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 cause 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-O-α-L-rhamnopyranoside (1), quercetin 3-0-β-D-glucopyranoside (2), quercetin 4’-O-α-L-rhamnopyranoside (3), quercetin 3-0-2’-O-galloyl)-α-L-rhamnopyranoside (4), and kaempferol 3-0-α-L-rhamnopyranoside (5). Compounds 1, 2, and 3 were isolated from *P. capitatum* for the first time.

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

**Introduction**

The genus *Phytophthora*, known as “plant destroyer,” consists of approximately 120 pathogenic species.¹² 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.¹ 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.² 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.³ 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.⁴ The asexual cycle is the driving force of rapid polyecyclic 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.⁵

**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 δ (ppm) were referenced to the solvent peaks of δH 3.30 ppm for 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.
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 (10:1, 9:1, 7:3, 5:3, 3:1, 1:1, and 0:1), CHCl₃/MeOH (9:1, 7:3, 5:3, 3:1, and 0:1), and MeOH (100%) to obtain eight fractions. The active fraction eluted with 100% MeOH was separated through an open column and eluted with MeOH/H₂O step gradient (30:70, 35:65, 40:60, 50:50, 60:50, 70:50, 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. Firstly, the aldose thiocarbamate standards were synthesized: L-rhamnose was derivatized with L-cysteine methyl ester, D-cysteine methyl ester, and o-tolylisothiocyanate, according to the published papers. Then, compound 3 (0.5 mg) was hydrolyzed at 80 °C, for 4 h in 2 M HCl (CH₃OH, 1 mL). The product was concentrated and dried in vacuo, and re-dissolved in pyridine (0.2 mL) containing 2.5 mg/mL L-cysteine methyl ester. The reaction was heated at 60 °C for 1 h. Approximately 0.1 mL of o-tolylisothiocyanate solution (2.5 mg/mL) in pyridine was added to the mixtures, which were then further heated at 60 °C for 1 h. The reaction products (2 μL) were diluted by methanol (98 μL) and directly analyzed by Agilent technologies 6224A accurate mass LC-TOF-MS under the following conditions: Agilent Extend C18 column (3.5 μm, 3.0 mm × 100 mm); detected at 210 nm; t = 0 min MeOH/0.02% trifluoroacetic acid (30:70:1), t = 15 min MeOH/0.02% trifluoroacetic acid (95:5:0.1); and flow rate: 0.45 mL/min.

The L-rhamnose was identified as the sugar moiety of 3 by comparing the retention time of its aldose thiocarbamate derivative (ts = 7.42 min) with those of the aldose thiocarbamate standards: D-cysteine-L- rhamnose (ts = 6.11 min), L-cysteine-L-rhamnose (ts = 7.36 min).

**Bioassay Method**

More than 100 kinds of methanol (MeOH) extracts of plants were screened for inhibition of the sporangia formation of P. capsici. For the bioassay, the A2 mating type of P. capsici was inoculated on a solid medium containing 10% V8 juice, 0.02% CaCO₃, and 2% agar. The inoculated solid medium was incubated at 25 °C for 3–4 days. A piece of hypha was cut from the edge of the Phytophthora colony and placed in the center of a Petri dish containing liquid medium, that is, 10 mL of distilled water containing either the sample at various concentrations or the solvent control. After 24 h, the number of sporangia was counted under a microscope to evaluate inhibitory activity. Inhibition ratio was calculated according to the following equation:

$$I_{rat} = (N_s - N_c) / N_c \times 100\%$$

where Is is the inhibition rate, Nc is the number of sporangia after treatment with the solvent control, and Ns is the number of sporangia after treatment with the samples. Metalaxyl, a well-known fungicide against Phytophthora, was used as positive control to evaluate the reliability of the bioassay system.

**Statistical Analysis**

The data was presented as means ± SE values of three replications. Data was analyzed with paired Student t-test using GraphPad Prism software (GraphPad Software). P < 0.05 was considered statistically significant.

**Results and Discussion**

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

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 (a)-(g). Scale bar, 50 μm. (a) Negative control, (b) positive control (30 μg/mL), (c) 1 (30 μM), (d) 2 (30 μM), (e) 3 (3 μM), (f) 4 (3 μM), and (g) 5 (10 μM).

**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.
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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