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Original Article

Characterization of *Diaporthe* fungal extract composition and phytotoxicity on the aquatic noxious weed *Eichhornia crassipes*: inhibitory effects on photosynthetic machinery and membrane integrity

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Abstract

Fungal extract-based natural herbicides are a promising avenue for the development of sustainable weed management. The study investigates the herbicidal activity of fungal extracts against the prominent aquatic invasive weed *Eichhornia crassipes* (water hyacinth). The ethanol crude fraction derived from *Diaporthe* sp. strain EC010 demonstrated the highest phytotoxicity. Chemical characterization using gas chromatography-mass spectrometry revealed 2(3H)-furanone, dihydro-4-hydroxy- (22.81%), linoelaidic acid (6.87%), and hordenine (6.62%) as major constituents. Phytotoxicity was evaluated by wrapping bioassay under greenhouse conditions. Observable foliar damage, such as necrosis, chlorosis, and depigmentation, occurred within 1 day after treatment (DAT). The rapid onset of visible damage within 1 DAT and the detailed analysis of tissue damage are particularly noteworthy. Lesion progression reflected the phytotoxicity of the extract, increasing to 77.33% visible phytotoxicity at 14 DAT for the highest concentration (8.0% w/v). Microscopic analysis revealed disintegration and complete deformation of epidermal and parenchymal tissues, and treated plants featured extensive aerenchyma spaces. Furthermore, the *Diaporthe* sp. extract decreased chlorophyll a, b, and carotenoid concentrations while increasing electrolyte leakage and malondialdehyde, indicative of weed deterioration. The current work offers valuable insights for sustainable and eco-friendly strategies in the management of water hyacinth populations in a lentic ecosystem. The findings suggest that *Diaporthe* sp. extract could serve as a natural herbicide, offering an environmentally friendly alternative to synthetic chemicals in managing water hyacinth.

Keywords: Allelopathy, fungi extract, natural herbicide, water hyacinth

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Introduction

Aquatic plants that predominantly inhabit lentic ecosystems, termed macrophytes, are key in natural processes, including nutrient cycling and metabolism (Bowden et al., 2017). However, recent years have highlighted problems inherent to aquatic weeds, whose excessive proliferation poses threats to navigation, fish production, and electricity generation and contributes to dissemination of disease vectors (Pereira et al., 2012). One noteworthy example is *Eichhornia crassipes*, commonly known as water hyacinth, which is particularly problematic in tropical climates. Originally hailing from the Amazon region in Brazil, *E. crassipes* now stands as the most extensively disseminated aquatic plant (Ayanda et al., 2020), present mainly in Africa, Europe, North America, and Asia (Hussner et al., 2017). In Thailand, its proliferation in key rivers has escalated to a monthly increase of up to 5.6 million tons (Jirawattanasomkul et al., 2021). The government of Thailand, as reported by the Department of Public Works and Town Planning, has spent millions from its annual budget to enact various management strategies, including incineration, landfilling, and recycling. This approach involves large financial investment, recurrent implementation every 1-2 months, and generates significant environmental waste. Concerning weed management in general, a variety of approaches have been developed: mechanical harvesting, *in situ* cutting, and chemical methods; however, these are not effective measures due to their severe ecological and health effects (Jawed et al., 2022). Water hyacinth and other invasive floating plants are frequently controlled with the liquid herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and diquat (6,7-dihydrodipyrido[1,2-a:2',1'-c]pyrazinediium ion) (Mudge and Netherland, 2014). Chemical herbicides provide aquatic weed control, but may result in water pollution due to contamination. Ultimately, there are presently no approaches effective for long-term sustainable suppression. There is growing interest in natural product-based herbicides because of the problems associated with applying herbicides on water. Therefore, our study aims to provide alternative methods to control *E. crassipes* through natural products.

Natural herbicides offer myriad advantages such as enhanced target specificity and rapid degradation (Laosinwattana et al., 2007), the fungal extracts possess a diverse array of biological properties and the isolation of fungi capable of producing herbicides presents novel opportunities, particularly for developing herbicides with novel modes of action (Cimmino et al., 2013). Additionally, the challenges posed by live microbe pesticides, such as short shelf life and specific environmental demands, can be solved by using natural metabolites instead (Manichart et al., 2023; Zhang et al., 2010). Phytopathogenic fungi produce toxins capable of inducing lesions and impairing weed development (Souza et al., 2017), especially by depressing photosynthetic rate (Sutton et al., 2016).

In a previous study, we isolated 21 phytopathogenic fungi from infected weed tissues and assayed their potential for producing herbicidal metabolites that affect seed germination (Manichart et al., 2023). To the best of our knowledge for sustainable aquatic weed management, this study produced fungal extract-based natural herbicide and evaluated the collected fungi for visual phytotoxic effects, identified the phytotoxic compounds using gas chromatography-mass spectrometry (GC-MS), and examined the physiological processes of their activities, specifically membrane integrity and photosynthetic rate. The study's findings encourage the extract's continued use as a valued product in sustainable agriculture.

Material and Methods

Fungi and hydroethanolic extracts of fungi

The phytopathogenic fungi used in this study were previously collected by Manichart et al. (2023) in September 2021 from the Ladkrabang district of Bangkok, Thailand (geographic coordinates: 13.726725, 100.780125). All 21 isolates, as PN001-1, PN001-2, PN001-3, PN001-4, PN001-5, DA002-1, DA002-2, DA002-3, EC005-1, EC005-2, EC005-3, IA008, EC010, MP011-2, GC012-1, GC012-2, TP013-1, AS014, RT015-1, GC016-1, and GC016-2, were cultured via submerged fermentation in potato dextrose broth for 30 days. After the culture period, the biomass was collected and incubated at 45 °C in a hot air oven (Binder World FP400UL-208V, Binder, Germany) until fully air-dried. Hydroethanolic extracts were prepared by soaking dried mycelia in a solution of ethanol and water (75:25) at a ratio of 1:10 (w/v) for 24 hours; this was repeated three times with the same material. The extracts were then filtered and rotary evaporated (BUCHI Rotavapor

R255, BUCHI, Lausanne, Switzerland) at 45 °C to remove the ethanol. All crudes were stored at 4 °C in dark conditions to minimize light and temperature degradation until further analysis.

Leaf disk phytotoxic assay on *E. crassipes* under laboratory conditions

Each sticky fungal crude extract was dissolved in surfactant mixture (Tween 80:dimethyl sulfoxide; 4:3 v/v ratio) at a 3:7 ratio (crude extract [ai]: surfactant mixture; v/v) following to Manichart et al. (2023). Homogenized, this served as the stock solution for each strain extract. Leaf disk bioassays were performed to evaluate *E. crassipes* sensitivity to the extracts. Each formulation was added to a Whatman No.1 filter paper (Whatman Inc., Clifton, USA) inside a glass Petri dish. Leaf disks (1.00 cm diameter) were obtained from fully unfolded mature leaves of using a paper puncher, and individual disks were gently placed in the dish using a spatula, floating on the extract solution with the adaxial surface facing upward. Distilled water and surfactant mixture were used as negative controls. The phytotoxic effects were evaluated according to the modified scale adopted by the European Weed Research Council (EWRC) (Meseldžija et al., 2020). Leaf disks were visually inspected after 48 and 96 hours and scored on a scale of 1 to 5 based on the development of necrotic lesions and chlorosis, with 1 representing no damage and 5 complete destruction (see Table 1 and Fig. 2C). The experiment used a completely random design (CRD) with four replicates. The phytotoxic effect (% over control) was calculated as follows:

$$\text{Phytotoxic effect (\%)} = \frac{T - C}{C} \times 100 \qquad (1)$$

where T = scoring value of treatment
C = scoring value of control (distilled water treatment)

Partial separation of *Diaporthe* sp. via sequential extraction

Of the phytotoxic strains tested, EC010 showed the highest potential and hence was selected for partial separation using organic solvents with increasing polarity. According to Manichart et al. (2023), this strain was identified as *Diaporthe* sp. with GenBank accession number **OR143425**. Dried mycelia of *Diaporthe* were first soaked in hexane for 24 hours; the resulting mixture was filtered through Whatman No. 1 filter paper and the residue re-extracted twice more with hexane. The three solutions were combined and evaporated to obtain the crude hexane extract fraction, after which the remaining residue was similarly extracted with ethyl acetate (EtOAc) and then absolute ethanol (EtOH) to obtain the EtOAc and EtOH crude fractions. The phytotoxicity of each crude fraction was assessed by leaf disk assay as described above.

Table-1. Scoring scale used to guide visual assessment of phytotoxicity in the leaf disk bioassay

Scoring	Category	Description of phytotoxicity assessment
1	No effect	No damage on leaf disk
2	Slight effect	Slight visible symptoms, clearly yellowing localized in spots, and slight bruising lesions
3	Moderate effect	Chlorosis lesions covering more than 1/3 of the disk and lasting bruising in 1/3 of the disk area
4	Severe effect	Heavy chlorosis, paler green, and lasting necrosis along the disk margin
5	Complete effect	Total burning, general dark brown color, and deformation of disk (curling/twisting)

Gas chromatography-mass spectrometry (GC-MS) analysis of *Diaporthe* fungal ethanolic extracts

GC-MS was employed to identify the components of *Diaporthe* fungal ethanolic extracts. A Scion 436 gas chromatograph was coupled to a Gerstel multipurpose sampler autosampler and a triple quad (Bruker, USA) mass spectrometer. Operating conditions were as follows: initial oven temperature 50 °C (2 min), ramp to 250 °C (20 °C /min), hold for 18 min; constant helium flow rate of 1 ml/min; detection range 30–500 amu. The *Diaporthe* fungal ethanolic extracts (1 mL) was filtered through CN-CA syringe filter of 0.45 µm pore size, 13 mm diameter and injected into the HP-5MS capillary column (30 m., film 0.25 µm, ID. 0.25 mm) in splitless mode. Injector temperature was 250 °C. Ion source and transfer line temperatures were 230 °C and 250 °C, respectively. Individual constituents were distinguished via comparison of mass spectra

(molecular mass and fragmentation pattern) with the internal reference library (National Institute of Standards and Technology, NIST, 2014). Components were quantified in terms of the percentage peak area relative to total peak area. Peaks with a relative area less than 0.1% were excluded.

Greenhouse experiment

E. crassipes was collected from the Ladkrabang district of Bangkok, Thailand, in October 2023. This study complied with relevant institutional, national, and international guidelines and legislation. The aquatic macrophytes were carefully arranged and kept in water-filled containers (10.0 L) for three days, then were placed in buckets under greenhouse conditions (average temperature 28-30 °C, natural light, relative humidity 64-69%). *Diaporthe* EtOH crude extract was applied manually with the wrapping technique, in which leaf surfaces (adaxial and abaxial) and petioles were coated with extract preparations (2.0, 4.0, and 8.0% w/v). Water and a surfactant solution were used as control agents. The experiment utilized a CRD with ten replicates. After treatment, plants were continuously monitored and visual toxicity symptoms assessed based on the Rao (2000) methodology on days 1, 7, 14, and 28. Weed control efficacy was graded on a scale of 0% to 100%, where '0' means a lack of control and '100' total control resulting in the death of all plants. Photosynthetic pigment content and lipid oxidation were assessed on the assumption of phototoxicity being mediated by photosynthetic machinery inhibition and membrane disruption. At 28 DAT, plant fresh weight (FW) and dry weight were determined.

Anatomical analysis

Treated leaf samples were sectioned by hand. Briefly, leaves were sectioned into thin pieces using a razor blade, then treated with 0.5% NaOCl for 5 min to remove chlorophyll pigments. All samples were stained with 0.1% safranin for 10 min, then rinsed with 70% alcohol to remove excess pigment. The slides were examined and photomicrographs captured at 4X and 10X magnification using an EP50 digital microscope camera (Olympus CX23, Japan) and the EPview software.

Mechanism of action

Chlorophyll determination

To extract chlorophyll, treated leaves (0.5 g FW) were mashed in 80.00% (v/v) acetone for three hours under dark conditions at room temperature. After incubation, the suspension was filtered through a Whatman filter paper No. 1. Then, chlorophyll a, b, and carotenoid contents were determined by spectrophotometric analysis by Thermo Genesys 30 Spectrophotometer (Thermo Fisher Scientific, USA) at the absorption of 663, 647, and 470 nm and calculated according to the Lichtenthaler (1987) equation.

chlorophyll a = $12.25 \times A_{663} - 2.79 \times A_{647}$
chlorophyll b = $21.50 \times A_{647} - 5.10 \times A_{663}$
carotenoids = $(1000 \times A_{470} - 1.82 \times$ chlorophyll a $- 85.02 \times$ chlorophyll b$) / 198$

Relative electrolyte leakage (REL)

Electrolyte leakage was assessed. Five leaf discs were floated on 5.0 mL of deionized water after 30 min at 40 °C (EC1) and after boiling at 100 °C for 15 min (EC2), and conductivity measured using a Consort C3010 multi-parameter analyzer (Consort, Belgium). REL was calculated by the formula:

$$\text{REL (\%)} = \frac{EC1}{EC2} \times 100 \qquad (2)$$

Malondialdehyde (MDA) determination

Oxidative damage in *E. crassipes* were assessed as lipid peroxidation, determined by estimating malondialdehyde (MDA) concentration using the thiobarbituric acid (TBARS) assay. Firstly, leaf samples (0.5 g FW) were ground with a mortar and pestle in 6.0 mL of 3.0% (w/v) trichloroacetic acid (TCA), then transferred to a centrifuge tube and centrifuged at 10,000 rpm for 20 min. Subsequently, 1.0 mL of supernatant was combined with 2.0 mL of 0.5% (w/v) thiobarbituric acid and 20% (w/v) TCA, then heated in a boiling water bath. A UV/Vis spectrophotometer (Thermo Fisher Scientific, USA) then measured the absorbance at wavelengths of 532 nm and 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 m/M·cm.

Statistical analysis

The results are shown as means. All data were analyzed using SAS and subjected to analysis of variance (ANOVA) and comparison of means by Tukey's multiple range test ($p \leq 0.05$). Means followed by the same letter(s) are not significantly different.

Results and Discussion

The effect of fungal aqueous ethanolic crude extract on *E. crassipes* leaf tissue

Among *in vitro* techniques for evaluating phytotoxic effects, leaf disk bioassays are widely acknowledged for their effectiveness in assessing physiological responses, for example the accumulation of shikimic acid or tolerance to oxidative stress (Shaner et al., 2005). Compared to whole-plant assays, leaf disk assays require less preparation time, offer rapid results, and allow more replicates. In this study, leaf disk assays were used to assess the phytotoxicity of extracts from 21 isolated fungi. After 96 hours, strain EC010 (identified as *Diaporthe* sp.) showed the highest visible toxicity of 85.19% (Fig. 1). The different phytotoxic potentials of fungal extracts may depend on the chemical compounds that comprise them (Chotsaeng et al., 2019; Manichart et al., 2023). Previously, a screening bioassay demonstrated EC010 to be effective in inhibiting weed seed germination (Manichart et al., 2023). Our present observations support that *Diaporthe* sp. EC010 extract, with its complex mixture of phytotoxic compounds, can have multiple mechanisms of action. Taken together, these findings provide strong evidence to suggest that *Diaporthe* metabolites are promising candidates for non-selective and multiple-mode herbicidal potential.

Effect of sequential solvent extraction of *Diaporthe* sp. on *E. crassipes* leaf disks

To assess the phytotoxic effects of crude fractional extracts from *Diaporthe* sp., each fraction was diluted to 1.00 mg/mL and applied to *E. crassipes* leaf disks. During the initial 24 hours, treatments did not differ significantly in visible toxic effects (p>0.05; data not shown). However, by 48 hours, the EtOH crude extract exhibited noticeable phytotoxicity, with mild chlorosis and slight necrosis along the disk margin. At 96 hours, dark brown discoloration and deformation were evident, a 61.11% effect compared to the control (Fig. 2A and Table. 2). The surfactant mixture (1.0%, v/v) demonstrated only slight toxic potential, with an effect score of 6.48% (Fig. 2B). As the EtOH fraction exhibited the highest visible phytotoxicity, it was selected for further investigation.

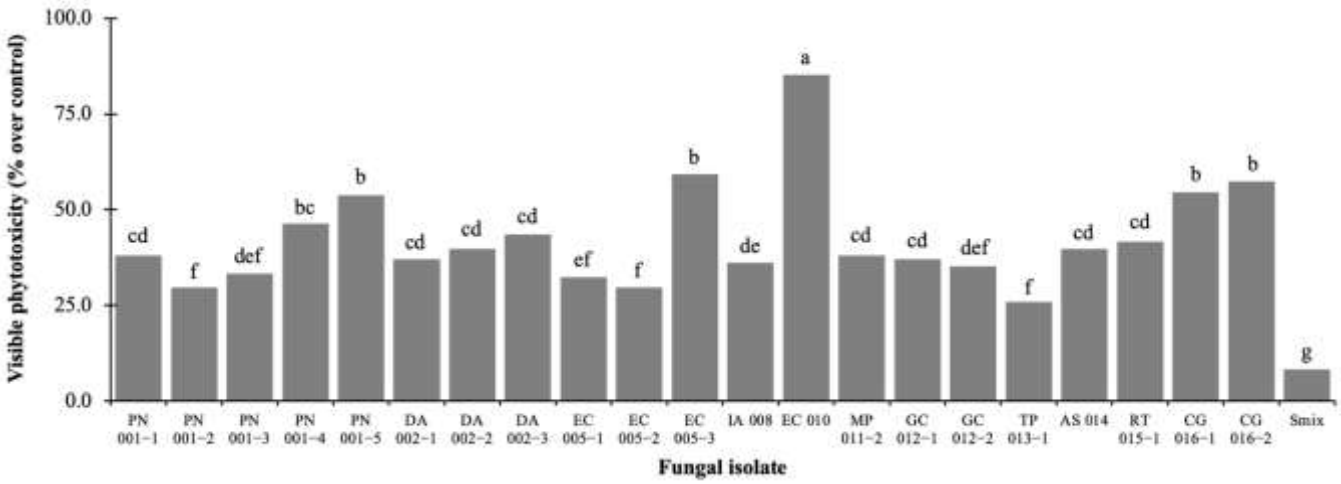

Figure-1. Visible phytotoxicity of 75% (v/v) ethanol crude fungal extracts (2.0 mg/mL) on *E. crassipes* leaf disks at 96 hours after treatment. Different letter(s) indicate significant difference by Tukey's multiple range test (p<0.05).

Table-2. Phytotoxicity of crude sequential extracts of *Diaporthe* sp. on *E. crassipes* leaf disks

Solvent (Concentration; mg/mL)	Visible phytotoxicity level (% over control)	
	48 hours	96 hours
Hexane (C_6H_{14}) fraction		
0.25 mg/mL	7.41 e	14.81 e
0.50 mg/mL	10.19 de	21.81 de
1.00 mg/mL	14.81 d	26.85 d

Ethyl acetate ($C_4H_8O_2$) fraction		
0.25 mg/mL	19.44 c	26.85 d
0.50 mg/mL	20.37 c	33.33 c
1.00 mg/mL	27.78 b	40.74 bc
Ethanol (C_2H_6O) fraction		
0.25 mg/mL	14.81 d	35.19 c
0.50 mg/mL	25.00 bc	50.00 b
1.00 mg/mL	37.96 a	61.11 a
Surfactant mixture	2.78 f	6.48 f

The data are presented as mean. Different letter(s) in the same column indicated significant differences according to Tukey's multiple range tests at $p<0.05$ level.

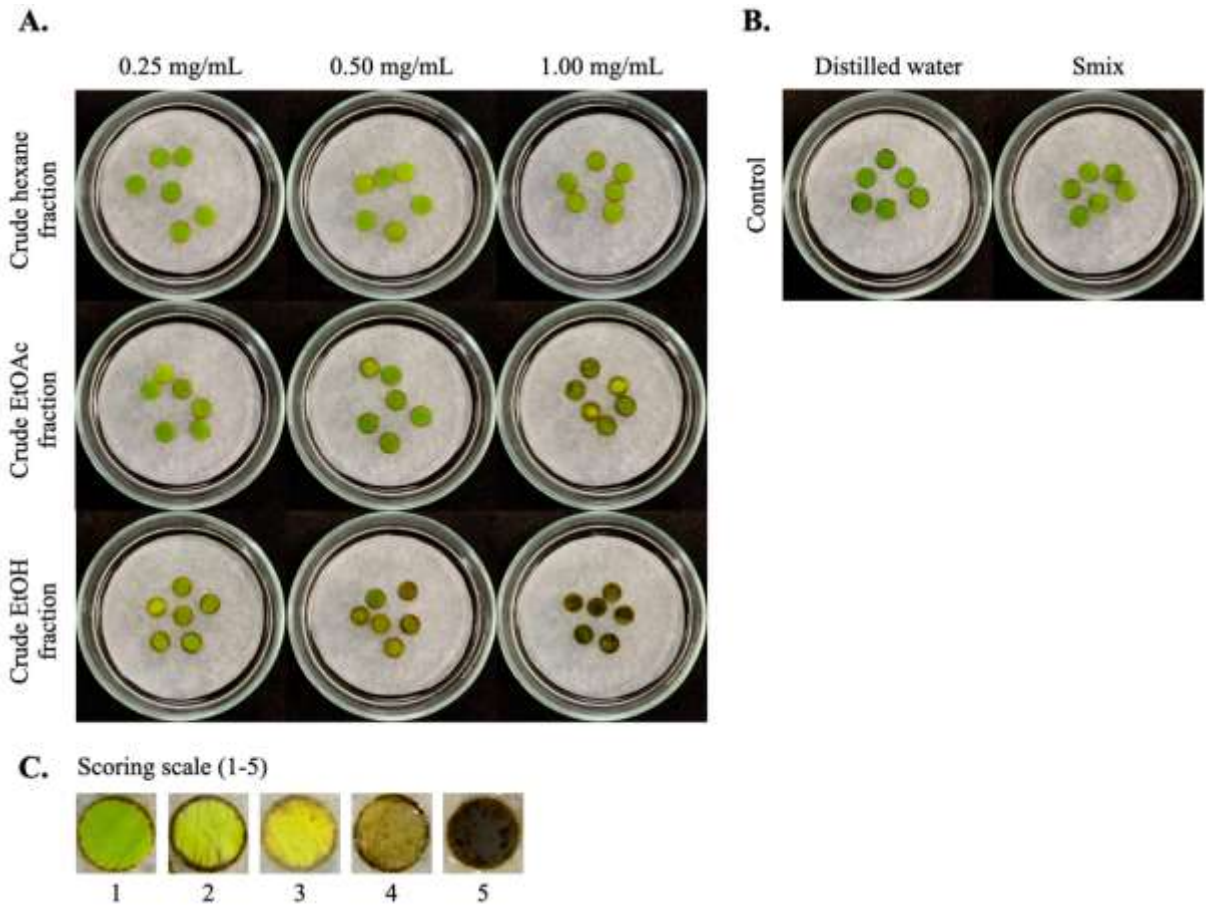

Figure-2. Representative images of phytotoxic effects on *E. crassipes* leaf disks from crude sequential extracts of *Diaporthe* sp. (A) and control solution (B) at 96 hours after treatment. Visual guide for the 1-5 scoring scale used in phytotoxic assessments (C).

Chemical constituents of *Diaporthe* fungal extracts

A comprehensive GC-MS analysis was performed for the EtOH fungal crude extract derived from *Diaporthe* sp. EC010; the 57 identified chemical components, comprising 89.051% of the extract, are detailed in Table 3. The primary components were 2(3H)-furanone, dihydro-4-hydroxy- (22.81%), linoelaidic acid (6.87%), and hordenine (6.62%), consistent with the results of Rosa et al. (2021), who identified fatty acid methyl esters and diaza-compounds as components of *D. schini* extract. The differentiation of chemical constituents may be influenced by specific enzyme activities within biosynthesis pathways (Winter et al., 2011) and variation among fungal species. Production of secondary metabolites can be affected by factors such

as the evolutionary history between host and fungus and nutrients in the growth media (Tanapichatsakul et al., 2019). Consequently, the application of fungal-based products should be approached with caution. The compound 2(3H)-furanone, dihydro-4-hydroxy- was previously identified in extracts from *Tabebuia rosea* and *Euphorbia serrata* (Sanchez et al., 2023), which exhibited phytotoxic activity against *Sorghum bicolor* and *Lactuca sativa* (Silva et al., 2019). Linoelaidic acid is an unsaturated fatty acid. Fatty acids frequently serve as the foundation for natural herbicides (Manthey et al., 1992) due to their effect on the epicuticular wax (Crmaric et al., 2018), a protective layer that protects leaves from allelochemicals. Erida et al. (2023) identified linoelaidic acid (5.93%) in *lmperata cylindica* crude extract, which exerted herbicidal activity on *Amaranthus spinosus*. Hordenine alkaloids were observed to be released from barley root extracts by Liu and Lovett (1993), and the herbicidal activity of hordenine has been demonstrated by numerous studies (Lebecque et al., 2018; Maver et al., 2020). In white mustard, the impact of hordenine manifests as decreased radicle length and an apparent decline in the overall health and vigor of radicle tips. Transmission electron microscopy revealed damage to cell walls, autophagy, and disorganization of organelles in root tissues (Lovett et al., 1994). Ultimately, the phytotoxic activity observed in the current study may be linked to the presence of these compounds and their potential synergistic interactions.

Table-3. Constituents of *Diaporthe* sp. EC010 crude EtOH extract by gas chromatography-mass spectrometry

No.	Constituent	RT[a]	% of total[b]
1	Hordenine	3.253	6.623
2	Betaine	4.159	0.417
3	1-Hydroxysulfonyl-3,4,4-trimethyl-2-azetidinone	4.601	0.315
4	2(5H)-Furanone	4.907	3.507
5	2,6-Dihydroxyacetophenone, 2TMS derivative	5.567	0.217
6	1,8-Cineole	5.954	1.018
7	1,6-Anhydro-2,4-dideoxy-β-D-ribo-hexopyranose	6.069	0.197
8	12-Dimethylamino-10-oxododecanoic acid	6.834	0.640
9	2(3H)-Furanone, dihydro-4-hydroxy-	6.974	22.812
10	Vinyl formate	7.082	2.257
11	dl-Citrulline	7.272	0.854
12	(S)-(1-2H)3-Methyl-2-butenyl acetate	7.330	1.035
13	1,4:3,6-Dianhydro-α-d-glucopyranose	7.431	0.974
14	Geranial	7.592	0.383
15	2-Coumaranone	7.642	1.206
16	Butanoic acid, pentyl ester	7.860	0.612
17	9,12,15-octadecatrienoic acid, methyl ester	7.999	0.122
18	1H-indole	8.076	0.220
19	Eugenol	8.486	0.419
20	cis-9-Octadecenoic acid	8.911	0.554
21	Pterin-6-carboxylic acid	8.970	0.590
22	Carbonic acid, (ethyl)(1,2,4-triazol-1-ylmethyl) diester	9.199	0.838
23	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)-	9.234	0.462
24	Arachidonic acid, TMS derivative	9.254	0.579
25	α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-O-(trimethylsilyl)-, cyclic methylboronate	9.310	2.735
26	Dihydroxanthin	9.405	0.588

27	Flavonol 3',4',5,7-OH,3-O-araglucoside	9.509	2.273
28	Adenosine-3-phosphoric acid	9.595	0.452
29	1-Tetradecanol	9.707	0.895
30	Ascaridole epoxide	9.811	1.004
31	2-Hexadecanol	10.002	1.998
32	Octadecanoic acid, 9,10-epoxy-18-(trimethylsiloxy)-, methyl ester, cis-	10.170	1.028
33	Perhydrocyclopropa[e]azulene-4,5,6-triol, 1,1,4,6-tetramethyl	10.277	0.927
34	Benzoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	10.324	0.363
35	Thieno[2,3-c]furan-3-carbonitrile, 2-amino-4,6-dihydro-4,4,6,6-tetramethyl-	10.646	0.461
36	Uric acid	10.716	1.476
37	1,4-Diaza-2,5-dioxobicyclo[4.3.0]nonane	10.962	3.972
38	1-Decanol	11.107	0.519
39	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	11.208	0.385
40	2-Myristynoyl pantetheine	11.231	0.146
41	Cyclo(Pro-Leu)	11.293	0.496
42	Actinomycin C2	11.423	0.696
43	Butyl octyl phthalate	11.507	0.340
44	1-Oxaspiro[4.4]nonan-4-one, 2-isopropyl-	11.808	0.904
45	Hexadecanoic acid	11.894	3.710
46	1,2-Benzenedicarboxylic acid, butyl octyl ester	11.979	1.639
47	Ethyl palmitate	12.078	2.154
48	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	12.359	0.399
49	Linoelaidic acid	12.863	6.868
50	Hexadecanoic acid, 1,1-dimethylethyl ester	13.003	1.071
51	Ethyl linoleate	13.063	2.182
52	Cyclo(Leu-Leu)	13.178	0.393
53	Quinoline-5,8-dione-6-ol, 7-[[(4-cyclohexylbutyl)amino]methyl]-	13.371	0.727
54	9,12,15-Octadecatrienoic acid, 2,3-bis(acetyloxy)propyl ester, (Z,Z,Z)-	17.927	0.581
55	9,12-Octadecadienoic acid (Z,Z)-, phenylmethyl ester	19.330	0.344
56	9,10-Dideuteriooctadecanal	27.219	0.166
57	Maytansine	27.482	0.920
		Total	**89.051%**

[a] = Retention time.
[b] = Relative area percentage (peak area relative to the total peak area, %).

Greenhouse experiment

Dose-dependent visual toxicity symptoms were observed in *E. crassipes* at 1, 7, 14, and 28 days after application (DAT) of the EtOH crude extract (Figure 3). The highest concentration of extract (8.0% ai) induced 42.22% toxicity by 1 DAT, which remarkably increased to 73.33% within 14 DAT; it also resulted in the lowest fresh and dry weights at the end of the experiment (Table 4), which differed significantly from the control (p<0.05). The visual symptoms included leaf burning, wilting, and necrosis, in keeping with the documented effects of herbicidal compounds from *Aspergillus flavus*, *Aspergillus miniscleroti*, and *Allophoma oligotrophica* (Karthick et al., 2023) on *E. crassipes*: severe growth decrease, chlorosis, and leaf burning with a maximum deterioration of 88-94%. Souza et al. (2015) and Bastos et al. (2017) likewise observed foliar lesions and occasional yellowing of *E. crassipes* plants exposed to *Diaporthe* sp. culture broth, and suggested the weed to be particularly susceptible to the extract, with a portion of the leaf tissue becoming necrotic. In another study, Souza et al. (2017) tested the herbicidal compounds produced from some phytopathogenic fungi via submerged fermentation on *Cucumis sativus* L., and obtained the

highest phytotoxicity of 60% for a culture filtrate from a fungus identified as *Diaporthe* sp. strain VP51. The toxicity observed in the present study was more pronounced, suggesting that herbicidal compounds may be efficiently extracted using organic solvents; moreover, our use of mycelia as source highlights the importance of mycelium in the production of highly toxic herbicidal compounds. Finally, Brun et al. (2022), evaluated *D. schini* broth for its efficacy against noxious weeds in Brazil and observed effective phytotoxicity against three weeds: *Bidens pilosa*, *Amaranthus viridis*, and *Echinocloa crusgalli*. Taken together, these findings suggest the fungal genus *Diaporthe* to have non-selective herbicidal potential. While this study suggests the herbicidal potential of *Diaporthe* sp. extract against water hyacinth, the field conditions, long-term effects, and environmental impact remain unexplored. Future research should focus on assessing efficacy under natural conditions, evaluating potential effects on non-target organisms and ecosystems, and developing formulation strategies to enhance stability and efficacy.

Table-4. Phytotoxicity of EtOH crude extract from *Diaporthe* sp. EC010 on *E. crassipes* weeds in a greenhouse setting.

Treatment	Visible phytotoxicity level (% over control)				FW increase[#]/ plant (%)	DW/ plant (g)
	1 DAT	7 DAT	14 DAT	28 DAT		
Control	–	–	–	–	115.97 a	55.90 a
Smix	0.00 c	7.22 c	8.06 d	10.56 d	97.74 ab	59.00 a
***Diaporthe* sp. extract**						
2.0%	0.00 c	11.67 c	25.83 c	37.78 c	85.35 b	53.60 a
4.0%	13.06 b	36.67 b	42.22 b	69.44 b	32.89 c	41.00 b
8.0%	42.22 a	59.44 a	73.33 a	85.00 a	0.00 d	25.00 c
C.V. (%)	32.71	16.57	15.95	11.27	40.09	19.49

Smix: surfactant mixture

Mean values with same letter(s) within a column are not significantly different according to Tukey's multiple range test ($p<0.05$)

[#] calculated as $[(FW_{30\ DAT} - FW_{0\ DAT})/ FW_{0\ DAT}] \times 100$

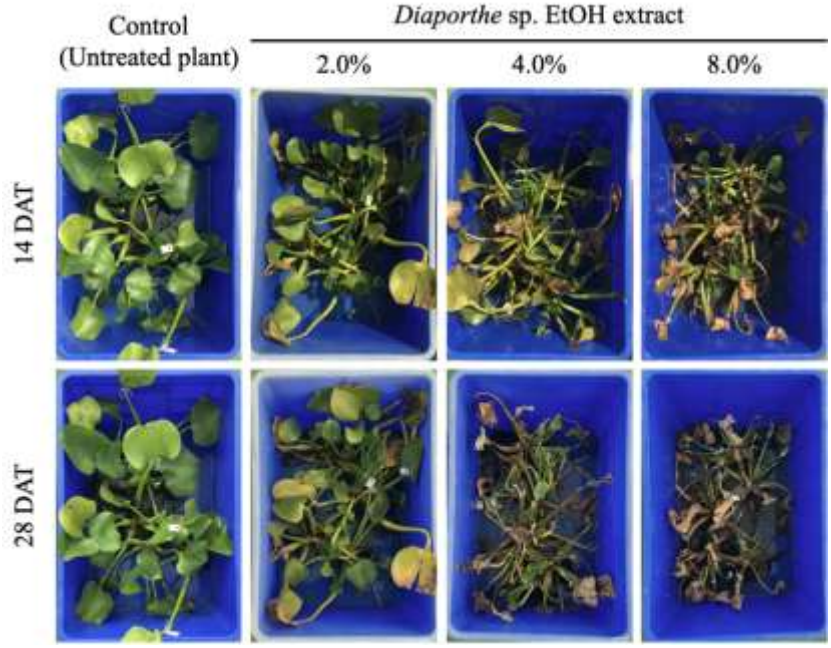

Figure-3. Representative images of phytotoxicity symptoms in *E. crassipes* treated with EtOH crude extract from *Diaporthe* sp.

***Diaporthe* sp. extract effected changes in *E. crassipes* leaf microstructure**

This study presents an anatomical analysis of the impact of *Diaporthe* EtOH crude extract on *E. crassipes*. In the normal condition, transverse sections of *E. crassipes* leaf blades reveal distinctive anatomical features, including a single layer of upper and lower epidermis with a very thin cuticle (Fig. 4A) (Zahoor et al., 2018). The leaves are amphistomatic, with stomata present on both upper and lower epidermal surfaces (Zhou et al., 2021) and situated at the same level as other epidermal cells. The leaf blade features two types of vascular bundles: smaller bundles in contact with the epidermis and larger bundles situated towards the leaf center. The mesophyll cells harbor numerous chloroplasts, randomly distributed along the cells. The leaf tissues contain styloid crystals (Fig. 4C) composed of calcium oxalate. One of the most characteristic features of *E. crassipes* is the aerenchyma tissue, predominantly in the ground meristem within the leaf mesophyll, characterized by large hexagonal air-filled chambers (Xiang et al., 2019). Leaves exposed to *Diaporthe* extract exhibited notable structure alterations. Anatomical analysis on 7 DAT revealed necrotic areas (Fig. 3) clearly associated with direct exposure to the extract. Plants treated with surfactant alone (data not shown) or with the lowest extract concentration (2.0% ai, Fig. 4B) showed unchanged epidermal tissue; conversely, tissue collapse increased drastically with extract concentration. In necrotic areas, upper and lower leaf blade thickness drastically decreased, consistent with the findings of Pereira et al. (2017), who demonstrated that application of 2,4-D herbicide led to decreased thickness of *E. crassipes* leaves. This reduction was accompanied by observable degradation of tissue (Fig. 4C, D), which could facilitate extract penetration (David et al., 2020). Treatment with 4.0 and 8.0% *Diaporthe* extract produced irreversible damage including total disruption of epidermal cells, larger aerenchyma chambers, and highly disorganized vascular bundles. The observed bundle damage may be attributed to degradation or damage of ground tissue, with the loss of supportive tissue resulting in bundle arrangement within a non-uniform plane. Pereira et al. (2017) previously observed disorganized vascular bundles in a Brachiaria grass (*Urochloa decumbens*) after application of sethoxydim. Ultimately, the tissue damage caused loss of membrane integrity, ion leakage (Table 5), and water loss (Grossmann et al., 2011; Langaro et al., 2017), as previously observed when applying clomazone and saflufenacil to *Setaria viridis* (David et al., 2020) and *S. italica* (Reddy et al., 2014).

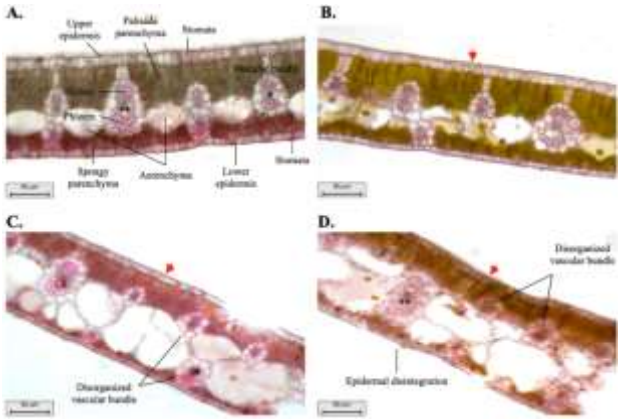

Figure-4. Cross-sectional structure of the *E. crassipes* leaf blade. Images show untreated plants (A) with smaller (asterisk) and larger (double asterisk) vascular bundles and *Diaporthe* extract-treated plants (2.0, 4.0, and 8.0% in B, C, and D, respectively) with reduced epidermis thickness (red arrow), vascular bundle disorganization, and large air pores.

Effect of the *Diaporthe* sp. EtOH crude extract on photosynthetic machinery and membrane integrity

Even at 1 DAT, the highest concentration *Diaporthe* extract induced a visible shift in *E. crassipes* leaves, from green to pale yellow (Fig. 3). Pigment content measurement revealed chlorophyll a, b, and carotenoid contents to decrease with increasing *Diaporthe* extract concentration and treatment time (Table 5), indicating inhibition of photosynthesis due to loss of pigment. This is consistent with prior studies highlighting the phytotoxic impact of natural chemicals on chlorophyll content (Laosinwattana et al., 2018; Ootani et al., 2017; Somala et al., 2022). The relative electrolyte leakage and TBARS content in *E. crassipes* leaves are also given in Table 5; both parameters increased with increasing concentration of *Diaporthe* extract, supporting that the extract disrupts cell membrane integrity and enhances electrolyte leakage. These results agree with the leaf anatomical changes in Fig. 4.

Table-5. Effect of EtOH crude extract from *Diaporthe* sp. EC010 on photosynthetic pigments, relative electrolyte leakage, and malondialdehyde in *E. crassipes* leaf after 1 and 7 days.

Treatment	Chlorophyll a (µg/ g FW)		Chlorophyll b (µg/ g FW)		Carotenoids (µg/ g FW)		Relative electrolyte leakage (REL; %)		Malondialdehyde (nmol/ g FW)	
	1 DAT	7 DAT	1 DAT	7 DAT	1 DAT	7 DAT	1 DAT	7 DAT	1 DAT	7 DAT
Control	105.10 a	120.68 a	36.40 a	43.72 a	21.43 a	31.35 a	7.28 c	30.48 cd	19.66 a	20.31 c
Smix	96.03 a	98.83 b	33.67 ab	33.79 b	21.02 a	21.56 b	8.14 c	29.26 d	22.45 a	23.76 c
***Diaporthe* sp. extract**										
2.0%	79.20 b	70.59 c	28.76 ab	27.05 bc	17.36 b	11.82 c	8.36 c	37.28 bc	24.70 a	29.16 bc
4.0%	76.77 b	61.88 c	26.68 b	20.68 c	15.59 b	9.85 c	11.32 b	41.55 b	25.96 a	37.23 b
8.0%	45.00 c	41.17 d	15.41 c	14.44 d	5.01 c	6.77 c	13.87 a	60.94 a	30.32 a	50.66 a
C.V. (%)	12.07	13.79	18.89	15.43	8.80	21.33	14.15	10.48	21.71	10.50

Smix, surfactant mixture

Values are means of ten replications. Means with same letter(s) within a column are not significantly different by Tukey's multiple range test (p<0.05).

As the plant cell membrane comprises fatty acids and lipids, membrane degradation leads to release of free lipids (Scrivanti et al., 2003), oxidation of which can be detected with the TBARS reaction. Penetration by the *Diaporthe* extract disrupts the cell membrane, impacting cellular function and photosynthesis. The decrease in observed chlorophyll levels may be caused by reduced biosynthesis, enhanced degradation, or both. Importantly, carotenoids potentially protect chlorophyll from photodamage; thus, reduced carotenoid levels could trigger photooxidative chlorophyll breakdown, leading to plant deterioration. This knowledge plays a pivotal role in the development of effective and sustainable herbicides. The *Diaprthe* sp. extract shows multiple modes of action, which is a key principle of herbicide resistance management. Herbicides with different modes of action could be combined to produce a synergistic effect. This improves weed control but reduces survivors that might develop resistance.

Conclusion

Our findings indicate the chemical composition of *Diaporthe* sp. EC010 mycelia EtOH extract, being mainly composed of 2(3H)-furanone, dihydro-4-hydroxy- and fatty acid methyl ester molecules, to align with its effective herbicidal properties. This extract has previously been shown to inhibit weed seed germination as a natural pre-emergence herbicide. In this study, foliar-applied *Diaporthe* sp. extract also demonstrated an extremely phytotoxic effect on the aquatic weed *E. crassipes*. This suggests that secondary metabolites from *Diaporthe* sp. mycelia have promise as multipurpose and contact herbicides for sustainable agriculture. However, elucidating the herbicidal mechanisms requires further in-depth physiological investigations. It also remains essential to conduct field experiments, cost analysis, and safety assessments. For further research, it is recommended to explore the specific physiological pathways affected by the secondary metabolites in *Diaporthe* sp., using advanced techniques such as transcriptomic and metabolomic analyses. In addition, it is necessary to carry out field tests to assess the efficacy of the extract in varied conditions, and a thorough cost-benefit analysis should be performed to determine the feasibility of large-scale application. Such comprehensive research will help in improving the formulation and application techniques, which may result in the development of a novel, environmentally friendly herbicide for methods of sustainable agriculture.

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Contribution of Authors

Somala N: Conceptualization, resources, manuscript review and editing
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Thongbang M: Investigations and literature review
Wichittrakarn P & Teerarak M: Literature review and manuscript editing

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