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Antifungal Property of Dihydroxyanthraquinones Against Phytopathogenic Fungi

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Abstract Fungicidal activities of *Cassia obtusifolia* extracts and their active principles were tested against *Botrytis cinerea*, *Erysiphe graminis*, *Phytophthora infestans*, *Puccinia recondita*, *Pyricularia grisea*, and *Rhizoctonia solani*, and compared with synthetic fungicides and two dihydroxyanthraquinones. At 1 g/l, the chloroform fraction of *C. obtusifolia* extracts showed fungicidal activity against *B. cinerea*, *E. graminis*, *P. infestans*, and *Py. grisea*, and the ethyl acetate fraction showed fungicidal activity against *E. graminis* and *P. infestans*. Danthrone was chromatographically isolated from the chloroform fraction and showed fungicidal activity against *B. cinerea*, *E. graminis*, *P. infestans*, and *Py. grisea* with 68, 100, 78, and 91% control values at 0.5 g/l, respectively. Specifically, alizarin and quinizarin inhibited *E. graminis*, *P. infestans*, and *Py. Grisea*, but did not inhibit the growth of *P. recondita* and *R. solani*. These results indicate at least one of the fungicidal actions of danthrone.

Key words: *Botrytis cinerea*, *Cassia obtusifolia*, danthrone, *Erysiphe graminis*, fungicidal activity, phytopathogenic fungi, *Phytophthora infestans*, *Puccinia recondita*, *Pyricularia grisea*, *Rhizoctonia solani*

Plant diseases are estimated to reduce yields worldwide by almost 20% in major food and cash crops [2, 4]. Synthetic fungicides have effectively controlled plant diseases for a number of years, but increasing concern over environmental effects of some of the fungicides has highlighted the need for the development of alternative types of selective control or of methods to protect crops with or without reduced use of conventional fungicides [4]. Research into plant-derived fungicides is now being intensified, as it becomes evident that plant-derived fungicides have enormous potential to influence modern agrochemical research [4].

Plant extracts may be an alternative to currently used fungicides to control phytopathogenic fungi, because they constitute a rich source of bioactive chemicals [5, 10, 15, 16, 18, 20]. Since these are often active against a limited number of specific target species, biodegradable to nontoxic products, and are potentially suitable for use in integrated management programs, they appear to promise development of new classes of ecologically safer disease control agents [5, 10, 18, 20, 21]. Therefore, efforts have been focused on secondary plant metabolites as lead compounds for potentially useful products [5, 3]. We earlier reported and confirmed that the extract of *Cassia obtusifolia* exhibits potent fungicidal activity against *Pyricularia grisea*, *Botrytis cinerea*, *Phytophthora infestans*, and *Erysiphe graminis* [18]. The *Cassia* species are not only important as a source of natural fungicides, but are also considered to possess biological properties, such as an antiseptic, antidiarrheal, antioxidant, and antimutagen [6, 14]. However, relatively little work has been done on management of fungi or their damage by using the *Cassia* species, in spite of their excellent biological actions. In this study, we assessed the fungicidal activities of *C. obtusifolia* seed-derived materials, the synthetic fungicides, and two dihydroxyanthraquinones against six phytopathogenic fungi.

Chemicals

Alizarin (1,2-dihydroxyanthraquinone), danthrone (1,8-dihydroxyanthraquinone), quinizarin (1,4-dihydroxy-anthraquinone), and Triton X-100 were purchased from Fluka Chemical Corp. (Milwaukee, WI, U.S.A.), anthraquinone, anthraquinone-2-sulfonic, 1-hydroxyanthraquinone, and 2-(hydroxymethyl)anthraquinone were from Sigma Chemical (St. Louis, MO, U.S.A.), and chlorothalonil ($C_8Cl_4N_2$, MW 265.90) and dichlofluanid ($C_9H_{11}Cl_2FN_2O_2S_2$, MW 333.24)

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were from Dongbu HanNong Chemical Co. All other chemicals were of reagent grade.

Extraction and Isolation

C. obtusifolia seeds (5.1 kg) were ground in a blender, extracted twice with methanol (10 l) at room temperature for 2 days, and filtered. The combined filtrate was then concentrated *in vacuo* at 45°C to yield 11.8%. Next, the extract (20 g) was sequentially partitioned into hexane (1.8 g), chloroform (4.3 g), ethyl acetate (2.2 g), butanol (3.3 g), and water-soluble (8.4 g) portions. The organic solvent portions were concentrated to dryness *in vacuo* at 45°C; the water portion was freeze-dried.

Because it exhibited good fungicidal activity against *B. cinerea*, *E. graminis*, *P. infestans*, and *Py. grisea*, the chloroform (13 g) portion was chromatographed on a silica gel column (Merck 70–230 mesh, 900 g, 6.0 i.d.×90 cm), and successively eluted with a stepwise gradient of chloroform/methanol (100:0, 90:10, 80:20, 70:30, and 60:40, v/v). The active 10% (chloroform/methanol, 90:10, v/v) fraction (5.6 g) was chromatographed on a silica gel column and eluted with chloroform/methanol (25:1). The column fractions were analyzed by TLC (chloroform/methanol, 20:1), and fractions with similar TLC patterns were combined. The combined fractions (2.8 g) showed fungicidal activities against *B. cinerea*, *E. graminis*, *P. infestans*, and *Py. grisea*, and they were successively chromatographed over a Sephadex LH-20 column (Pharmacia, 800×49 mm) using chloroform/acetone/methanol (50:2:1). This operation was repeated three times. The active fraction (895 mg) was chromatographed through a Polyclar AT column (Touzart and Matignon, 100 g) packed with chloroform/acetone (20:1), and the column was eluted with an increasing ratio of methanol (1, 2, 5, and 10%). The active fraction (404 mg) was finally purified on a Sephadex LH-20 column (Pharamacia) and cellulose (Merck) eluted with chloroform/methanol (3:7). Finally, 97 mg of the active compound was isolated. The structural determination of the active isolates was made by spectral analyses: The ^{1}H and ^{13}C NMR spectra were recorded using a Bruker AM-500 spectrometer, the UV spectra using a Waters 490 spectrometer, the IR spectra using a Biorad FT-80 spectrophotometer, and the mass spectra using a JEOL JMS-DX 30 spectrometer.

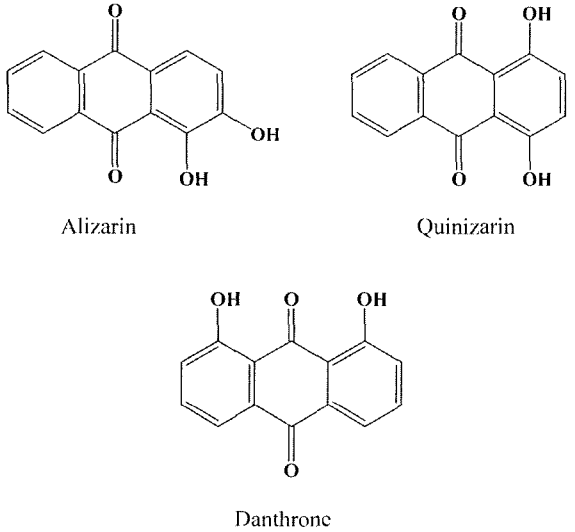

Fig. 1. Structure of dihydroxyanthraquinones tested.

Fungicidal Activity

The six plant diseases evaluated were rice blast, rice sheath blight, cucumber gray mold, tomato late blight, wheat leaf rust, and barley powdery mildew caused by *Py. grisea*, *R. solani*, *B. cinerea*, *P. infestans*, *P. recondita*, and *E. graminis*, respectively [18]. Except for *P. recondita* and *E. graminis*, the others were routinely maintained on potato dextrose agar (PDA) slants and V-8 agar slants, and kept at 4°C.

The fungicidal activities of test samples were determined by a whole plant method in a greenhouse, as previously described [5, 18, 19]. The initial concentration of the test solution was 2 g/l, in which over 60% of the compounds showed fungicidal activity against 6 test fungi. Further tests employed a dilution sequence of 1, 0.5, 0.25, and 0.125 g/l. To prepare test solutions at 2 g/l, 100 mg of the test sample was dissolved in 0.5 ml of dimethyl sulfoxide, followed by dilution with 49.5 ml of water containing Tween 20 (250 μg/ml). Fifty ml of each test sample solution were sprayed simultaneously onto two pots on a turntable. The treated plants were kept in a greenhouse for 1 day before being inoculated by each pathogen. Controls were sprayed with the Tween 20 solution. The condition tested on each disease is described in Table 1, as described by Kim *et al.* [5]. All tests were carried out in triplicate. The control effect of test samples on each disease was evaluated with control value (CV) calculated by the formula CV (%)=[(A-B)/A]×100, where A and B represent the disease area on the untreated and treated plants, respectively. LC_{50} values were calculated by probit analysis [8].

Statistical Analysis

Analysis of variance was performed using the PROC GLM procedure (SAS institute, Cary, NC, U.S.A.). If P was less than 0.01, means were separated with the least significant difference (LSD) test at the P=0.05 level.

In preliminary experiments, we observed that 2 g/l methanolic extract of *C. obtusifolia* seeds possessed significant fungicidal activities (>90% control value) against *E. graminis*, *Py. grisea*, *P. infestans*, and *B. cinerea* with 70% control value. Further solvent fractionation showed 90, 92, 60, and 73% control values in the chloroform fraction against *Py. grisea*, *E. graminis*, *B. cinerea*, and *P. infestans* at 1 g/l, respectively (Table 2). The ethyl acetate fraction had 47

Table 1. Test condition of phytopathogenic fungi in a greenhouse.

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

[a]Chamber temperature kept for treated and control plants.
[b]Period (days) from inoculation of pathogen to evaluation of disease severity on host plants.
[c]Inoculum of *Rhizoctonia 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.
[d]Preparation 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 [5].

and 58% control values against *P. infestans* and *E. graminis*, respectively, and the hexane fraction had 42% control values against *B. cinerea* at 1 g/l. However, little fungicidal activity was observed by the butanol and water fractions (not shown). One active isolate from the chloroform fraction showed strong fungicidal activities against *B. cinerea*, *E. graminis*, *Py. grisea*, and *P. infestans* (Table 3), and it was characterized by spectroscopic analyses as danthrone: The compound was identified on the basis of the following evidence. Orange needles; mp 193- 197°C; EI-MS (70 eV) *m/z* (% relative intensity): M^+ 240 (100), 212 (71), 184 (57), 155 (16), 138 (24), 128 (18), 92 (24), 63 (12), 51(7); ^{1}H-NMR (CD_3COCD_3, 400 MHz); δ 7.81 (*m*, 4H), 7.37 (*d*, 2H, *J*=8 Hz); ^{13}C-NMR (CD_3OD, 100 MHz): 182.1, 163.3, 138.4, 134.6, 125.1, 125.0, 120.4, 116.8. Elemental analysis calculated for $C_{14}H_8O_4$ (MW, 240): C, 70.00; H, 3.36; O, 26.64. Found: C, 69.96; H, 3.29; O, 26.49. The spectroscopic analyses of danthrone from *C. obtusifolia* seeds were identical with the data of danthrone isolated from *C. obtusifolia* roots [14].

The fungicidal activities of various concentrations of danthrone against six phytopathogenic fungi were determined (Table 3). Danthrone showed fungicidal activities against *B. cinerea*, *E. graminis*, *Py. grisea*, and *P. infestans in vivo*, except for wheat leaf rust and rice sheath blight. This study is the first to show the fungicidal function of the component isolated from *C. obtusifolia* seeds against *B. cinerea*, *P. infestans*, *P. recondita*, *Py. grisea*, and *R. solani*. On the basis against *B. cinerea*, *E. graminis*, *P. infestans*, and *Py. grisea*, danthrone was about 2.1- 4.2 times more toxic than

Table 2. Antifungal activities of *C. obtusifolia* seed-derived materials against phytopathogenic fungi[a].

Material	Conc. (g/l)	Control Values[b] (%)					
		RCB	RSB	CGM	TLB	WLR	BPM
Methanol extract	2	100	0	70	90	0	100
	1	87	0	56	73	0	88
Hexane fraction	2	0	0	65	0	0	0
	1	0	0	42	0	0	0
Chloroform fraction	2	100	0	72	92	0	100
	1	90	0	60	73	0	92
Ethyl acetate fraction	2	0	0	0	72	0	89
	1	0	0	0	47	0	58
LSD[c] (0.05)		9.3	-	4.1	8.6	-	9.8

[a]Activity is preventive value (%), 100% complete killing, 0% zero killing.
[b]RCB, rice blast caused by *Py. grisea* on rice; RSB, rice sheath blight caused by *R. solani* on rice; CGM, cucumber gray mold caused by *B. cinerea* on cucumber; TLB, tomato late blight caused by *P. infestans* on tomato; WLR, wheat leaf rust caused by *P. recondita* on wheat; BPM, barley powdery mildew caused by *E. graminis* on barley.
[c]LSD, least significant difference.

the chloroform fraction. In a previous study, the Hazardous Substances Data Bank (HSDB) indicated that danthrone is only used as a fungicide for control of barley powdery mildew (*E. graminis*) [12]. Our data for the control of powdery mildew are identical to the data of HSDB. Danthrone has been widely used since the beginning of this century as a laxative [13]. In 1987, the FDA ordered its withdrawal from the market for its use as a laxative [7] and U.S. manufacturers' production of all human drug products containing the compound [13]. It is currently used as an antioxidant in synthetic lubricants and in the synthesis of experimental antitumor agents [12]. Nevertheless, *C. obtusifolia* is a well known traditional Chinese medicinal plant. The seeds of the plant, called Juemingzi in Chinese, have been widely used in traditional Chinese medicine for the treatments of red and tearing eyes, headache, and dizziness [9]. It is also used as an essential ingredient in medicine as an antiseptic, antidiarrheal, and antimutagen [6, 14]. Among the components of *C. obtusifolia*, anthraquinones constitute a major group of secondary metabolites [9]. Aloe-emodin, chrysophanol, physcion, and rhein derived from *Rheum emodi* have been reported to exhibit antifungal activity against *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton mentagrophytes*, and *Aspergillus fumigatus* [1]. Furthermore, anthraquinone, anthraflavine, anthrarufin, and quinizarin strongly inhibit *Clostridium perfringens* and *Staphylococcus aureus* as well as microsomal conversion of aflatoxin B_1 [17].

To examine the fungicidal activity of anthraquinones, alizarin, anthraquinone, anthraquinone-2-sulfonic, 1-hydroxyanthraquinone, 2-(hydroxymethyl)anthraquinone, and quinizarin were tested against six phytopathogenic fungi (Table 3). Alizarin had 96, 68, and 93% control values at 0.5 g/l and 75, 55, and 79% control values at 0.25 g/l against *Py. grisea*, *P. infestans*, and *E. graminis*, respectively. On the basis of fungicidal activities of alizarin against three phytopathogenic fungi, alizarin was more toxic against *Py. grisea* and *E. graminis* than *P. infestans*. Furthermore, quinizarin had 91, 68, 78, and 100% control values at 0.5 g/l and 72, 41, 64, and 88% control values at

Table 3. Antifungal activities of danthrone isolated from *C. obtusifolia* seeds, two commercially available dihydroxyanthraquinones, and synthetic fungicides against phytopathogenic fungi[a].

Material	Conc. (g/l)	Control values[b] (%)					
		RCB	RSB	CGM	TLB	WLR	BPM
Alizarin	1	100	0	31	86	0	100
	0.5	96	0	17	68	0	93
	0.25	75	0	0	55	0	79
	0.125	59	0	0	38	0	60
	0.0625	41	0	0	17	0	39
	LC_{50} (g/l)	0.094	-	-	0.219	-	0.094
Quinizarin	1	100	0	64	90	0	100
	0.5	90	0	39	72	0	89
	0.25	77	0	18	60	0	75
	0.125	62	0	0	42	0	58
	0.0625	45	0	0	26	0	34
	LC_{50} (g/l)	0.080	-	0.688	0.172	-	0.101
Danthrone	1	100	0	87	91	0	100
	0.5	91	0	68	78	0	100
	0.25	72	0	41	64	0	88
	0.125	55	0	23	47	0	77
	0.0625	35	0	12	29	0	49
	LC_{50} (g/l)	0.109	-	0.323	0.146	-	0.064
Chlorothalonil[c]	0.05	0	0	0	94	0	0
Dichlofluanid	0.05	0	0	91	0	0	0
LSD[d] (0.05)		9.8	-	8.7	9.4	-	10.5

[a]Activity is preventive value (%), 100% complete killing, 0% zero killing.

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

[c]Commercial name.

[d]LSD, least significant difference.

0.25 g/l against *Py. grisea*, *B. cinerea*, *P. infestans*, and *E. graminis*, respectively. The fungicidal activities of quinizarin were stronger against *E. graminis* and *Py. grisea* than *B. cinerea* and *P. infestans*. However, little or no activity was observed with anthraquinone, anthraquinone-2-sulfonic, 1-hydroxyanthraquinone, and 2-(hydroxymethyl)anthraquinone, when 1 g/l was used against six phytopathogenic fungi (not shown). In this regard, it is still unclear which of two hydroxyl groups in one or both aromatic rings of dihydroxyanthraquinones was responsible for inhibiting the growth of *E. graminis*, *Py. grisea*, and *P. infestans*, and further work is necessary to clarify this point. Due to the fungicidal activity of dihydroxyanthraquinones against *B. cinerea*, *E. graminis*, *Py. grisea*, and *P. infestans*, these principles were compared with synthetic fungicides such as chlorothalonil and dichlofluanid which have fungicidal activity against *P. infestans* or *B. cinerea* (Table 3). Potent fungicidal activity was observed with chlorothalonil against *P. infestans* and dichlofluanid against *B. cinerea* at 0.05 g/l, whereas no fungicidal activity was observed by chlorothalonil against *B. cineria*, *Py. grisea*, *R. solani*, and *P. recondita* and dichlofluanid against *Py. grisea*, *P. infestans*, *P. recondita*, and *R. solani*. In this study, although the fungicidal activity of danthrone was found to be much less than synthetic fungicides used, danthrone may be useful as a lead compound to control *B. cinerea*, *E. graminis*, *Py. grisea*, and *P. infestans* in crops. Certain plant materials and phytochemicals act on various types of disease-causing agents in many ways, and may be applied to the crop in the same way as other agricultural chemicals. They are being considered as potential alternatives for synthetic fungicides or lead compounds for new classes of synthetic fungicides [10, 11, 19, 21].

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