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ISOLATION AND CHARACTERIZATION OF ALLELOCHEMICALS IN RYE HERBAGE*

JANE P. BARNES, ALAN R. PUTNAM, BASIL A. BURKE and ARNE J. AASEN

Michigan State University, East Lansing, MI 48824, U.S.A.; ARCO Plant Cell Research Institute, Dublin, CA 94568, U.S.A.

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Key Word Index—*Secale cereale*; Gramineae; winter rye; phytotoxicity; benzoxazinones; 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one; 2(3*H*)-benzoxazolinone; *Lepidium sativum*; *Echinochloa crusgalli*.

Abstract—Residues and aqueous extracts of rye (*Secale cereale* L. 'Wheeler') shoot herbage were previously shown to contain phytotoxic compounds. Sequential partitioning of aqueous extracts against a series of solvents of increasing polarity separated the most active compounds in the Et_2O fraction based on a cress (*Lepidium sativum* L. 'Curly') root growth assay. Bioassays after TLC indicated two major zones of toxicity. Further separation of the Et_2O extract revealed two new phytotoxic compounds in rye. The compounds were identified as 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one (DIBOA) and a breakdown product 2(3*H*)-benzoxazolinone (BOA). In the cress bioassay, DIBOA and BOA inhibited root growth 50% or more at concentrations of *ca* 0.37 and 1.05 mM, respectively. Only DIBOA showed significant activity on barnyardgrass (*Echinochloa crusgalli* L. Beauv.). Neither compound inhibited seed germination at concentrations tested. DIBOA stimulated root and shoot growth of cress at the lowest concentration tested (0.09 mM).

INTRODUCTION

Winter rye (*Scale cereale* L.) is often used as a cover crop to reduce soil erosion and increase organic matter. Although numerous reports of plant injury by rye and its decomposing residues appear in the literature [1–10], none have conclusively explained this phenomenon.

In no-tillage cropping systems, the residue tends to be concentrated near the seed germination zone where it has the potential to chemically interfere with seedling growth. The chemical influence of one plant upon another is termed allelopathy [11] and may account for the phytotoxicity associated with rye residues. Proof of allelopathy relies on isolation and identification of compounds responsible for the phytotoxicity.

We have previously reported reductions in weed biomass under a rye mulch in undisturbed soil [9] and have extensively evaluated and confirmed that residues and aqueous extracts of rye are toxic to several plant species [12]. As rye shoot herbage is twice as inhibitory as root herbage, isolation and characterization of allelopathic chemicals in rye were based on extraction of shoot herbage. Realizing that several compounds may be involved in allelopathy by rye, we directed the separations toward identification of the most toxic compounds. Our approach has been to monitor every stage and fraction of the separation through bioassays of their relative activity on the germination and seedling growth of cress (*Lepidium sativum* L. 'Curly').

RESULTS

Extraction efficiency and relative activity

Overall, H_2O removed more compounds from dried rye shoot tissue than MeOH–H_2O (Fig. 1). From 50 g of dried tissue, 16.8 g or 34% of the dried plant material was extracted with MeOH–H_2O in 24 hr. In contrast, extraction with H_2O in 24 hr yielded 24.6 g or 49% of the initial weight. There was little difference in recovery between the 0.5 and 24 hr aqueous extractions. Further extraction of residue previously extracted with H_2O for 24 hr yielded an additional 1.8 g.

Regardless of the duration or solvent of extraction, greatest toxicity partitioned into Et_2O. Toxicity of the Et_2O fraction obtained from the residue initially extracted with MeOH–H_2O was significantly less active than all other Et_2O extracts. As water is the solvent of extraction in nature, and efficiently removes many compounds from rye, all subsequent separations were based on a 24 hr aqueous extraction at 4°.

Depending on the concentration tested, all fractions exhibited some degree of activity in dose response bioassays. Therefore, the yield, I_{50}, and unit activity of each fraction were determined and compared (Table 1). The I_{50} for the initial crude extract was *ca* 1.9 mg or 1300 ppm. Based on the quantity of material recovered from the initial extraction, the aqueous crude extract contained *ca* 13 000 potential units of activity. After initial separations, 48% of the unit activity was associated with the protein precipitate and final aqueous extracts. Although these fractions accounted for 72% of the crude weight and 97% of the recovered material, their specific activity was considerably less than the organic extracts. Only 65% of the initial activity was recovered in the fractions after separation.

Overall, organic solvent fractions were much more active than the aqueous fractions. The Et_2O fraction had the greatest specific activity with an I_{50} of 150 μg or 100 ppm (w/v). The second most active fraction was the EtOAc extract followed by the CH_2Cl_2 and hexane fractions. The organic fractions accounted for 18% of the potential unit activity, but only 1.9% of the initial crude weight. The Et_2O fraction alone accounted for 12% of the

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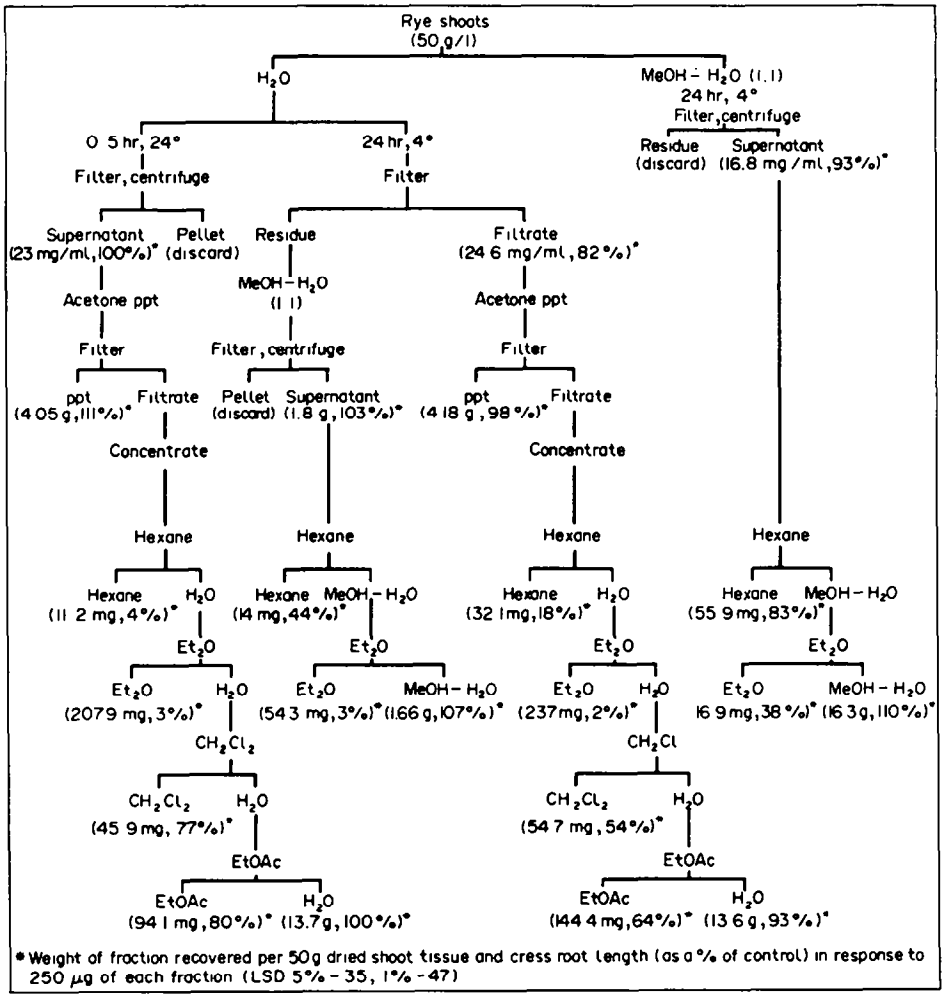

Fig. 1. Flow diagram for initial extractions of rye (*Secale cereale* L.) shoot herbage and bioassay on cress (*Lepidium sativum* L.).

unit activity and only 1% of the crude weight. Therefore, the Et_2O fraction was chosen for further isolation work.

Preliminary TLC bioassay of the ether extract

Initially, the Et_2O extract was separated by TLC and each R_f zone was bioassayed (Table 2). Activity on silica gel plates was spread out in two zones. The more polar toxin found at R_f zone 0.11–0.30 was detected by UV, $FeCl_3$–HCl, $CeSO_4$–H_2SO_4, and vanillin–H_2SO_4. A second, more non-polar, zone of toxicity was evident at R_f 0.61–0.70. This zone reacted with all previously mentioned detection reagents except $FeCl_3$. A second bioassay of these zones confirmed activity on cress.

Isolation, characterization and activity of compound 1

Column separation of the Et_2O fraction on a silica SEP-PAK resulted in a spread of activity from the non-polar to moderately polar range of the sample. An additional, extremely polar, inhibitory fraction was eluted later. All fractions with activity in the non-polar range reacted blue with $FeCl_3$ suggesting that this compound may be a good indicator of activity. To narrow the range of polarity of the sample and improve separation, a second column was run with two SEP-PAKs in series. TLC of the resultant fractions indicated that the higher R_f toxin eluted in the first volume of CH_2Cl_2–EtOAc (9:1) and the lower R_f toxin was eluted in the second volume. Later fractions also contained traces of the lower R_f toxin. After sample concentration and storage at $-70°$ overnight, the fraction containing the greatest quantity of the lower R_f toxin (compound 1) began to crystallize.

Compound 1 reacted to form a blue complex with $FeCl_3$ which is specific for phenols and hydroxamic acids. The UV spectrum of 1 in MeOH showed two major absorption bands at 281 nm and 254 nm. Upon addition of base (1 drop of 10% KOH in MeOH), the spectrum shifted to 301 and 217 nm supporting an enolic or phenolic type compound. NMR indicated the compound had a base structure of C_8H_7. The M^+ was determined to be 181 via EIMS, which also indicated an odd number of nitrogens in the compound. A library search of compounds with similar fragmentation patterns resulted in retrieval of a compound, 2(3*H*)-benzoxazolinone (BOA), structurely related to the unknown. The molecular for-

Table 1. Activity of rye (*Secale cereale* L.) shoot herbage on cress (*Lepidium sativum* L.) root elongation

Fraction*	Percentage of crude†	I_{50} (mg)‡	Units§
Initial aqueous‖	—	1.90	13 000
Acetone ppt	17.0	1.64	2550
Hexane	0.1	0.68	47
Et_2O	1.0	0.15	1580
CH_2Cl_2	0.2	0.38	144
EtOAc	0.6	0.29	498
Final aqueous	55.0	3.80	3570

*Obtained from extraction and partitioning of rye shoot herbage.

†73.9% of crude aqueous extract was recovered from initial separations.

‡Quantity of material necessary for 50% inhibition of root elongation.

§Based on weight (mg) of fractions recovered (per 1000 ml crude aqueous extract) per weight (mg) of fraction necessary for 50% inhibition of root elongation (mg).

‖49% of dried tissue weight was extracted with H_2O in 24 hr at 4°.

Table 2. Cress (*Lepidium sativum* L.) bioassay of TLC separation of the Et_2O extract

R_f*	Root length† (% of control)	± s.d.‡
Load zone	60	5
0–0.10	84	5
0.11–0.20§	4	1
0.21–0.30§	5	1
0.31–0.40	22	3
0.41–0.50	51	12
0.51–0.60	24	19
0.61–0.70‖	2	0
0.71–0.80	128	3
0.81–0.90	54	5
0.91–1.0	124	11

*TLC parameters: Whatman LK6D, silica gel, prewashed in EtOAc; the Et_2O extract was applied to plates which were developed (15 cm) in EtOAc.

†Bioassay parameters: treatments consisted of 6 channels of silica from the 1.5 cm R_f zone where 2.5 mg of extract was applied per channel; control treatments consisted of 6 channels of silica from unloaded plates similarly developed.

‡Standard deviation of root length relative to control based on 2 replications of 10 seeds per treatment.

§TLC: UV 254—absorbs; UV 366—fluoresce blue; $CeSo_4$–H_2SO_4—orange; vanillin–H_2SO_4—tan; $FeCl_3$–HCl—blue.

‖TLC: UV 254—absorbs; $CeSO_4$–H_2SO_4—purple; vanillin–H_2SO_4—many spots.

mula was subsequently determined to be $C_8H_7NO_4$ and the compound was identified as 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one or DIBOA (Fig. 2b).

DIBOA was originally identified primarily via non-spectral methods by Virtanen and Hietala [13]. The structure was subsequently confirmed through synthesis of the compound [14]. Compound 1 and DIBOA both react to form a blue complex with $FeCl_3$. Virtanen and Hietala [13] determined the major UV absorption bands of DIBOA in EtOH at 225 and 282 nm which correspond to those obtained for 1 in MeOH (254 and 281 nm). Due to lack of availability of similar EIMS for DIBOA in the literature, the spectra of compound 1 was compared with that obtained for 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) by Klun *et al.* [15]. DIMBOA differs by the presence of a methoxyl group on carbon-7 of the aromatic ring which should result in a similar mass spectrum in which fragments differ by 30 amu. The major fragments of DIMBOA at *m/z* 211, 195, 193, 165 do differ by 30 from the DIBOA ions of *m/z* 181, 165, 163, 135 confirming the structure previously determined.

The pure compound was subsequently assayed for activity on cress (Fig. 3). Increasing concentrations of DIBOA reduced both root and shoot length of cress, but showed little effect on seed germination at these concentrations. Cress root growth was more inhibited than shoot growth by DIBOA. I_{50} values for root and shoot length by DIBOA were *ca* 0.37 mM (100 μg) and 0.70 mM (190 μg), respectively. Both root and shoot growth of cress were stimulated by low concentrations (0.09 mM) of DIBOA.

Isolation, characterization and activity of compound 2

Additional separation of the crude Et_2O extract was directed toward isolation of the active, higher R_f, com-

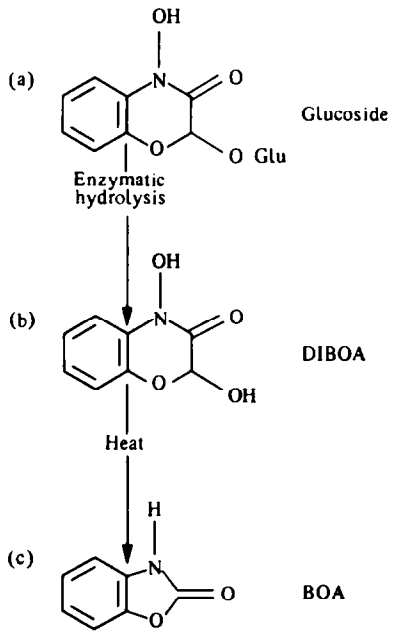

Fig. 2. Structures for (a) glycoside, previously reported in non-injured plants, (b) DIBOA and (c) BOA isolated from rye residues.

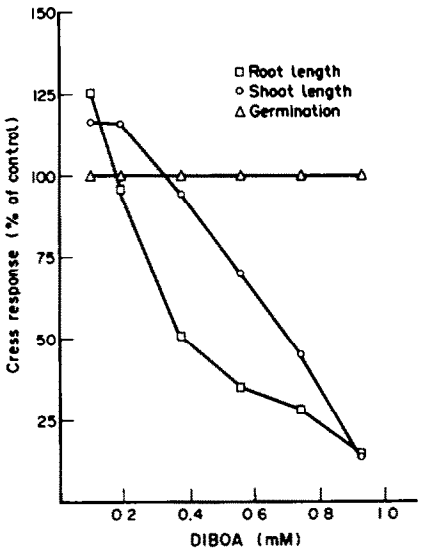

Fig. 3. Response of cress (*Lepidium sativum* L.) to various concentrations of DIBOA after 72 hr incubation. LSD relative to control for root length (5–20%), shoot length (5–12%), and germination (NS).

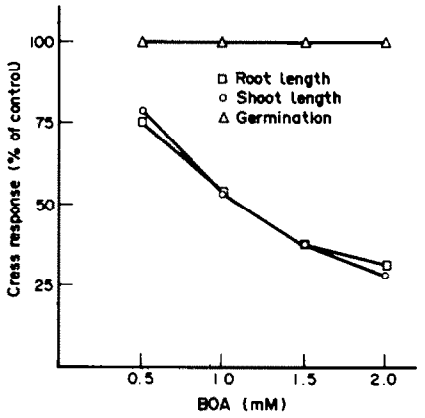

Fig. 4. Response of cress (*Lepidium sativum* L.) to various concentrations of BOA after 72 hr incubation. LSD relative to control for root length (5–16%), shoot length (5–11%), and germination (NS).

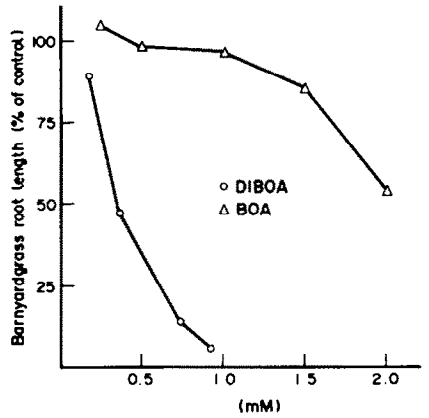

Fig. 5. Response of barnyardgrass (*Echinochloa crusgalli* L. Beauv.) to various concentrations of DIBOA and BOA after 72 hr incubation. LSD relative to control for both DIBOA and BOA (5–33%).

pound. Initially, the higher R_f zone (0.61–0.70) was separated from **1** by TLC. This sample contained many compounds when evaluated in a different solvent system on TLC and was active in the absence of **1**. Bioassay of the TLC plate indicated two zones of activity in the sample, of which one specifically reacted with $CeSO_4$.

Subsequently, the crude Et_2O extract was separated on a silica gel 'flash' column. All fractions were concentrated, evaluated by TLC, and bioassayed at 250 μg. Most of the activity was associated with **1** which specifically reacted blue with $FeCl_3$. Addition of hexane to the first fraction resulted in formation of insoluble crystals. The crystals were detected with $CeSO_4$, active when tested at higher concentrations, and, therefore, identified.

As **1** also reacted with $CeSO_4$, there was a good possibility that **1** and **2** were related. Compound **2** was hypothesized to be BOA (Fig. 2c), which is commercially available. For preliminary comparisons, the two compounds were co-chromatographed by TLC. They behaved similarly on silica gel when developed with several solvent systems and reacted similarily with detection reagents. Spectrometry further supported BOA as **2**. NMR indicated a $C_7H_5^-$ base for the compound. The M^+ was determined to be 135 and the fragmentation pattern resembled **1**. Upon comparison with a published spectrum [16], **2** was confirmed to be 2(3*H*)-benzoxazolinone ($C_7H_5NO_2$), first identified and implicated as an antifusarium factor in rye seedlings by Virtanen and Hietala [17].

BOA similarly reduced cress root and shoot growth (Fig. 4), but was less active than DIBOA. The I_{50} values for root and shoot inhibition in response to BOA are *ca* 1.05 mM (212 μg) and 1.24 mM (250 μg), respectively. BOA, like DIBOA, had little effect on seed germination at these concentrations.

Activity of pure compounds was also determined with a second test species, barnyardgrass (*Echinochloa crusgalli* L. Beauv.), which had previously shown growth reductions in field and greenhouse studies with rye residues (Fig. 5). The I_{50} for DIBOA on root length of barnyardgrass was *ca* 0.36 mM (98 μg) which is about equal to the I_{50} on cress. In contrast, the I_{50} for BOA is greater than 1.98 mM (400 μg).

DISCUSSION

Benzoxazinones were first identified in rye [17], but are now known to occur in many plant species [18]. While rye contains primarily DIBOA, others, including corn (*Zea mays* L.) and wheat (*Triticum durum* L.), contain higher concentrations of the methoxylated compound, DIMBOA [18]. The chemical differences between plant species are probably more quantitative than qualitative [19]. These compounds were originally identified and postulated as 'resistance' factors in plants, since they are found in several varieties of crop plants and exhibit

antifungal and insectistat properties [20]. Willard and Penner [21] have extensively reviewed the role and importance of benzoxazinones as inhibitors of pathogen and insect activity. DIMBOA contributes to the tolerance of corn to triazine herbicides [21–24].

Benzoxazinones can be regarded as complex hydroxamates which occur as glycosides in intact plants [19, 25–28] and seeds [29]. In rye, the sugar moeity is attached to the oxygen of carbon-2 (Fig. 2a). The aglycones are released through action of the hydrolytic enzyme, β-glucosidase, when plant tissue is injured. Upon hydrolysis, the aglycones tend to rearrange forming oxazolinones [28]. The amount of these compounds present varies greatly depending upon species, age, and plant part analysed [18, 30–34].

The biological properties of DIMBOA, and its related compounds, have been studied much more extensively than DIBOA, although its herbicidal activity is cited only indirectly. Several years ago, Hofman and Hofmanova [27] concluded that DIMBOA from corn could not occur in the non-conjugated form because corn plants grown in its presence ceased growth and later died. Wolf *et al.* [29] recently discovered the glycoside of DIBOA in seeds of a dicotyledonous plant, bear's breech (*Acanthus mollis* L.). Enzymatic hydrolysis yielded DIBOA which completely inhibited velvetleaf (*Abutilon theophrastis* Medic.) germination at a concentration of 2 mM. BOA was less active and the glucoside had little effect on germination. The glucoside did cause 'unusual' growth in velvetleaf seedlings.

Our drying and extraction techniques have precluded isolation of the glycoside which is found in intact plants. As a result we are not sure that the glycoside contributes to phytotoxicity. There is some evidence for reduced activity with the glycoside [29], which may explain how rye safely stores the more toxic aglycone during normal growth. Many of the literature citations regarding phytotoxicity have been reported for green rye turned into the soil [2, 4, 6, 35] and rye residues in no-till cropping systems [9, 10, 36]. As both of these situations result in injury to the rye plant, they may induce activity of the β-glucosidase and release of the toxic aglycone.

In our bioassays, DIBOA was by far the most active chemical and appeared to be present in high concentrations. This confirms previous reports of high concentrations of DIBOA in rye [37]. As our previous no-till and soil/residue bioassays implicate a chemical interference by rye residues, we believe DIBOA and BOA play a role in the allelopathic activity exhibited by rye. Further, it is possible that benzoxazinones, as a group, could be important in allelopathy exhibited by other cereals in similar no-till situations.

EXPERIMENTAL

Extraction. 'Wheeler' rye shoot tissue for extraction was greenhouse grown [500 μE/m/sec; 27°/18° (day/night)] for 35 days, cut, dried at 50°, and ground through a 40 mesh screen in a Wiley mill. Extraction and partitioning procedures are presented in Fig. 1. All initial extractions were based on 50 mg tissue per ml solvent and all fractions were saved for bioassay.

Bioassay. A cress seed germination and root growth bioassay was used to detect activity of all fractions. For bioassay of aq. fractions, known wts of material were applied in 1.5 ml H_2O to Whatman No. 1 filter paper in Petri dishes (60 by 15 mm). For bioassay of organic soluble fractions, filter papers were impregnated with equivalent, known sample wts applied in 0.5 ml solvent. Pure solvent similarily applied to filter paper, and H_2O, were used as controls. After solvent evaporation, 1.5 ml of H_2O was added to all dishes. After application of sample to filter paper, all bioassay procedures for aq. and organic fractions were similar. Ten seeds were added and dishes were dark incubated in a high humidity growth chamber (27°). After 72 hr, root lengths were measured. Values of 50% inhibition of root growth (I_{50}) were calculated from dose response bioassays.

Isolation of unknown compounds. The Et_2O extract was determined to be most active in the preliminary assays. It was further separated by TLC on silica gel and bioassayed to locate regions of activity (Table 2). Each R_f zone was scraped and added to Petri dishes for bioassay. Plate scrapings from an equivalent number of unloaded channels treated similarily were used as controls. Results of this bioassay indicated two zones of activity (lower R_f zone—1; higher R_f zone—2) and were used as a base for activity found in column separations.

The Et_2O extract was further separated by column chromatography for isolation and identification of the two active zones. For 1, the Et_2O extract was applied to a silica SEP PAK and eluted with increasing volumes of EtOAc in CH_2Cl_2, followed by MeOH. After bioassay and TLC detection, crystals of 1 were obtained with a second separation on two silica SEP PAKs in series. Crystals of 2 were obtained after separation of the Et_2O on a silica 'flash' column eluted with increasing proportions of EtOAc in hexane. All fractions obtained during column separations were bioassayed for activity on cress and qualitatively evaluated by TLC. Pure compounds were tested for toxicity to both cress and barnyardgrass.

Identification of pure compounds. The biologically active unknown compounds (1 and 2) were subjected to mass, ^1H NMR, and ^{13}C NMR spectrometry for identification. UV spectrometry was also used to identify 1.

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