Inhibitors of Sporangia Formation of Phytophthora capsici from Polygonum capitatum

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ABSTRACT

The genus Phytophthora comprises of nearly 120 pathogenic species which causes both economic and environmental damage. This study aims to isolate active compounds from natural products for inhibition of sporangia formation of Phytophthora capsici. The ethyl acetate (EtOAc) layer of the methanol (MeOH) extract of Polygonum capitatum exhibited significant activity. The ethyl acetate (EtOAc) fraction was then subjected to bioassay-guided separation and purification to afford five active flavonoids which significantly inhibited sporangia formation on Phytophthora capsici. The structures were elucidated by spectroscopic analysis and comparison of spectroscopic data with those reported. The active compounds were identified as myricetin 7-α-L-rhamnopyranoside (1), quercetin 3-O-β-D-glucopyranoside (2), quercetin 4′-O-α-L-rhamnopyranoside (3), quercetin 3-O-β-D-galloyl)-α-L-rhamnopyranoside (4), and kaempferol 3-O-α-L-rhamnopyranoside (5). Compounds 1, 2, and 3 were isolated from P. capitatum for the first time. Moreover, the stereo structure of sugar moiety of 3 was corrected by chemical derivatization means. Compounds (1-5) isolated from P. capitatum significantly inhibited sporangia formation of Phytophthora capsici.

Keywords: Flavonoids, Inhibitors, Phytophthora capsici, Polygonum capitatum, Sporangia formation

Introduction

The genus Phytophthora, known as “plant destroyer,” consists of approximately 120 pathogenic species.¹ ² These species could bring serious effects on agriculture, environment, and related industries. Potato late blight caused by Phytophthora infestans is economically the most important and most destructive potato and tomato disease worldwide. The disease causes annual losses of several billion dollars and globally threatens the potato market.³ In the middle of the 19th century, P. infestans destroyed a significant part of potato crop plantations in the USA and Europe; this pathogen is widely known as the cause of the Irish potato famine in 1845, resulting in the death of more than a million people.⁴ Various preventive control strategies are used to prevent Phytophthora infection. Metalaxyl, is the most effective and commonly used fungicide against Phytophthora. However, the long-term use of this fungicide led to serious resistance and environmental issues.⁵ Therefore, development of new methods to control Phytophthora is a very urgent task for researchers. The life cycle of Phytophthora can be separated into asexual and sexual cycles. To control the sexual reproduction of Phytophthora, we previously determined the chemical structures of two signaling molecules, namely, hormones α1 and α2, which stimulate sexual reproduction in heterothallic species.⁶ The asexual cycle is the driving force of rapid polycyclic epidemics in crops and forest trees during the growing season. The infected plants produce and release numerous sporangia into the atmosphere. These sporangia germinate directly or indirectly to produce zoospores, which not only directly infect the plants but also grow to hypha and complete the asexual cycle. Hence, screening of inhibitors of sporangia formation of Phytophthora species will be a promising way to control the disease.⁷

Materials and Methods

General

Optical rotations of isolated compounds were obtained on a JASCO P-1030 digital polarimeter. NMR spectra were recorded on Bruker AV III-500 spectrometer. Chemical shifts in ppm were referenced to the solvent peaks of CD3OD. Mass spectra were obtained on 6224A accurate mass TOF LC/MS system. Preparative HPLC analysis was conducted using ELITE P-230 pumps. Column chromatography was performed using silica gel (Qingdao Haiyang Chemical Co. Ltd., Qingdao, China) and ODS (Cosmosil 75 C18-OPN, Nacalai Tesque, Japan). TLC analysis was conducted using pre-coated silica gel (0.25 mm) and RP-18 plates (0.25 mm).

Plant Material

The whole plant of P. capitatum was purchased from Bozhou City, Anhui Province, China in January 2016. The plant was authenticated by Professor Jianhua Qi from the College of Pharmaceutical Sciences, Zhejiang University. A voucher specimen (20160103) was deposited at the Institute of Materia Medica, Zhejiang University.

Extraction and Isolation

The dried plant of P. capitatum (250 g) was powdered, soaked in methanol (MeOH), and continuously stirred at room temperature (25 °C) for 2 days. The supernatant was separated by filtration and concentrated under reduced pressure to obtain crude methanol (MeOH) extract. The crude methanol (MeOH) extract was partitioned between ethyl acetate (EtOAc) and water to obtain ethyl acetate (EtOAc) and...
water layers. The active ethyl acetate (EtOAc) fraction (Figure 1) was loaded on silica gel column and successively eluted with stepwise gradients of n-hexane/CHCl₃ (10:0, 9:1, 7:3, 5:5, 3:7, 1:9, and 0:10), CHCl₃/EtOAc (9:1, 7:3, 5:5, 3:7, and 0:10), and MeOH (100%) to yield eight fractions. The active fraction eluted with 100% MeOH was separated through an open column and eluted with MeOH/H₂O (30:70, 40:60, 50:50, 60:40, 70:30, 90:10, and 100:0) to yield eight fractions. Fractions 3 and 4 were active. The active fraction 3 was separated by ODS open column and eluted with MeOH/H₂O (30:70, 50:50, and 100:0) to obtain four fractions. The active fraction eluted with MeOH/H₂O (50:50) was further purified by HPLC using solvent MeOH/H₂O (38:62) to yield active compounds 1, 2, and 3. Similarly, the active fraction 4 was separated through ODS open column and eluted with MeOH/H₂O (30:70, 50:50, and 100:0) to afford 3 fractions. The active fraction eluted with MeOH/H₂O (50:50) was further purified by HPLC using solvent MeOH/H₂O (48:52) to obtain active compounds 4 and 5.

Acid Hydrolysis of 3 and Sugar Analysis

The absolute configuration of sugar moiety of compound 3 was determined according to the reported method:10-11 Firstly, the aldose thiocarbamate standards were synthesized: L-rhamnose was derivatized with L-cysteine methyl ester, D-cysteine methyl ester, and α-tolylisothiocyanate, according to the published papers. 13

Results and Discussion

During our screening study, the ethyl acetate (EtOAc) layer, obtained from the partitioning of the methanol (MeOH) extract of P. capitatum, exhibited a potential inhibitory activity against the sporangia formation of P. capitatum. Figure 1. P. capitatum is used as a traditional medicine for treatment of urinary tract infection, hematuria, eczema, diarrhea, and dysentery.12 The major constituents isolated from P. capitatum include volatile oils, glycosides, lignins, chromosome glycosides, and flavonoids.13

This research focused on bioassay guided separation and purification of inhibitors of sporangia formation of Phytophthora capsici from the ethyl acetate (EtOAc) layer of P. capitatum. As a result, five active compounds (1-5) were obtained.

Structure Elucidation

Structures of the isolated compounds (1-5) (Figure 2) were determined by spectroscopic analyses, particularly MS and 1H NMR spectroscopy, and comparison of spectroscopic data with published literature.14-19 The isolated compounds were identified as myricetin 7-O-α-L-rhamnopyranoside (1),14 quercetin 3-O-β-D-glucopyranoside (2),15 queretin 4'-O-α-L-rhamnopyranoside (3),16 quercetin 3-O(2''-galloyl)-α-L-rhamnopyranoside (4),17 and kaempferol 3-O-α-L-rhamnopyranoside (5),18-19 respectively (Figure 2). Although 1H NMR data reported for literature for compound 3 was the same as we obtained,18 the configuration of the sugar moiety was incorrect. Therefore, to confirm the absolute configuration of sugar moiety of compound 3, chemical derivatization was conducted according to the reported method.14-15 Finally, the configuration of sugar moiety was identified as L-rhamnose and the structure of compound 3 was revised (Figure 2). Compounds 1-3 were isolated for the first time from P. capitatum.

Spectroscopic data of compounds 1-5

Myricetin 7-O-α-L-rhamnopyranoside (1): yellow solid, [α]D =2.00° (c 0.14, MeOH), 1H NMR (500 MHz, CD3OD): 0.89 (3H, d, J = 6.0 Hz, H-6''), 3.33-3.79 (9H, m, H-3''), 4.22 (1H, br s, H-2''), 5.31 (1H, s, H-1''), 6.20 (1H, d, J = 2.0 Hz, H-6), 6.36 (1H, d, J = 2.0 Hz, H-8), 6.95 (2H, s, H-2' and 6''), ESI-TOF-MS m/z 465 [M+H]+.

Quercetin 3-O-β-D-glucopyranoside (2): yellow solid, [α]D = -15.5° (c 0.08, MeOH), 1H NMR (500 MHz, CD3OD): 3.20-3.47 (4H, m, H-3'' and 5''), 3.78 (1H, d, J = 12.0 Hz, H-8''), 3.70 (1H, d, J = 1.5 Hz, H-6''), 6.37 (1H, d, J = 8.0 Hz, H-1''), 6.39 (1H, d, J = 2.0 Hz, H-8), 6.48 (1H, d, J = 8.5 Hz, H-5'), 7.58 (1H, d, J = 8.5, 2.0 Hz, H-6'), 7.70 (1H, d, J = 2.0 Hz, H-2''), ESI-TOF-MS m/z 465 [M+H]+.

Quercetin 4'-O-α-L-rhamnopyranoside (3): yellow solid, [α]D = -179.2° (c 0.3, MeOH), 1H NMR (500 MHz, CD3OD): 0.94 (3H, d, J = 6.0 Hz, H-6''), 3.39-3.51 (2H, m, H-4'' and 5''), 3.75 (1H, dd, J = 9.5, 3.0 Hz, H-3''), 4.22 (1H, br s, H-2''), 5.35 (1H, s, H-1''), 6.20 (1H, d, J = 1.25 Hz, H-6), 6.37 (1H, d, J = 1.25 Hz, H-8), 6.91 (1H, d, J = 8.0 Hz, H-5'), 7.31 (1H, dd, J = 8.0, 2.0 Hz, H-6'), 7.34 (1H, d, J = 2.0 Hz, H-2''), ESI-TOF-MS m/z 449 [M+H]+.

Quercetin 3-O(2''-gallyl)-α-L-rhamnopyranoside (4): yellow solid, [α]D = -9.5° (c 0.32, MeOH), 1H NMR (500 MHz, CD3OD): 1.02 (3H, d, J = 6.0 Hz, H-6''), 3.46 (2H, m, H-4'' and 5''), 4.00 (1H, dd, J = 9.0, 3.4 Hz, H-3''), 5.50 (1H, d, J = 1.5 Hz, H-1''), 5.62 (1H, dd, J = 3.4, 1.5 Hz, H-2''), 6.19 (1H, d, J = 2.0 Hz, H-6), 6.36 (1H, d, J = 2.0 Hz, H-8), 6.93 (1H, d, J = 8.5 Hz, H-5'), 7.06 (2H, s, H-2'' and 6'') 7.34 (1H, dd, J = 8.5, 2.0 Hz, H-6'), 7.36 (1H, d, J = 2.0 Hz, H-2''), ESI-TOF-MS m/z 601 [M+H]+.

Kaempferol 3-O-α-L-rhamnopyranoside (5): yellow solid, [α]D = -149.6° (c 0.21, MeOH), 1H NMR (500 MHz, CD3OD): 0.92 (3H, d, J = 5.5 Hz, H-6''), 3.33-3.35 (2H, m, H-4'' and 5''), 3.71 (1H, dd, J = 9.0, 3.3 Hz, H-3''), 4.22 (1H, dd, J = 3.3, 1.5 Hz, H-2''), 5.37 (1H, d, J = 1.5 Hz, H-1''), 6.18 (1H, d, J = 2.0 Hz, H-6), 6.35 (1H, d, J = 2.0 Hz, H-8), 6.93 (2H, d, J = 9.0 Hz, H-3' and 5''), 7.77 (2H, d, J = 9.0 Hz, H-2' and 6''), ESI-TOF-MS m/z 433 [M+H]+.

Bioassay results of compounds 1-5

Compounds 1-5 significantly inhibited the sporangia formation of Phytophthora in comparison with the positive (metalaxyl) and negative controls (0.2% DMSO) (Figure 3). Compounds 1 and 2 (30 μM) showed 74% and 65% inhibition rates, respectively. Similarly, Compounds 3 and 4 (5 μM) showed inhibition rates of 86% and 78%, respectively. Compound 5 showed 90% inhibition rate at 10 μM. All of these compounds exhibited inhibitory activity 24 h after the treatment.

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Figure 1: Inhibition rate of the EtOAc and H$_2$O layers of MeOH extract of *P. capitatum* in comparison with metalaxyl (positive control) and DMSO (negative control).

**Conclusion**

These results indicated that methanol (MeOH) extract of *P. capitatum* contains active components possessing inhibition activity on sporangia formation of *Phytophthora*. *P. capitatum* is a traditional Chinese medicinal plant and with wide distribution in China. Therefore, methanol (MeOH) extract of *P. capitatum* and its active components could be potential agents for development of fungicides to control *Phytophthora* infection which is a big threat to forests, crops and ecosystem in the world. The mechanism of action of the most active compound (3) will be investigated in future study.

Figure 2: Chemical structures of myricetin 7-O-α-L-rhamnopyranoside (1), quercetin 3-O- β-D-glucopyranoside (2), quercetin 4’-O-α-L-rhamnopyranoside (3), quercetin 3-O-(2”-galloyl)-α-L-rhamnopyranoside, (4) and kaempferol 3-O-α-L-rhamnopyranoside (5).

Figure 3: Inhibition rate of compounds 1-5 in comparison with P (positive control) and C (negative control).

Figure 4: Photomicrographs of *P. capsici* mycelia under phase-contrast microscope 24 h after treatment of negative control, positive control and compounds 1-5 at the optimal dose, respectively. Few sporangia were observed on mycelia after treatment of positive control, compounds 3 and 5 at indicated doses, respectively.
Conflict of interest  
The authors declare no conflict of interest.

Authors’ Declaration  
The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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