

In vivo fungicidal properties of *Diospyros kaki*-isolated compound and its analogues

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Abstract Fungicidal effects of active component purified from *Diospyros kaki* roots and its analogues against *Erysiphe graminis*, *Botrytis cinerea*, *Pyricularia grisea*, *Puccinia recondite*, *Rhizoctonia solani*, and *Phytophthora infestans* were investigated using a whole-plant method. Active constituents isolated from the chloroform fraction of *D. kaki* roots were characterized as plumbagin, using various spectroscopic analyses. To establish the structure–activity relationships, the fungicidal effects of plumbagin and its structural analogues were bioassayed against phytopathogenic fungi. At 0.25, 0.125, and 0.0625 g/L, plumbagin had a greater fungicidal effect than *p*-naphthoquinone, juglone, lawsone, 2-methoxy-*p*-naphthoquinone, or menadione against *P. grisea*, *B. cinerea*, *R. solani*, and *E. graminis*. Our results suggest that the methyl and hydroxyl functional groups at the 5'- and 2'-positions of *p*-naphthoquinone play key roles in its fungicidal effect against six phytopathogenic fungi. Therefore, plumbagin and its analogues may be suitable as antifungal agents to control plant diseases.

Keywords *Diospyros kaki* roots · Functional group · Fungicidal effect · Phytopathogenic fungi · Structure–activity relationships

Introduction

Plant pathogens, such as bacteria, fungi, nematodes, and viruses, cause several diseases or damages in plants [1]. Among these plant pathogens, fungi are the major plant pathogen and cause many serious diseases of plants. Plant pathogenic fungi also cause pre- and post-harvest losses in economically important agricultural crops [2, 3]. For many years, synthetic chemicals have been used as fungicidal agents to control plant diseases [4]. Synthetic fungicides, such as benzimidazole and difenoconazole, are widely used to control plant diseases caused by phytopathogenic fungi [5, 6]. However, the intensive use of synthetic fungicides has adverse effects, including fungicide resistance and residual toxicity [7]. Thus, there is a need for the development of environmentally safe fungicides. One possible answer is to use plant-derived metabolites as sources for the development of fungicidal agents [8, 9].

Diospyros kaki L., in the Ebenaceae family, is traditionally used to treat burns, frostbite, hiccup, and paralysis [10]. Various biological activities, including antimicrobial, acaricidal, and fungicidal activities of this species, have been reported previously [11–14]. Although there are several studies of the antifungal effects of *D. kaki* against phytopathogenic fungi in vitro, to our knowledge the in vivo efficacy of *D. kaki* against phytopathogenic fungi has not been studied. The aim of our work was to study in vivo antifungal effects of the isolated component from *D. kaki* and its analogues in a greenhouse.

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Materials and methods

Chemicals

2-Hydroxy-1,4-naphthalenedione (lawsone), 2-methyl-1,4-naphthalenedione (menadione), 2-methoxy-*p*-naphthoquinone, and 1,4-naphthalenedione (*p*-naphthoquinone) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dichlofluanid (C₉H₁₁Cl₂FN₂O₂S₂) was purchased from Dongbu Hitek Co.

Isolation and identification

D. kaki roots (1 kg) were dried at 35 °C and homogenized with a grinder. Afterward, *D. kaki* roots were extracted for 48 h with methanol; this extraction was repeated three times. The extract was filtered over Toyo filter paper No. 2 (Tokyo, Japan), and methanol was completely removed from the residual *D. kaki* roots by evaporation with a rotary vacuum evaporator. The methanol extract (18.4 g) was suspended in H₂O and partitioned successively with hexane, chloroform, ethyl acetate, and butanol to obtain chloroform (3.94 g), hexane (5.41 g), ethyl acetate (2.96 g), and butanol (6.09 g), respectively.

The active component from the chloroform fraction derived from the *D. kaki* extract was purified by silica-gel chromatography (5.5 i.d. × 68 cm L, Merck 70-230 mash). Hexane and chloroform (30:70–0:100 v/v) were the elution solvents. The column chromatographic analysis yielded six fractions (K1–K6). The K1 showed the strongest fungicidal activity against six fungi. The K1 fraction was rechromatographed using a silica-gel column. Chloroform and hexane (70:30 v/v) were the elution solvents. From K1, six fractions (K11–K16) were obtained. The K11 was purified by prep HPLC (JAI Co., Tokyo, Japan) to separate the active component, and the eluates were tested. From K11, six fractions (K111–K116) were obtained using a Jaigel GS Series Column (21.4 mm i.d. × 500 mm L, JAI Co., Tokyo, Japan) with chloroform–hexane–isopropanol (70:30:2) at 4.8 mL/min (268 nm). The K114 was isolated using a Jaigel W Series Column (20.1 mm i.d. × 500 mm L, JAI Co., Tokyo, Japan) with chloroform–hexane–isopropanol (70:30:2) at 4.6 mL/min, resulting in four fractions (K1141–K1144). Finally, the K1144 was purified. The structure of K1144 was studied by spectroscopic analyses. ¹H (600 MHz), ¹³C (150 MHz), and DEPT (100 MHz) NMR were studied using JNM-LA 400F7 (Jeol Ltd., Japan) in CDCl₃. HMQC and ¹H–¹H COSY was measured to study the relationships between carbons and protons. Tetramethylsilane was used as the internal standard. EI-MS spectra were studied with a spectrometer (JEOL JMS-DX 30).

Pathogens and cultures

Pyricularia grisea, *Erysiphe graminis*, *Botrytis cinerea*, *Phytophthora infestans*, *Rhizoctonia solani*, and *Puccinia recondita* were used (Table 1). *Erysiphe graminis* and *P. recondita* were maintained on barley and wheat, respectively. *Rhizoctonia solani*, *P. grisea*, *P. infestans*, and *B. cinerea* were grown on potato dextrose agar (PDA) slants and V-8 agar slants and kept at 4 °C.

In vivo bioassay for fungicidal effect

Fungicidal effects of samples against six phytopathogenic fungi using various concentrations (2.0, 1.0, 0.5, 0.25, 0.125, and 0.0625 g/L) were evaluated by a whole-plant method, as described by Kim et al. [15]. Each test sample was dissolved in DMSO (0.5 mL) and diluted in water (49.5 mL) containing of Tween 20 (0.25 mg/mL) to obtain a 2 g/L concentration. Samples (50 mL) were sprayed onto test plants at the same time. The treated plants were placed in a greenhouse for 24 h before inoculation with each pathogen. Negative controls were applied with the DMSO–Tween solution. Experiments were replicated three times. Test conditions of cucumber gray mold (CGM), rice sheath blight (RSB), rice blast (RCB), barley powdery mildew (BPM), wheat leaf rust (WLR), and tomato late blight (TLB) caused by *B. cinerea*, *R. solani*, *P. grisea*, *E. graminis*, *P. recondita*, and *P. infestans*, respectively, in a greenhouse are presented in Table 1.

Statistical analysis

The fungicidal effect of test materials on CGM, RSB, RCB, BPM, WLR, and TLB was measured by a control value (CV) calculated by the equation

$$CV(\%) = [(A - B)/A] \times 100,$$

where *A* and *B* represent the disease area on the untreated and treated plants, respectively. The plant disease area was studied with a Color Image Microscope System (Samsung MW-200B1). CV was transformed to arcsine square-root values for ANOVA. A Scheffe's test was performed to compare and separate treatment means.

Result and discussion

The in vivo fungicidal effects of the methanol extract and four fractions (hexane, chloroform, ethyl acetate, and butanol) derived from *D. kaki* roots against six phytopathogenic fungi when treated with 1.0 and 2.0 g/L are shown in Table 2. In a test with *E. graminis*, *B. cinerea*, *P. grisea*, and *R. solani*, strong fungicidal effects were

Table 1 Test conditions of six phytopathogenic fungi in a greenhouse

Disease	Pathogen	Keeping period in humidity chamber	Inoculation; inoculum dosage	Plant/stage	Plant no./pot	Chamber temp ^a /period ^b
Barley powdery mildew (BPM)	<i>Erysiphe graminis</i>	Not need	Dusting ^c the conidia on barley plant	Barley/1-leaf	4	20 °C/10 days
Cucumber gray mold (CGM)	<i>Botrytis cinerea</i>	3 days	Leaf spray; 1 × 10 ⁶ spore/mL	Cucumber/1-leaf	1	20 °C/3 days
Rice blast (RCB)	<i>Pyricularia grisea</i>	1 day	Leaf spray; 1 × 10 ⁶ spore/mL	Rice/2-leaf	3	25 °C/5 days
Rice sheath blight (RSB)	<i>Rhizoctonia solani</i>	7 days	Pouring inoculum ^d on the soil; 10 mL/pot	Rice/3-leaf	3	28 °C/7 days
Tomato late blight (TLB)	<i>Phytophthora infestans</i>	4 days	Leaf spray; 1 × 10 ⁵ zoosporangia/mL	Tomato/2-leaf	2	18 °C/4 days
Wheat leaf rust (WLR)	<i>Puccinia recondita</i>	1 day	Leaf spray; 1.5 mg of uredospores/pot	Wheat/1-leaf	4	20 °C/10 days

^aChamber temperature kept for treated and control plants

^bPeriod (days) from inoculation of pathogen to evaluation of disease severity on host plants. It was included the days keeping plants applied test solution in humidity chamber

^cPreparation of *E. graminis* conidia, known as obligate parasite, was made by dusting inoculation of conidia on 10 barley plants cultivated in the pot (ϕ 7.5 cm). Treated plants were dusted with *E. graminis* conidia formed on leaves of barley by the ratio of 8 tested pots/a maintained pot

^dInoculum of *R. solani* was made by inoculating mycelial plugs in wheat bran medium at 25 °C for 7 days, and macerated at the ratio of 500 g of medium incubated *R. solani* per 1 L of distilled water into the mixer

Table 2 Antifungal effects of *D. kaki* root-derived materials against phytopathogenic fungi¹ in a greenhouse

Material	Conc. (g/L)	Control value (mean ± SE %) ²					
		BPM	CGM	RCB	RSB	TLB	WLR
Methanol extract	2.0	100 ± 0.0 ^a	86 ± 5.3 ^a	91 ± 2.3 ^a	100 ± 0.0 ^a	0	0
	1.0	76 ± 2.8 ^b	59 ± 3.2 ^b	63 ± 3.4 ^b	70 ± 4.6 ^c	0	0
Hexane fraction	2.0	0 ^c	0 ^c	0 ^c	0 ^d	0	0
	1.0	0 ^c	0 ^c	0 ^c	0 ^d	0	0
Chloroform fraction	2.0	100 ± 0.0 ^a	88 ± 3.6 ^a	95 ± 2.6 ^a	100 ± 0.0 ^a	0	0
	1.0	80 ± 4.0 ^{ab}	64 ± 3.5 ^b	68 ± 3.8 ^b	81 ± 2.8 ^b	0	0
Ethyl acetate fraction	2.0	0 ^c	0 ^c	0 ^c	0 ^d	0	0
	1.0	0 ^c	0 ^c	0 ^c	0 ^d	0	0
Butanol fraction	2.0	0 ^c	0 ^c	0 ^c	0 ^d	0	0
	1.0	0 ^c	0 ^c	0 ^c	0 ^d	0	0

¹BPM barley powdery mildew caused by *E. graminis* on barley, CGM cucumber gray mold caused by *B. cinerea* on cucumber, RCB rice blast caused by *Py. grisea* on rice, RSB rice sheath blight caused by *R. solani* on rice, TLB tomato late blight caused by *P. infestans* on tomato, WLR wheat leaf rust caused by *P. recondita* on wheat

²Means within a column followed by same letters are not significantly different ($P = 0.05$, Scheffe's test)

produced by the methanol extract (86–100%) and chloroform fraction (88–100%) at 2.0 g/L. However, hexane, ethyl acetate, and butanol fractions had no fungicidal effect against six phytopathogenic fungi. A negative control containing DMSO-Tween 20 solution did not show fungicidal effects against six phytopathogenic fungi. However, the chloroform fraction partitioned from the methanol extract of *D. kaki* roots was selected to isolate the fungicidal compound of *D. kaki* roots.

To isolate the active compound of chloroform fraction, chromatographic analyses were performed. The active isolate (K1144) was identified by spectroscopic analyses (¹H NMR, ¹³C NMR, ¹H–¹H COSY, EI-MS, DEPT, and ¹H–¹³C HMQC). The fungicidal compound was identified as 5-hydroxy-2-methyl-1,4-naphthalenedione (plumbagin, C₁₁H₈O₃, MW 188) based on the following evidence: ¹H-NMR (600 MHz, CDCl₃): δ 7.60–7.61 (1H, d), 7.56–7.59 (1H, d), 7.24–7.25 (1H, m), 6.78–6.79 (1H, d), 2.17–2.18

Table 3 Antifungal effects of plumbagin analogues and synthetic fungicides against phytopathogenic fungi¹ in a greenhouse

Chemical	Conc. (g/L)	Control value (mean ± SE %) ²					
		BPM	CGM	RCB	RSB	TLB	WLR
1,4-Naphthalenedione (<i>p</i> -naphthoquinone)	0.5	0 ^f	86 ± 3.5 ^{ab}	0 ^e	100 ^a	0 ^b	0
	0.25	0 ^f	62 ± 3.0 ^{cd}	0 ^e	80 ± 2.6 ^{bc}	0 ^b	0
	0.125	0 ^f	44 ± 4.4 ^f	0 ^e	55 ± 4.0 ^{fg}	0 ^b	0
	0.0625	0 ^f	21 ± 3.0 ^j	0 ^e	23 ± 2.2 ^{ij}	0 ^b	0
5-Hydroxy-1,4-naphthalenedione (juglone)	0.5	0 ^f	85 ± 3.6 ^{ab}	95 ± 2.0 ^a	93 ± 4.0 ^{ab}	0 ^b	0
	0.25	0 ^f	59 ± 2.2 ^{de}	71 ± 2.6 ^a	68 ± 4.4 ^{def}	0 ^b	0
	0.125	0 ^f	37 ± 2.8 ^{gh}	48 ± 1.8 ^c	49 ± 4.6 ^{gh}	0 ^b	0
	0.0625	0 ^f	16 ± 2.6 ^j	32 ± 3.0 ^d	24 ± 1.8 ^{ij}	0 ^b	0
2-Hydroxy-1,4-naphthalenedione (lawsone)	0.5	0 ^f	87 ± 2.0 ^a	90 ± 2.6 ^a	89 ± 3.5 ^{ab}	0 ^b	0
	0.25	0 ^f	56 ± 3.6 ^{de}	65 ± 2.8 ^b	59 ± 4.0 ^{efg}	0 ^b	0
	0.125	0 ^f	35 ± 2.5 ^{gh}	45 ± 2.4 ^c	37 ± 3.6 ^{hi}	0 ^b	0
	0.0625	0 ^f	13 ± 1.0 ^k	27 ± 2.6 ^d	19 ± 1.7 ^j	0 ^b	0
2-Methoxy-1,4-naphthalenedione (2-methoxy- <i>p</i> -naphthoquinone)	0.5	0 ^f	81 ± 3.6 ^{ab}	0 ^e	85 ± 2.6 ^{abc}	24 ± 2.0 ^a	0
	0.25	0 ^f	54 ± 1.0 ^{de}	0 ^e	57 ± 1.8 ^{fg}	0 ^b	0
	0.125	0 ^f	22 ± 1.6 ^{ij}	0 ^e	37 ± 2.2 ^{hi}	0 ^b	0
	0.0625	0 ^f	0 ^k	0 ^e	12 ± 1.7 ^{jk}	0 ^b	0
2-Methyl-1,4-naphthalenedione (menadione)	0.5	100 ± 0.0 ^a	82 ± 2.6 ^{ab}	0 ^e	100 ^a	0 ^b	0
	0.25	84 ± 1.7 ^b	63 ± 2.0 ^{cd}	0 ^e	75 ± 4.0 ^{cde}	0 ^b	0
	0.125	63 ± 4.3 ^d	41 ± 2.2 ^e	0 ^e	51 ± 1.7 ^{gh}	0 ^b	0
	0.0625	39 ± 2.0 ^e	23 ± 1.6 ⁱ	0 ^e	27 ± 1.7 ^{ij}	0 ^b	0
5-Hydroxy-2-methyl-1,4-naphthoquinone (plumbagin)	0.5	100 ± 0.0 ^a	89 ± 1.7 ^a	92 ± 1.8 ^a	100 ^a	0 ^b	0
	0.25	90 ± 2.6 ^b	65 ± 2.8 ^{cd}	69 ± 4.0 ^b	82 ± 1.8 ^{bcd}	0 ^b	0
	0.125	75 ± 1.5 ^c	46 ± 2.8 ^{efg}	51 ± 2.6 ^c	61 ± 2.0 ^{efg}	0 ^b	0
	0.0625	47 ± 4.0 ^c	25 ± 3.1 ^{hij}	29 ± 1.6 ^d	37 ± 1.8 ^{hi}	0 ^b	0
Dichlofluanid	0.05	0 ^f	90 ± 2.8 ^a	0 ^e	0 ^k	0 ^b	0

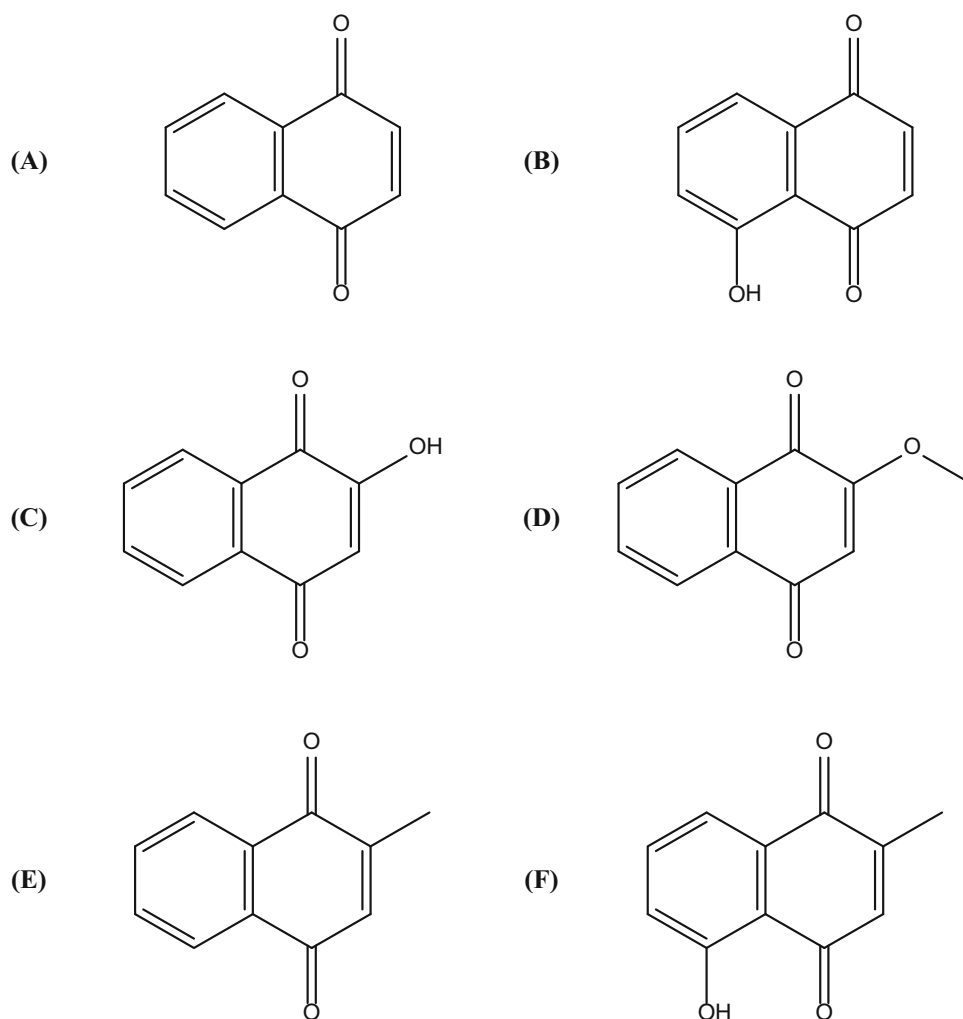
¹See Table 2²Means within a column followed by same letters are not significantly different ($P = 0.05$, Scheffe's test)

(3H, d). ¹³C-NMR (150 MHz, CDCl₃): δ 190.0, 184.6, 161.0, 149.5, 135.9, 135.3, 131.9, 124.0, 119.2, 115.0, 16.5. EI-MS (70 eV) m/z (% relative intensity): M⁺ 188 (100, base peak), 173 (18), 160 (19), 131 (16), 120 (12), 92 (9), 63 (4). The spectroscopic properties of plumbagin were compared with those of previous studies [11, 16].

To measure the fungicidal activity of plumbagin isolated from *D. kaki* roots, its fungicidal activities against six phytopathogenic fungi at 0.5, 0.25, 0.125, and 0.0625 g/L were measured in vivo and compared with that of dichlofluanid, which served as a positive control (Table 3). At 0.5 and 0.25 m/L, plumbagin exhibited strong (80–100%) and moderate (60–80%) fungicidal effects against *E. graminis*, *B. cinerea*, *P. grisea*, and *R. solani*, but not against *P. infestans* and *P. recondite*. At 0.125 and 0.0625 g/L, plumbagin showed moderate and weak (< 60%) fungicidal effects against *E. graminis*, *B. cinerea*, *P. grisea*, and *R. solani*. Strong fungicidal effects were

observed with dichlofluanid against *B. cinerea* at 0.05 g/L, whereas no fungicidal effect was produced by dichlofluanid against *E. graminis*, *P. grisea*, *P. infestans*, *P. recondite*, or *R. solani*. The in vivo trials in this study showed that plumbagin exhibited consistent fungicidal effects against *E. graminis*, *B. cinerea*, *P. grisea*, and *R. solani*. Our results agreed well with those reported in previous studies. Indeed, Shin et al. [16] found that the methanol extract of *Nepenthes ventricosa* leaves inhibited the growth of eight plant pathogenic fungi (*Aspergillus niger*, *Alternaria alternata*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Bipolaris oryzae*, *Rhizoctonia solani*, *Phytophthora capsici*, and *Rhizopus stolonifera*). According to these authors, the antifungal effect of *N. ventricosa* extract resulted from the presence of plumbagin. Furthermore, Dzoyem et al. [17] studied the antifungal effect of plumbagin against five yeast fungi (*Candida tropicalis*, *Candida glabrata*, *Candida albicans*, *Candida neoformans*, and *Candida krusei*)

Fig. 1 Structures of plumbagin and its analogues. (A) 1,4-Naphthalenedione (*p*-naphthoquinone); (B) 5-Hydroxy-1,4-naphthalenedione (juglone); (C) 2-Hydroxy-1,4-naphthalenedione (lawsone); (D) 2-Methoxy-1,4-naphthalenedione (2-methoxy-*p*-naphthoquinone); (E) 2-Methyl-1,4-naphthalenedione (menadione); (F) 5-Hydroxy-2-methyl-1,4-naphthalenedione (plumbagin)



and seven filamentous fungi (*Aspergillus flavus*, *Aspergillus niger*, *Cladosporium* sp., *Alternaria* sp., *Fusarium* sp., *Penicillium* sp., and *Geotrichum candidum*). These authors suggested that plumbagin might be a better fungicidal agent than ketoconazole, which was used as the standard fungicidal agent.

To establish the structure–activity relationships between plumbagin analogues and fungicidal effects against phytopathogenic fungi, *p*-naphthoquinone, juglone, lawsone, 2-methoxy-*p*-naphthoquinone, and menadione were selected as plumbagin analogues for testing (Fig. 1). The fungicidal effects of analogues structurally related to plumbagin, and how toxicity varies with structure was examined (Table 3). In a test with *E. graminis*, strong fungicidal effects (100, 90%, respectively) were produced by menadione at 0.5 and 0.25 g/L. Moderate or weak fungicidal effect was obtained from menadione at 0.125 and 0.0625 g/L. However, no activity was observed for *p*-naphthalenedione, juglone, lawsone, or 2-methoxy-*p*-naphthoquinone when treated at 0.5, 0.25, 0.125, and

0.0625 g/L. With *B. cinerea*, control values over 80% were obtained with *p*-naphthoquinone, juglone, lawsone, 2-methoxy-*p*-naphthoquinone, and menadione at 0.5 g/L. Moderate or weak fungicidal effect was obtained in *p*-naphthoquinone, juglone, lawsone, 2-methoxy-*p*-naphthoquinone, and menadione at 0.25, 0.125, and 0.0625 g/L. In a test with *P. grisea*, juglone and lawsone revealed a strong fungicidal effect (95, 90%, respectively) at 0.5 g/L, whereas a moderate or weak fungicidal effect was observed with juglone and lawsone at 0.25, 0.125, and 0.0625 g/L. The other analogues showed no antifungal effects. With *R. solani*, control values over 85% were obtained with *p*-naphthoquinone, juglone, lawsone, 2-methoxy-*p*-naphthoquinone, and menadione at 0.5 g/L. A moderate or weak fungicidal effect was obtained in *p*-naphthoquinone, juglone, lawsone, 2-methoxy-*p*-naphthoquinone, and menadione at 0.25, 0.125, and 0.0625 g/L. In a test with *P. infestans*, 2-methoxy-*p*-naphthoquinone showed a weak effect at 0.5 g/L, and the other plumbagin analogues showed no fungicidal effect. In a test with *P. recondita*, no

fungicidal effect was obtained with *p*-naphthoquinone, juglone, lawsone, 2-methoxy-*p*-naphthoquinone, or menadione at 0.5, 0.25, 0.125, and 0.0625%.

In our study, the fungicidal effects of plumbagin and its analogues against phytopathogenic fungi were affected by the position and number of functional groups as well as by the various types of functional groups in the chemical structure. Jeon et al. [18] suggested that the insecticidal effects of plumbagin that contains the hydroxyl and methyl functional groups on *p*-naphthoquinone were greater against two species of planthoppers than that of menadione containing the methyl functional group on *p*-naphthoquinone, *Nilaparvata lugens*, and *Laodelphax striatellus*. Furthermore, Park et al. [19] reported that the antimicrobial effects of 2,6-dimethoxy-1,4-benzoquinone analogues against the *Bacillus cereus*, *Salmonella enterica*, and *S. typhimurium* are related to the different functional groups on the 1,4-benzoquinone. That the plumbagin analogues lack fungicidal effects may be connected to the lack of hydroxyl or methyl functional groups. In this regard, plumbagin containing hydroxyl and methyl functional groups at positions 5'- and 2'- exhibited broad fungicidal effects against phytopathogenic fungi. In conclusion, plumbagin and its analogues may be used as natural fungicides to control phytopathogenic fungi that cause plant disease.

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