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Antimicrobial Activity of Quinoline Derivatives Isolated from *Ruta chalepensis* Toward Human Intestinal Bacteria

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Abstract The growth responses of *Ruta chalepensis* leaf-derived materials toward human intestinal bacteria were examined. The biologically active constituent of the *R. chalepensis* extract was characterized as quinoline-4-carboxaldehyde ($C_{10}H_7NO$). The growth responses varied depending on the bacterial strain, chemicals, and dose tested. At 0.25 and 0.1 mg/disk, quinoline-4-carboxaldehyde strongly inhibited the growth of *Clostridium perfringens* and weakly inhibited the growth of *Escherichia coli* without any adverse effects on the growth of three lactic acid bacteria. Furthermore, at 0.05 and 0.025 mg/disk, this isolate showed moderate activity against *C. perfringens*. In comparison, chloramphenicol at as low as 0.01 mg/disk significantly inhibited the growth of all bacteria tested, and cinnamaldehyde at 0.25 mg/disk did not inhibit *Bifidobacterium bifidum*, *B. longum*, *E. coli*, and *Lactobacillus acidophilus*, with the exception of *C. perfringens*. The structure-activity relationship revealed that quinoline-3-carboxaldehyde had strong growth inhibition against *C. perfringens*, but quinoline, quinoline-3-carboxylic acid, and quinoline-4-carboxylic acid did not inhibit the growth of *B. bifidum*, *B. longum*, *C. perfringens*, *E. coli*, and *L. acidophilus*. These results indicate that the carboxyl aldehyde functional group of quinolines seems to be required for growth-inhibiting activity against *C. perfringens*, thus indicating at least one of the pharmacological actions of *R. chalepensis* leaf.

Key words: *Clostridium perfringens*, inhibition, intestinal bacteria, quinoline-4-carboxaldehyde, *Ruta chalepensis*

The concept of ingesting live microorganisms for the purpose of improving one's intestinal health and general well-being can be traced back to the beginning of the 20th century [26]. Although numerous genera of bacteria are currently being marketed as probiotic cultures throughout the world, the two most commonly used genera are *Bifidobacterium* and *Lactobacillus*. It is widely accepted that the normal gastrointestinal microflora exert a protective role against attack by enteric pathogens [22]. Bifidobacteria have been successfully used to treat intestinal disorders [21] and to prevent rotaviral diarrhea in children [20]. Some studies also support the use of a *Lactobacillus* strain, specifically *L. rhamnosus* GG, for the prevention [18] and treatment [5, 20] of diarrhea in children. Antibiotic-associated gastrointestinal disturbances are well-recognized problems, and Black *et al.* [2] observed that *Bifidobacterium longum*, delivered with *Lactobacillus acidophilus*, decreased the incidence of ampicillin-associated diarrhea and the time required for recolonization. In contrast, clostridia are commonly found in the gastrointestinal tract of both humans and other animals, as well as in soil and sewage, and have been shown to be a cause of several human diseases, including necrotizing enterocolitis of infants, enteritis necroticans, and food poisoning [4, 13]. These diseases are mediated through the production of toxins or extracellular enzymes such as phospholipase C, collagenase, and hyaluronidase. *C. perfringens* is also known to be one of the most powerful gas producers among human bacteria of fecal origin [15] that can cause potential flatulence [17], and also produces harmful enzymes like β-glucuronidase [23] as well as putrefactive products in the intestine [7].

As the relationships between the microbial community structure and the health of the host continue to be 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 from harmful adverse effects [6, 8, 9]. However,

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despite their excellent pharmacological activity [3], relatively little work has been carried out on the effect of *Ruta chalepensis* leaf-derived components toward the growth of intestinal microorganisms. In this study, in order to develop new and safer types of antimicrobial agents, we assessed the growth-inhibitory effects of *R. chalepensis* against intestinal bacteria. Additionally, the antimicrobial activities of commercially available quinoline derivatives and antimicrobial agents (chloramphenicol, cinnamaldehyde) were also assessed for comparison.

Sample Preparation

The *R. chalepensis* leaves (3 kg), belonging to the family Rutaceae, were purchased from a local market in Seoul, Korea. The leaves were ground in a blender, extracted twice with methanol (15 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 (20 g) was sequentially partitioned into hexane- (2.4 g), chloroform- (3.5 g), ethyl acetate- (2.7 g), butanol- (3.1 g), and water-soluble (8.3 g) portions for subsequent bioassay. The isolation procedures used to extract antimicrobial constituents from *Ruta* leaf are shown in Fig. 1. The structural determination of the active isolate was based on a spectroscopic analysis. The ^{1}H and ^{13}C NMR spectra were recorded in methanol, using a Bruker AM-500 spectrometer (Rheinspettem, Germany)

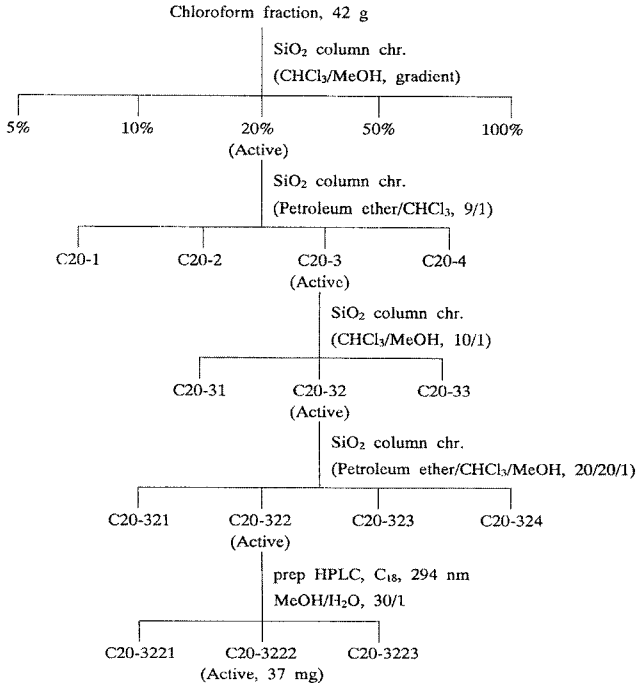

Fig. 1. Procedures for isolation of antimicrobial constituents from the leaf of *Ruta chalepensis*.

at 400 and 100 MHz, respectively. The ultraviolet spectra were obtained in methanol by using a Waters 490 spectrometer (Massachusetts, U.S.A.), and mass spectra were obtained by using a JEOL JMS-AX 302 spectrometer (Tokyo, Japan).

Identification

Bioassay-guided fractionation of the *R. chalepensis* extract afforded an active constituent identified by spectroscopic analyses, including EI-MS, ^{13}C and ^{1}H NMR, and by direct comparison with an authentic reference compound. The active constituent was characterized as quinoline-4-carboxaldehyde. The compound was identified based on the following evidence: quinoline-4-carboxaldehyde ($C_{10}H_7NO$, MW, 157.1); EI-MS (70 eV) *m/z* (% relative intensity): M^+ 157 (100), 129 (90), 128 (55), 101 (30), 75 (25), 51 (15); ^{1}H-NMR (CD_3OD, 400 MHz); d 8.82–8.83 (*d*, 1H, *J*=4 Hz), 8.26–8.28 (*d*, 1H, *J*=8 Hz), 8.02–8.04 (*d*, 1H, *J*=8 Hz), 7.76–7.77 (*d*, 1H, *J*=4 Hz), 7.72–7.75 (*m*, 1H), 7.59–7.63 (*m*, 1H), 6.13 (*s*, 1H); ^{13}C-NMR (CD_3OD, 100 MHz); 151.0, 149.0, 148.1, 130.6, 129.5, 127.9, 127.2, 126.0, 119.1, 95.7.

Antimicrobial Activity

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* ATCC 4356, and *L. casei* ATCC 27216, isolated from human feces. 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 BHI broth (pH 7.6) and MRS broth.

The growth-inhibiting activities of the various fractions obtained from the methanol extract of *R. chalepensis* leaves against human intestinal bacteria were assayed by the impregnated paper disk method. To assay the effect of the test material 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 (100 μl) of the 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 solvent, 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 against the organisms used. All tests were performed in triplicate. In routine screening, the methanol extract at 5 mg/disk exhibited a potent inhibiting

Table 1. Growth-inhibiting responses of *R. chalepensis* leaf-derived materials against intestinal bacteria.

Material[a]	Bacterial strain[b]					
	B. bifidum	*B. longum*	*L. acidophilus*	*L. casei*	*C. perfringens*	*E. coli*
Methanol extract	11[c]	0	10	11	31	22
Hexane fraction	0	0	0	0	0	0
Chloroform fraction	14	0	15	13	35	27
Ethyl acetate fraction	0	0	0	0	7	0
Butanol fraction	0	0	0	0	0	0
Water fraction	0	0	0	0	0	0

[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, mm.

activity against *Clostridium perfringens* and *Escherichia coli*. On the other hand, the methanol extracts showed no or weak inhibitory response against *B. bifidum*, *B. longum*, *L. acidophilus*, and *L. casei* (Table 1). In fractionation guided by the growth-inhibiting activity at a dose of 5 mg/disk, the chloroform fraction exhibited a potent and strong growth-inhibiting activity toward *C. perfringens* and *E. coli* (Table 1). However, no activity was observed in the hexane, ethyl acetate, butanol, and water fractions. Therefore, further purification of the active compound

Table 2. Growth-inhibiting responses of isolated compound, quinoline derivatives, and antimicrobial agents against intestinal bacteria.

Compound	Dose (mg/disk)	Bacterial Strain[a]				
		B. bifidum	*B. longum*	*L. acidophilus*	*C. perfringens*	*E. coli*
Quinoline-3-carboxaldehyde	2.0	21[b]	21	0	24	0
	1.0	12	16	0	17	0
	0.5	0	10	0	12	0
	0.25	0	0	0	6	0
Quinoline-4-carboxaldehyde	2.0	16	0	17	52	28
	1.0	10	0	11	45	20
	0.5	0	0	0	39	16
	0.25	0	0	0	30	14
	0.1	0	0	0	24	10
	0.05	0	0	0	20	0
	0.025	0	0	0	16	0
Quinoline	2.0	0	0	0	0	0
	1.0	0	0	0	0	0
Quinoline-3-carboxylic acid	2.0	0	0	0	0	0
	1.0	0	0	0	0	0
Quinoline-4-carboxylic acid	2.0	0	0	0	0	0
	1.0	0	0	0	0	0
Cinnamaldehyde	2.0	25	0	0	38	0
	1.0	18	0	0	25	0
	0.5	8	0	0	17	0
	0.25	0	0	0	13	0
Chloramphenicol	2.0	65	52	64	71	65
	1.0	54	43	56	60	57
	0.5	46	31	49	52	49
	0.25	32	18	42	44	38
	0.1	25	12	34	36	31
	0.025	16	0	24	28	22

[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, mm.

was performed from the chloroform fraction by silica gel column chromatography and HPLC.

The growth-inhibiting activity of the isolate against the five intestinal bacteria was examined (Table 2). Growth responses varied depending on the chemical, dose, and bacterial strain tested. In a test with *C. perfringens*, quinoline-4-carboxaldehyde produced strong inhibition at 2, 1, 0.5, 0.25, and 0.1 mg/disk and moderate inhibition at 0.05 and 0.025 mg/disk. Furthermore, this isolate revealed a strong activity against *E. coli* at 2 mg/disk and showed moderate and weak activites against *E. coli* at 0.5 and 0.25 mg/disk, respectively. However, at a dose of 1 mg/disk, weak and no growth inhibition against *B. bifidum*, *B. longum*, and *L. acidophilus* was observed with quinoline-4-carboxaldehyde (Table 2). Growth-promoting activities of the isolated compound and quinoline derivatives were assayed toward the beneficial bacteria on a modified György broth and modified RCM broth (data not shown). Quinoline derivatives exhibited no promoting activity at a concentration of 0.1%. The effect of antimicrobial agents as a positive control on intestinal bacterial growth was compared with that of quinoline derivatives (Table 2). Chloramphenicol and cinnamaldehyde were all purchased from Sigma (Sigma Chemical Co., St. Louis, MO, U.S.A.) and quinoline, quinoline-3-carboxaldehyde, quinoline-3-carboxylic acid, and quinoline-4-carboxylic acid were provided from Fluka (Fluka Chemcial Co., Milwaukee, WI, U.S.A.). Chloramphenicol at 0.025 mg/disk significantly inhibited the growth of all bacteria with the exception of *B. longum*, but cinnamaldehyde at 0.25 mg/disk did not inhibit *B. bifidum*, *B. longum*, *E. coli*, and *L. acidophilus* with the exception of *C. perfringens*. The growth-inhibiting effect of chloramphenicol against *C. perfringens* was more pronounced than that of quinoline-4-carboxaldehyde. However, quinoline-4-carboxaldehyde did not cause any adverse effects on the growth of bifidobacteria at 0.5 mg/disk, indicating that this compound has selective activity against human beneficial and harmful bacteria.

According to Mitsuoka's [14], the maintenance of high levels of *Bifidobacterium* spp. and low levels of *C. perfringens* helps ensure good health and longevity in humans. Therefore, we focused on the microorganisms of *Bifidobacterium* spp. and *C. perfringens* for use in screening for growth-modulator function that might improve the intestinal environment. Although little is known about the autogenic factors within the intestinal ecosystem, some of the health-promoting effects of several dietary components effective in the control of normal microflora growth have been studied [1, 12]. Recently, attention has been focused on the inhibitory roles of natural compounds in suppressing the carcinogenic and mutagenic effects of clostridia. Many naturally occurring compounds have been well documented as modifiers of the human intestinal bacterial populations [6, 8, 9]. In this study, the inhibitory activity of quinoline-4-carboxaldehyde isolated from *R. chalepensis* confirmed their superiority and usefulness as the lead compound of antimicrobial agents. *Ruta chalepensis* L. (Rutaceae) is a perennial folk medicine widely used as an antirheumatic, antispasmodic, and aphrodisiac agent, and treatment for snakebites, headaches, and wounds [3]. The plant is a rich source of several acridone and quinoline alkaloids, as well as coumarins [27]. A few of the coumarins from *R. chalepensis* exhibited antifertility effects [28], and some quinoline alkaloids isolated from *Ruta* species displayed mutagenic [19], ganglionic-blocking, curare-like [24], and spasmolytic activities [16]. Quinoline and furoquinoline alkaloids are also found in *Ruta* spp., such as τ-fagrine, kokusaginine, ardorinine, akimmianine, dictamnine, and graveoline [28], and this was interpreted as a chemical defense barrier to potential pathogens.

As for the structure-activity relationships against *C. perfringens*, quinoline-3-carboxaldehyde and quinoline-4-carboxaldehyde revealed strong and moderate growth-inhibiting activity at 2 and 1 mg/disk, respectively, but no growth inhibition toward *B. bifidum*, *B. longum*, and *L. acidophilus* at any concentrations. For intestinal microorganisms, five compounds derived from *Cinnamomum cassia* bark were tested for their growth inhibitory effect against *Bacteroides fragilis* and *C. perfringens*. In a test using 1 and 0.5 mg/disk, cinnamaldehyde and salicylaldehyde, including the aldehyde functional group, revealed potent inhibition against *B. fragilis* and *C. perfringens*, whereas weak or no inhibitory activity was obtained against *B. longum* and *L. acidophilus* [10]. It appears that reuterin is the first low-molecular-weight aldehyde antimicrobial substance produced by this group of bacteria to be chemically identified [25]. In the current study, the results indicated that the growth-inhibiting activity of two carboxaldehydes against *C. perfringens* and *E. coli* was much more pronounced in quinoline, including the aldehyde group, than quinoline without the aldehyde group.

It is highly desirable to inhibit the growth of potential pathogens such as clostridia, while increasing 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 would normalize disturbed physiological functions, thereby resulting in 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 plant-derived materials are relatively nontoxic to humans. For example, extracts from *Phellodendron amurense* and *Pinus densiflora* have been shown not only to enhance the growth of bifidobacteria, but also to selectively inhibit various clostridia [6, 8]. In the present study, the growth inhibitory constituent of *R. chalepensis* leaves was identified as quinoline-4-carboxaldehyde with a species selectivity.

In conclusion, the present results indicate that *R. chalepensis* leaf-derived material has inhibitory effect against specific human intestinal bacteria *in vitro*. This information is expected to help elucidate and augment the positive biological effects of *R. chalepensis* and its products. In a previous study, the oral LD_{50} value of quinoline-4-carboxaldehyde for mouse was reported as 1.26 g/kg, indicating its low acute toxicity to mammals [11]. More importantly, the inhibitory action of quinoline-4-carboxaldehyde against *C. perfringens* and *E. coli* could be as potential therapeutics for the treatment of diseases caused by harmful bacteria. It is highly possibile that the materials of *R. chalepensis* extract would alter the microflora and metabolites in a manner that would promote human health.

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