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ALLELOPATHIC POTENTIAL OF *Phormidium angustissimum*

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ABSTRACT

In a laboratory bioassay, the allelopathic effects of Phormidium angustissimum were investigated on seed germination, shoot length and root length of Chinese amaranth (Amaranthus tricolor L.) and barnyard grass (Echinochloa crus-galli [L] P. Beauv.). The seeds were germinated in Petri dishes with aqueous extract (1.25 to 10% concentrations) and crude organic extracts (500 to 4000 ppm concentrations) with distilled water used as control. The inhibitory effect on these test plants was directly proportional to the increasing concentration of aqueous extracts of P. angustissimum. At the highest concentration studied, the aqueous extract completely inhibited the germination of Chinese amaranth. In barnyard grass, the aqueous extract had a moderate effect on seed germination and seedling growth. To investigate the allelochemicals, P. angustissimum was extracted with organic solvents, hexane, ethyl acetate and methanol. The crude ethyl acetate was most inhibitory to Chinese amaranth, but all three crude organic extracts had low inhibitory effect on barnyard grass. Bioactivity guided extraction and chromatographic techniques were used to isolate and purify the allelochemicals from the crude ethyl acetate extract of P. angustissimum. The pure compounds were studied by extensive GC-MS and NMR spectroscopic methods in order to determine their structures. The crude ethyl acetate extract of P. angustissimum yielded C_{11} norisoprenoid, dihydroactinidiolide and a mixture of long chain fatty acids. Dihydroactinidiolide displayed the highest inhibitory activity against seed germination and seedling growth of Chinese amaranth and barnyard grass.

Keywords: *Phormidium angustissimum*, allelopathic effects, aqueous extract, crude organic extract, *Amaranthus tricolor*, *Echinochloa crus-galli*

INTRODUCTION

Plants are known to produce secondary metabolites that affect germination and the growth of other plants. Allelopathy is commonly

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defined as any direct or indirect, stimulatory or inhibitory, influence on plants due to chemicals released into the environment by plants (Rice, 1984). The chemical exudates from allelopathic plants play a major role in the allelopathy mode of action. These chemicals, called allelochemicals are natural products or phytogrowth inhibitors that help to regulate the structure of plant communities (Smith & Martin, 1994). Evidence shows that higher plants release various allelochemicals into the environment, which includes phenolics, alkaloids, long-chain fatty acids, terpenoids, and flavonoids (Rice, 1984; Chou, 1995). Allelochemic natural products offer excellent opportunities for new herbicidal solutions or lead compounds for new herbicides (Duke *et al*., 2000; Vyvyan, 2002). Allelopathic phenomena are well recognized in the terrestrial plant kingdom, but very little is known about algae. Microalgae such as cyanobacteria (blue-green algae) from marine and freshwater habitats are known to produce a diverse array of toxic or bioactive metabolites (Cohen, 1999; Kreitlow *et al*., 1999; Legrand *et al*., 2003; Charoenying *et al*., 2010). A limited number of studies suggest that some of the compounds may have ecological roles as allelochemicals. *Phormidium angustissimum* is a photosynthetic cyanobacterium with known biological activity. Several studies have shown that cyanobacterias in the genus *Phormidium* produce a wide array of biologically active constituents with different bioactivities (Rodríguez-Meizoso *et al*., 2008). The objectives of this research were to determine the allelopathic potential of *P. angustissimum* and to isolate and identify the allelopathic constituents.

MATERIALS AND METHODS

Algal Culture and Test Plants

Dried *P. angustissimum* was obtained from the Division of Animal Production Technology and Fisheries, King Mongkut's Institute of Technology Ladkrabang, Bangkok. Algal cultures were grown photoautotrophically in a BG11 medium (Vonshak and Maske 1982). Cultures were bubbled with air, at 25°C, and the cultivar flasks were illuminated under 400 $\mu molm^{-2}s^{-1}$ light intensity. Biomass was harvested by filtration on a 40 μm sieve shaker, dried in an oven at 50°C, and stored in an inert atmosphere until extraction. The seeds of Chinese amaranth were purchased from Thai Seed & Agriculture Co., Ltd., Bangkok, and barnyard grass seeds were collected (in August 2009) from paddy fields in the Minburi district of Bangkok, Thailand. Empty and undeveloped seeds were discarded by floating them in tap water. The remaining seeds were air-dried and hermetically stored at room temperature (28-32°C). In germination tests, germination activity of these seeds was randomly checked and found to be > 80%.

Effects of the Aqueous Extracts on Seed Germination and Growth

This experiment was conducted in order to determine the water-soluble phytotoxic constituents in *P. angustissimum*. First, 10 g of dried algae were soaked in a 125 mL Erlenmeyer flask containing 90 mL distilled water, and agitated for 24 hours on a shake at room temperature. Next, the extract was strained through two layers of cheese cloth and then through a Whatman No. 1 filter paper. Then, the extract was refrigerated at 4^{o}C until use. Four concentrations of the aqueous extract were used in the experiment: 10, 5, 2.5 1.25% w/v. Five mL of each concentration was added to each Petri dishes (9 cm diameter) containing germination paper, and then 20 seeds of test plant were placed on the germination paper as per treatments. The control received only distilled water. The treatments were replicated four times in a completely randomized design. All Petri dishes were covered and placed at room temperature (32^{o}C day and 28^{o}C night). After seven days, germination percentage, shoot and root length were recorded in all treatments. Inhibition percentage relative to control, was calculated as:

Inhibition (% of control) = [1-(sample extracts/control)] x 100

Effects of Crude Organic Extracts

The dried *P. angustissimum* (300g) was extracted by hexane-treatment for seven days at room temperature. The extract was then filtered through a Whatman No. 1 paper. The collected filtrate was evaporated to dryness under reducing pressure at 40^{o}C using a rotary evaporator to yield the crude hexane extract. The residue was then similarly extracted with ethyl acetate (EtOAc) and methanol (MeOH) to yield crude ethyl acetate and methanol extracts, respectively. The three dried samples concentrated from hexane, ethyl acetate and methanol were again dissolved in each solvent to compare their inhibitory effects. Next, 500 μL of each crude extract solution (5000, 10000, 20000 and 40000 ppm concenrtrations) were added to Petri dishes (9 cm diameter) lined with germination paper, the solutions were allowed to evaporate for three hours at room temperature. After evaporation, 5 mL of distilled water was added on the germination paper to obtain 500, 1000, 2000 and 4000 ppm concentrations. Then, 20 seeds of each test plant were placed on the treated germination paper for seven days. All germination tests were conducted under similar conditions as described above.

Isolation and Identification of Allelopathic Substances

Each crude extract obtained was submitted for a test of allelopathic activity with Chinese amaranth and barnyard grass. From

this bioassay, the crude ethyl acetate was found to be the most active. Bioassay-guided fractionation of crude ethyl acetate (8 g) was subjected to silica gel column chromatography (Scharlau GE 0048), eluted stepwise with hexane with increasing amounts of ethyl acetate, yielding nine fractions (F_1-F_9) on the basis of TLC (Thin Layer Chromatography) analysis. These fractions were again evaluated in the allelopathic bioassay. The most interesting active principal was found in the fractions obtained by elution with 5% ethyl acetate in hexane (F_6). The semipure active fraction, F_6 (220 mg) was further purified on a silica column chromatography (MERCK silica gel 60) using hexane:ethyl acetate (95:5) as the eluting solvent, which resulted in the isolation of dihydroactinidiolide as the major active compound. The structure of this active principal was determined by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and by comparison of the spectral data with the data reported in the literature.

Analysis of Active Compounds

Analytical TLC was performed on a MERCK silica gel 60 F_{254} aluminum sheet. Spots were visualized by UV light (254 and 366 nm) or by spraying with anisaldehyde reagent. The plates were then heated for five minutes at 110°C. Column chromatography was performed on Scharlau GE 0048 silica gel 60, 0.02-0.06 mm. Flash column chromatography was performed on MERCK silica gel (<0.063 mm). Nuclear magnetic resonance (^{1}H, ^{13}C and distortionless enhancement by polarization transfer) spectra were recorded at 300 and 75.5 MHz, respectively, on a spectrometer (Bruker Avance 300 DPX) in deuterated chloroform using tetramethylsilane as an internal reference. The chemical composition of the fatty acids in crude ethyl acetate was analyzed by a gas chromatography-mass spectrometer (GC:Agilent Technologies Model 6890N, MS:Agilent Technologies Model 7683) using a DB-Wax column. The fractions were converted to fatty acid methyl esters by Yayli's (2001) method: the detector was FID. Each peak was identified by comparing the peak retention times with those of the authentic fatty acid methyl esters samples.

Seed Germination and Seedling Growth Bioassay

Seeds of Chinese amaranth and barnyard grass were used to assess the effects of dihydroactinidiolide. A stock solution of dihydroactinidiolide was prepared in ethyl acetate as the initial solvent. Next, 50 μL of solution at 10000, 5000, 2500, 1250 and 625 ppm concentrations was added in a vial (4.5 X 2 cm) lined with germination paper and evaporated to dryness for 3 hours at room temperature. After evaporation, 0.5 mL of distilled water was added to the germination paper to yield the final concentrations at 1000, 500, 250, 125 and 62.5 ppm, and then 10 seeds per species were placed on the germination paper. The vials were sealed with Parafilm to prevent loss

of moisture and maintained at room temperature. All germination tests were conducted under similar conditions as described above.

Statistical Analysis

Differences in the percentages of seed germination and root and shoot length were assessed by analysis of variance statistical methods. Comparisons between treatments were made at a 0.05 probability level using Duncan's Multiple Range Test (DMRT).

RESULTS

Effect of the Aqueous Extracts on Seed Germination and Growth

As shown in Table-1, the 1.25% and 2.5% concentrations had no allelopathic effects on seed germination and shoot length of Chinese amaranth. The 5% concentration had a moderate effect on the seed germination but a high inhibitory effect on seedling growth. Moreover, the highest applied concentration (10%) completely inhibited the germination and seedling growth of Chinese amaranth. In barnyard grass, the 1.25% and 2.5% aqueous extract did not influence the germination and shoot length but remarkably stimulated the root length. The 5% concentration significantly reduced the seed germination and seedling growth, while the highest concentration (10%) had a moderate effect on the seed germination and shoot length and completely inhibited the root length. Thus, the potent inhibitory activity of *P. angustissimum* extracts on seed germination and seedling growth of test plants depended on the extract concentration.

Table-1. Allelopathic effects of aqueous extract of *P. angustissimum* on seed germination and seedling growth of Chinese amaranth and barnyard grass.

Concentrations (% W/V)	Chinese amaranth (% Inhibition)			Barnyard grass (% Inhibition)		
	Seed germination	Shoot length	Root length	Seed germination	Shoot length	Root length
Control	0c	0c	0e	0c	0c	0c
1.25	0c	-6.92d	4.02d	0c	0.11c	-5.03e
2.5	0c	1.65c	51.75c	2.5c	0c	-0.41d
5	77.5b	90.23b	95.12b	12.5b	18.65b	2.51b
10	100a	100a	100a	35a	77.28a	100a

Mean values in each column followed by the same letter are not significantly different at *P*=0.05 according to the Duncan's multiple range test.

Effects of Crude Organic Extracts

The effects of the crude organic extracts of *P. angustissimum* on Chinese amaranth became apparent when applied at high

concentrations (Table-2). The crude hexane and ethyl acetate extracts at 4000 ppm completely inhibited the germination and seedling growth of Chinese amaranth, while crude methanol highly inhibited the germination and seedling growth. In barnyard grass, crude hexane and methanol extracts at 4000 ppm concentration slightly inhibited seed germination. All concentrations of crude hexane and methanol stimulated the root length of barnyard grass. The crude ethyl acetate at 2000 to 4000 ppm concentrations reduced seed germination and seedling growth.

Isolation and Identification of Allelopathic Substances

All crude extracts obtained from solvent extraction were evaluated for their allelopathic potential (Table-2). The most noticeable inhibition was observed in the bioassay of crude ethyl acetate extract, which showed the highest activity on the germination and seedling growth in both test plants. Successive bioassay directed chromatography of the ethyl acetate extract on the silica gel column gave nine main fractions (F_1 to F_9), among which F_6 was found to be the most inhibitory at 1000 ppm (data not shown). Repeated column chromatography and preparative TLC led to the isolation of a plant growth inhibitor identified as a norisoprenoid compound, dihydroactinidiolide (9.6 mg). The structure was assigned using NMR spectroscopic techniques (Fig. 1.).

Table 2. Allelopathic effects of crude organic extracts of *P. angustissimum* on seed germination and seedling growth of Chinese amaranth and barnyard grass.

Concentrations (ppm)	Chinese amaranth (% Inhibition)			Barnyard grass (% Inhibition)		
	Seed germination	Shoot length	Root length	Seed germination	Shoot length	Root length
Control	0f	0ef	0f	0d	0b	0cd
Hexane 500	2.56f	-1.70ef	-20.72g	3.03cd	-1.06bc	-14.26e
Hexane 1000	0f	6.23e	39.62d	0d	-5.80c	-30.59f
Hexane 2000	69.23c	54.67c	55.51c	3.03cd	0.22b	-45.32g
Hexane 4000	100a	100a	100a	15.15bc	0.61b	-41.68g
EtOAc 500	0f	4.82e	-22.05g	0d	-0.33b	-1.90d
EtOAc 1000	12.82e	31.73d	19.58e	0d	2.45b	3.49bc
EtOAc 2000	87.18b	84.14b	93.16b	15.15bc	12.88a	5.55b
EtOAc 4000	100a	100a	100a	30.30a	16.06a	53.57a
MeOH 500	2.56f	-7.93f	-39.92h	0d	0.613b	42.95g
MeOH 1000	2.56f	1.70e	-37.64h	3.03cd	1.17b	-51.1h
MeOH 2000	53.85d	49.01c	54.18c	6.06b-d	0.89b	51.35h
MeOH 4000	94.87a	88.67b	96.20ab	18.18ab	0.06b	-3.65d

Mean values in each column followed by the same letter are not significantly different at *P*=0.05 according to the Duncan's multiple range test.

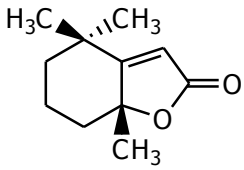

Figure 1. Chemical structure of dihydroactinidiolide.

The ^{1}H NMR (300 MHz, $CDCl_3$) spectrum of the compound showed δ 1.22-1.34 (1H, m, H2-α), 1.62-1.72 (1H, br.m, H-2β), 1.72-1.77 (2H, br.m, H-3), 1.46 (1H, m, H-4α), 2.24 (1H, dq, *J* = 12.47, 2.05 Hz, H-4β), 5.64 (1H, s, H-7) 1.22 (3H, s, H-9), 1.27 (3H, s, H-10) and 1.55 (3H, s, H-11). The ^{13}C NMR (75.5 MHz, $CDCl_3$) spectrum of the compound showed δ 36.50, 41.66, 19.65, 40.09, 87.26, 171.99, 112.38, 182.51, 24.18, 29.83 and 24.3. From the comparison of these data with those reported in the literature (Mori and Khlebnikov 1993; Yao *et al* 1998; Eidman and MacDougall, 2006). The GC-MS analysis of active constituents in other fractions (combination of F_3 and F_4) of crude ethyl acetate extract showed the presence of fatty acids. The results found palmitic acid (46.24%) to be a major constituent, oleic acid, 8-octadecenoic acid, linoleic acid, palmitoleic acid, stearic acid, 7,10-hexadecadienoic acid and 7-hexadecenoic acid, respectively.

Effect of Dihydroactinidiolide on Seed Germination

The inhibitory activity of dihydroactinidiolide on the germination of Chinese amaranth and barnyard grass is shown in Table-3. The compound caused significant inhibition of seed germination on the two species. Germination of Chinese amaranth was inhibited at a concentration of 250 ppm by 54.55%. In particular, dihydroactinidiolide at concentrations of 500 and 1000 ppm exhibited complete inhibition on seed germination of Chinese amaranth. For barnyard grass, the concentrations of 250 and 500 ppm treatments, dihydroactinidiolide inhibited seed germination by 12.12% and 54.55%, respectively. Similar to the effect on barnyard grass, the 1000 ppm concentration completely inhibited the seed germination. It should be noted that dihydroactinidiolide had a greater effect on Chinese amaranth than on barnyard grass. In general, inhibition increased with greater concentrations of dihydroactinidiolide.

Effect of Dihydroactinidiolide on Seedling Growth

Dihydroactinidiolide showed significant shoot growth inhibition on Chinese amaranth (Table-). The 62.5 ppm concentration slightly inhibited the shoot length but stimulated the root length. In concentrations of 125 and 250 ppm, shoot growth was inhibited by 9.35% and 64.03%, respectively and completely inhibited in concentrations of 500 and 1000 ppm. Comparing barnyard grass with

control, 62.5, 125, 250 and 500 ppm treatments significant reduced the shoot growth by 0, 13.02%, 19.23%, 45.82% and 71.65%, respectively. In the 1000 ppm concentration dihydroactinidiolide completely inhibited the shoot length of barnyard grass. This data indicate that dihydroactinidiolide had more shoot inhibition on Chinese amaranth than on barnyard grass. In both plants, significant reduction of root length was observed in the concentration of 62.5 ppm dihydroactinidiolide. In the 500 ppm concentration, Chinese amaranth was more sensitive to dihydroactinidiolide than barnyard grass, completely inhibiting on the root growth of Chinese amaranth.

Table-3. Allelopathic effects of dihydroactinidiolide from *P. angustissimum* on seed germination and seedling growth of Chinese amaranth and barnyard grass.

Concentrations (ppm)	Chinese amaranth (% Inhibition)			Barnyard grass (% Inhibition)		
	Seed germination	Shoot length	Root length	Seed germination	Shoot length	Root length
Control	0d	0d	0d	0d	0d	0d
62.5	0d	3.96a	-0.46a	0d	13.02a	38.20a
125	6.06a	9.35cd	13.36b	3.03d	19.23a	51.26a
250	54.55a	64.03a	66.06a	12.12a	45.82a	75.42a
500	100a	100a	100a	54.55a	71.65a	92.13a
1000	100a	100a	100a	100a	100a	100a

Mean values in each column followed by the same letter are not significantly different at *P*=0.05 according to the Duncan's multiple range test.

DISCUSSION

The aqueous extract of *P. angustissimum* showed allelopathic potential in terms of seed germination and seedling growth inhibition. Seed germination, shoot length and root length of test plants decreased proportionally with the increasing concentration of aqueous extracts. The results showed that root growth was slightly more sensitive than shoot growth to the presence of allelochemicals in aqueous extracts. In this study, we attempt to identify the bioactive substances associated with the toxicity observed in the crude ethyl acetate extract of *P. angustissimum*. Bioassay-guided fractionation, purification, and spectroscopic analysis led to the isolation of dihydroactinidiolide as one of the potential allelochemicals. The allelopathic effects of dihydroactinidiolide isolated from spikerush have been previously reported (Stevens and Merrill 1980). This compound proved toxic at 50 ppm and completely inhibited raddish seed germination after five days. The active constituents in other fractions were identified as fatty acids. It might be possible that the activity observed in our initial experiment with the aqueous extract or crude organic extracts is related to a synergistic combination of

dihydroactinidiolide, fatty acids and some other water soluble chemical constituents, which might also occur in substantial quantities that have not yet been identified. It is also possible that dihydroactinidiolide, fatty acids, or other unidentified components, might influence the overall allelopathic potential of *P. angustissimum* in an additive fashion.

CONCLUSION

The *P. angustissimum* aqueous extract and crude organic extracts had moderate inhibitory activity. The biological activity of extracts increased at higher concentrations. All extracts of *P. angustissimum* were more inhibitory to the root length than shoot length. Using bioassay-guided fractionation, dihydroactinidiolide could be isolated from the ethyl acetate extract of *P. angustissimum* together with fatty acids. Chinese amaranth was more sensitive to the extracts and dihydroactinidiolide than barnyard grass. The results of this study provide evidence that *P. angustissimum* has allelopathic potential.

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REFERENCES CITED

Charoenying, P., P. Chotsaeng and C. Laosinwattana. 2010. Effects of *Spirulina platensis* and C-Phycocyanin on seed germination and seedling growth of two monocot and dicot plants. Allelopathy J. 25(2): 453-460.

Chou, C.H. 1995. Allelopathy and sustainable agriculture. In : Inderjit, Dakshini, K.M.M., Einhrlling, F.A. (eds.). *Allelopathy: Organisms, Process and Applications*. ACS Symposium Series 582. Am. Chem. Soc., Washington, DC. pp. 211-233.

Cohen, Z. 1999. *Chemicals from Microalgae*. Taylor & Francis, London.

Duke, S.O., J.G. Romangi and F.E. Dayan. 2000. Natural products as sources for new mechanisms of herbicidal action. Crop Prot. 19: 583-589.

Eidman, K.F. and B.S. MacDougall. 2006. Synthesis of loliolide, actinidiolide, dihydroactinidiolide and aeginetolide *via* cerium enolate chemistry. J. Org. Chem. 71(5): 9513-9516.

Mori, K. and V. Khlebnikov. 1993. Carotenoids and degrade carotenoids, viii-synthesis of (+)-dihydroactinidiolide, (+)-and (-)-actinidiolide, (+)- and (-)-loliolide as well as (+)- and (-)-

epiloliolide. Justus Liebigs Annalen der Chemie. 1993(1): 77-82.

Kreitlow, S., S. Mundt and U. Lindequist. 1999. Cyanobacteria-a potential source of new biologically active substances. J. Biotechnol. 70: 61-63.

Legrand, C., K. Rengefors, G.O. Fistarol and E. Graneli. 2003. Allelopathy in phytoplankton biochemical, ecological and evalutionary aspects. Phycologia. 42: 406-419.

Rice, E.L. 1984. *Allelopathy*. 2nd Ed. Acadamic Publishers. New York. USA. 424 pp.

Rodríguez-Meizoso, I., L. Jamie, S. Santoyo, A. Cifuentes, R.G. García-Blairsy, F.J. Señoráns and E. Ibáñez. 2008. Pressurized fluid extraction of bioactive compounds from phormidium species. J. Agric. Food Chem. 56(10): 3517-3523.

Smith, A.E. and L.D. Martin. 1994. Allelopathic characteristic of three cool-season grass species in forage ecosystem. Agron. J. 86: 243-246.

Stevens, K.L. and G.B. Merrill. 1980. Growth inhibitors from spikerush. J. Agric. Food Chem. 28(3): 644-646.

Vyvyan. J.R. 2002. Allelochemicals as leads for new herbicides and agrochemicals. Tetrahedron 58: 1631-1636.

Vonshak, A. and H. Maske. 1982. Algae: Growth Techniques and Biomass Production. In: Coombs, J., Hall, D.O. (Eds.), *Techniques in Bioproductivity and Photosynthesis*. Pergamon Press, Oxford, pp. 66-77.

Yao, S., Johannsen. M, Hazell, R.G. and Jørgensen, K.A. (1998). Total synthesis of (*r*)-dihydroactinidiolide and (*r*)- actinidiolide using asymmertic catalytic hetero-diels-alder methodology. J. Org. Chem. 63(1): 118-121.

Yayli, N., Z. Kiran, H. Seymen and H. Genc. 2001. Characterization of lipids and fatty acids methyl ester contents in leaves and roots of *Crocus vallicola*. Turkish J. Chem. 25: 391-395.