Effect of Gallotannins Derived from *Sedum takesimense* on Tomato Bacterial Wilt

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Abstract

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In the process of searching antibacterial agents from plants, we discovered that the methanol extract of *Sedum takesimense* showed potent antibacterial activity against *Ralstonia solanacearum* in vitro and in vivo. Eight antibacterial gallotannins were isolated from the aerial parts of *S. takesimense* and identified as gallic acid, methyl gallate, 4,6-di-O-galloylarbutin, 2,6-di-O-galloylarbutin, 2,4,6-tri-O-galloyl-glucose, 1,3,4,6-tetra-O-galloyl- β -glucose, 1,2,4,6-tetra-O-galloyl- β -glucose, and 1,2,3,6-tetra-O-galloyl- β -glucose based on electrospray ionization mass spectrometry and proton nuclear magnetic resonance spectroscopy. These gallotannins displayed broad-spectrum activity against various plant-pathogenic bacteria, and the strongest in vitro antibacterial activities of these gallotannins were against *R. solanacearum* (minimum inhibitory concentration = 0.02 to 0.10 g/liter). Among these gallotannins, methyl gallate and 1,2,3,6-tetra-O-galloyl- β -glucose showed the strongest activities. In addition, synergistic or partial synergistic effects were observed in most combinations between major antibacterial compounds. The wettable powder formulation of the *S. takesimense* crude extract effectively reduced the development of tomato bacterial wilt caused by *R. solanacearum* under greenhouse conditions for 14 days after infection. This is the first report on the isolation of antibacterial compounds from *S. takesimense*. These results suggest that the extract from *S. takesimense* or the isolated gallotannins could be used as natural bactericides for the control of tomato bacterial wilt.

Ralstonia solanacearum (Smith) Yabuuchi et al. is one of the most important soilborne plant-pathogenic bacteria. Bacterial wilt caused by *R. solanacearum* is a common and serious disease that reduces the yield of tomato and many other crops in tropical, subtropical, and warm-temperature regions of the world. This soil bacterium has been recognized as one of the leading models in pathogenicity and the causative agent of bacterial wilt disease because of worldwide distribution and large host range of more than 200 plant species, including tomato, potato, pepper, peanut, tobacco, and banana (17). This bacterium can be free-living as a saprophyte in water or in the soil in the absence of host plants (15). In an established region, the bacterium can contaminate soil and surface waterways as well as infect plants through roots and wounds (17,39). The symptoms start as drooping followed by wilting of whole plants within a few days and then total plant collapse (30).

Due to a lack of commercially available pathogen-resistant tomato cultivars, several research groups have focused on the development of effective and safe bactericides to reduce soil populations. The use of plant-derived compounds to control this bacterial disease has been the subject of increasing interest (1,8,19,35,50). The management of pathogens by the direct use of plant extracts is now considered to be one component of integrated disease management programs (7,48).

Sedum is a large genus belonging to the family Crassulaceae. Plants of the *Sedum* genus have been used for a long time in traditional medicine in various countries for treatment of many diseases as antitumor, anti-inflammatory, radioprotective, vasodilating,

wound-healing, and hypotensive agents (22,23,37,38,43). According to previous studies, the chemical compositions of *Sedum* spp. consist of flavonoids, phenolic acids (mainly gallic), coumarins, alkanoids, carbohydrates, polysaccharides, and tannins (22,23,29,37). However, there is no report describing antibacterial activity of *Sedum takesimense* Nakai and isolation of antibacterial compounds from *S. takesimense*.

In search of antibacterial compounds from plants, we observed that the methanol extract of *S. takesimense* had potent in vitro antibacterial activity against *R. solanacearum*. There have been many reports on the control of bacterial wilt disease using different antibacterial agents (16,32,33,39,42) but information on the antibacterial effects of the *S. takesimense* extract and determination of its antibacterial components are lacking. This study reports the isolation of the active compounds and their in vitro antibacterial activities against *R. solanacearum* and various plant-pathogenic bacteria as treated both independently and in combination. In addition, this study reveals the structure activity relationships of these compounds.

Materials and Methods

Bacterial strains and culture conditions. The following nine bacteria strains were used: *Acidovorax avenae* subsp. *cattleiae* SL4351 (Pavarino) Willems et al. (bacterial brown spot of *Phalenopsis* [*Phalenopsis* sp.]), *Agrobacterium tumefaciens* SL2434 (Smith and Townsend) Conn (crown gall of apple [*Malus domestica* L. Borkh.]), *Burkholderia glumae* SL4269 (Kurita and Tabei) Urakami et al. (bacterial panicle blight of rice [*Oryza sativa* L.]), *Clavibacter michiganensis* subsp. *michiganensis* SL4135 (Smith) Davis et al. (bacterial wilt and canker of tomato [*Solanum lycopersicum* L.]), *Pectobacterium carotovorum* subsp. *carotovorum* SL290 (Jones) Bergey et al. (bacterial soft rot of potato [*S. tuberosum* L.]), *Pseudomonas syringae* pv. *actinidiae* SL2339 Takikawa et al. (bacterial canker of kiwifruit [*Actinidia arguta* (Sieb. et Zucc.) Planch. ex Miq.]), *R. solanacearum* SL1944 (bacterial wilt of tomato), *Xanthomonas euvesicatoria* SL4000 Jones et al. (bacte-

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rial spot of tomato), and *X. arboricola* pv. *pruni* SL4370 (Smith Vauterin et al. (bacterial spot of peach [*Prunus persica* (L.) Batsch])). All of these bacteria strains were isolated from the infected tissues by Dr. S. D. Lee of the National Academy of Agricultural Sciences, except *R. solanacearum*, which was isolated by Prof. S.-W. Lee of Dong-A University (26). They were kindly supplied and then preserved at -80°C at the Korea Research Institute of Chemical Technology. Tryptic soy broth (TSB) and tryptic soy agar (TSA) (Difco, Becton Dickinson) were used to culture all of the bacteria. *Pseudomonas syringae* pv. *actinidiae* and *Xanthomonas* spp. were cultured at 25°C for 18 to 36 h, and the remaining strains were cultured at 30°C for 18 to 36 h.

Plant extract. The aerial parts of *S. takesimense* were purchased from the Arim SG botanical garden in Gyeongbuk province, Korea in July 2011 and dried at 80°C for 3 days. The dried *S. takesimense* were extracted with methanol (1:9 [wt/vol]), evaporated, and stored at -20°C for further purification and the bioactivity test.

Isolation and determination of active compounds. The methanol extract of *S. takesimense* (7 g) was suspended in distilled water and partitioned twice with ethyl acetate. The ethyl acetate layer (5 g) showed strong activity against *R. solanacearum* determined by disc diffusion assay (21) and was subjected to a silica gel column (3.5 by 60.0 cm; Kiesel gel 60, 150 g, 230 to 400 mesh; E. Merck) with an eluting solvent of chloroform/methanol/distilled water/acetic acid (55:36:8:1 by volume) to give four fractions (F1 to F4) (21,48).

Fraction F1 (2.2 g) was separated on a silica gel column (3.0 by 60.0 cm; Kiesel gel 60, 100 g, 230 to 400 mesh) with an eluting solvent of chloroform/methanol/distilled water/acetic acid (55:36:8:1 by volume) to afford two fractions (F11 and F12). Fraction F11 (440 mg) containing compounds 1 and 2 was successfully separated on a Sephadex LH20 column (3.5 by 60 cm; 130 g, 70 to 100 μm ; Sigma-Aldrich) using methanol (48,49). Compounds 1 and 2 were further purified using a preparative high-performance liquid chromatographic (prep-HPLC) system (Capcell Pak C18, 20 by 250 mm; Shiseido). The substances were eluted with an isocratic solvent system of methanol/water (20:80 by volume) at a flow rate of 6.0 ml/min and detected at a wavelength of 275 nm using Waters 996 PDA detector. The prep-HPLC yielded the pure compounds 1 (120 mg) and 2 (14 mg) (48,49). A portion (440 mg) of fraction F12 (1.7 g) was then separated using prep-HPLC as described above, except the mobile phase consisted of an isocratic solvent system of methanol/water (22.5:77.5 by volume). The prep-HPLC yielded the pure compounds 3 (16 mg) and 4 (170 mg).

Fraction F2 (1.2 g) was chromatographed on a Sephadex LH20 column (3.5 by 60 cm; 130 g, 70 to 100 μm) with an eluting solvent of methanol. The F23 fraction (80 mg) was further purified by prep-HPLC as described above for the purification of compound 5. The prep-HPLC yielded the pure compound 5 (14 mg).

Fraction F3 (720 mg) was separated on a Sephadex LH20 column (2.5 by 60 cm; 80 g, 70 to 100 μm) using methanol to yield three fractions. From fraction F33 (450 mg), three compounds—namely, compound 6 (16 mg), 7 (150 mg), and 8 (14 mg)—were purified using prep-HPLC as described above for the isolation of compounds 1 and 2.

The chemical structures of the purified compounds were determined by mass and proton nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy. Electrospray ionization mass spectra (ESI-MS) of the purified compounds were recorded on an MSD1100 single-quadrupole mass spectrometer equipped with an ESI (Hewlett-Packard Co.). $^1\text{H-NMR}$ spectra were recorded in CD_3OD (Merck) with a Bruker AMX-500 spectrometer (Bruker Analytische Messtechnik GmbH) operated at 500 MHz for ^1H . Chemical shifts (parts per million) are relative to tetramethylsilane, which was used as an internal reference (49). Coupling constants are reported in hertz. The chemical structures of the purified substances were analyzed on the basis of ESI-MS and $^1\text{H-NMR}$ spectral data and comparisons of reported values in the literature (13,28,40,41,43,45–47,50).

In vitro antibacterial activity. The purified compounds were dissolved in dimethyl sulfoxide (DMSO) at the desired concentration (200.00 g/liter). Twofold serial dilutions of the compounds were conducted through wells of sterile 96-well plates containing 100 μl of TSB, and 100 μl of inoculated TSB medium containing 2×10^5 CFU/ml, except for *Xanthomonas* spp. and *P. syringae* pv. *actinidiae* (2×10^6 CFU/ml), was then added to each well. The final concentrations of the three major compounds purified in amounts over 120 mg from 7 g of the methanol extract of *S. takesimense*—namely, compounds 1, 4, and 7, as well as compound 2, which was commercially obtained (Sigma-Aldrich)—were 0.02 to 2.00 g/liter. The final concentrations of the minor compounds purified in amounts less than 16 mg from 7 g of the methanol extract of *S. takesimense* were 0.01 to 1.00 g/liter. The final concentration of DMSO was less than 1%. The final density of the bacteria in the assay was 10^5 CFU/ml, except for *Xanthomonas* spp. and *P. syringae* pv. *actinidiae* (10^6 CFU/ml). The microtiter plates were incubated at 30°C , except for *Xanthomonas* spp. and *P. syringae* pv. *actinidiae* at 25°C , for 18 to 36 h. Bacterial growth was determined by reading the absorbance at 600 nm using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad). Minimum inhibitory concentration (MIC) values were defined as the lowest concentration of tested compounds inhibiting bacterial growth by 100% according to the microdilution method published by National Committee for Clinical Laboratory Standards (31). Each treatment was replicated three times and the experiment was repeated three times.

Cho et al. (4) reported that the antibacterial activity of 1,2,3,4,6-penta-O-galloyl- β -D-glucose but not gallic acid from *Rhus trichocarpa* Miq. is restored by the addition of Fe^{2+} , indicating that the antibacterial activity of pentagalloylglucose is different from gallic acid and attributable to the chelation of iron. To investigate the effects of combinations of the simple gallate compounds (gallic acid and methyl gallate), galloylarbutin (compound 4), or galloylglucose (compound 7), a checkerboard assay was performed and the fractional inhibitory concentration (FIC) index was then determined, as described previously (6,10,27,44). Twofold serial dilutions of the four compounds—namely, gallic acid (compound 1), methyl gallate (compound 2), 2,6-di-O-galloylarbutin (compound 4), and 1,2,4,6-tetra-O-galloyl- β -glucose (compound 7)—were prepared in TSB medium for the combinations tested, and 50- μl aliquots of each compound were placed into wells of a sterile 96-well microtiter plate. The inocula were prepared using the MIC determination method. The final concentrations of the broth antibacterial agents was 1/16 to 2 times the MIC for each compound. After agitation, the plates were incubated under the same conditions as described above.

The FIC index for all combinations was determined by the following equation: $\text{FIC index} = \text{FIC}_A + \text{FIC}_B = [\text{A}]/\text{MIC}_A + [\text{B}]/\text{MIC}_B$ (6,10,27,44), where A is the concentration of compound A, MIC_A is the MIC of compound A, and FIC_A is the FIC of compound A for the organism; and B, MIC_B , and FIC_B are defined in the same fashion as for compound A. The FIC index (FICI) according to the checkerboard method is interpreted as follows: $\text{FICI} \leq 0.5$, synergy; $0.5 < \text{FICI} \leq 0.75$, partial synergy; $0.75 < \text{FICI} \leq 1.0$, additive effect; $1.0 < \text{FICI} \leq 4.0$, indifference; and $\text{FICI} > 4.0$, antagonism (6). Each concentration was replicated three times for each compound and bacterial strain, and the experiment was performed twice.

Disease control efficacy of the wettable powder formulation of the ethyl acetate layer of *S. takesimense* against tomato bacterial wilt. To evaluate the potential of the crude extract of *S. takesimense* as a natural bactericide for the control of tomato bacterial wilt caused by *Ralstonia solanacearum* SL1944 (race 1, biovar 4) (26), the methanol extract (14 g) of *S. takesimense* was dissolved in 500 ml of distilled water and then partitioned twice with an equal volume of ethyl acetate. After drying, 10 g of the ethyl acetate layer was dissolved in a minimal volume of acetone and then mixed with 3 g of synthetic hydrated silicon dioxide (white carbon; Rhodia Asia Pacific Pte Ltd.). Acetone was removed in a fume hood, and the white carbon was then mixed with

1 g of sodium dodecylsulfate (CR-SDS; Yoosung Chemical R&T Co., Ltd.) as a wetting agent, 1 g of sodium poly(naphthalene formaldehyde) sulfonate (CR-100; Yoosung Chemical R&T Co., Ltd.) as a dispersal agent, and 5 g of kaoline. The wettable powder (ST-WP) was milled in a blender.

Three-week-old tomato plants at the four- to five-true-leaf stage of 'Seokwang' grown in vinyl pots with a volume of 90 ml in a greenhouse were transplanted into vinyl pots with a volume of 180 ml and then transferred to a growth chamber. The soil used was a commercial horticulture nursery soil manufactured by Punong Co. The ST-WP was diluted 200- or 400-fold in distilled water containing Tween 20 at a concentration of 0.25 g/liter. Potted plants were irrigated with 20 ml of the solutions. Plants treated with the Tween 20 solution (0.25 g/liter) served as controls, while agricultural streptomycin sulfate (0.20 g/liter) was used as the bactericide control. The tomato plants were inoculated with 20 ml of an *R. solanacearum* suspension (10^8 CFU/ml) 1 day after treatment. Pots were arranged as a randomized complete block with five replicates per treatment. The experiment was repeated three times. The plants were kept in a controlled climate at $30 \pm 2^\circ\text{C}$ and a relative humidity of 70 to 80%.

The severity of wilting was ranked daily for 14 days according to the scale of He et al. (18): no symptoms (class 0), one leaf wilted (class 1), two or three leaves wilted (class 2), four or more leaves wilted (class 3), and plant dead (class 4). Disease development was presented using wilt incidence (WI; %) calculated as the percentage of leaves that were completely wilted according to the following equation: [(number of plants in class 1 $\times$ 1) + (number of plants in class 2 $\times$ 2) + (number of plants in class 3 $\times$ 3) + (number of plants in class 4 $\times$ 4)]/(total number of plants in the treatment $\times$ 4) (9,34). The control efficacy was calculated using the following formula 7 and 14 days after inoculation: control efficacy (%) = $100 \times (\text{WI of control} - \text{WI of treatment})/\text{WI of control}$.

Statistical analysis. Data were subjected to analysis of variance (ANOVA) using SAS software (version 9.3; SAS Institute). MIC values were analyzed by ANOVA as a function of the treated chemical (covariable), the pathogenic bacterium (factor), and their interaction. FIC and FICI values were analyzed by ANOVA as a function of the pathogenic bacterium (covariable), the combination of the chemicals (factor), and their interaction. The values of wilt incidence and control efficacy of ST-WP were analyzed by ANOVA as a function of the concentration of ST-WP (covariable), time after inoculation (factor), and their interaction. Mean comparisons were conducted using a least significant difference (Fisher's least significant difference [LSD]) test at $P = 0.05$. Standard error and LSD results were recorded.

Results

Structure determination. Repeated chromatography of the ethyl acetate layer of *S. takesimensense* on silica gel columns, Sephadex LH-20 columns, or prep-HPLC led to the purification of eight compounds (compounds 1 to 8). These compounds were identified as gallic acid (compound 1; 13), methyl gallate (compound 2; 28,50), 4,6-di-O-galloylarbutin (compound 3; 46), 2,6-di-O-galloylarbutin (compound 4; 43), 2,4,6-tri-O-galloyl-glucose (compound 5; 46), 1,3,4,6-tetra-O-galloyl- β -glucose (compound 6; 47), 1,2,4,6-tetra-O-galloyl- β -glucose (compound 7; 41,46), and 1,2,3,6-tetra-O-galloyl- β -glucose (compound 8; 40,45) by comparing their spectroscopic data with data previously reported (Fig. 1).

In vitro antibacterial activity. The in vitro antibacterial activity of the three major substances—namely, compounds 1, 4, and 7—as well as compound 2 was evaluated against nine plant-pathogenic bacteria strains. The in vitro activity of the remaining minor compounds (compounds 3, 5, 6, and 8) was investigated using the plant-pathogenic bacteria, except for *Agrobacterium tumefaciens* and *Pectobacterium carotovorum* subsp. *carotovorum*. Cell growth of all bacteria strains was inhibited by the eight gallotannin compounds (Table 1). Their inhibitory activity was different according to species and chemical type. Among the eight compounds, methyl gallate (compound 2) and 1,2,3,6-tetra-O-galloyl- β -glucose (com-

pound 8) showed the most potent in vitro antibacterial activity against the bacteria tested, with MIC values of 0.03 to 0.56 and 0.02 to 0.13 g/liter, respectively. Compound 7, which was 1,2,4,6-tetra-O-galloyl- β -glucose, also strongly inhibited the cell growth of the pathogenic bacteria, with MIC values of 0.03 to 1.78 g/liter.

Of the nine bacteria strains tested, *R. solanacearum* was most sensitive to the eight isolated compounds followed by *Pseudomonas syringae* pv. *actinidiae*, *X. arboricola* pv. *pruni*, *Acidovorax avenae* subsp. *cattleyae*, and *C. michiganensis* subsp. *michiganensis*. The MIC values of the eight chemicals against *R. solanacearum* were 0.02 to 0.10 g/liter, respectively. In comparison, *Pectobacterium carotovorum* subsp. *carotovorum* and *Agrobacterium tumefaciens* were most sensitive to methyl gallate (MIC = 0.25 g/liter) but were not sensitive to the other antibacterial compounds, with MIC values of approximately 2.00 g/liter or more. Interestingly, *Xanthomonas* spp. were insensitive to gallic acid and digalloylarbutins (compounds 3 and 4) but were highly sensitive to methyl gallate and tetragalloylglucoses (compounds 7 and 8).

Methyl gallate was more active than gallic acid (Table 1). Moreover, of the two galloylarbutin compounds, 2,6-di-O-galloylarbutin (compound 4) was more active than 4,6-di-O-galloylarbutin (compound 3). With regard to the galloylglucose compounds, the in vitro antibacterial activity of 1,2,3,6-tetra-O-galloyl- β -glucose (compound 8) was strongest, followed by 1,2,4,6-tetra-O-galloyl- β -glucose (compound 7), 1,3,4,6-tetra-O-galloyl- β -glucose (compound 6), and 2,4,6-tri-O-galloyl-glucose (compound 5).

Combination effect of simple gallate compounds and galloylarbutin or galloylglucose. The combinations of gallic acid (compound 1) + 2,6-di-O-galloylarbutin (compound 4), methyl gallate (compound 2) + 1,2,4,6-tetra-O-galloyl- β -glucose (compound 7), and methyl gallate (compound 2) + 2,6-di-O-galloylarbutin (compound 4) had synergistic or partial synergistic modes of interac-

Fig. 1. Chemical structures of eight antibacterial compounds isolated from the aerial parts of *Sedum takesimensense*: compound 1, gallic acid; compound 2, methyl gallate; compound 3, 4,6-di-O-galloylarbutin; compound 4, 2,6-di-O-galloylarbutin; compound 5, 2,4,6-tri-O-galloyl-glucose; compound 6, 1,3,4,6-tetra-O-galloyl- β -glucose; compound 7, 1,2,4,6-tetra-O-galloyl- β -glucose; and compound 8, 1,2,3,6-tetra-O-galloyl- β -glucose.

Table 1. Minimum inhibitory concentration (MIC) values of isolated compounds against plant-pathogenic bacteria

Pathogen	MIC (g/liter) for compounds 1–8 ^z							
	1	2	3	4	5	6	7	8
<i>Acidovorax avenae</i>								
subsp. <i>cattleyae</i>	0.17 ± 0.07 d	0.56 ± 0.17 ab	0.72 ± 0.26 a	0.50 ± 0.00 b	0.44 ± 0.11 bc	0.25 ± 0.00 cd	0.13 ± 0.00 d	0.10 ± 0.03 d
<i>Agrobacterium tumefaciens</i>	0.42 ± 0.13 b	0.25 ± 0.00 b	–	1.67 ± 0.50 a	–	–	1.33 ± 0.50 a	–
<i>Burkholderia glumae</i>	0.83 ± 0.25 b	0.05 ± 0.02 d	1.00 ± 0.00 a	1.00 ± 0.00 a	0.44 ± 0.11 c	0.31 ± 0.11 c	0.19 ± 0.07 cd	0.11 ± 0.03 d
<i>Clavibacter michiganensis</i>								
subsp. <i>michiganensis</i>	0.78 ± 0.26 a	0.04 ± 0.02 d	0.83 ± 0.25 a	0.50 ± 0.00 b	0.42 ± 0.13 b	0.33 ± 0.13 bc	0.15 ± 0.06 cd	0.13 ± 0.00 cd
<i>Pectobacterium carotovorum</i>								
subsp. <i>carotovorum</i>	>2.00	0.25 ± 0.00 b	–	>2.00	–	–	1.78 ± 0.44 a	–
<i>Pseudomonas syringae</i>								
pv. <i>actinidiae</i>	0.33 ± 0.13 b	0.17 ± 0.06 cd	0.47 ± 0.08 a	0.28 ± 0.08 bc	0.25 ± 0.00 bc	0.17 ± 0.06 cd	0.17 ± 0.06 cd	0.13 ± 0.00 d
<i>Ralstonia solanacearum</i>	0.05 ± 0.02 b	0.03 ± 0.01 bc	0.10 ± 0.03 a	0.08 ± 0.03 a	0.04 ± 0.02 bc	0.03 ± 0.00 bc	0.03 ± 0.00 bc	0.02 ± 0.01 c
<i>Xanthomonas arboricola</i>								
pv. <i>pruni</i>	0.39 ± 0.13 b	0.06 ± 0.00 c	1.00 ± 0.00 a	0.83 ± 0.25 a	0.33 ± 0.13 b	0.10 ± 0.03 c	0.04 ± 0.02 c	0.03 ± 0.00 c
<i>X. euvesicatoria</i>	>2.00	0.05 ± 0.02 d	0.94 ± 0.17 a	0.56 ± 0.17 b	0.50 ± 0.00 b	0.50 ± 0.00 b	0.21 ± 0.06 c	0.13 ± 0.00 cd

^z MIC = minimum concentration inhibiting bacterial growth by 100%. Compound 1, gallic acid; compound 2, methyl gallate; compound 3, 4,6-di-O-galloylarbutin; compound 4, 2,6-di-O-galloylarbutin; compound 5, 2,4,6-tri-O-galloyl-glucose; compound 6, 1,3,4,6-tetra-O-galloyl-β-glucose; compound 7, 1,2,4,6-tetra-O-galloyl-β-glucose; compound 8, 1,2,3,6-tetra-O-galloyl-β-glucose. Means within the same row followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's least significant difference test; – = not tested.

Table 2. Fractional inhibitory concentration (FIC) indexes (FICIs) of combined effects among major active compounds (compounds 1, 4, and 7) and methyl gallate (compound 2) against plant-pathogenic bacteria^z

Pathogen	FIC			FIC			FIC			FIC		
	1	4	FICI	2	7	FICI	2	4	FICI	1	7	FICI
<i>Acidovorax avenae</i>												
subsp. <i>cattleyae</i>	0.13 cd	0.50 a	0.63 ± 0.13ab	0.50 a	0.38 cd	0.88 ± 0.14 ab	0.21 b	0.42 b	0.63 ± 0.11 c	0.50 b	0.05 d	0.55 ± 0.02 d
<i>Agrobacterium tumefaciens</i>	0.50 a	0.09 cd	0.59 ± 0.03 ab	0.05 d	1.00 a	1.05 ± 0.02 a	0.50 a	0.15 d	0.65 ± 0.09 c	0.33 bc	0.50 bc	0.83 ± 0.13 c
<i>Burkholderia glumae</i>	0.31 b	0.28 bc	0.59 ± 0.03 ab	0.09 cd	0.50 c	0.59 ± 0.03 c	0.21 b	0.33 bc	0.58 ± 0.05 c	0.38 bc	0.19 cd	0.57 ± 0.07 d
<i>Clavibacter michiganensis</i>												
subsp. <i>michiganensis</i>	0.19 bc	0.06 d	0.25 ± 0.07 cd	0.10 cd	0.50 c	0.60 ± 0.03 c	0.50 a	0.05 d	0.55 ± 0.02 c	0.50 b	0.09 d	0.59 ± 0.03 d
<i>Pectobacterium carotovorum</i>												
subsp. <i>carotovorum</i>	0.16 cd	0.38 ab	0.53 ± 0.03 b	0.25 c	0.06 d	0.31 ± 0.00 d	0.13 bc	0.19 cd	0.31 ± 0.07 d	0.38 bc	0.75 ab	1.13 ± 0.14 b
<i>Pseudomonas syringae</i>												
pv. <i>actinidiae</i>	0.25 bc	0.21c	0.46 ± 0.06 c	0.33 bc	0.33 cd	0.67 ± 0.13 bc	0.50 a	0.13 d	0.63 ± 0.00 c	0.25 c	0.50 bc	0.75 ± 0.00 c
<i>Ralstonia solanacearum</i>	0.50 a	0.19 c	0.69 ± 0.07 a	0.42 ab	0.15 d	0.53 ± 0.03 c	0.25 b	0.19 cd	0.44 ± 0.07 cd	1.00 a	0.38 c	1.38 ± 0.14 a
<i>Xanthomonas arboricola</i>												
pv. <i>pruni</i>	0.25 bc	0.09 cd	0.34 ± 0.03 cd	0.13 cd	0.75 b	0.88 ± 0.27 ab	0.09 c	1.0a	1.09 ± 0.03 a	0.10 d	1.0 a	1.10 ± 0.03 b
<i>Xanthomonas euvesicatoria</i>	0.04 d	0.06 d	0.10 ± 0.03 d	0.21 c	0.50 c	0.71 ± 0.06 bc	0.67 a	0.14 d	0.81 ± 0.20 b	0.33 bc	0.83 a	1.13 ± 0.14 b

^z FICI ≤ 0.5, synergy; 0.5 < FICI ≤ 0.75, partial synergy; 0.75 < FICI ≤ 1.0, additive effect; 1.0 < FICI ≤ 4.0, indifference and FICI > 4.0, antagonism. Compound 1, gallic acid; compound 2, methyl gallate; compound 4, 2,6-di-O-galloylarbutin; and compound 7, 1,2,4,6-tetra-O-galloyl-β-glucose. Means within the same column followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's least significant difference test.

tions for most of the bacteria tested, showing FICI ranges of 0.01 to 0.88. 2,6-Di-O-galloylarbutin (compound 4) was strongly synergistic with gallic acid (compound 1) against *X. euvesicatoria* (FIC = 0.10), followed by *X. arboricola* pv. *pruni*, *C. michiganensis* subsp. *michiganensis*, and *Pseudomonas syringae* pv. *actinidiae* (Table 2). 2,6-Di-O-galloylarbutin (compound 4) was strongly synergistic with methyl gallate (compound 2) against *Pectobacterium carotovorum* subsp. *carotovorum* and *R. solanacearum*. The combination of 1,2,4,6-tetra-O-galloyl-β-glucose (compound 7) and methyl gallate (compound 2) was synergistic against *P. carotovorum* subsp. *carotovorum* and had partial synergistic effects against all of the remaining bacteria, except *A. tumefaciens*. The combination of gallic acid (compound 1) and 1,2,4,6-tetra-O-galloyl-β-glucose (compound 7) did not show synergistic effects but had partial synergistic or indifference effects. No combinations were found to have antagonistic effects.

Effect of the wettable powder on tomato bacterial wilt. In the greenhouse experiment, wilt symptoms were first observed 5 days after inoculation. ST-WP effectively suppressed the development of bacterial wilt caused by *R. solanacearum* in a dose-dependent manner (Table 3). At 200- and 400-fold dilutions, the control efficacies of the formulations were 100 and 93.9%, respectively, 7 days after inoculation and 78.3 and 53.9%, respectively, 14 days after inoculation. ST-WP had a 400-fold higher disease control efficacy than agricultural streptomycin sulfate (0.20 g/liter) at a significance level of $P = 0.05$.

Discussion

We isolated eight compounds with antibacterial properties against *R. solanacearum* race 1, biovar 4 and eight other plant-pathogenic bacteria. Gallic acid (compound 1) and its related compounds, methyl gallate (compound 2), galloylarbutins (compounds 3 and 4), and galloylglucoses (compounds 5–8), with galloyl groups ranging from two to four, belong to the gallotannin class (36). There have been several reports on the biological activities, including antioxidant and antimicrobial activities, of gallic acid and methyl gallate (3,5,6,14,20,33,50). Except for 2,6-di-O-galloylarbutin (compound 4), the remaining seven gallotannin compounds from *S. takesimensis* were first reported in this study. To our knowledge, information on antibacterial activity of these galloylarbutin and galloylglucose compounds against plant-pathogenic bacteria has not been previously reported.

In the present study, gallic acid (compound 1) had weak antimicrobial activity against plant-pathogenic bacteria compared with methyl gallate (compound 2). Methyl gallate showed a broad antibacterial spectrum and potent activity against all of the pathogenic bacteria, showing MIC ranges of 0.03 to 0.56 g/liter. Ooshiro et al. (33) reported the in vitro antibacterial activity of gallic acid and methyl gallate against *R. solanacearum*. In a paper disc method, gallic acid showed approximately 1/100 of the activity of methyl gallate as estimated from the dose-response curve of methyl gallate (33). In addition, methyl gallate isolated from *Toxicodendron syl-*

Table 3. Effect of the wettable powder formulation of the ethyl acetate layer of *Sedum takesimense* containing eight compounds on the incidence and control of tomato bacterial wilt under greenhouse conditions^z

Treatment	7 days		14 days	
	Wilt incidence (%)	Control efficacy (%)	Wilt incidence (%)	Control efficacy (%)
ST-WP 400-fold	3.3 ± 2.9 c	93.9 ± 5.4 a	38.3 ± 2.9 b	53.9 ± 9.8 b
ST-WP 200-fold	0.0 ± 0.0 c	100.0 ± 0.0 a	18.3 ± 2.9 c	78.3 ± 2.9 a
Agricultural streptomycin sulfate	20.0 ± 5.0 b	62.8 ± 6.3 b	43.3 ± 7.6 b	48.2 ± 12.9 b
Control	53.3 ± 5.8 a	...	85.0 ± 13.2 a	...

^z Wilt incidence (WI) was assessed 7 and 14 days after inoculation, and WI was calculated as the percentage of leaves that were completely wilted. Control efficacy was calculated using the following formula: control efficacy (%) = 100 × (WI of control – WI of treatment)/WI of control. Data shown are mean of three replications. Means within the same column followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's least significant difference test.

vestre (Siebold & Zucc.) Kuntze displayed significant control efficacy against tomato bacterial wilt in planta (50).

Methyl gallate is one of the most active compounds among the eight compounds isolated from *S. takesimense*, which suggested that the esterification of the carboxylic group with the alkyl group may be increased, and enhanced the antibacterial spectrum of gallic acid. Our results are in agreement with those of previous studies (3,20,24,25,33). In general, the antibacterial ability of alkyl gallates depends on the carbon number of esters. For example, Kubo et al. (24,25) observed that the length of the alkyl group is not largely associated with the potency of antibacterial activity. The results suggested that the antibacterial activity of alkyl gallates is a parabolic function of their lipophilicity and is maximized with an alkyl chain length between C₈ and C₁₁. In contrast, Ooshiro et al. (33) reported that methyl gallate is the most active against *R. solanacearum* and that the activity weakens as the carbon number of esters increases. Thus, the antibacterial spectra and potency of alkyl gallates depend mainly on the hydrophobic portion of the molecules (24).

The remaining six compounds were identified as galloylarbutins (compounds 3 and 4) and galloylglucoses (compounds 5–8), with degrees of galloylation ranging from two to four. Among them, some compounds have been reported present in root extracts of plants, including *Sanguisorba officinalis* L. (40) and *Bergenia purpurascens* (Hook. f. & Thomson) Engl. (46). Several papers have recently reported the antimicrobial activity of larger galloylglucoses, with galloyl groups ranging from 5 to 10, in addition to mechanisms responsible for their antimicrobial activity, as follows: complexation with essential mineral micronutrients, including iron ions (Fe²⁺ and Fe³⁺); precipitation with proteins; enzyme inhibition; substrate deprivation; and action on membranes (11,12,36). In addition, Cho et al. (4) reported that 1,2,3,4,6-penta-O-galloyl-β-D-glucose isolated from *Rhus trichocarpa* inhibited the growth of both gram-negative and gram-positive bacteria and that their bacterial activity is restored by the addition of Fe²⁺.

The antibacterial effects of six compounds from twofold to eightfold are arranged in the following increasing order: compound 3 < compound 4 < compound 5 < compound 6 < compound 7 < compound 8. This arrangement indicates that an increasing number of galloyl groups and substitution at the 1 or 2 position on the glucose ring further enhances the activity of the galloylarbutins and galloylglucoses. To our knowledge, this is the first report showing the effects of the number and position of galloyl groups of smaller galloylglucoses on the growth of plant-pathogenic bacteria. The inhibitory activities of gallotannins from mango kernels are attributable to their iron complexing properties and relate to the inactivation of membrane-bound proteins (11). Larger and more hydrophobic gallotannins bind proteins more effectively than smaller gallotannins (2). In contrast, the number of galloyl groups generally does not affect the antibacterial activities of gallotannins from mango kernels with galloyl groups ranging from 5 to 10 (12). The structure activity relationship of galloylglucose compounds with galloyl groups ranging from 2 to 10 remains to be determined.

Synergistic or partial synergistic effects were observed in the following combinations gallic acid (compound 1) + 2,6-di-O-galloylarbutin (compound 4), methyl gallate (compound 2) + 1,2,4,6-

tetra-O-galloyl-β-glucose (compound 7), and methyl gallate (compound 2) + 2,6-di-O-galloylarbutin (compound 4). Among the four combinations tested, three showed partial synergistic or synergistic effects on most of the tested bacteria, including *Ralstonia solanacearum*. These results support the potent antibacterial activity of the ethyl acetate layer of *Sedum takesimense* against various plant-pathogenic bacteria.

The wettable powder formulation made from the ethyl acetate layer of *S. takesimense* effectively suppressed the development of tomato bacterial wilt in the greenhouse experiment. We did not observe phytotoxic symptoms on plants. In order to develop new botanical bacteriocides using the products derived from *S. takesimense*, further studies are necessary for the discovery of chemicals to enhance the antibacterial activity, the development of optimum formulation, and the determination of effective dose in field crops. This study suggests that the extract from *S. takesimense* or the isolated gallotannins could be used as natural bactericides for the control of tomato bacterial wilt.

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