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Antibacterial Activity of Pharbitin, Isolated from the Seeds of *Pharbitis nil*, against Various Plant Pathogenic Bacteria

Hoa Thi Nguyen[1], Nan Hee Yu[1], Ae Ran Park[1], Hae Woong Park[2], In Seon Kim[1], and Jin-Cheol Kim[1]*

[1]*Department of Agricultural Chemistry, Institute of Environmentally Friendly Agriculture, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 61186, Republic of Korea*
[2]*R&D Division, World Institute of Kimchi, Gwangju 61755, Republic of Korea*

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**Corresponding author*
Phone: +82-10-2823-7640;
Fax: +82-62-530-2139;
E-mail: kjinc@jnu.ac.kr

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This study aimed to isolate and characterize antibacterial metabolites from *Pharbitis nil* seeds and investigate their antibacterial activity against various plant pathogenic bacteria. The methanol extract of *P. nil* seeds showed the strongest activity against *Xanthomonas arboricola* pv. *pruni* (Xap) with a minimum inhibition concentration (MIC) value of 250 μg/ml. Among the three solvent layers obtained from the methanol extract of *P. nil* seeds, only the butanol layer displayed the activity with an MIC value of 125 μg/ml against Xap. An antibacterial fraction was obtained from *P. nil* seeds by repeated column chromatography and identified as pharbitin, a crude resin glycoside, by instrumental analysis. The antibacterial activity of pharbitin was tested in vitro against 14 phytopathogenic bacteria, and it was found to inhibit *Ralstonia solanacearum* and four *Xanthomonas* species. The minimum inhibitory concentration values against the five bacteria were 125–500 μg/ml for the n-butanol layer and 31.25–125 μg/ml for pharbitin. In a detached peach leaf assay, it effectively suppressed the development of bacterial leaf spot, with a control value of 87.5% at 500 μg/ml. In addition, pharbitin strongly reduced the development of bacterial wilt on tomato seedlings by 97.4% at 250 μg/ml, 7 days after inoculation. These findings suggest that the crude extract of *P. nil* seeds can be used as an alternative biopesticide for the control of plant diseases caused by *R. solanacearum* and *Xanthomonas* spp. This is the first report on the antibacterial activity of pharbitin against phytopathogenic bacteria.

Keywords: Antibacterial activity, *Pharbitis nil* seeds, pharbitin, bacterial leaf spot, tomato wilt

Introduction

Pathogenic bacteria are a major cause of disease in plants and reduce the yield of crops worldwide. *Xanthomonas arboricola* pv. *pruni* (Xap) is an important invasive phytopathogenic bacterium, with a broad host range including peach, nectarine, prune, plum, and apricot. Symptoms of this disease are fruit spots, leaf spots, and twig cankers [1]. According to Stefani [2], damage caused to plum orchards in northern Italy by Xap has been estimated at 11.200 € per ha (cv. Golden Plum) or 9,500 € per ha (cv. Angeleno). Yield losses of plums, peaches, and nectarines in Japan were up to 10,000 € per ha [2].

Ralstonia solanacearum (Rs) is a soil- and water-borne gram-negative phytopathogenic bacterium, and causes bacterial wilt diseases, affecting hundreds of species. Plants are infected through either the wounds or at the site of emergent root tips [3, 4]. It causes bacterial wilt in over 200 plant species, including tomato, potato, pepper, peanut, tobacco, eggplant, and banana [5], and has a major impact on crop production all over the world. Control of bacterial wilt is difficult, and the most effective approach is to use crops resistant to the pathogen. The use of commercial antibiotics, copper compounds, and synthetic pesticides to control various plant pathogenic bacteria has improved the situation, but their long-term use has resulted in environmental pollution, residual toxicity, and the development of resistance in the target pathogens. Accordingly,

there is a need for natural biopesticides based on plants and microbial-derived materials that can replace synthetic bactericides [6–9].

The largest genus of the morning glory family Convolvulaceae is *Pharbitis* (*Ipomoea*), with approximately 600 species [10, 11]. One of these, *P. nil* (*I. nil*), is widely distributed in Southeast Asia [12]. The chemical compositions of *Pharbitis* spp. have been investigated, and it was found to consist of resin glycosides, phenolic amides, gibberellins, flavonoids, and hevein-like proteins [13–17]. The seeds of *P. nil* have been used for a long time in the traditional medicines of Korea, China, and Japan as purgative drugs [18, 19]. The major ingredients of the genus *Pharbitis* are well known as "resin glycosides," including pharbitin and convolvulin [20]. The substances derived from the seeds of *P. nil* were reported to exhibit antitumor [12, 21, 22], antifungal [16, 23, 24], anticancer [25], and anthelmintic [26] activities, but no information regarding their antibacterial activity against plant pathogenic bacteria has been disclosed.

In order to discover new antibacterial active substances, 225 medicinal plant extracts were tested in vitro for antibacterial activity against two plant pathogenic bacteria. The methanol extract of *Pharbitis nil* seeds showed the strongest activity against Rs and Xap. Therefore, this study aimed to isolate and characterize the antibacterial metabolites contained in this extract, and to examine their in vitro antibacterial activity against various plant pathogenic bacteria. Additionally, their disease control efficacy was evaluated against bacterial leaf spots on peach leaves in a detached leaf bioassay, and bacterial wilt on tomato seedlings.

Materials and Methods

Bacterial Strains and Culture Conditions

The following 14 phytopathogenic bacterial strains were used: *Acidovorax avenae* subsp. *cattleyae* (Aac; bacterial brown spot of *Phalaenopsis*), *Acidovorax konjaci* (Ak; bacterial blight of konjac), *Agrobacterium tumefaciens* (At; crown gall of apple), *Burkholderia glumae* (Bg; bacterial panicle blight of rice), *Clavibacter michiganensis* subsp. *michiganensis* (Cmm; bacterial wilt and canker of tomato), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc; bacterial soft rot of potato), *Pectobacterium chrysanthemi* (Pc; bacterial soft rot of aloe), *Pseudomonas syringae* pv. *actinidiae* (Psa; bacterial canker of kiwifruit), *Pseudomonas syringae* pv. *lachrymans* (Psl; angular leaf spot of cucumber), *Ralstonia solanacearum* (Rs; bacterial wilt of tomato), *Xanthomonas arboricola* pv. *pruni* (Xap; bacterial leaf spot of peach), *Xanthomonas axonopodis* pv. *citri* (Xac; citrus canker), *Xanthomonas euvesicatoria* (Xe; bacterial spot of tomato), and *Xanthomonas oryzae* pv. *oryzae* (Xoo; bacterial leaf blight of rice). Rs and Psa were isolated by Prof. S.-W. Lee of Dong-A University [27] and Prof. Y. J. Koh of Sunchon National University [28], respectively, who kindly supplied us with samples, which were preserved at −80°C at Chonnam National University. All other bacterial strains were acquired from the Korean Agricultural Culture Collection (KACC), National Academy of Agricultural Sciences. All strains were grown on tryptic soy broth (TSB) and tryptic soy agar (TSA) (Becton, Dickinson and Co., USA) and incubated at 30°C, expect for Psa, which was incubated at 25°C, and *Xanthomonas* spp., which was incubated at 28°C.

Chemicals

Streptomycin sulfate was purchased from Sigma-Aldrich Co. (USA). Tween-20 and all the chemicals and solvents used, including methanol (MeOH), ethyl acetate (EtOAc), *n*-butanol (BuOH), chloroform ($CHCl_3$), acetone, potassium carbonate (K_2CO_3), hydrochloric acid (HCl), and sodium sulfate (Na_2SO_4), were of analytical grade and acquired from either E. Merck (Germany) or Daejung Chemicals (Korea).

Plant Material

The MeOH extract of *P. nil* seeds used in the antibacterial activity screening was acquired from the Korean Plant Extract Bank (Korea). The seeds of *P. nil* used for isolation of the active compounds were purchased from a Korean herbal medicine shop.

Isolation of Antibacterial Substances

The seeds of *P. nil* (150 g) were ground and extracted twice with MeOH (2 × 2 L) at room temperature. The extracts were filtered through a Whatman No. 2 filter paper and the filtrate was concentrated in vacuo to afford a brown syrup (8 g). This syrup was then suspended in distilled water (500 ml) and then successively extracted with equal volumes of EtOAc and BuOH (×2 for each). The organic layers and aqueous layer were concentrated in vacuo. The in vitro antibacterial activity of each layer was tested against Xap, and the BuOH layer was found to exhibit the strongest antibacterial activity. Accordingly, isolation of the bioactive compounds from the BuOH layer was pursued.

The BuOH layer (3.2 g) was subjected to column chromatography on-silica gel column (3.5-cm inner diameter and 60-cm length, Kiesel gel 60, 150 g, 70–230 mesh; E. Merck) by successive elution with $CHCl_3$ / MeOH / water / acetic acid (14/6/1/0.1 (v/v/v/v), 800 ml), to give seven fractions, F1–F7. Each fraction was analyzed using thin-layer chromatography (TLC) (Kiesel gel 60GF 254, 0.25-mm layer thickness; E. Merck) with the developing solvent $CHCl_3$/MeOH/water (6/4/1 (v/v/v)). The fractions were individually assayed in vitro for growth inhibition of Xap. F1 was not found to inhibit growth. Fractions F2–F5 all inhibited growth to some extent. TLC of each showed a similar pattern of primary components when visualized under ultraviolet light (254 and 365 nm). Visualization with *p*-anisaldehyde reagent showed one major component with an R_f value of 0.8. The F2–F5 fractions (1.47 g) were combined, concentrated, and subjected to Sephadex LH 20 column chromatography (3 cm inner diameter, 80 cm

length, 100 g, bead size 18–111 μm; GE Healthcare, Sweden), eluting with 100% MeOH. Four fractions (F21–F24) were obtained. In a TLC analysis using the solvent systems $CHCl_3$ / MeOH / water / acetic acid (30/9/1/0.1 (v/v/v/v)), $CHCl_3$ / MeOH / water / acetic acid (12/8/1/0.1 (v/v/v/v)), and $CHCl_3$ / MeOH / water / acetic acid (6/4/1/0.1 (v/v/v/v)), the active fraction of F21 appeared as a single spot when sprayed with *p*-anisaldehyde and charred. F21 was obtained as a pale yellow power.

Alkaline Hydrolysis of F21

The ^{1}H NMR spectrum of F21 showed the presence of olefinic, sugar, and saturated hydrocarbon protons. The spectrum was similar to that of pharbitin, a mixture of seven oligoglycosides of hydroxyl fatty acid [29]. In order to get more information by chemical degradation, F21 was hydrolyzed using alkaline. F21 (470 mg) was suspended in 1% aqueous K_2CO_3 (10 ml) and heated at 90°C for 2.5 h. The mixture was adjusted to pH 4 with 1N HCl and extracted with EtOAc (10 ml). The organic phase was washed with H_2O and dried over Na_2SO_4 [30]. The aqueous layer was concentrated at 60°C and then chromatographed on a Sephadex LH-20 column (3.5-cm inner diameter and 60-cm length, 100 g, bead size 18–111 μm; GE Healthcare), eluting with 100% MeOH. Four fractions (F21W1–F21W4) were obtained. The fractions were monitored using TLC with the developing solvent $CHCl_3$/ MeOH/water (6/4/1 (v/v/v)). The residue of F21W4 was separated by preparative TLC using a mixture of $CHCl_3$/MeOH/ water/acetic acid (55/ 36/8/1 (v/v/v/v)) to yield 85.3 mg of a pure compound, called compound **1**.

Structure Determination of the Antibacterial Substance

The chemical structure of compound **1** was determined by nuclear magnetic resonance (NMR) spectroscopy and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) analyses. ^{1}H-NMR spectra were measured in pyridine d_5 (Cambridge Isotope Laboratories, Inc., USA) with a Bruker Avance III HD 500 MHz instrument (Bruker Biospin GmbH, Germany) at 500 MHz. Chemical shifts were measured using tetramethylsilane as the internal standard. HR-ESI-MS was determined by a Synapt G2 HDMS quadrupole time-of-flight (QTOF) mass spectrometer equipped with an electrospray ion source (Waters, UK). Coupling constants are reported in Hertz unit. The chemical structure of compound **1** was analyzed on the basis of ^{1}H-NMR spectral data and HR-ESI-MS.

In Vitro Antibacterial Activity

In vitro antibacterial activity was determined in 96-well microtiter plates using the broth microdilution method [6]. The EtOAc, BuOH, and aqueous layers were dissolved in acetone, MeOH, and distilled water, respectively, at a concentration of 500 mg/ml. The fractions obtained during the isolation of the active compound, F21 and compound **1**, were dissolved in MeOH at a concentration of 500 mg/ml. The bioassays were conducted using 96-well plates, each well containing 100 μl of TSB medium inoculated with pathogenic bacteria at 1×10^5 CFU/ml, except for *Xanthomonas* spp. which was inoculated at 1×10^6 CFU/ml. The final concentrations of the samples of F21 and compound **1** ranged from 7.8 to 500 μg/ml. The final concentration of MeOH was less than 1% (v/v), and the negative controls were treated with 1% MeOH. Streptomycin sulfate was used as a positive control. It was dissolved in distilled water at a concentration of 10 mg/ml and the final concentrations of the treated streptomycin sulfate ranged from 1.56 to 100 μg/ml. The blank wells were prepared with culture medium containing each extract at the same test concentration. All samples were incubated for 24–72 h at 30°C, except for *Xanthomonas* spp., which was incubated at 28°C, and Psa, which was incubated at 25°C. Bacterial growth was measured using a microplate reader (Benchmark Plus; Bio-Rad, USA) at 600 nm (OD_{600}). The minimum inhibitory concentration (MIC) was defined as the lowest concentration that completely inhibited bacterial growth. The assay was repeated three times in triplicate for each sample, against the individual bacterial species at all test concentrations.

Disease Control Efficacy against Peach Bacterial Leaf Spot

To evaluate the disease control efficacy of the BuOH layer and F21 against bacterial leaf spot caused by Xap, a detached leaf assay was conducted using leaves of peach trees [31]. Young apical leaves (6–9 cm long) were collected from the top of branches and transferred to a moist plastic chamber. The leaves were rinsed with sterile distilled water three times and dried using sterilized paper towels. The BuOH layer and F21 were dissolved in MeOH at concentrations of 12.5, 25, and 50 mg/ml. Each stock solution was diluted 100-fold in distilled water containing 250 μg/ml Tween-20 to obtain concentrations of 125, 250, and 500 μg/ml, respectively. Leaves treated with 1% MeOH in Tween-20 (250 μg/ml) served as negative controls. Streptomycin sulfate at a concentration of 200 μg/ml in Tween-20 (250 μg/ml) was used as a positive control.

Xap was cultured on a TSB test tube at 28°C for 36–48 h. Bacterial suspensions were adjusted in sterile distilled water to obtain an OD_{600} of 0.1 (approximately 1×10^8 CFU/ml). Two leaves were dipped for 10 sec in each chemical solution and then placed on a plastic dish at room temperature. After 4 h, six wounds were made in each leaf using a forceps clamp, and then Xap (10^8 CFU/ml) was inoculated by dipping in the pathogen suspension. The two inoculated leaves were placed on 0.5% water agar in a petri dish (diameter 90 mm, height 15 mm) (20 ml per dish). The petri dishes were maintained at 28°C for 16 h in the light, and 8 h in the dark. Bacteria spot symptom development was recorded daily until day 5 post inoculation. Each experiment was repeated twice with two petri dishes per treatment. The disease incidence and control value were calculated using the following formulae:

% Disease incidence (DI) = 100 × [number of bacterial leaf spots / number of all wounds]

% Control value = 100 – [100 × % DI of treatment / % DI of control]

Disease Control Efficacy against Tomato Bacterial Wilt

The disease control efficacy of the BuOH layer of *P. nil* and F21 against tomato bacterial wilt caused by Rs was evaluated in a growth room. The tomato seeds ("Seokwang"; FarmHannong Co., Korea) were grown in the greenhouse in vinyl pots filled with commercial horticulture nursery soil manufactured by the Punong Co. (Korea). Four-week-old tomato plants at the 4–5 true-leaf stage were transplanted into vinyl pots with a diameter of 7 cm (one plant per pot). Samples of the BuOH layer and F21 were dissolved in MeOH at concentrations of 50, 100, and 200 mg, and 12.5, 25, and 50 mg, respectively. The chemical solutions were then diluted with distilled water containing 250 μg/ml Tween-20 to obtain test concentrations of 500, 1,000, and 2,000 μg/ml for the BuOH layer, and 125, 250, and 500 μg/ml for F21. Rs was cultured on TSA at 30°C for 48 h and then the cells were harvested with sterile distilled water. The inoculum suspension was adjusted to 1×10^8 CFU/ml.

Each chemical solution (20 ml) was evenly applied to each pot by soil drench. After 6 h, Rs was inoculated by applying 20 ml of the inoculum suspension into the soil. Potted plants treated with Tween-20 solution containing 1% MeOH served as controls, and streptomycin sulfate (200 μg/ml) was used as the bactericide control. The plants were maintained in a controlled climate at 30 ± 2°C with a relative humidity of 70–80%. The pots were arranged as a randomized complete block with five replicates per treatment. The experiment was repeated three times.

The disease severity (DS) was recorded on a scale of 0–5 as described by Winstead and Kelman [32]. The following scales were used: 0 = no symptoms, 1 = one leaf partially wilted, 2 = one to two leaves wilted, 3 = two to three leaves wilted, 4 = four or more leaves wilted, and 5 = death of the entire plant. The control value was calculated using the following formula:

% Control value = 100 × [DS of control – DS of treatment / DS of control]

Statistical Analysis

The data obtained in this study were evaluated by ANOVA, and statistical analysis of the significance of mean differences was performed using Duncan's HSD test for multiple comparisons ($p \leq 0{\cdot}05$) with the SAS software (ver. 9.3; SAS Institute, USA).

Results

In Vitro Antibacterial Activity of Crude Extracts of *P. nil* Seeds

The MeOH extract of *P. nil* seeds showed in vitro antibacterial activity against Xap with MIC values of 250 μg/ml (Fig. 1). The in vitro antibacterial activity of the two organic layers and one aqueous layer obtained from the MeOH extract were also evaluated. Of the three layers, the BuOH layer showed the strongest antibacterial activity against Xap with an MIC value of 250 μg/ml (Fig. 1). The other two layers were virtually inactive.

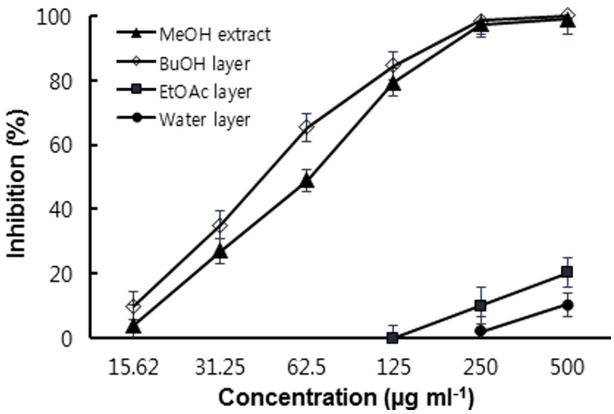


Fig. 1. Antibacterial activity of the methanol extract and three layers of *Pharbitis nil* seeds against *Xanthomonas aboricola* pv. *pruni*.

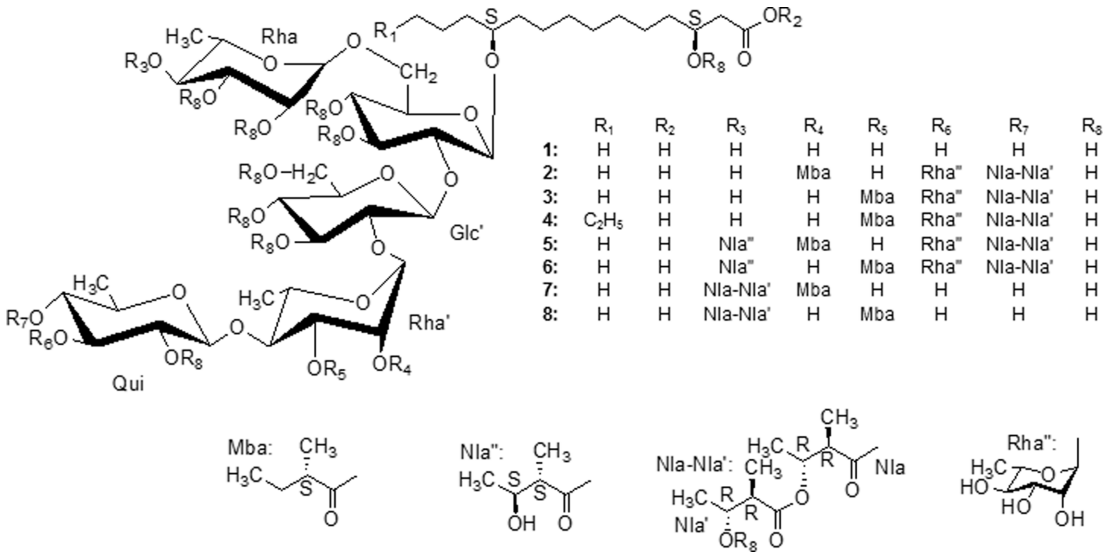
Each value represents the mean ± standard deviation of three runs with two replicates per run (MeOH: methanol; BuOH: butanol; EtOAc: ethyl acetate).

Characterization of the Antibacterial Metabolite

F21 was isolated from the BuOH layer of the *P. nil* seed extract, and appeared as a single spot on TLC (Fig. S1). However, the ^{1}H-NMR spectrum of F21 clearly indicated it to be a mixture of resin glycosides (data not shown). F21 was hypothesized to be pharitin, a known mixture of seven oligoglycosides of hydroxyl fatty acid (Fig. 2) [29]. To confirm the identity of F21, it was hydrolyzed using 1% aqueous K_2CO_3. One major compound (compound **1**) was purified from the alkaline hydrolysis products by Sephadex LH-20 and prep-TLC chromatographic methods as a white powder. The HR-ESI-MS results of compound **1** displayed ion peaks $[M+Na]^+$ at *m/z* 1,045.4694 and $[M-H]^-$ at *m/z* 1,021.4698 in positive and negative modes, respectively, suggesting a molecular formula of $C_{44}H_{78}O_{26}$ with six degrees of unsaturation (Fig. S2). The ^{1}H-NMR spectrum of compound **1** exhibited signals corresponding to five anomeric protons (δ 6.26, 5.89, 5.40, 5.21, and 4.87), three secondary methyls, perhaps corresponding to 6-deoxyhexose (δ 1.86, 1.63, and 1.52), a nonequivalent 2-methylene (δ 2.72 and 2.87), and a primary methyl (δ 0.95) ascribable to an ipurolic acid moiety (Table S1). Both ^{1}H-NMR and HR-ESI-MS data were identical to those of pharbitic acid C reported by Ono *et al.* [30]. Thus, compound **1** was identified as pharbitic acid C (Fig. 2), and F21 as pharbitin.

In Vitro Antibacterial Activity

The in vitro antibacterial activity of the BuOH layer and



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
1:	H	H	H	H	H	H	H	H
2:	H	H	H	Mba	H	Rha"	Nla-Nla'	H
3:	H	H	H	H	Mba	Rha"	Nla-Nla'	H
4:	C_2H_5	H	H	H	Mba	Rha"	Nla-Nla'	H
5:	H	H	Nla"	Mba	H	Rha"	Nla-Nla'	H
6:	H	H	Nla"	H	Mba	Rha"	Nla-Nla'	H
7:	H	H	Nla-Nla'	Mba	H	H	H	H
8:	H	H	Nla-Nla'	H	Mba	H	H	H

Fig. 2. Chemical structures of pharbitin (mixture of compounds **2**–**8**) (Ono *et al*. [30]) and pharbitic acid C (compound **1**).

F21 (pharbitin) was tested against 14 plant pathogenic bacteria (Table 1). The two samples exhibited specific in vitro antibacterial activity against Rs and *Xanthomonas* spp. BuOH and F21 displayed MIC values with a range of 125–500 μg/ml and 31.25–125 μg/ml against the five sensitive bacteria, respectively. Of the 14 bacteria, Rs and Xoo were most sensitive to pharbitin, with an MIC value of 31.25 μg/ml, followed by Xap and Xe, which had MIC values of 125 μg/ml for the BuOH layer and 62.5 μg/ml for F21. Neither the BuOH nor the F21 layers were active against the other bacteria. In contrast, compound **1** did not show any activity against any of the 14 phytopathogenic bacteria tested, even at 500 μg/ml.

Disease Control Efficacy against Peach Bacterial Leaf Spot

In a detached leaf bioassay, both the BuOH layer and F21 reduced the development of bacterial leaf spot on peach leaves in a dose-dependent manner. F21, at concentrations of 125, 250, and 500 μg/ml, suppressed the development of bacterial leaf spot by 50%, 70.8%, and 87.5%, respectively. The control values of the BuOH layer against peach bacterial leaf spot were 29.2%, 54.2%, and 75.0% at concentrations of 125, 250, and 500 μg/ml, respectively. Streptomycin sulfate, used as the positive control, showed a control value of 91.7% at a concentration of 200 μg/ml (Fig. 3).

Disease Control Efficacy against Tomato Bacterial Wilt

The BuOH layer and F21 were evaluated for in vivo antibacterial activity against Rs on 4-week-old tomato seedlings. F21 significantly reduced the disease severity compared with untreated control plants sprayed with water in a dose-dependent manner (Fig. 4). Its disease control efficacy was comparable to or stronger than streptomycin sulfate. At 7 days after inoculation, the control values of pharbitin were 79.5%, 97.4%, and 100% at 125, 250, and 500 μg/ml, respectively. In comparison, the control value of streptomycin sulfate at 200 μg/ml was 76.9%. The disease control efficacy of pharbitin was decreased 14 days after inoculation. The BuOH layer also strongly reduced the disease; its control values were 76.9%, 100%, and 100% at 500, 1,000, and 2,000 μg/ml, respectively, at 7 days after inoculation. However, the BuOH layer at 2,000 μg/ml caused phytotoxic symptoms of yellow chlorosis lesions on the leaves at 2 days after treatment.

Discussion

This study focused on the identification of an antibacterial principle in the MeOH extract of *P. nil* seeds, and the evaluation of its in vitro and in vivo activities against phytopathogenic bacteria. The hevein-like proteins from *P. nil* significantly suppressed plant pathogenic fungi, including *Phytophthora capsici* and *Fusarium oxysporum* in tomato (*Solanum lycopersicum*) [16]. Koo *et al.* [23] also reported antifungal activity of two hevein homologs isolated from the seeds of *P. nil* against *Botrytis cinerea* and *Phytophthora parasitica*. Anticancer activity of *P. nil* seed extracts and metabolites therefrom was observed against human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-15) [12, 21, 22]. Treatment of the crude extract of *P. nil*

Table 1. Minimum inhibitory concentration (MIC) values of the butanol layer and pharbitin against plant pathogenic bacteria.

Pathogen	MIC (μg/ml)		
	Butanol layer	Pharbitin	Streptomycin sulfate
Acidovorax avenae subsp. *cattlyae*	-	-	>100
Agrobacterium konjaci	-	-	6.25
Agrobacterium tumefaciens	-	-	>100
Burkholderia glumae	-	-	12.5
Clavibacter michiganensis subsp. *michiganensis*	-	-	25
Pectobacterium carotovorum subsp. *carotovorum*	-	-	6.25
Pectobacterium chrysanthemi	-	-	>100
Pseudomonas syringae pv. *actinidae*	-	-	12.5
Pseudomonas syringae pv. *lachrymans*	-	-	12.5
Ralstonia solanacearum	125	31.25	6.25
Xanthomonas arboricola pv. *pruni*	125	62.5	12.5
Xanthomonas axonopodis pv. *citri*	500	125	50
Xanthomonas euvesicatoria	125	62.5	12.5
Xanthomonas oryzae pv. *oryzae*	125	31.25	6.25

- : No activity.

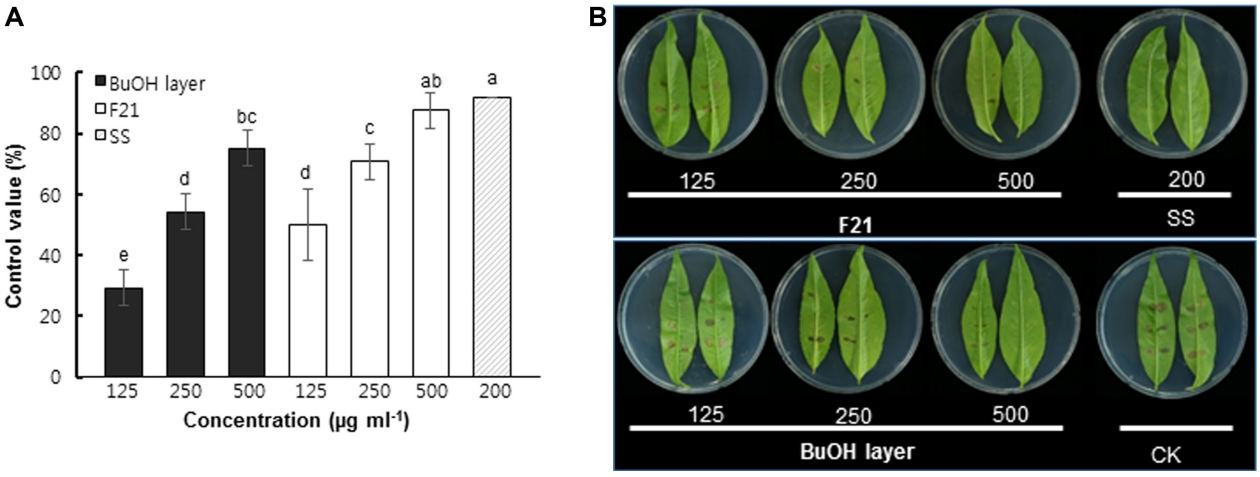


Fig. 3. Disease control efficacy of the BuOH layer and F21 (pharbitin) obtained from *Pharbitis nil* seeds against bacterial leaf spot caused by *Xanthomonas arboricola* pv. *pruni* on detached peach leaves.

(**A**) Mean percentage disease control value of the BuOH layer and F21 against bacterial leaf spot on a peach tree leaf after 5 days. Each value represents the mean ± standard deviation of two runs with three replicates per run. Lowercase letters indicate that values are not significantly different from other values with the same letter according to Duncan's HSD test for multiple comparisons ($p \leq 0.05$) with the SAS software (ver. 9.3; SAS Institute, USA). (**B**) Symptoms of bacterial leaf spot on detached peach leaves at 5 days after inoculation. BuOH: butanol layer; SS: streptomycin sulfate; CK: untreated control; unit: μg/ml.

induced growth inhibition and apoptosis of an AGS gastric cancer cell line [25].

Antibacterial activity of the other species of the *Pharbitis* genus has been reported against animal and human pathogenic bacteria. *Pharbitis bahiensis*-derived resin glycosides showed antibacterial activity against several bacteria, including *Bacillus subtilis* and *Staphylococcus aureus* [33]. The resin glycoside fractions isolated from *Pharbitis stans* exhibited antibacterial activity against *S. aureus* and *B. sublilis* [34]. Tricolorin A isolated from *Pharbitis tricolor* displayed

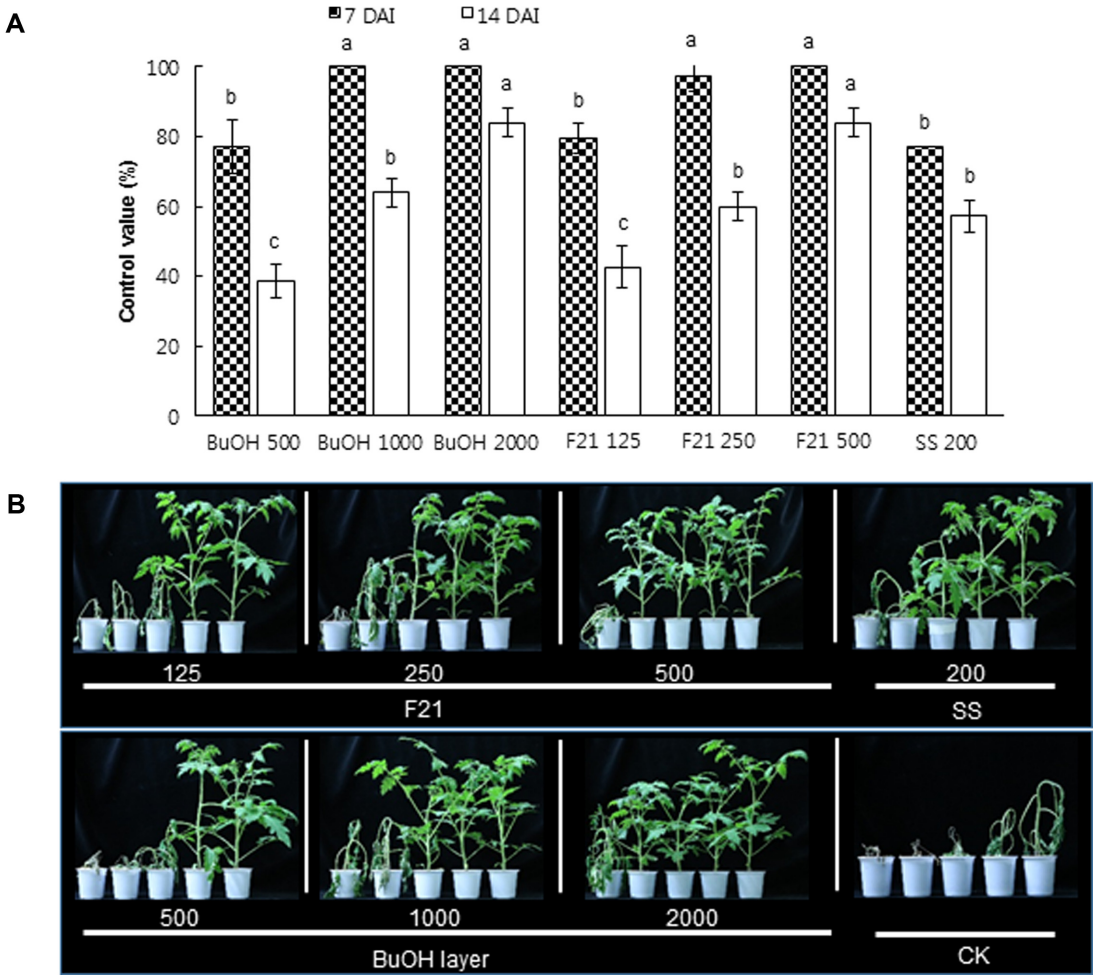


Fig. 4. Control efficacy of the BuOH layer and F21 (pharbitin) obtained from *Pharbitis nil* seeds in reducing wilting disease severity caused by *Ralstonia solanacearum* on tomato seedlings.

(**A**) Mean percentage disease control values of the BuOH layer and F21 against bacterial wilt on tomato seedlings at 7 and 14 days after inoculation. Each value represents the mean ± standard deviation of two runs with three replicates per run. Lowercase letters indicate that values are not significantly different from other values with the same letter according to Duncan's HSD test for multiple comparisons ($p \leq 0.05$) with the SAS software (ver. 9.3; SAS Institute, USA). (**B**) Symptoms of bacterial wilt on tomato seedlings at 14 days after inoculation. DAI: days after inoculation; BuOH: butanol layer; SS: streptomycin sulfate; CK: untreated control; unit: μg/ml.

strong antibacterial activity against *S. aureus* with an MIC value of 1.8 μg/ml, and significant cytotoxic activity against human breast cancer cells with an ED_{50} of 2.2 μg/ml [35]. However, the antibacterial activity of the crude extract of *P. nil* seeds has yet to be reported. To the best of our knowledge, this is the first report of the antibacterial activity of the crude extract of *P. nil* seeds.

In this study, pharbitin was isolated as an antibacterial principle from *P. nil* seeds. It is an ether-insoluble resin glycoside, present as a mixture of various compounds. Two glycosidic acids, named pharbitic acids C and D, along with valeric, tiglic, nilic, and (+)-2-methylbutyric acids, were isolated from the alkaline hydrolysis product of pharbitin [17, 30, 36]. In addition, Ono *et al.* [29] isolated seven acylated glycosidic acid methyl esters and proposed that pharbitin appears to be a mixture of seven monomers composed of free carboxylic acid forms (Fig. 2). In this study, we isolated one major compound from the alkaline product of the active fraction and it was identified as pharbitic acid C. This indicates that the active fraction obtained from *P. nil* seeds is pharbitin.

When the in vitro antibacterial activities of pharbitin and pharbitic acid C were tested against various phytopathogenic bacteria, pharbitin was found to be active against *Xanthomonas*

spp. and Rs. However, pharbitic acid C was not active, suggesting aglycone groups to be crucial to the antibacterial activity of pharbitin. Both the BuOH layer and pharbitin showed the same antibacterial spectrum against phytopathogenic bacteria; both samples were active only toward *Xanthomonas* spp. and Rs among the 14 phytopathogenic bacteria tested. This indicates that pharbitin is mainly responsible for the antibacterial activity of the BuOH layer.

Both the BuOH layer of *P. nil* seeds and isolated pharbitin controlled the development of bacterial leaf spot in a detached leaf bioassay using peach leaves, in a dose-dependent manner. There are few studies on the biological control of bacteria spots on peach leaves. Kawaguchi *et al.* [1] reported the use of *X. campestri* strains AZ98101 and AZ98106 for controlling Xap in the field. Penicillic acid isolated from *Aspergillus persii* EML-HPB1-11 was shown to significantly decrease the development of bacterial leaf spot on peach leaf by 95.0% at a concentration of 333.3 μg/ml [9].

In this study, both the BuOH layer of *P. nil* seeds and pharbitin significantly reduced the development of tomato bacterial wilt caused by Rs. Although the in vitro antibacterial activity of pharbitin is four times weaker than that of streptomycin sulfate, the disease control efficacy of pharbitin is comparable to that of streptomycin sulfate; the control value of the former at 125 μg/ml was similar to that of the latter at 200 μg/ml, 7 days after inoculation. At 14 days after inoculation, the control value of pharbitin at 250 μg/ml was similar to that of streptomycin sulfate at 200 μg/ml. On the other hand, the disease control efficacies of the BuOH layer at 500, 1,000, and 2,000 μg/ml were almost similar to those of pharbitin at 125, 250, and 500 μg/ml. The in vivo antibacterial activities of the BuOH layer and pharbitin against Rs were almost identical to the in vitro antibacterial activities of the two samples. These results indicate that about 25% of the BuOH layer was pharbitin, and pharbitin is a major compound in the BuOH layer of *P. nil* seeds.

The use of plant products to control the development of tomato bacterial wilt caused by Rs under greenhouse conditions has been reported by research groups. Indian neem tree leaves have been successfully used for the control of tomato bacterial wilt in a greenhouse. Dry and fresh neem leaves displayed strong disease control efficacy for tomato bacterial wilt inhibition at a concentration of 60 g leaves/l of soil with control values of 100% and 78%, respectively, 30 days after incorporation in the soil [37]. Methyl gallate isolated from the methanol extract of *Toxicodendron sylvestre* significantly decreased the development of bacterial wilt on tomatoes by 78.6% at a concentration 1 g/l at 26 days after inoculation [38]. Eight gallotannins isolated from *Sedum takesimense* effectively inhibited the growth of various plant pathogenic bacteria [6]. Furthermore, at a 200-fold dilution, the wettable powder-type formulation of the ethyl acetate layer of the aerial part of the plant also effectively suppressed the development of tomato bacterial wilt in plants by 78.3%, 14 days after inoculation. Deberdt *et al*. [39] reported that the soil treatment of *Allium fistulosum* extract was effective in reducing tomato bacterial wilt. On the other hand, several plant essential oils, such as thymol, palmarosa, lemongrass, clove, and cinnamon oils, also significantly reduced the incidence of tomato bacterial wilt in greenhouse experiment [40–42]. Despite these interesting studies, to the best of our best knowledge, there is no commercially available botanical bacteriocide for the control of plant bacterial diseases.

In conclusion, pharbitin was isolated from *P. nil* seeds and determined to be the main antibacterial metabolite present. Both the BuOH layer and pharbitin showed specific antibacterial activity against Rs and *Xanthomonas* spp. They also reduced the development of bacterial leaf spot in a detached leaf assay using peach leaves, and tomato bacterial wilt in a pot experiment. These results suggest *P. nil* seed extract to be a promising candidate for the development of a new biopesticide for the control of plant diseases caused by Rs and *Xanthomonas* spp. However, further studies to develop an optimum extraction process and formulation, as well as to investigate the toxicity and determine the effective dose in field crops, are necessary before such a product can be commercialized.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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