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Notes

Control Efficacy of Phloretin Isolated from Apple Fruits Against Several Plant Diseases

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In the course of a searching natural antifungal compounds from plant sources, we found that the methanol extract (3,000 μg/ml) of *Malus domestica* fruits had potential of control against rice blast (*Magnaporthe grisea*) and tomato late blight (*Phytophthora infestans*). Under bioassay-guided purification, we isolated phloretin, a phenolic compound, with *in vivo* antifungal activity against *M. grisea*. By 1-day protective application of phloretin (500 μg/ml), the compound strongly inhibited the disease development of *M. grisea* and *P. infestans* on rice and tomato seedlings, respectively. And red pepper anthracnose caused by *Colletotrichum coccodes* also was moderately suppressed. However, rice sheath blight (*Rhizoctonia solani* AG1), and barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) were hardly controlled. In addition, the compound showed *in vitro* antifungal activity against some plant pathogenic fungi including *Phytophthora capsici*, *Alternaria panax*, *Sclerotinia sclerotiorum*, *R. solani* AG4, and *M. grisea*. This is the first report on the antifungal activity of phloretin against plant pathogenic fungi.

Keywords **:** antifungal activity, *Magnaporthe grisea*, phloretin, *Phytophthora infestans*, rice blast, tomato late blight

Crops are consistently threatened by various pathogenic micro-organisms present in environment. *Magnaporthe grisea* (T. T. Hebert) M. E. Barr classified to Magnaporthaceae, Magnaporthales, Sordariomycetes, Ascomycota, Fungi is an important plant pathogenic fungus that causes rice blast, rice rotten neck, and rice seedling blight in rice cultivation areas. *Phytophthora infestans* (Mont.) de Bary, previously classified to the Kingdom Fungi, belongs to Pythiaceae, Peronosporales, Oomycetes, Oomycota, Chromista. The oomycete pathogen causing late blight disease of potato and tomato is one of the most destructive pathogens with a worldwide distribution. In case of foliar diseases such as rice blast and late blight of tomato and potato, chemical control remains the main measure to reduce the incidence of the disease in most crops. However, field isolates of *M. grisea* and *P. infestans* that are resistant to fungicides such as dithiolane, carpropamid, azoxystrobin, metalaxyl, dimethomorph and famoxadone have developed rapidly and have become widespread worldwide (Avila-Adame and Köller, 2003; Cohen and Reuveni, 1983; Gisi et al., 2000; Ishii et al., 2001; Shattock, 2002; Takagaki et al., 2004; Uesugi, 1981). Due to concerns about human health and environmental quality, the demand for organic foods cultivated without treatment of synthetic fungicides is rapidly increasing. Biological control using plant-derived natural products is regarded as an alternative to fungicides for protection of plant diseases.

The apple is the pomaceous fruit of *Malus domestica* B. (Rosaceae), one of the most widely cultivated tree fruits. The general perception that apples are good for health has encouraged many researchers to search for the magic ingredients in apples. Consumption of apples has been associated with prevention of cardiovascular disease, lung dysfuntions and various cancers (Knekt et al., 1996; Eberhardt et al., 2000; Le-Marchand et al., 2000; Xing et al., 2001). The apple flavonoids such as quercetin, epicatechin, and procyanidin B2 showed potent antioxidant activity and some phytochemicals of this plant represented antiproliferative effects on cancer cells (Eberhardt et al., 2000; He and Liu, 2007; Lee et al., 2003; Yoon and Liu, 2007).

Recently, an antifungal protein, Mal d 2, isolated from *M. domestica* fruits was reported to inhibit the spore germination of *Fusarium oxysporum* and *Penicillium expansum* (Krebitz et al., 2004). On the other hand, a few phenolics of the plants showed antibacterial activities. In particular, phloretin represented minimum inhibitory concentration (MIC) of 274 μg/ml against *Erwinia amylovora*, which causes fire blight of *Maloideae* (Pontais et al., 2008).

In order to search natural antifungal compounds from plants, we tested the *in vivo* antifungal activity of various plant fruits against plant pathogenic fungi (Choi et al., 2006). In the course of the research, the methanol extract of *M. domestica* fruits was found to show remarkable control

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efficacy against rice blast (*M. grisea*) and tomato late blight (*P. infestans*). By bioassay-guided phytochemical investigation, we then isolated an antifungal compound from the plant and determined its chemical structure by instrumental analyses. In this study, we report the isolation and identification of antifungal compound from the methanol extract of *M. domestica* fruits. In addition, the *in vitro* and *in vivo* antifungal activities of the purified compound were investigated against several plant pathogenic fungi.

Apples (*M. domestica*) were purchased from Gyeongsan market in South Korea. A voucher specimen has been deposited in Natural Products Chemistry Laboratory of School of Biotechnology, Yeungnam University. Ground-up apples (1 kg) were homogenized for 10 minutes with 70% *aq* MeOH and the slurry was then filtered through a Whatman No.1 filter paper. The filtrate was evaporated *in vacuo* to yield 30 g of extract. The methanolic extracts were successively partitioned with *n*-Hexane, $CHCl_3$, and EtOAc, affording 5.0 g, 5.4 g, and 10.4 g of the fraction, respectively. The $CHCl_3$ fraction with potent activity was subjected to silica gel column chromatography eluting with *n*-hexane and EtOAc gradient system (10:1 → 10:5), affording ten fractions (Fr. 1 ~ Fr. 10). Among them, fraction 4 gave a colorless crystal, compound **1** (70 mg). In addition, Fr. 5 was subjected to semi-preparative HPLC (20 to 100% CH_3CN in H_2O over 40 min) to afford compound **1** (10 mg).

In the ^{1}H-NMR spectrum of **1**, the signals for a 1,4-disubstituted benzene ring appeared at δ 7.02 and 6.66 (each 2H, J = 8.1 Hz). In addition, two aromatic singlet protons at δ 5.81 together with a chelated hydroxyl proton at δ 12.21 indicated that 1,3,5-trihydroxylbenzene moiety was attached to a carbonyl group. A mutually coupled CH_2-CH_2 spin system δ_H 3.22 and 2.77 together with a ketone carbon at δ_C 204.1 suggested a linkage between the 1,4-disubstituted benzene ring and the 1,3,5-trihydroxylbenzene moiety, indicative of the presence of a dihydrochalcone, a type of polyphenolic compounds. On the basis of the spectral data, compound **1** was identified as phloretin, which was confirmed by comparison with the data previously reported in the literature (Nakamura et al., 2003).

The methanol extract of *M. domestica* fruits, its solvent-soluble fractions, and compound **1** were tested *in vivo* for antifungal activity against the following eight plant diseases: rice blast (*M. grisea*), rice sheath blight (*Rhizoctonia solani* J. G. Kühn AG1), tomato gray mold (*Botrytis cinerea* Pers.:Fr.), tomato late blight (*P. infestans*), barley powdery mildew (*Blumeria graminis* (DC.) Golovin ex Speer f. sp. *hordei*), wheat leaf rust (*Puccinia recondita* Rob ex Desm), red pepper anthracnose (*Colletotrichum coccodes* Wallr.) and red pepper Phytophthora blight (*Phytophthora capsici* Leonian). The *in vivo* antifungal bioassays of seven plant pathogens except for *P. capsici* were performed as described previously (Kim et al., 2001; Cho et al., 2006). Rice (*Oryza sativa* L., cv. Nakdong), tomato (*Solanum lycopersicum* L., cv. Seokwang), barley (*Hordeum sativum* Jessen, cv. Dongbori), wheat (*Triticum aestivum* L., cv. Chokwang), and red pepper (*Capsicum annuum* L., cv. Bugang) plants were grown in plastic pots (4.5-cm diameter) in a greenhouse at 25±5°C for 1 to 5 weeks. To evaluate *in vivo* antifungal activity of samples, the methanol extract of *M. domestica* fruits was dissolved in methanol, and the other fractions obtained during the purification and the purified compound were diluted in acetone. Each solution was then added to Tween 20 (250 μg/ml in distilled water) solution at final concentrations of 5% (v/v) for methanol and 10% (v/v) for acetone. The plant seedlings were sprayed with each solution until run-off. Control plants were also sprayed with Tween 20 solution containing 5% methanol or 10% acetone without chemical. After 24 hr, the treated plant seedlings were inoculated with spores or mycelial suspensions of one of the eight plant pathogens, followed by rating disease symptoms 3-7 days after inoculation.

For red pepper Phytophthora blight, the treated red pepper seedlings with four fully expanded leaves were inoculated with *P. capsici* by spraying with a zoospore suspension released from a sporangial suspension (1×10^4 sporangia/ml). The inoculated plants were kept in the dark at 25°C and 100% RH for 24 hr. And then the infected plants were transferred to a growth chamber maintained at 25°C and 70-80% RH. Disease severity was determined as the percentage of infected leaf area 3 days after inoculation.

Pots were arranged as a randomized complete block with three replicates per treatment. Blasticidin-S for rice blast, validamycin for rice sheath blight, fludioxonil for tomato gray mold, dimethomorph for tomato late blight, flusilazole for wheat leaf rust, benomyl for barley powdery mildew, dithianon and metalaxyl for anthracnose and Phytophthora blight of red pepper, respectively were applied as positive controls. Experiments were conducted in a growth chamber with 9-hr light a day, and the mean value of three estimates for each treatment was converted into percentage fungal control. The percentage of disease control was determined using the following equation: control value (%) = 100[1 − B/A], where A = the diseased area (%) on leaves or sheaths of control plants, and B = the diseased area (%) on treated leaves or sheaths.

As shown in Fig. 1, the methanol extract (at 3,000 μg/ml) of *M. domestica* fruits exhibited potent control efficacy against tomato late blight caused by *P. infestans* as well as rice blast caused by *M. grisea*. It also moderately reduced the development of anthracnose caused by *C. coccodes* and Phytophthora blight by *P. capsici* on red pepper seedlings. The other plant diseases including rice sheath blight (*R.*

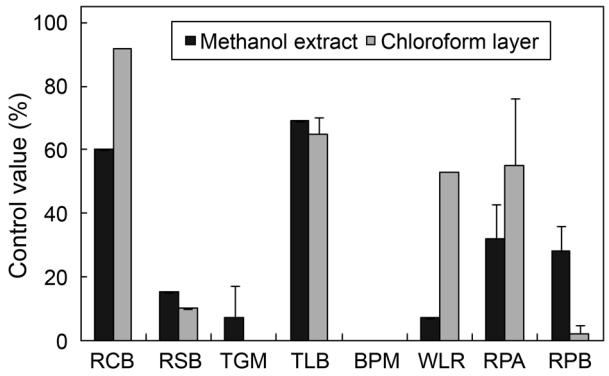

Fig. 1. Control efficacy of the methanol extract (3,000 μg/ml) from *Malus domestica* fruits and the chloroform-soluble fraction (1,000 μg/ml) partitioned from the methanol extract against eight plant diseases. Seedlings were inoculated with spores or mycelia suspensions of the test organisms 1 day after spraying with the chemical solutions. RCB, rice blast; RSB, rice sheath blight; TGM, tomato gray mold; TLB, tomato late blight; BPM, barley powdery mildew; WLR, wheat leaf rust; RPA, red pepper anthracnose; and RPB, red pepper Phytophthora blight. Disease severities of untreated control plants were 25% for RCB, 100% for RSB, 70% for TGM, 65% for TLB, 15% for WLR, 30% for BPM, 33% for RPA, and 95% for RPB.

solani AG1), tomato gray mold (*B. cinerea*), wheat leaf rust (*P. recondita*), and barley powdery mildew (*B. graminis* f. sp. *hordei*) were hardly controlled by the methanol extract. Among the three fractions partitioned from the methanol extract, including *n*-hexane-, chloroform-, and ethyl acetate-soluble layers, the chloroform layer (at 1,000 μg/ml) showed the most activity against rice blast. The fraction was also moderately active to tomato late blight, wheat leaf rust and red pepper anthracnose. Thus, rice blast caused by *M. grisea* was selected as the target disease for bioassay to isolate the antifungal compound.

Consumption of apples has been associated with prevention of cancer and chronic diseases (Le Marchand et al., 2000; Boyer and Liu, 2004). Compared to many other fruits and vegetables, apples contain relatively low amounts of vitamin C, but are a rich source of other antioxidant compounds. According to some reports, apples possess phenolic compounds, which may be cancer protective, and demonstrate antioxidant activity (Lee et al., 2004). Major phenolic compounds of apples are quercetin, epicatechin, epicatechin, and procyanidin (Lee et al., 2003). They may also have a positive effect on heart disease, weight loss, and control of cholesterol. A few studies have demonstrated that *M. domestica* cv. Edward VII, which is resistant to *Sclerotinia fructigena*, a brown rot pathogen, produced antifungal compounds when the plants were infected with the fungus (Fawcett and Spencer, 1966, 1967, 1968). On the other hand, we found that the methanol extract of *M.*

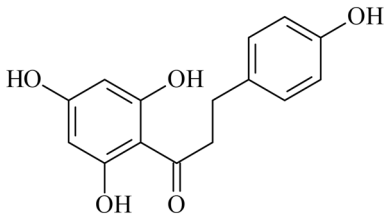

Fig. 2. Chemical structure of phloretin.

domestica fruits without infection of any plant pathogenic fungus exhibited potent control efficacy against rice blast and tomato late blight caused by *M. grisea* and *P. infestans*, respectively.

Bioassay-guided isolation using an *in vivo* antifungal assay against *M. grisea* yielded phloretin, a dihydrochalcone (Fig. 2). By 1-day protective application, phloretin (at a concentration of 500 μg/ml) strongly suppressed the disease development of *P. infestans* on tomato seedlings as well as that of *M. grisea* on rice plants (Fig. 1). The compound also demonstrated moderate control efficacy against red pepper anthracnose caused by *C. coccodes*. However, the compound (500 μg/ml) did not show *in vivo* antifungal activity against rice sheath blight (*R. solani* AG1), and barley powdery mildew (*B. graminis* f. sp. *hordei*). The antifungal spectrum of phloretin is similar to that of the methanol extract of *M. domesticia* fruits. Thus, it is likely that phloretin is one of the antifungal principals of the plant.

A few studies have demonstrated antifungal phenolics from the fruits of *M. domestica* cv. Edward VII infected with brown rot pathogen *S. fructigena* (Fawcett and Spencer, 1966, 1967, 1968). The cultivar possessed moderate resistance to the plant pathogenic fungus. Using a spore germination assay against *B. cinerea*, *Alternaria brassicicola*, and *Glomerella cingulata*, two antifungal compounds, 4-hydroxybenzoic acid and 4-hydroxy-3-methoxybenzoic acid, were isolated from the variety infected by *S. fructigena*.

Phloretin is a natural polyphenolic compound with an abundant presence in apples and pears, especially in the peel (Escarpa and Gonzalez 1998; Tsao et al., 2003). The compound is known to exert anti-tumor activity due to inhibition of protein kinase C, induction of apoptosis, suppression of glucose transport across the plasma membrane by specific inhibition of glucose transporter 2, and inhibition of human leukemia cell growth (Devi and Das, 1993; Kern et al., 2007; Jordan and Holman, 1992; Salter et al., 1978). In addition, the compound was reported to inhibit the growth of bladder cancer and rat mammary adenocarcinoma cells *in vivo* (Nelson and Falk, 1993). On the other hand, several studies have shown that phloretin

Table 1. Control efficacy of phloretin isolated from *Malus domestica* fruits against eight plant diseases[a]

Chemical	Conc. (μg/ml)	Disease Control (%)							
		RCB[b]	RSB	TGM	TLB	BPM	WLR	RPA	RPB
Psoralren	100	40±0.0[c]	0±0.0	27±0.0	14±0.0	0±0.0	3±4.7	7±10	2±5.0
	500	96±0.0	0±0.0	36±13	88±3.0	8±12	20±0.0	50±10	24±5.0
Blasticidin-S	1	83±0.0	–	–	–	–	–	–	–
	50	100	–	–	–	–	–	–	–
Validamycin	5	–[d]	90±0.0	–	–	–	–	–	–
	50	–	100	–	–	–	–	–	–
Fludioxonil	5	–	–	85±3.9	–	–	–	–	–
	50	–	–	100	–	–	–	–	–
Dimethomorph	2	–	–	–	94±2.0	–	–	–	–
	10	–	–	–	100	–	–	–	–
Benomyl	1	–	–	–	–	95±2.4	–	–	–
	100	–	–	–	–	100	–	–	–
Flusilazole	2	–	–	–	–	–	77±14	–	–
	10	–	–	–	–	–	100	–	–
Dithianon	10	–	–	–	–	–	–	29±0.0	–
	50	–	–	–	–	–	–	94±2.0	–
Metalaxyl	10	–	–	–	–	–	–	–	80±5.0
	50	–	–	–	–	–	–	–	100

[a] The compounds were dissolved in dimethyl sulfoxide (1%) and Tween 20 (250 μg/ml), and they were then sprayed to run off on the following seedlings: rice (3- leaf stage for RCB, 4-leaf stage for RSB), tomato (3-leaf stage), barley (1-leaf stage), wheat (1-leaf stage), and red pepper (4-leaf stage for RPA, 6-leaf stage for RPB). After 24 hr, the treated seedlings were inoculated with spores or mycelial suspensions of the pathogens. The disease severity of untreated control plants was 50% for RCB, 100% for RSB, 55% for TGM, 70% for TLB, 30% for BPM, 15% for WLR, 23% for RPA, and 95% for RPB.

[b] RCB, rice blast (*Magnaporthe grisea*); RSB, rice sheath blight (*Rhizoctonia solani* AG1); TGM, tomato gray mold (*Botrytis cinerea*); TLB, tomato late blight (*Phytophthora infestans*); BPM, barley powdery mildew (*Blumeria graminis* f. sp. *hordei*); WLR, wheat leaf rust (*Puccinia recondita*); RPA, red pepper anthracnose (*Colletotrichum coccodes*) and RPB, red pepper Phytophthora blight (*Phytophthora capsici*).

[c] Each value represents the mean ± standard deviation of three replicates.

[d] Not tested

has antidiabetic effects by inhibition of intestinal glucose absorption (Minami et al., 1993; Murphy and Lumsden, 1984). And the phytochemical has been reported to act as an antioxidant through significant scavenging of the hydroxyl radical in cultured primary human melanocytes (Lee et al., 2003; Lin et al., 2007). To the best of our knowledge, antifungal activity of phloretin isolated from *M. domestica* fruits against plant pathogenic fungi has not been reported.

In vitro antifungal activity of phloretin was tested against the seven plant pathogenic fungi such as *M. grisea*, *R. solani* AG4, *B. cinerea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp. *raphani*, *S. sclerotiorum*, and *Alternaria panax* and two oomycetes (*P. infestans* and *P. capsici*). The compound was dissolved in dimethylsulfoxide (DMSO) to give the concentrations of 0.082, 0.25, 0.74, 2.2, 6.7, and 20 mg/ml. A 10-μl aliquot of each solution was loaded into each well of a 96-well microtiter plate. And broth medium (100 μl) including the spores of seven test fungi except for *R. solani* AG4 and *S. sclerotinia* at a concentration of $1x10^4$ spores/ml was then added to each well. For *R. solani* AG4 and *S. sclerotiorum,* fresh medium (100 μl) including 1% of mature seed broth was also loaded. Broth media were potato dextrose broth for eight test fungi (except for *P. infestans*) and clarified V-8 juice broth for *P. infestans*. Controls containing 1% DMSO without the chemical were also included. The plates were incubated at 25°C (for six test fungi except for *P. infestans*, *S. sclerotinia*, and *B. cinerea*) and 20°C (for *P. infestans*, *S. sclerotinia*, and *B. cinerea*) for 3-5 days. Minimum inhibitory concentration (MIC) of phloretin against each fungus was investigated by visual observation.

Among nine plant pathogenic fungi, phloretin showed the most antifungal activity against *P. capsici* (MIC value of 67 μg/ml), although the compound (500 μg/ml) slightly suppressed the development of Phytophthora blight on red pepper plants (Table 2). This result may be due to disease severity of Phytophthora blight on red pepper seedlings in our *in vivo* assay system. On the other hand, the growth of some fungi including *M. grisea*, *R. solani* AG4, *S. sclero-*

Table 2. Minimum inhibitory concentration (MIC) of phloretin isolated from *Malus domestica* fruits against several plant pathogenic fungi[a]

Plant pathogen	MIC (μg/ml)
Phytophthora capsici	67
Alternaria panax	200
Magnaporthe grisea	200
Rhizoctonia solani AG4	200
Sclerotinia sclerotiorum	200
Botrytis cinerea	>200
Colletotrichum gloeosporioides	>200
Fusarium oxysporum f. sp. *raphani*	>200
Phytophthora infestans	>200

[a] Phloretin was dissolved in dimethylsulfoxide and a 10-μl aliquot was loaded in each well of 96-well plates to give the final concentrations of 0.82, 2.5, 7.4, 22, 67, and 200 μg/ml. And then, broth medium (100 μl) including spores or mycelia of each plant pathogenic microorganisms was added to the well. And the plate was incubated at 25°C or 20°C with 150 rpm for 3-5 days. MIC value of the compound against the test fungi was determined by observation of the growth of each fungus.

tinia, and *A. panax* was also inhibited by the compound (Table 2).

Phloretin (500 μg/ml) showed potent disease control efficacy against tomato late blight caused by *P. infestans* and rice blast by *M. grisea* (Table 1). Phloretin (200 μg/ml) inhibited the growth of *M. grisea*, but the compound did not suppress the growth of *P. infestans in vitro* (Table 2). Thus, these results suggest that the control of phloretin on tomato late blight may not be due to inhibition of microbial growth, but indirect suppression such as induction of systemic acquired resistance in plants and/or other mechanisms including the secretion inhibition of fungal cell wall degrading enzymes as fungicide mepanipyrim and pyrimethanil (Pieterse et al., 1998; Sticher et al., 1997; Jeun et al., 2001; Milling and Richardson, 1995; Miura et al., 1994). To the best of our knowledge, this is the first report on the antifungal activity of phloretin from *M. domestica* fruits. Also, the compound did not show phytotoxicity on some crops such as tomato, rice, wheat, barley, and red pepper (data not shown). Results of this study suggest that the chloroform extract of *M. domestica* fruits and a phenolic compound phloretin could be used as biopesticides for control of rice blast as well as tomato late blight.

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