Isolation and Identification of Allelochemical in *Dicranopteris linearis* (Burm. f.) Underw.

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Abstract: An allelochemical, 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester, in aerial tissues of *Dicranopteris linearis* was identified using a bioactivity-guided isolation method. The structure of the allelochemical was determined by mass spectrometry, infrared spectra and 1H NMR spectroscopy. This compound exhibited strong phytotoxic effects on leaf discs of *Crassocephalum crepidioides*.

Keywords: Allelochemical · *Dicranopteris linearis* · 1,2-benzenedicarboxylic acid · Bis(2-ethylhexyl) ester

INTRODUCTION

*Dicranopteris linearis* (Burm.f.) Underw. is a tropical fern. In Malaysia, it is a problematic weed widely distributed in agricultural land and along the roadside. This species usually demonstrates the typical allelopathic characteristic of tending to form dense, pure patches under natural conditions.


The allelopathic effects of ferns have not been studied as intensely as compared to other plants. According to Northup et al. [4], *D. pectinata* (Willd.) contained several polyphenol allelochemicals. Aoki et al. [5] found that the diterpenes allelochemical from *D. pedata* inhibited the root growth of *Lactuca sativa*. Allelochemicals that suppress or prevent the growth of plant species are receiving increased attention currently as they can be used as natural herbicides. Allelochemicals from weeds have the potential to be explored as natural herbicides. As they have relatively short half-lives they could be considered as being more environment friendly [6,7].

The objective of this study was to isolate and identify the allelochemicals in *D. linearis*. In this study, the bioassay-guided isolation method was used for identifying the allelochemicals of *D. linearis*. This method had been employed by Rimando et al. [8] to identify allelochemicals from rice. *Crassocephalum crepidioides* was chosen as the bioassay species, it was found in previous studies to be highly susceptible to the allelopathic effects of *D. linearis* [1-3].

MATERIALS AND METHODS

Plant Materials: Aerial tissues of *Dicranopteris linearis* were collected from the field adjacent to the greenhouse of the Universiti Kebangsaan Malaysia (UKM). The samples were air-dried at room temperature (27±3°C) for 72 hours and then ground (1 cm size) using a commercial blender. The bioassay seed (*Crassocephalum crepidioides*) were also collected from the same location but from an area free of *D. linearis* and they were grown in the greenhouse of UKM.

Extraction, Isolation and Phytotoxicity Assay: Air-dried tissues (1 kg) of *D. linearis* were soaked in distilled water (10 L), divided into 40 parts and poured into 250 mL conical flasks and shaken on an orbital shaker for 24 hours (Model S102, Fristek Scientific, Taiwan ROC ) at
room temperature (27±3°C). The aqueous extract was filtered with Whatman filter paper (No. 42) and then centrifuged at 15000 rpm (RC-5B Sorvall, DuPont Instrument Co, USA) for 15 min. The supernatant was collected and concentrated to dryness, to obtain a crude extract, using a rotary vacuum evaporator at 40°C (WB2000 & VV2000, Heidolph Instruments, Germany). The resulting dried, crude extract was dissolved in 250 mL distilled water and then fractionated with n-hexane (5 x 250 mL), ethyl acetate (5 x 250 mL) and n-butanol (5 x 250 mL) successively. The collected organic phase was dried over anhydrous MgSO₄. Each fraction which included the final aqueous fraction was then evaporated to dryness using a rotary vacuum evaporator (40°C) and then dissolved in distilled water in order to obtain a concentration of 500 ppm. The fractions were filtered using a 0.22 μm membrane filter (Minisart®-RC/SRP, Sartorius).

Five leaf discs were placed into Petri dishes that lined with 9 cm Whatman filter paper No. 1. Eight milliliters of each fraction were added into the Petri dishes separately. The Petri dishes were kept in the laboratory (temperature: 27±3°C, light intensity: 50 μEm “s”-1). After 48 hours, the level of the phytotoxic effects on the leaf discs was evaluated using “visual scoring”, in which a score of 0 corresponded to no observed symptoms of injury (leaf disc greenish color), a score of 1 corresponded to slight injury (brownish color appeared in small portions on the leaf disc), a score of 2 corresponded to moderate injury (a large portion of the leaf disc turned brownish color) and a score of 3 corresponded to severe injury (the whole leaf disc turned brown).

**Column Chromatography:** The n-butanol fraction (based on results, the n-butanol fraction showed the strongest phytotoxic activity) was further purified on a silica gel column [2.5 cm i.d x 45 cm length, Bio-Rad, USA; Silica gel 60 (0.063-0.200 mm)] Elution will be performed with ethyl acetate-MeOH mixtures as follows: 100:0 (300 mL), 95:5 (200 mL), 90:10 (300 mL), 85:15 (200 mL), 80:20 (300 mL), 75:25 (400 mL), 70:30 (300 mL), 65:35 (200 mL), 60:40 (200 mL), 50:50 (200 mL), 40:60 (400 mL) and 0:100 (200 mL). The column chromatographic fraction (each 100 mL) was evaporated to dryness using a rotary vacuum evaporator (40°C) and then dissolved in distilled water in order to obtain a concentration of 500 ppm. It was subjected to the phytotoxicity assay using the above-mentioned method for determining the phytotoxic activity.

**Thin Layer Chromatography (TLC):** The column chromatographic fraction no 10, 11 and 12 (based on results, these fractions demonstrated the highest phytotoxic activity) were combined and further fractionated using TLC (Silica gel 60 F₂₅₄ 2mm; 25% chloroform:75% methanol) separation. The compounds on TLC were detected using UV light (Transilluminator UVP, San Gabriel, USA). Each compound was then dissolved in distilled water in order to obtain a concentration of 500 ppm and subjected to the phytotoxicity assay using the above-mentioned method for determining the phytotoxic activity. The compound that showed the strongest phytotoxic activity was isolated for determination of its chemical structure.

**Chemical Identification:** The compound was analyzed by gas chromatography (GC; Hewlett Packard, System 5890) -mass spectrometry (MS; Hewlett Packard, 5971A Mass Selective Detector, database: Wiley) [column: DB-1, 30 m length x 0.25 mm i.d., film 0.25 μm; temperature: 100°C (3°C/min)-230°C (15 min); gas: helium (50 cm³/min); injection temperature: 250°C; injection volume: 10 μL]. The infrared (IR) spectra of the active compound was obtained using the Spectrum One FT-IR Spectrometer, PerkinElmer, USA, (FT-IR software: Spectrum for Windows- SPECTRUM). The number of protons (H) of the compound was determined by NMR Bruker (300 MHz, CD₃OD), Avance DRX.

**RESULTS AND DISCUSSION**

The n-butanol fraction of the aqueous solution of *Diancoperis linearis* clearly exhibited the highest phytotoxic effects on the leaf discs of *Crassocephalum crepidioides* (Table 1). As for the n-hexane fraction, it totally did not have any destructive effect on the leaf discs of *C. crepidioides*. This suggested that the n-butanol fraction contained some substances highly phytotoxic to leaf disc of *C. crepidioides*. The phytotoxic activity of column chromatographic fractions is shown in Fig.1. Column chromatographic fractions no 11, 12 and 13 exhibited the highest phytotoxic activity on the leaf discs of *C. crepidioides*. These fractions contained 7 compounds as observed when further separated using TLC (Table 2). Among the 7 compounds separated by TLC, the R₆ 0.67 compound demonstrated the highest phytotoxic activity on the leaf discs of *C. crepidioides* (Table 2).
Fig. 1: Phytotoxic effects of column chromatographic fractions on leaf discs of *Crassocephalum crepidioide*.

Table 1: Phytotoxic effects of *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions of *Dictamnus albus* on leaf discs of *Crassocephalum crepidioide*.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Phytotoxic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>n</em>-hexane</td>
<td>0</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>1</td>
</tr>
<tr>
<td><em>n</em>-butanol</td>
<td>3</td>
</tr>
<tr>
<td>aqueous</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2: Compounds that appeared on thin layer chromatography and their phytotoxic effects on leaf discs of *Crassocephalum crepidioide*.

<table>
<thead>
<tr>
<th>Compound noted on TLC</th>
<th>Distance of the compound moved (cm)</th>
<th>Phytotoxic effects</th>
<th>Distance of the Solvent moved (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.97</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.67</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.58</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.35</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.06</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

The GC-MS analysis of the *R*$_y$ 0.67 compound of *Dictamnus albus* required approximately 55 minutes. Only a major peak appeared at retention time 39.55 min (Fig. 2). This result showed that the isolated compound was pure. The mass spectrum when compared with spectra from the database of GC-MS (Wiley) it appeared that the *R*$_y$ 0.67 compound was 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester. Based on the database of the IR Spectrum One FT-IR Spectrometer, PerkinElmer, the IR spectrum (Fig. 3) the presence of the basic functional group carbonyl was revealed, corresponding to the analysis of GC-MS. The ¹H NMR spectral data of *R*$_y$ 0.67 compound is shown in Fig. 4. The ¹H NMR spectrum indicated that the *R*$_y$ 0.67 compound contained 38 H and this spectrum verified that the *R*$_y$ 0.67 compound was 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester (Fig. 4).

Rimando et al. [8] also reported that 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester was found in the rice (Oryza sativa L.) cv. Taichung Native 1 and it could inhibit the germination of lettuce (Lactuca sativa L.). The information of the allelopathic activity of this compound is not well documented. Several other benzenedicarboxylic acids like 1,2-benzenedicarboxylic acid in Cucumis sativus [9] and 1,3-benzenedicarboxylic acid [10] also demonstrated allelopathic effects.

The substance, 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester, may at least partly to be responsible for the allelopathic effects, namely the suppressed growth and germination of the bioassay species in previous studies done by the authors. Combination of 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester and other allelochemicals may act synergistically in inhibiting germination and growth of other species. Allelopathic activity in the field is thought to be a joint action of a mixture of allelochemicals [11, 12].

In summary, the results of this study showed that *D. linearis* contained allelochemicals. The finding supports and confirms the previous reports that *D. linearis* is an allelopathic plant. Further studies are needed to identify other allelochemicals and investigate the allelopathic effects of the mixture of allelochemicals of *D. linearis*. 

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Fig. 2: GC-MS chromatogram of the $R_f 0.67$ compound.

Fig. 3. Infrared spectrum (IR) of the $R_f 0.67$ compound of *Dicranopteris linearis*. 
Fig. 4: Structure of 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester. $^1$H NMR Shift (ppm): 7.23 (A), 6.23 (B), 3.26 (C), 1.87 (D), 1.20-1.31 (E), 0.97 (F), 0.89 (G).

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REFERENCES


