxins *in vivo*.

In conclusion, the current results indicate that *C. tora* seed-derived materials have growth-inhibitory effects *in vitro* against specific bacteria from the human intestine. On the basis of this data, the inhibitory actions of quinizarin against *C. perfringens*, *S. aureus*, and the mouse liver microsomal conversion of aflatoxin B_1 into aflatoxin B_1-8,9-epoxide may be an indication of at least one pharmacological action of *C. tora* seeds [36]. Further work is necessary to establish whether this activity still occurs *in vivo* after the consumption of *C. tora* seeds by humans.

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