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Effects of *Rosa rugosa* Petals on Intestinal Bacteria

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The effects of pulverized petal of *Rosa rugosa* on the growth of 10 species of intestinal and pathogenic bacteria were investigated. Growth of bifidobacteria and lactobacilli was not affected by the addition of the petal in plate cultivation. However, the growth of *Bacteroides vulgatus*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* was completely inhibited by the addition of 0.1, 0.5, 0.1, and 0.05% (w/v) of the petal respectively. In liquid cultivation, the addition of the petal (0.5%) stimulated the growth of *Bifidobacterium breve* and slightly inhibited the growth of *Lactobacillus salivarius*. But the growth of *E. coli*, *S. aureus*, *B. cereus*, and *Salmonella* sp. was inhibited by nearly 50%. Hydrolyzable tannins isolated from *R. rugosa*, rugosin D, and tellimagradin II showed antibacterial activities against *E. coli*, *S. aureus*, *B. cereus*, and *Salmonella* sp., but little or no effect against *Bif. breve* and *L. salivarius*. *R. rugosa* petal showed selective antibacterial activities against intestinal and pathogenic bacteria, and the selectivity resembled that of prebiotics such as oligosaccharides and dietary fiber. Hydrolyzable tannins in *R. rugosa*, such as rugosin D and tellimagradin II, must be active constituents.

Key words: *Rosa rugosa*; petal; intestinal bacteria; bifidobacteria; hydrolyzable tannins

Rosa rugosa is distributed mainly along the coast of Hokkaido, Japan, and is called Japanese rose. In China, the flowers of *R. rugosa* var. *plena* and related plants are used as a medicine, "mei gui hua," for diarrhea, bleeding, and diseases of women.[1)] It is mainly used as an ingredient in tea or a source of rose oil in China. In Japan, utilization of *R. rugosa* is limited to cases such as the production of jam from fruits, but our research on old documents written by doctors on Ezo island (the old name of Hokkaido) at the end of Edo period revealed that the Ainu people used the flower petal of *R. rugosa* as a medicinal tea, probably as a supply of vitamin C in the winter season ("Kochi-Yojyo-Ko" written by Shotatsu Iwaya in 1857).

Since there has been no investigation utilizing *R. rugosa* petals as a functional food, we have been studying the petal to develop new functional foodstuffs. The petal is known to contain many hydrolyzable tannins,[2,3)] and the health-promoting effects of tannins and related polyphenols has attracted attention recently. In the course of our studies, oral administration of the pulverized petal of *R. rugosa* reduced the odor of rat feces. The odor of feces is derived from metabolic products of intestinal flora, and hence the intestinal flora in rats must be affected by the administration of *R. rugosa* petal. In this paper, we report the effects of *R. rugosa* petal and its hydrolyzable tannins on human intestinal and pathogenic bacteria.

Materials and Methods

Materials and chemicals. R. rugosa petal, collected at Monbetsu, Hokkaido in 2004, were dried and pulverized to under 200 μm in diameter with a mill pot rotator (Mitsubishi, Tokyo) and an automatic mill (West, Tokyo). The pulverized petal was sterilized by spraying of 70% ethanol followed by drying at 80 °C for 18 h. All chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

Bacterial strains. Bifidobacterium breve, *Bif. adolescentis*, *Bif. bifidum*, *Lactobacillus acidophilus*, *L. casei*, *L. salivarius*, *Bacteroides vulgatus*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* were obtained from the Japan Collection of Microorganisms (JCM) and the American Type Culture Collection (ATCC). *Salmonella* sp. (wild type) was obtained from the Hokkaido Okhotsk Area Regional Food Processing Technology Center, and was identified with SMID agar media (BioMérieux Japan, Tokyo). Each strain was isolated by single-colony formation on a GAM (Nissui, Tokyo) agar plate, and cultivated overnight in 5 ml of GAM broth.[4)] After they were washed twice with

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150 mM NaCl, the cells were preserved in 10% glycerol at −80 °C. The glycerol stocks were inoculated in 5 ml of GAM broth in test tubes, and the test tubes were incubated at 37 °C for 18 h as pre-culture. Bifidobacteria were incubated under a CO_2 atmosphere.

Culture media. An MRS medium of the following composition was used; glucose, 20 g/l; Bacto proteose peptone, 10 g/l; Bacto beef extract, 10 g/l; Bacto yeast extract, 5 g/l; Tween 80, 1 g/l; triammonium citrate, 2 g/l; sodium acetate, 5 g/l; magnesium sulfate, 0.1 g/l; manganese sulfate, 0.05 g/l; dipotassium phosphate, 2 g/l; agar, 15 g/l. The medium was adjusted to pH 6.5 and sterilized in an autoclave (115 °C, 15 min). Mannit agar (Eiken, Tokyo) was used in the detection of *S. aureus*, and TOS propionate agar (Eiken) was used in the detection of bifidobacteria.[5,6]

Plate and liquid cultivation. Pre-culture broth was diluted with 150 mM NaCl and spread on an agar plate with and without test samples. Pulverized petals were added to MRS agar plates or broth at concentrations of 0.01, 0.05, 0.1, and 0.5% (w/v). The plate was incubated at 37 °C for 48 h in a jar pack with disposable O_2 absorbing and CO_2 generating agent (Mitsubishi, Tokyo) for bifidobacteria. Pre-culture broth (50 µl) was inoculated in 5 ml of liquid media with or without the test sample, and was cultivated at 37 °C for 18 h. The pulverized petals were added to MRS agar plates or broth at concentrations of 0.1, 0.3, and 0.5% (w/v). The antibacterial activities of the test samples were monitored by counting colony-forming units (CFUs).[6,7] The average CFU and standard deviation was calculated from three experiments at each dilution rate. The inhibition rate was calculated by the following equation: inhibition rate = 100 × [(CFU in test culture after incubation) − (CFU in control culture before incubation)]/[(CFU in control culture after incubation) − (CFU in control culture before incubation)].

Preparation of hydrolyzable tannins in Rosa rugosa. Dried, pulverized petals of *R. rugosa* (500 g) were extracted with 70% acetone 3 times. The extract was evaporated to remove the acetone, and the aqueous solution was extracted with ethyl acetate (EtOAc) 3 times. The combined EtOAc layer was evaporated to dryness to obtain an EtOAc soluble fraction (40.4 g). The EtOAc soluble fraction (25 g) was dissolved in ethanol (EtOH) and applied to a column of Sephadex LH-20 (8.5 cm i.d. × 18 cm). The column was eluted with a solvent system of EtOH-H_2O-acetone to obtain fractions containing hydrolyzable tannins at different degrees of galloylation.[8] These fractions were further purified by reversed phase chromatography and preparative HPLC. Tellimagrandin I (235 mg) was isolated from a fraction eluted with 60% EtOH, Tellimagrandin II (77 mg) and Rugosin A (136 mg) were isolated from a fraction eluted with EtOH:H_2O:acetone = 54:36:10, and Rugosin D (122 mg) was isolated from a fraction eluted with EtOH:H_2O:acetone = 42:28:30. The structures of these hydrolyzable tannins were identified by comparison of ^{1}H- and ^{13}C-NMR spectra, and high resolution mass spectra with those in the literature.[2,3,9]

Statistical analysis. Differences among groups were examined for statistical significance by one-way analysis of variance (ANOVA), and then determined by the least significant difference test. The criterion for significance was $p < 0.01$.

Results and Discussion

Effects of pulverized petal of Rosa rugosa *on the growth of bacteria*

The effects of the pulverized petals of *R. rugosa* on the growth of eight intestinal and two pathogenic bacteria are summarized in Table 1. The addition of pulverized petals in a range of 0.01–0.5% (w/v) did not affect the growth of bifidobacteria (*Bifidobacterium breve*, *Bif. adolescentis*, and *Bif. bifidum*) or lactobacilli (*Lactobacillus acidophilus*, *L. casei*, and *L. salivarius*). However, the growth of *Bacteroides vulgatus* was almost entirely inhibited (82%) by the addition of 0.05% of pulverized petals, and was entirely inhibited at 0.1%. The growth of *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* was entirely inhibited by the addition of 0.5, 0.1, and 0.05% of pulverized petals respectively.

These results suggest that the soluble constituents in the petals of *R. rugosa* suppressed the growth of four intestinal bacteria with pathogenic natures in plate culture, but did not affect the growth of bifidobacteria or lactobacilli. These two genera of bacteria are known to be beneficial to human health and have been reported to enhance the immune system and to produce short-chain fatty acids.[10–13]

The effects of the pulverized petals on the growth of six bacteria, including *Salmonella* sp., in liquid media (MRS broth) are summarized in Table 2. The growth of *Bif. breve* was dose-dependently promoted by the addition of the petals. The growth of *L. salivarius* was slightly affected by the addition of 0.5% of the petals in the culture, but was promoted 9.4 and 26.2% by the addition of 0.1 and 0.3% of the petals respectively. On the contrary, the growth of *E. coli*, *S. aureus*, *B. cereus*, and *Salmonella* sp. was significantly inhibited as compared with that of *Bif. breve* and *L. salivarius*. The inhibitory effects of the petals on the growth of *E. coli*, *S. aureus*, *B. cereus*, and *Salmonella* sp. was dose-dependent, and the growth of these bacteria was nearly 50% inhibited in the presence of 0.5% of the petals.

Compared with the results in plate culture, the effects of the pulverized petals were not distinct in liquid culture. This difference might be due to the availability of a carbon source as between the plate media and the liquid media.

Table 1. Effects of Pulverized Petals of *Rosa rugosa* on the Growth of Intestinal and Pathogenic Bacteria in Plate Cultivation

	CFU				
Conc. of the petals	0%	0.01%	0.05%	0.1%	0.5%
Bifdobacterium breve JCM1192^T	1.43×10^9	1.50×10^9	1.54×10^9	1.41×10^9	1.43×10^9
Inhibition rate (%)	—	—	—	—	—
Bifdobacterium adolescentis JCM1275^T	8.33×10^8	8.40×10^8	8.28×10^8	8.38×10^8	8.45×10^8
Inhibition rate (%)	—	—	—	—	—
Bifdobacterium bifidum JCM1255^T	3.44×10^8	3.37×10^8	3.54×10^8	3.55×10^8	3.40×10^8
Inhibition rate (%)	—	—	—	—	—
Lactobacillus acidophilus JCM1132^T	5.77×10^8	5.83×10^8	5.79×10^8	5.80×10^8	5.69×10^8
Inhibition rate (%)	—	—	—	—	—
Lactobacillus casei JCM1134^T	2.57×10^9	2.67×10^9	2.66×10^9	2.55×10^9	2.57×10^9
Inhibition rate (%)	—	—	—	—	—
Lactobacillus salivarius JCM1044	1.11×10^9	1.20×10^9	1.17×10^9	1.10×10^9	1.17×10^9
Inhibition rate (%)	—	—	—	—	—
Bacteroides vulgatus JCM5826^T [a]	1.57×10^9	1.67×10^9	2.87×10^8	0	0
Inhibition rate (%)	—	—	82	100	100
Escherichia coli JCM1649^T	5.37×10^8	5.29×10^8	2.66×10^8	3.41×10^7	0
Inhibition rate (%)	—	—	51	94	100
Staphylococcus aureus JCM2413	3.03×10^8	3.16×10^8	2.00×10^5	0	0
Inhibition rate (%)	—	—	66	100	100
Bacillus cereus ATCC14579^T	3.11×10^7	1.24×10^6	0	0	0
Inhibition rate (%)	—	96	100	100	100

Values are means of triplicate experiments, and the coefficients of variation of the values were less than 3%.
[a] Bacteroidaceae were cultivated on GAM agar plates (pH 6.5).

Table 2. Effects of Pulverized Petals of *Rosa rugosa* on the Growth of Intestinal and Pathogenic Bacteria in Liquid Cultivation

	Inhibition rate of Growth (%)		
	0.1% Petal	0.3% Petal	0.5% Petal
Bifdobacterium breve	-13.8 ± 8.4^a	-16.4 ± 4.4^a	-20.8 ± 5.9^a
Lactobacillus salivarius	-9.4 ± 3.5^a	-26.2 ± 14.5^a	5.1 ± 13.7^b
Escherichia coli	25.5 ± 10.0^b	37.1 ± 4.2^b	53.5 ± 7.1^c
Staphylococcus aureus	18.4 ± 9.0^b	26.2 ± 9.0^b	41.5 ± 10.0^{cd}
Bacillus cereus	24.2 ± 4.4^b	33.8 ± 1.9^b	46.8 ± 3.8^{ce}
Salmonella sp.	28.4 ± 5.9^b	45.2 ± 11.3^b	57.1 ± 2.6^{ce}

Values are mean ± SD (n = 3).
[a-e] Values in the same column with no common superscript letter differ significantly ($p < 0.01$).
The initial and final CFU/ml of each strain as control in culture broth was as follows: *Bif. breve*, $3.11–3.71 \times 10^7$, $2.71–3.12 \times 10^9$; *L. salivarius*, $3.73–4.59 \times 10^7$, $9.13–13.7 \times 10^8$; *E. coli*, $6.25–7.54 \times 10^6$, $5.16–7.98 \times 10^8$; *S. aureus*, $8.23–11.9 \times 10^6$, $5.91–9.86 \times 10^8$; *B. cereus*, $1.16–1.46 \times 10^6$, $0.90–1.25 \times 10^8$; *Salmonella* sp., $7.30–8.54 \times 10^6$, $5.81–8.64 \times 10^8$.

It is known that tannins and related polyphenols show antibacterial activities against *Helicobactor pylori*, *S. aureus*, *E. coli*, *Salmonella* sp., and streptococci.[14–20] Since the petals of *R. rugosa* are known to contain many hydrolyzable tannins, such as rugosins,[2,3] the effects of the petals on *Bac. vulgatus*, *E. coli*, *S. aureus*, *B. cereus*, and *Salmonella* sp. must be related to hydrolyzable tannins.

There has been no report on the antibacterial activities of tannins and related polyphenols against bifidobacteria and lactobacilli. In our experiments in plate and liquid media, petals of *R. rugosa* showed selective antibacterial activities; the petals promoted or had little effect on the growth of bifidobacteria and lactobacilli, but inhibited intestinal bacteria of a pathogenic nature. This selectivity suggests the effects of prebiotics such as oligosaccharides and dietary fibers, which are known to promote the growth of bifidobacteria and lactobacilli.[5–7,21,22]

Effects of hydrolyzable tannins on the growth of intestinal and pathogenic bacteria

The ethyl acetate soluble portion obtained from aqueous acetone extract of the petals of *R. rugosa* was mainly composed of hydrolyzable tannins.[2,3] The yields of the ethyl acetate soluble portion were in the range of 15–20%, and the main constituents were rugosin A and D and tellimagrandin I and II.

To determine the active constituents in the petals of *R. rugosa*, the antibacterial activities of four hydrolyzable tannins isolated from the petals were tested against *Bif. breve*, *L. salivarius*, *E. coli*, *S. aureus*, *B. cereus*, and *Salmonella* sp. Epigallocatechin gallate (EGCG) was used as a control, because it has been reported to show antibacterial activity.[16–19]

As shown in Table 3, the growth of *Bif. breve* and *L. salivarius* was not affected in the presence of 1 mM of rugosin D, but that of *E. coli*, *S. aureus*, *B. cereus*, and *Salmonella* sp. was significantly inhibited. The inhibition rates were 29.6, 74.9, 55.3, and 43.5% respectively. Rugosin A showed the same tendency, but the growth of *Bif. breve* and *L. salivarius* was inhibited 25.4 and

Table 3. Effects of Hydrolyzable Tannins Isolated from Petals of *Rosa rugosa* on the Growth of Intestinal and Pathogenic Bacteria in Liquid Cultivation

	Inhibition rate of Growth (%) at 1 mM				
	EGCG*	Tellimagrandin I	Tellimagrandin II	Rugosin A	Rugosin D
Bifdobacterium breve	3.8 ± 2.2	27.6 ± 15.8[a]	−14.3 ± 16.2[a]	25.4 ± 9.1[a]	3.0 ± 1.8[a]
Lactobacillus salivarius	7.0 ± 7.0	−4.9 ± 10.8[b]	9.6 ± 7.5[a]	21.7 ± 8.3[a]	−0.9 ± 0.8[b]
Escherichia coli	4.3 ± 5.8	44.1 ± 11.2[bc]	52.0 ± 12.3[b]	41.0 ± 4.7[b]	29.6 ± 6.4[c]
Staphylococcus aureus	2.8 ± 8.8	15.3 ± 2.0[ad]	65.7 ± 5.5[bd]	90.5 ± 3.0[c]	74.9 ± 11.1[d]
Bacillus cereus	−2.8 ± 2.8	42.8 ± 5.2[acce]	55.5 ± 3.5[be]	79.9 ± 7.7[d]	55.3 ± 9.3[dc]
Salmonella sp.	3.5 ± 4.2	34.9 ± 1.7[acce]	43.7 ± 5.2[bf]	63.4 ± 8.0[d]	43.5 ± 9.1[ce]

Values are mean ± SD (n = 3).
[a–f]Values in the same column with no common superscript letter differ significantly ($p < 0.01$).
*EGCG: epigallocatechin gallate
The initial and final CFU/ml of each strain as control in culture broth was as follows: *Bif. breve*, 3.32–3.89 × 10^7, 2.84–3.82 × 10^9; *L. salivarius*, 1.23–1.84 × 10^7, 2.11–2.51 × 10^9; *E. coli*, 3.88–7.69 × 10^6, 3.61–4.47 × 10^8; *S. aureus*, 5.13–9.30 × 10^6, 3.64–6.81 × 10^8; *B. cereus*, 0.70–1.26 × 10^6, 6.04–9.36 × 10^7; *Salmonella* sp., 7.07–8.86 × 10^6, 7.08–9.75 × 10^8.

21.7% respectively. In the case of tellimagrandin II, the growth of *Bif. breve* was enhanced and that of *L. salivarius* was slightly inhibited, and that of the other bacteria was significantly inhibited. The inhibitory rates were higher than 50%, except for *Salmonella* sp. Tellimagrandin I also inhibited the growth of *E. coli*, *B. cereus*, and *Salmonella* sp. more than 30%. It showed no inhibitory effect against *L. salivarius*, but the growth of *Bif. breve* was inhibited 27.6%. The addition of EGCG showed almost no effect at 1 mM.

Our experiments indicated that rugosin D and tellimagrandin II showed selective antibacterial activities. These hydrolyzable tannins showed a little or no effect on the growth of *Bif. breve* or *L. salivarius*, and a significant inhibitory effect against *E. coli*, *S. aureus*, *B. cereus*, and *Salmonella* sp. Rugosin A and tellimagrandin I showed the same tendency, but were not as selective as rugosin D and tellimagrandin II.

Ruupponen-Pimiä *et al.*[15] reported that extracts of Nordic berry showed selective antibacterial activities; the extracts showed antibacterial activities against *Salmonella enterica* and *S. aureus* at a concentration of 1 mg/ml, but did not affect the growth of *L. rhamnosus*. They concluded that phenolic compounds such as ellagitannins were strongest inhibitors of staphylococci. Ahn *et al.*[21] reported that methanol extracts of green tea showed moderate stimulating effects on the growth of bifidobacteria but did not stimulate the growth of clostridia, bacteroides, eubacteria, or *E. coli*.

The effects of rugosin D and tellimagrandin II on intestinal bacteria are different from those indicated in these reports, and resemble to those of Whelan *et al.*[22] and Wang *et al.*[23] In these reports, fructooligosaccharides and dietary fiber increased the growth of a health-promoting genus (bifidobacteria) and reduced those of pathogens (*E. coli* and clostridia). Our studies *in vitro* indicate that the petals of *R. rugosa* and its constituents show similar effects on intestinal flora as prebiotics, such as fructooligosaccharide, and that the active constituents must be hydrolyzable tannins such as rugosin D and tellimagrandin II.

Rugosin D is a dimeric hydrolyzable tannin including the rugosin A moiety, and the structural difference between tellimagrandin I and II is the absence of the galloyl group at the anomeric position in the former. Our results do not explain the relation between the selective antibacterial activities and structures of hydrolyzable tannins. Moreover, the mechanism of selective antibacterial activities is unsolved, and more investigation is needed.

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