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Isolation and Identification of Antibacterial and Antifungal Compounds from *Praxelis clematidea* R. M. King & H. Robinson as an Effective Potential Treatment against Rice Pathogens

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Abstract: Bacterial leaf blight and blast diseases caused by the bacterium *Xanthomonas oryzae* pv. *oryzae* and the fungus *Pyricularia oryzae*, respectively, are among the most important infectious diseases affecting rice. We evaluated the antimicrobial effects of compounds derived from *Praxelis clematidea* on *Xanthomonas oryzae* and *Pyricularia oryzae*. The dried aerial parts of *Praxelis clematidea* were subjected to ethanol extraction, separated by solvent partitioning using hexane, chloroform, ethyl acetate, and water. In vitro assays demonstrated that the main antibacterial and antifungal activities were distributed in the ethyl acetate and chloroform fractions, respectively. These fractions were further separated using silica gel chromatography and reversed-phase chromatography. Finally, we isolated five compounds, **1**–**5**, that inhibited the growth of *Xanthomonas oryzae* in vitro and four compounds, **6**–**9**, that exhibited in vitro antifungal activity against *Pyricularia oryzae*. We evaluated their antimicrobial activities and identified their chemical structures by NMR and mass spectrometry analyses. This is the first study to isolate compound **2** (4,4′,4′′-nitrilotriphenol) as an alternative microbial from natural resources and evaluate its physiological activity. Moreover, this is the first report to demonstrate antibacterial activity in comparison with flavonoids. *Praxelis clematidea* extracts plausibly exert both antibacterial and antifungal effects, which should be further validated in field trials.

Keywords: *Xanthomonas oryzae* pv. *oryzae*; *Pyricularia oryzae*; plant extracts; *Praxelis clematidea*; natural antimicrobials; alternative antimicrobials; 4,4′,4′′-nitrilotriphenol

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1. Introduction

Rice (*Oryza sativa* L.) is an essential cereal crop worldwide [1,2]. Various infectious diseases caused by fungi, bacteria, viruses, mycoplasma-like organisms, and nematodes affect rice [3–5]. The bacterium *Xanthomonas oryzae* pv. *oryzae* (Fang et al. 1957) Swings et al. 1990 (*Xoo*), the causative pathogen of leaf blight disease, and the fungus *Pyricularia oryzae* Cavara 1892 (*P. oryzae*), the causative pathogen of rice blast disease, have widespread distribution and cause severe crop destruction in rice-growing regions globally [3,5,6]. *Xoo* can reduce the rice yield by approximately 50–70% under conditions favorable for the bacteria [5,7]. Seedlings are easily infected by a vertical infection [5,8,9]. In tropical regions, high temperatures, humidity, and an abundance of host plants allow *Xoo* to persist throughout the year [5]. *P. oryzae* infects leaves, collars, nodes, internodes, necks, and other parts of the rice panicle and sometimes the leaf sheath [3,5,10]. Neck blast and panicle blast

are the most damaging stages of the disease [5,6,11]. The rice leaf harboring the lesion is gray or whitish in the center, has a brown border, and is elliptical [3,5]. The annual losses of the rice yield may be up to 30%, depending on the variety of rice [12]. Thus, devising control strategies for these two pathogens is imperative.

Several methods such as the use of antimicrobial chemicals, resistant cultivars, biocontrols, and natural products have been used to suppress infectious diseases [3,5,10,12–14]. Synthetic chemicals are the most widely used antimicrobial agents in the field [3,5,10]; however, they negatively impact the environment and have long degradation intervals [12,14–18]. In contrast, natural antimicrobial products are considered to be more environmentally friendly and easily decompose in a sustainable agricultural system [12,19,20]. Many groups have reported the efficacy of plant extracts against infectious diseases in vitro and in vivo together with their efficacy on agronomic traits. Naqvi et al. showed that the aqueous extracts of various plants, individually or in combination with others agents, suppressed bacterial blight disease caused by *Xoo* infections in three rice varieties in a greenhouse [21]. Moreover, the treatments improved the agronomic traits such as the plant height, panicle length, number of tillers and grains/panicle, weight of grains, and paddy yield of the rice plants compared with the control. Another group reported that three plant aqueous extracts enhanced seed germination and seedling vigor, and suppressed bacterial blight disease in five rice varieties grown in greenhouses [22]. They also demonstrated that *Adhatoda vasica* leaf extract can activate the accumulation of enzymes involved in the plant defense, which may confer a resistance against rice bacterial leaf blight. Water and ethanol leaf extracts and an oil extract of *Azadirachta indica* seeds reduced the in vitro growth of *P. oryzae* and suppressed the development and spread of blast disease in rice plants grown in a greenhouse [23].

Praxelis clematidea R. M. King & H. Robinson (Asteraceae) (*P. clematidea*) belongs to the Eupatorieae tribe of the aster family [24]. It is a common, invasive, alien weed that is harmful to native plants and causes vast ecological destruction and economic losses [25,26]. The Asteraceae family has been studied for its chemical composition and biological activities including antifungal, antiviral, and antibacterial activities and several new drugs and insecticides have been developed [24].

In a previous screening experiment using extracts of 30 kinds of species we observed a relatively stronger antibacterial activity against *Xoo* and antifungal activity against *P. oryzae* in the ethanol extract of *P. clematidea*. Therefore, in this study, we aimed to isolate and identify anti-*Xoo* and anti-*P. oryzae* compounds and evaluate their antimicrobial efficacy.

2. Materials and Methods

2.1. Materials

The aerial parts of wild *P. clematidea* typically have flowers and achenes. However, as the plant samples were collected at a mature stage but before flowering in the present study, the plants did not have flowers and achenes. The samples were collected at Can Tho, Vietnam (10°02′23.7″ N, 105°45′05.8″ E) from March to April 2018. The plant was identified by Professor Tran Vu Phen and kept in the Department of Plant Protection, College of Agriculture, Can Tho University (code number CCHLRC-100). Microorganism *Xoo* H87135 was kindly given by Professor Seiji Tsuge of Kyoto Prefectural University, Japan, and *P. oryzae* Cavara (MAFF 101512) was purchased from the Genebank Project, National Agriculture and Food Research Organization, Ibaraki, Japan.

Solvents (ethanol, hexane, chloroform, ethyl acetate, methanol, and acetonitrile), dimethyl sulfoxide (DMSO), agar, and sucrose were purchased from Fujifilm Wako Chemicals (Osaka, Japan). Filtration paper No. 1 was obtained from Advantec (Osaka, Japan). Silica gel 60 (0.040–0.063 mm, 230–400 mesh ASTM), thin-layer chromatography (TLC) plates, and silica gel 60 coated with fluorescent indicator F-254 were purchased from Merck (Darmstadt, Germany). Potato dextrose agar (PDA) and polypeptone were purchased from Nissui and Nihon Seiyaku, Japan, respectively. Streptomycin sulfate salt, used as a positive control, was purchased from Sigma-Aldrich (Darmstadt, Germany).

2.2. Methods

2.2.1. Extraction and Solvent Partition of *P. clematidea*

The antimicrobial compounds were isolated using general extraction methods using ethanol, a solvent partition, silica gel chromatography, and reversed-phase chromatography [27–32]. The aerial parts of *P. clematidea* were washed with tap water and chopped into small pieces. After air-drying for 14 days in a shaded net-house, they were ground into a fine powder. The *P. clematidea* powder (1.5 kg) was soaked in ethanol (95%, 7.5 L) and incubated at 25 °C for 24 h in the dark. The supernatant was filtered using a filter paper and the residue was extracted six times. The supernatant was evaporated using a rotary evaporator (Iwaki, Japan) and 97.2 g of the extract was finally obtained.

The ethanol extract was separated into four fractions by solvent partitioning using hexane, chloroform, ethyl acetate, and water. Each fraction was evaporated and then lyophilized. The yields of hexane, chloroform, ethyl acetate, and water fractions were 45.8, 18.3, 11.5, and 16.0 g, respectively.

2.2.2. Silica Gel Column Chromatography

The ethyl acetate and chloroform fractions were separated using silica gel column chromatography. The ethyl acetate fraction (3 g) was loaded on a silica gel column (2 × 70 cm) equilibrated with hexane. Eleven fractions were collected following silica gel column chromatography and elution with the following solvent ratios: hexane-ethyl acetate, 80–20 (E1–E3), 60–40 (E4), 40–60 (E5), 20–80 (E6), and 0–100 (E7 and E8); and ethyl acetate-methanol, 100–10 (E9) and 0–100 (E10 and E11). The chloroform fraction (3.26 g) was loaded onto a silica gel column (3 × 38 cm) equilibrated with hexane. After elution with a hexane and ethyl acetate mixture at a ratio of 3:1, the fractions were eluted with ethyl acetate and methanol at a ratio of 100:10. After evaporation, the eluate (1.4 g) was re-chromatographed using a silica gel column and separated into 16 fractions. The fractions were eluted by silica gel column chromatography using the following solvents: hexane-ethyl acetate, 100–40 (C5), 100–40 (C7), 100–50 (C9), 100–75 (C10), and 100–100 (C12); and ethyl acetate-methanol, 100–10 (C15) and 0–100 (C16).

2.2.3. Thin-Layer Chromatography (TLC)

TLC was performed using silica gel 60 F-254 plates to check the purity of the compounds and determine the separation conditions using silica gel chromatography. After development, the TLC plates were observed under a UV light. The plates were soaked in a sodium phosphomolybdate n-hydrate solution (Fujifilm Wako Chemicals, Osaka, Japan) concentration 36 g in 500 mL ethanol) for 2 s, heated for 2 min at 95 °C, and observed.

2.2.4. Reversed-Phase HPLC

An HPLC system comprising a Pump L-2130 and a UV-VIS detector L-2420 (Hitachi, Japan) was used. The *P. clematidea* fractions were loaded onto a Cosmosil $5C_{18}$ MS-II column (150 × 4.6 mm, Nacalai Tesque, Inc., Kyoto, Japan) equilibrated with 5% acetonitrile and eluted with a linear gradient of acetonitrile at a flow rate of 1 mL/min. The eluted compounds were detected at 260 nm.

2.2.5. Identification of the Antimicrobial Compounds

The chemical structures of the isolated compounds were identified using a combination of multistage mass spectroscopy (MS) and one-dimensional and two-dimensional (1D and 2D) NMR spectroscopy [33]. The electrospray ionization time-of-flight (ESI-TOF) MS spectra were acquired using a Bruker micrOTOF spectrometer (Ibaraki, Japan), and the 1D and 2D NMR spectra were acquired on a Bruker AV-600 NMR spectrometer.

2.2.6. Antibacterial Assay against *Xoo*

We used two methods to assay the antibacterial activity against *Xoo:* an agar well diffusion method [7] and a spectrophotometric assay [34]. For the assay, 200 mg of the

P. clematidea sample as dissolved in 1 mL DMSO and then diluted to appropriate concentrations with distilled water before use.

Xoo was cultured in 5 mL a polypeptone sucrose (PS) medium for 24 h at 150 rpm and 28 °C. The bacterial suspension was diluted in the PS medium to obtain an absorbance of 0.1 at 600 nm (~1 × 10^8 colony-forming units (CFU) /mL). The suspension (200 µL) was spread onto a PS medium containing 1.5% agar on a Petri dish (86 mm diameter), dried briefly, and then six wells were punched on the agar with a sterile cork-borer (6 mm in diameter). *P. clematidea* fractions (50 µL; 5 and 10 mg/mL) containing 10% DMSO were added to each well. DMSO (10%) was used as the negative control. All dishes were incubated at room temperature for 1 h to allow for the sample diffusion and then incubated at 30 °C for 3 days. Thereafter, the diameter of the clear zone (mm) was measured as an inhibition zone for bacterial growth. The experiment was conducted with five replicates.

A spectrophotometric assay was performed in a 96-well flat-bottom microliter plate. Each well contained 100 µL of the PS medium, 15 µL water, 35 µL of the *Xoo* suspension (~1 × 10^8 CFU/mL), and 50 µL samples at appropriate concentrations. Streptomycin (10 µg/mL in water) and 0.5% DMSO were used as positive and negative controls, respectively. The plate was shaken at 30 °C and 150 rpm for 24 h in a plate shaker (MBI 100-2A thermoshaker, Allsheng, China), and the absorbance at 600 nm ($A_{600\,nm}$) was measured using a microplate reader (Model SH-1200 Lab, Corona Electric, Ibaraki, Japan). Subsequently, 50 µL 2,3,5-tetrazolium chloride (1.5% in water w/v, Sigma-Aldrich) was added to the wells as a redox indicator and shaken for an additional 4 h under the same conditions. The red color of the mixture indicated that the bacteria were alive [7].

The percent inhibition (PI) of the bacterial growth was calculated using the following equation:

$$\%\ \text{inhibition} = \frac{(A_{600\text{nm}}\ \text{of negative control} - A_{600\text{nm}}\ \text{of test})}{A_{600\text{nm}}\ \text{of negative control}} \times 100 \quad (1)$$

2.2.7. Assay for Inhibitory Activity against the Mycelial Growth of *P. oryzae*

The poisoned food assay was used to examine the antifungal activity with slight modifications [35–37]. Briefly, *P. oryzae* was cultured on a PDA medium (39 g in 1 L water) at 26 °C and 500 mg of *P. clematidea* fractions were dissolved in 1 mL DMSO to prepare the stock solution. The stock solution was diluted to obtain appropriate concentrations with a PDB medium. The DMSO was used at a final concentration of 0.2%. For a larger- or a smaller-scale assay, 100 or 24 µL of *P. clematidea* extract or fractions (125 µg/mL) were mixed in 50 or 6 mL of the PDA medium at 55 °C, and then 10 or 2 mL of the medium was dispensed into Petri dishes (8.6 or 3.5 cm dia.). After solidification, a mycelial plug (6 or 4 mm diameter) of *P. oryzae* (5 days old) was placed on the central agar medium and the dishes were incubated at 26 °C for 14 or 7 days. The diameter (mm) of the radial *P. oryzae* growth was measured and the PI (%) was calculated using the equation of Astiti and Surapta [36–38]:

$$\%\ \text{PI} = \frac{(\text{DC} - \text{DT})}{\text{DC}} \times 100 \quad (2)$$

where PI (%) is the inhibitory activity against the radial growth, DC is the diameter (mm) of the fungal colonies treated without the extract (control), and DT is the diameter (mm) of the fungal colonies treated with the extract.

In the assay using the isolated compounds, 5 mg of each compound was dissolved in a mixture of 100 µL DMSO and 100 µL methanol then serially diluted 2-fold from 320 to 5 µg/mL using the culture medium. The final concentrations of DMSO and methanol in the assay mixture were maintained at 0.2%. A 77 µL sample was then used for the smaller-scale assay. The antifungal activity of the compound was expressed as the half-maximal inhibitory concentration (IC_{50}), which was the concentration of the antifungal compound required to suppress the radial growth of the fungi by 50%.

2.3. Statistical Analysis

The experiments were repeated at least three times. The results are expressed as the mean ± standard deviation (SD) or standard error (SE). Differences among the means were tested for a statistical significance using a one-way analysis of variance (ANOVA), followed by Tukey's multiple tests. The statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of P. clematidea Extract on the Growth of Xoo and P. oryzae

We extracted the aerial parts of *P. clematidea* with ethanol and then separated them into hexane, chloroform, ethyl acetate, and water fractions via a solvent partition. The antibacterial and antifungal activities of the extract and the four fractions against *Xoo* and *P. oryzae*, respectively, are shown in Figure 1. The ethyl acetate fraction showed the highest anti-*Xoo* activity, expressed as an inhibition zone (mm) of *Xoo* growth (Figure 1A). The chloroform fraction exhibited the highest anti-*P. oryzae* activity, which was expressed as the PI of the radial *P. oryzae* growth at 125 μg/mL (Figure 1B). These results indicated that *P. clematidea* contains multiple anti-*Xoo* compounds mainly in the ethyl acetate fraction and anti-*P. oryzae* compounds in the chloroform fraction.

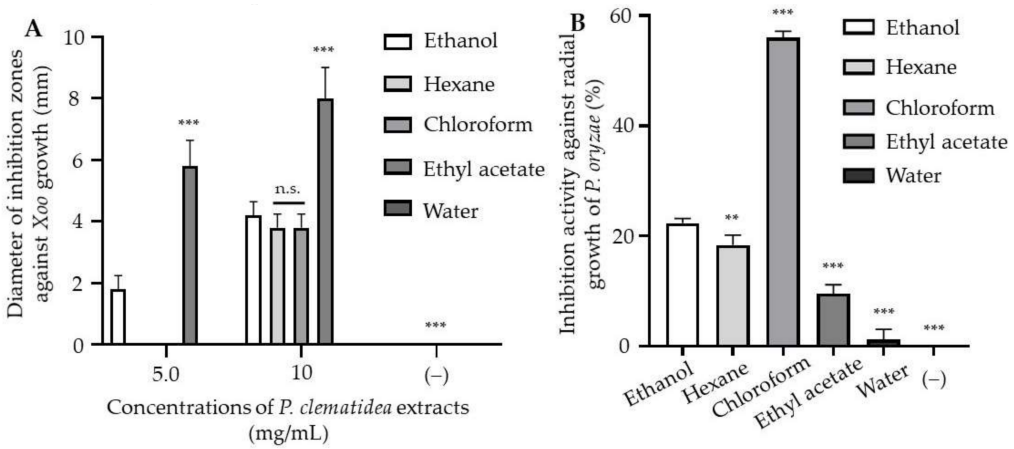

Figure 1. Anti-*Xoo* (**A**) and anti-*P. oryzae* (**B**) activities of *P. clematidea* extract and four partitioned fractions. The values represent the mean ± SD ($n = 5$). A statistical significance was calculated between the ethanol extract with the other fractions using a one-way ANOVA. **, $p < 0.01$; ***, $p < 0.001$.

3.2. Isolation of Anti-Xoo Compounds from the Ethyl Acetate Fraction

To isolate the anti-*Xoo* compounds, we separated the ethyl acetate fraction (3 g) using silica gel column chromatography and obtained 11 sub-fractions. Based on the results of the anti-*Xoo* assay shown in Figure 2A, we selected three fractions E4, E5, and E7 for further purification using reversed-phase HPLC. Finally, we obtained five antibacterial compounds against *Xoo*: compounds 1 and 2 from fractions E4; 3 and 4 from fraction E5; and 5 from fraction E7. The yields from the ethyl acetate fraction (3 g) were 5.01 mg for compound 1, 8.43 mg for 2, 7.23 mg for 3, 2.33 mg for 4, and 3.05 mg for 5.

3.3. Isolation of the Antifungal Compounds from the Chloroform Fraction

To isolate the antifungal compounds, the chloroform fraction (1.4 g) was separated into 16 sub-fractions using silica gel column chromatography; seven of them with larger yields were tested for their ability to inhibit the growth of *P. oryzae*. Based on the results shown in Figure 2B, three fractions C9, C12, and C15 were further separated by reversed-phase HPLC. Finally, four compounds were isolated from these fractions, namely, compound 6 from C9, 7 from C12, and 8 and 9 from C15. The yields (%) of compounds 6, 7, 8, and 9 from the chloroform fraction (1.4 g) were 29.05, 8.06, 17.80, and 7.84 mg, respectively.

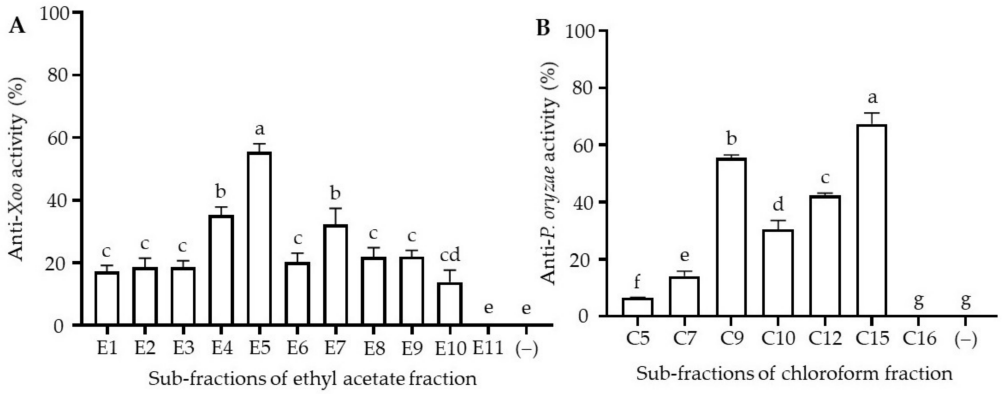

Figure 2. The antimicrobial activities of the ethyl acetate and chloroform fractions. (**A**) The 11 fractions (100 μg/mL) obtained by silica gel chromatography of the ethyl acetate fraction were assayed for anti-*Xoo* activities. (−): negative control (0.5% DMSO). (**B**) Anti-*P. oryzae* activities of seven fractions (125 μg/mL) obtained by silica gel chromatography of the chloroform fraction with larger yields. (−); negative control (0.2% DMSO). Means ± SD (n = 3) with different letters indicate significantly different results at $p < 0.05$ whereas means with similar letters are not different from each other.

3.4. Identification of Isolated Anti-Xoo and Anti-P. oryzae Compounds except for Compound **5**

After verifying the purity using TLC and reversed-phase HPLC, the chemical structures of the isolated compounds were analyzed using MS and NMR spectroscopy. Most of the compounds were classified as flavonoids and their molecular masses were consistent with their chemical formula. As shown in Figure 3, all anti-*Xoo* compounds except for compound **5** were identified as follows: compound **1**, apigenin (270 Da) (Supplementary Table S1, Figures S1–S3) [38,39]; 2, 4,4′,4′′-nitrilotriphenol (293 Da) (Table S2, Figures S4–S6) [40]; 3, luteolin (286 Da) (Table S3, Figures S7–S9) [39]; and 4, 3-*O*-methylquercetin (316 Da) (Table S4, Figures S10–S13) [41]. The anti-*P. oryzae* compounds were compound **6**, scutellarein tetramethylether (4′,5,6,7-tetramethoxyflavone, 342 Da) (Table S6, Figures S20–S23) [42]; 7, apigenin trimethylether (4′,5,7-trimethoxyflavone, 312 Da) (Table S7, Figures S24–S28) [43]; 8, sinensetin (3′,4′,5,6,7-pentamethoxyflavone, 372 Da) (Table S8, Figures S29–S31) [42]; and 9, luteolin tetramethylether (3′,4′,5,7-tetramethoxyflavone, 342 Da) (Table S9, Figures S32–S35) [44]. The ^{1}H and ^{13}C NMR chemical shifts and J-coupling constants are listed in Tables S1–S4 and Tables S6–S9 in the Supplementary Materials along with the full spectral assignment. The NMR parameters were consistent with those reported previously.

3.5. Determination of The Chemical Structure of Compound **5**

Compound **5** has not been reported previously and the data obtained by analyzing the chemical structure of compound **5** are detailed below. Compound **5** in methanol was a light purple solution, crystal, with a melting temperature from 148 to 152 °C. Its UV spectrum (λ_{max} = 295, 360, 550 nm) was similar to that of compound **2**, 4,4′,4′′-nitrilotriphenol (λ_{max} = 295, 360, 568 nm). The ESI-TOF MS of 5 gave a negative peak of $(M - H)^{-}$ at 454 m/z and a positive peak of $(M + Na)^{+}$ at 478 m/z, which corresponded with the chemical formula $C_{24}H_{25}NO_8$ (calculated molecular weight, 455). The ^{1}H NMR, DQF-COSY, and ROESY showed two pairs of doublet signals with a *β*-D-glucopyranose moiety; one pair of the doublet (J = 9.0 Hz, 2H) at 6.95 and 6.82 ppm and the other of the doublet (J = 9.0 Hz, 4H) at 6.83 and 6.68 ppm (Table S5, Figures S14 and S15). The ^{13}C NMR, ^{1}H-^{13}C HSQC, and HMBC spectra (Figures S16–S18) indicated that 5 contained a 4,4′,4′′-nitrilotriphenyl group. In the ^{1}H-^{13}C HMBC spectrum of 5, a cross peak of glc H-1 at 4.80 ppm to C-1 at 153.6 ppm was observed and a ROESY cross peak was observed between the glc H-1 and H-2, 6 protons. These results demonstrated that the glucopyranose moiety was connected to one of the three phenyl groups of 4,4′,4′′-nitrilotriphenol. The nitrogen atom was confirmed by a ^{1}H-^{15}N HMBC spectrum (Figure S19); a single ^{15}N peak at 89.7 ppm connected with the H-3, 5 protons at 6.82 ppm with the H-3′, 3′′, 5′, 5′′ protons at 6.83 ppm. The ^{15}N

chemical shift of 5 coincided with that of 1 (88.5 ppm). A hydrolysis of 5 yielded compound **2**. Thus, compound **5** was identified as 4,4′,4′′-nitrilotriphenyl *β*-D-glucopyranoside, as shown in Figure 3.

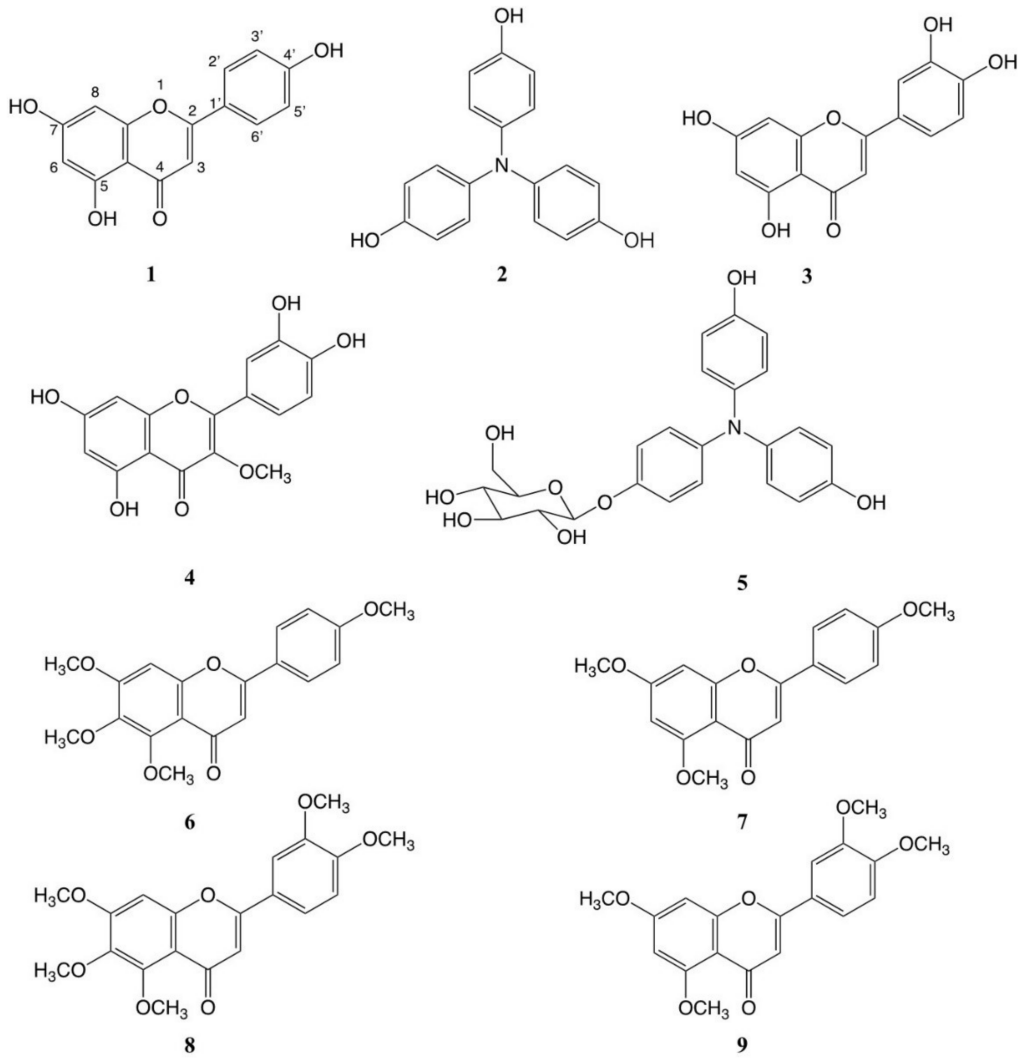

Figure 3. Chemical structures of the anti-*Xoo* compounds **1**–**5** and anti-*P. oryzae* compounds **6**–**9**. The compounds were identified using ESI-TOF MS and NMR spectroscopy. Compound **1**, apigenin; 2, 4,4′,4′′-nitrilotriphenol; 3, luteolin; 4, 3-*O*-methylquercetin; 5, 4,4′,4′′-nitrilotriphenyl *β*-D-glucopyranoside; 6, scutellarein tetramethylether (4′,5,6,7-tetramethoxyflavone); 7**,** apigenin trimethylether (4′,5,7-trimethoxyflavone); 8, sinensetin (3′,4′,5,6,7-pentamethoxy flavone); and 9, luteolin tetramethylether (3′,4′,5,7-tetramethoxyflavone).

Compound **5**, ^{1}H-NMR (in MeOD-d_4, 600.2 MHz): δH (ppm/TMS) 6.95 (*d*, 9.0 Hz, 2H, H-2, 6), 6.83 (*d*, 9.0 Hz, 4H, H-3′, 3′′, 5′, 5′′), 6.82 (*d*, 9.0 Hz, 2H, H-3, 5), 6.68 (*d*, 9.0 Hz, 4H, H-2′, 2′′, 6′, 6′′), 4.80 (*d*, 7.4 Hz, 1H, glc H-1), 3.88 (*dd*, 11.9, 1.5 Hz, 1H, glc H-6), 3.69 (*dd*, 11.9, 5.1 Hz, 1H, glc H-6′), 3.44 (*m*, 1H, glc H-3), 3.42 (*m*, 1H, glc H-2), 3.38 (*m*, 2H, glc H-4), 3.38 (*m*, 2H, glc H-5). ^{13}C NMR (in MeOD-d_4, 150.9 MHz): δC (ppm/TMS) 154.2 (C-1′, 1′′), 153.6 (C-1), 145.3 (C-4), 142.3 (C-4′,4′′), 126.8 (C-3′, 3′′, 5′, 5′′), 124.0 (C-3, 5), 118.8 (C-2, 6), 116.9 (C-2′, 2′′, 6′, 6′′), 103.1 (glc C-1), 78.1 (glc C-3), 78.1 (glc C-5), 75.0 (glc C-2), 71.5 (glc C-4), 62.6 (glc C-6). ^{15}N NMR (in MeOD-d_4, 60.8 MHz): δN (ppm/NH_3) 89.7. (Table S5, Figures S14–S19).

3.6. Antibacterial and Antifungal Activities of the Isolated Compounds

The antibacterial and antifungal activities of the isolated compounds were analyzed by spectrophotometric and poisoned food assays, respectively. The IC_{50} values, which were the concentrations required to suppress the *Xoo* and *P. oryzae* growth by 50%, were calculated (Figure 4) and the summary can be seen in Table 1. Compounds **1**–**5** isolated from the ethyl acetate fraction suppressed *Xoo* growth in a dose-dependent manner but did not inhibit *P. oryzae* growth between concentrations of 5–320 μg/mL. In contrast, compounds **6**–**9** isolated from the chloroform fraction suppressed *P. oryzae* growth but did not inhibit *Xoo* growth between concentrations of 2–256 μg/mL.

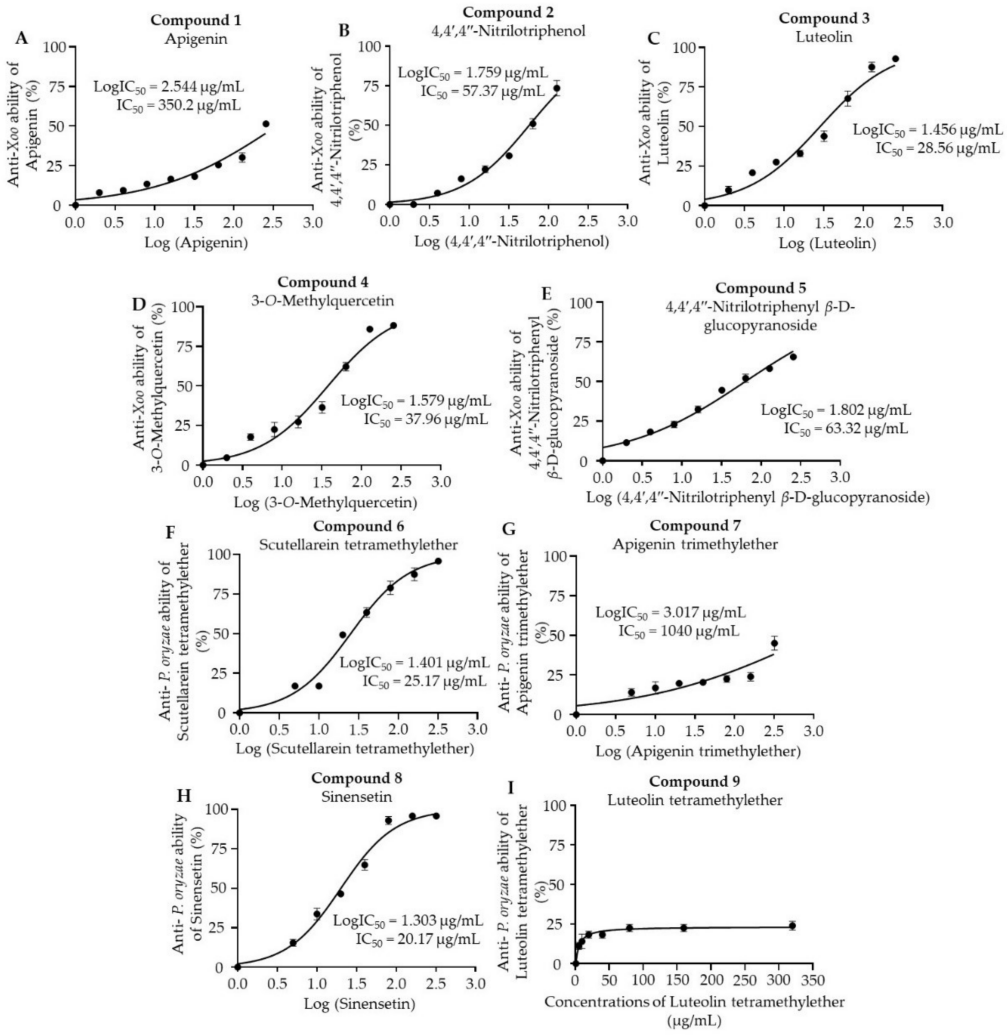

Figure 4. Antibacterial and antifungal activities of anti-*Xoo* compounds **1**–**5** and anti-*P. oryzae* compounds **6**–**9**. (**A**–**E**) The antibacterial activities against *Xoo* were assayed by the spectrophotometric method. *Xoo* suspensions (~1×10^8 CFU/mL) were mixed with various concentrations of isolated compounds (0, 2, 4, 8, 16, 32, 64, 128, and 256 μg/mL). After culturing for 24 h, the absorbance at 600 nm was measured and the percent inhibition was calculated. (F–I) The antifungal activities against *P. oryzae* were assayed by the poisoned food method. The diameter (mm) of the radial fungal growth was measured at 7 days of culture on an agar medium containing various concentrations of the isolated compounds (0, 5, 10, 20, 40, 80, 160, and 320 μg/mL) and then the percent inhibition was calculated (n = 3).

Table 1. The antibacterial activities against *Xoo* and the antifungal activities against *P. oryzae* of compounds **1**–**9**. Based on the results shown in Figure 4, the IC_{50} values (μg/mL) were calculated and expressed as mean ± SE. ∞ indicates that the IC_{50} values were higher than 1040 μg/mL. N.D. indicates that the activities were not detected in the range of 2–256 μg/mL and 5–320 μg/mL for *Xoo* and *P. oryzae*, respectively.

	Compounds	IC_{50} (μg/mL)	
		Anti-*Xoo*	**Anti-*P. oryzae***
Ethyl acetate fraction	1. apigenin	350.20 ± 11.43	N.D.
	2. 4,4′,4′′-nitrilotriphenol	57.37 ± 1.15	N.D.
	3. luteolin	28.56 ± 2.73	N.D.
	4. 3-*O*-methylquercetin	37.96 ± 1.27	N.D.
	5. 4,4′,4′′-nitrilotriphenyl *β*-D-glucopyranoside	63.32 ± 2.13	N.D.
Chloroform fraction	6. scutellarein tetramethylether	N.D.	25.17 ± 3.09
	7. apigenin trimethylether	N.D.	1040 ± 114.30
	8. sinensetin	N.D.	20.17 ± 2.47
	9. luteolin tetramethylether	N.D.	∞

4. Discussion

In this study, we demonstrated that the ethanol extract of *P. clematidea* suppressed the growth of *Xoo* and *P. oryzae,* the main pathogens of rice. Our promising results warrant that this extract should be first tested on diseased plants grown in a greenhouse before testing it in the field. The fractions containing the compounds with antibacterial and antifungal activities were separated by solvent partitioning mainly into ethyl acetate and chloroform fractions, respectively. Thus, we decided to isolate anti-*Xoo* compounds from the ethyl acetate fraction and anti-*P. oryzae* compounds from chloroform fraction. We identified nine antimicrobial compounds: compounds **1**–**5** possessed anti-*Xoo* activities and compounds **6**–**9** showed antifungal activities against *P. oryzae.* To the best of our knowledge, this is the first study to report the isolation of compound **2** (4,4′,4′′-nitrilotriphenol) from natural resources and its physiological activity. Its glucose derivative, compound 5 (4,4′,4′′-nitrilotriphenyl *β*-D-glucopyranoside), was identified as a novel compound. Compound **5** indicated that the 4,4′,4′′-nitrilotriphenyl moiety was produced in the plant; 4,4′,4′′-nitrilotriphenol and its derivatives are known to be excellent hole-transport materials that constitute organic light-emitting diodes or solar cells [45–47]. Compound **2** may display an antioxidant activity similar to other flavonoids. Interestingly, the biological activities of 2 and 5 were almost comparable with those of flavonoids, which are not suitable for mass production. Therefore, the 4,4′,4′′-nitrilotriphenyl group may be a suitable candidate for developing alternative antibacterial agents and designing new antimicrobials. However, the toxicity of this group needs to be clarified with regard to environment and human health before its commercial use is explored.

Compounds **1** (apigenin) and **3** (luteolin) exhibit antibacterial activities against pathogenic bacteria whereas compound **4** (3-*O*-methylquercetin) displays antibacterial activities against animal and plant pathogens. Apigenin extracted from *Mentha longifolia* exhibited antimicrobial activities against *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* with a minimum inhibitory concentration (MIC) of 30–90 μg/mL [48]. Additionally, apigenin and luteolin extracted from *Acacia cochliacantha* showed an antibacterial activity against *S. aureus* with an MIC value of approximately 0.8 mg/mL [49]. Kim et al. demonstrated that apigenin induced bacterial apoptosis via the activation of the cellular oxidative pathways and induced the production and accumulation of reactive nitrogen species/reactive oxygen species [50]. A previous study reported the mechanism of luteolin against *Staphylococcus aureus* and *Listeria monocytogenes*. These results indicate that luteolin exerts antimicrobial effects by impairing the bacterial cell membranes inducing cell morphological alterations in the planktonic state and inhibiting biofilm formation [51]. 3-*O*-methylquercetin isolated from *Inula viscosa* (L.) inhibited *B. cereus* and *Salmonella*

typhimurium at an MIC of 125 μg/mL [52]. This compound displays strong antibacterial activities against plant pathogens such as *Agrobacterium tumefaciens*, *Pseudomonas lachrymans*, and *Xanthomonas vesicatoria* and antifungal activities against the plant fungi *Candida albicans* and *Magnaporthe oryzae* [53]. Thus, the mechanisms underlying antibacterial activities of compounds **1**, **3**, and **4** against *Xoo* may be similar to those reported; however, further studies are needed to clarify these mechanisms. Compound **1** exhibited a weaker anti-*Xoo* activity than that of compound **3**. This suggests that the hydroxyl group at position 3 of the B ring may be important for antibacterial activity, which should be clarified.

Three antibacterial compounds, **1**, **3**, and **4**, are flavones with none or only one methoxy group; in contrast, the four antifungal compounds, **6**–**9**, are polymethoxylated flavones. This suggests that an increased hydrophobicity imparted by a conjugation with methoxy groups may be required for antifungal activity. A comparison of the structures and anti-*P. oryzae* activities of compounds **6**–**9** suggested that the methoxy group at position 6 on the A ring is essential for the antifungal activity of flavones. The antifungal activities of plant extracts containing polymethoxyflavones have been reported; however, only a few reports of specific isolated compounds have been published. Yenjai et al. demonstrated that apigenin trimethyl ether (compound **7**) from *Kaempferia parviflora* possessed an antifungal activity against *Candida albicans* yeast with 17.63 μg/mL of IC_{50} and demonstrated no cytotoxicity against human cells [54]. In our study, the anti-*P. oryzae* activity of compound **7** was much weaker than that reported previously. We cannot exclude the possibility that the different components of the membranes of *C. albicans* and *P. oryzae* inhibited the membrane permeability of apigenin trimethyl ether. In addition, the IC_{50} value of apigenin trimethyl ether from their study was the same as that of compound **6** (scutellarein tetramethyl ether) and compound **8** (sinensetin) against *P. oryzae.* Sinensetin has been shown to possess a wide range of pharmacological activities including an antimicrobial activity [55]. Wu et al. showed that the fraction containing polymethoxyflavones from *Citrus reticulata* suppressed *Aspergillus niger* by altering the permeability of the cytomembrane and impairing the integrity of the cell walls via a chitin inhibition [56].

The isolated antimicrobial compounds may have limited applicability as alternative antimicrobials in the field as their yields from the plant extract are small. Therefore, *P. clematidea* extract may have an increased application in net-houses and fields. As *P. clematidea* extract contains compounds with antibacterial and antifungal properties, we hypothesize that it could simultaneously suppress infectious diseases afflicting rice that are caused by bacteria and fungi. *P. clematidea* extract also contains two different types of antibacterial compounds, flavonoid derivatives, and compound **5** and its glycoside, which may have different antibacterial effects and underlying mechanisms. Therefore, their use may inhibit the generation of bacteria resistant to *P. clematidea* extract. These results provide novel insights for further evaluating the efficacy of *P. clematidea* extract to control bacterial leaf blight and blast diseases in net-houses and in the field.

5. Conclusions

We isolated and characterized five antibacterial and four antifungal compounds derived from *P. clematidea* and evaluated their antimicrobial activities. Among them, flavones, apigenin, luteolin, and 3-*O*-methylquercetin which have no or only one methoxy group exhibited anti-*Xoo* activities whereas polymethoxylated flavones, sinensetin, and scutellarein tetramethylether were the anti-*P. oryzae* compounds. We also discovered a new antibacterial compound, 4,4′,4′′-nitrilotriphenol, which was purified from a natural organism for the first time and its glucose conjugate; a novel compound. Although we need to evaluate their toxicity to the environment and human health, these novel compounds are promising candidates as alternative antibacterial agents and seed compounds for the development of new antimicrobials.

P. clematidea extract might be applicable to control bacterial leaf blight and blast diseases in the field; however, it needs to be tested on plants growing in greenhouses first before assessing its applicability in the field.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11112366/s1: Table S1: NMR data of compound **1**, apigenin; Figure S1: Full assignment ^{1}H NMR spectrum of compound **1** recorded in CD_3OD; Figure S2: Full assignment ^{13}C NMR spectrum of compound **1** recorded in CD_3OD; Figure S3: ^{1}H-^{13}C HMBC spectrum of compound **1** recorded in CD_3OD; Table S2: NMR data of compound **2**, 4,4′,4′′-nitrilotriphenol; Figure S4: Full assignment ^{1}H NMR spectrum of compound 2 recorded in CD_3OD; Figure S5: ^{1}H-^{13}C HMBC spectrum of compound **2** recorded in CD_3OD; Figure S6: ^{1}H-^{15}N HMBC spectrum of compound **2** recorded in CD_3OD; Table S3: NMR data of compound 3, luteolin; Figure S7: Full assignment ^{1}H NMR spectrum of compound **3** recorded in CD_3OD; Figure S8: ^{13}C NMR spectrum of compound **3** recorded in CD_3OD; Figure S9: ^{1}H-^{13}C HMBC spectrum of compound **3** recorded in CD_3OD; Table S4: NMR data of compound **4**, 3-*O*-methylquercetin; Figure S10: Full assignment ^{1}H NMR spectrum of compound **4** recorded in CD_3OD; Figure S11: NOESY spectrum of compound **4** recorded in CD_3OD; Figure S12: Full assignment ^{13}C NMR spectrum of compound **4** recorded in CD_3OD; Figure S13: ^{1}H-^{13}C HMBC spectrum of compound **4** recorded in CD_3OD; Table S5: NMR data of compound **5**, 4,4′,4′′-nitrilotriphenyl β-D-glucopyranoside; Figure S14: Full assignment ^{1}H NMR spectrum of compound **5** recorded in CD_3OD; Figure S15: DQF-COSY spectrum of compound **5** recorded in CD_3OD; Figure S16: ^{13}C NMR spectrum of compound **5** recorded in CD_3OD; Figure S17: ^{1}H-^{13}C HSQC spectrum of compound **5** recorded in CD_3OD; Figure S18: ^{1}H-^{13}C HMBC spectrum of compound **5** recorded in CD_3OD; Figure S19: ^{1}H-^{15}N HMBC spectrum of compound **5** recorded in CD_3OD; Table S6: NMR data of compound **6**, scutellarein tetramethyl ether (4′,5,6,7-Tetramethoxyflavone); Figure S20: Full assignment ^{1}H NMR spectrum of compound **6** recorded in CD_3OD; Figure S21: NOESY spectrum of compound **6** recorded in CD_3OD; Figure S22: ^{1}H-^{13}C HSQC spectrum of compound **6** recorded in CD_3OD; Figure S23: ^{1}H-^{13}C HMBC spectrum of compound **6** recorded in CD_3OD; Table S7: NMR data of compound **7**, apigenin trimethyl ether (4′,5,7-Trimethoxyflavone); Figure S24: Full assignment ^{1}H NMR spectrum of compound **7** recorded in CD_3OD; Figure S25: NOESY spectrum of compound **7** recorded in CD_3OD; Figure S26: ^{13}C NMR spectrum of compound 7 recorded in CD_3OD; Figure S27: ^{1}H-^{13}C HSQC spectrum of compound **7** recorded in CD_3OD; Figure S28: ^{1}H-^{13}C HMBC spectrum of compound **7** recorded in CD_3OD; Table S8: NMR data of compound **8**, sinensetin (3′,4′,5,6,7-pentamethoxy flavone); Figure S29: Full assignment ^{1}H NMR spectrum of compound **8** recorded in CD_3OD; Figure S30: ^{1}H-^{13}C HSQC spectrum of compound **8** recorded in CD_3OD; Figure S31: ^{1}H-^{13}C HMBC spectrum of compound **8** recorded in CD_3OD; Table S9: NMR data of compound **9**, luteolin tetramethyl ether (3′,4′,5,7-Tetramethoxyflavone); Figure S32: Full assignment ^{1}H NMR spectrum of compound **9** recorded in CD_3OD; Figure S33: NOESY spectrum of compound **9** recorded in CD_3OD; Figure S34: ^{1}H-^{13}C HSQC spectrum of compound **9** recorded in CD_3OD; Figure S35: ^{1}H-^{13}C HMBC spectrum of compound **9** recorded in CD_3OD.

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