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Table: 1, Figs : 5

Isolation and characterization of allelopathic compounds from the indigenous rice variety 'Boterswar' and their biological activity against *Echinochloa crus-galli* L

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ABSTRACT

Aqueous methanol extracts of the Bangladesh indigenous rice (*Oryza sativa* L.ssp. *indica*) variety 'Boterswar' inhibited the germination and seedling growth of *Lepidium sativum* L. and *Echinochloa crus-galli* L. Beauv which suggested that this variety may contain phytotoxic substance(s). Four biologically active compounds, syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde), (-) loliolide, 3*β*-hydroxy-5*α*,6*α*-epoxy-7-megastigmen-9-one and 3-hydroxy-*β* –ionone, were isolated from the ethyl acetate phase using several chromatographic steps. The chemical structures of the compounds were determined through electrospray ionization and spectroscopic analyses. The biological activity of these compounds showed that concentration > 10 µM significantly inhibited the root and shoot growth of *E. crus-galli* seedlings, and the I_{50} (50% growth inhibition) values ranged from 16.03 to 27.23 µM and 23.94 to 75.49 µM for root and shoot growth, respectively. The four compounds synergistically suppressed the growth of *E. crus-galli* more strongly than the individual compounds. Thus, the indigenous rice 'Boterswar' has potential use for weed management and this indigenous variety could be used to develop a new commercial rice variety that may suppress weeds.

Key words. Allelochemicals, Bangladesh local rice, *Echinochloa crus-galli*, germination, *Lepidium sativum*, *Oryza sativa*, phytotoxicity, root growth, shoot growth, weed management

INTRODUCTION

Rice (*Oryza sativa* L.) is a major staple crop worldwide particularly in Bangladesh. Weeds are the key biotic threat to rice productivity hence, in rice production herbicides are used (36), but their harmful impacts makes it desirable to search for other eco-friendly weed management options such as allelopathy (1,43,53). Allelopathy refers to the direct or indirect harmful or beneficial effects of one plant to another plant from the release of biochemicals, known as allelochemicals into the environment (47). Thus, allelopathy is a phytotoxic interference in most circumstances (49). Allelochemicals are present in all plant parts such as the root, stem, leaf, bud and flower (21). Under certain conditions, allelochemicals are released into the environment as exudates from living plants and from the decomposition of plant residues in abundant quantities to inhibit the growth of adjacent and successive plants (11,51). Herbicide use rates in rice can be minimized by exploiting

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weed suppressive allelopathic rice varieties (14) which may not add any extra cost (25). Many rice varieties have been studied (7,30,44) and it has been found that some are weed suppressive (25). An allelopathic rice variety could release water-soluble chemicals which can suppress the growth of adjacent and successive weeds (57). Many researchers have endeavored to recognize that allelochemicals are released from rice (31,48). Among the Bangladeshi rice varieties 2,9-dihydroxy-4-megastigmen-3-one from 'BR17' (50) and 9-hydroxy-β-ionone and 9-hydroxy-4-megastigmen-3-one from 'kartikshail' (27) were identified as allelochemicals. Several studies showed that a conventional breeding method could possibly be used to develop commercially cultivable allelopathic rice varieties (3,35). The discovery of the allelopathic compounds will allow the efficient production of more allelopathic rice varieties through conventional breeding or biological-based genetic modifications, which may be less dependent on herbicides (1,12). Another approach involves the isolation, characterization, and elucidation of the specific mode of action of phytotoxic natural products from allelopathic rice to develop eco-friendly herbicides (5,23). In addition, allelopathic rice can initiate its weed resistance mechanism through the production and release of allelochemicals (32). On the other hand, a specific plant response to allelochemicals is to trigger a cell death cascade in susceptible plants, while these allelochemicals are not very toxic themselves and they induce a toxic response (54).

Kong (33) reported that a few rice varieties produce and release allelochemicals into the paddy fields and suppresses the growth of adjacent or successive weeds. There are eight thousand indigenous rice varieties in Bangladesh, and farmers still cultivate more than one thousand rice varieties (19). Determination of allelochemicals in Bangladesh rice varieties and their use for weed control could be advantageous for those who primarily depend on human labour or herbicide uses. We previously reported that the Bangladesh indigenous rice variety 'Boterswar' had the highest allelopathic potentiality out of several test species and weeds (41), which suggested that this variety has a higher concentration of allelochemicals. Our objectives in current research were to isolate and identify the allelopathic compound(s) present in 'Boterswar' indigenous rice variety.

MATERIALS AND METHODS

The Bangladesh indigenous rice (*Oryza sativa* L. ssp. *indica*) variety 'Boterswar' was grown hydroponically (48) in glasshouse at the University of Ryukyus for 55 days. At this stage, the average tiller per hill was 16, and the plants developed an extensive and strong root system and obtained an average of 143 g of fresh biomass per hill. After harvesting the rice plants were stored at -20°C until use. Seeds of *L. sativum* L. were purchased from the Green Field Project (Kumamoto, Japan) and seeds of *E. crus-galli* L. were collected from the rice field of the Okinawa Agricultural Research Centre, Nago, Okinawa, Japan. Because of its known germination behavior, *L. sativum* was used as a model test plant for the bioassay (55), and *E. crus-galli*, which has developed resistance to many herbicides (17), is considered one of the worst weeds in rice production in 61 countries, including Bangladesh (18).

Aqueous methanol extraction

Extracts were prepared using the method described by Salam *et al.* (50) for isolating allelochemicals. A total of 3 kg of fresh rice plants (roots, stems and leaves) were

blended and extracted with 15 L of 80% (v/v) aqueous methanol for 48 h. The extract was filtered through one layer of filter paper (no. 2; Toyo Roshi Kaisha Ltd., Tokyo, Japan), and the filtrate was extracted again with the same volume of methanol for another 48 h and filtered then, both filtrates were stirred and concentrated at 40°C *in-vacuo* to prepare the aqueous concentrate (100 mL).

Plant extract bioassay

Rice plants (100 g fresh weight) were extracted and concentrated as described above for bioassay experiment. An aliquot of the aqueous concentrate (1, 3, 10, 50 and 100 mg fresh weight [FW] equivalent extract per mL final assay concentration) was evaporated on an evaporator at 40°C until dry. Then, the dried sample was dissolved in cold methanol (0.2 mL) placed on a sheet of filter paper (no. 2) in a 3 cm Petri-dish, desiccated in a draft chamber and then soaked in 0.8 mL of 0.05% (v/v) an aqueous solution of Tween 20 (polyoxyethylene sorbitan monolaurate, Nacalai, Tesque, Inc., Kyoto, Japan) as a surfactant. For the control treatment, methanol (0.2 mL) was added to a sheet of filter paper in the Petri-dish and evaporated, as described above. Ten seeds of *L. sativum* or *E. crus-galli* were placed on the filter paper and then incubated at 25°C in a dark incubator. Germination was assessed every 12 h with a magnifying glass by counting the germinating seeds, searching for the rupture of the seed coats and the emergence of a radicle ≥ 1 mm (42), until no further seeds germinated (48 h for *L. sativum* and 72 h for *E. crus-galli*). The germination (%) was determined for the control (without extracts) according to methods by Salam *et al.* (50). For the seedling growth bioassay, ten uniform germinated seedlings of *L. sativum* and *E. crus-galli* were placed in the Petri-dishes and then incubated using the aforementioned procedure. The root and shoot lengths of the test species were determined after 48 h of incubation. The growth inhibition (%) was calculated with respect to control (without extracts) seedlings.

Purification of active substances in the ethyl acetate fraction

According to Salam *et al.* (50), the aqueous concentrate was adjusted to pH 7.0 with 1 M phosphate buffer, separated five times against the same volume of ethyl acetate to obtain aqueous and ethyl acetate fractions. The biological activity of the aqueous and ethyl acetate fractions was determined by germination and seedling growth bioassays using *L. sativum* and *E. crus-galli*. The active ethyl acetate fraction was evaporated until dryness after standing with added anhydrous Na_2SO_4 overnight and then chromatographed on a column of silica gel (70 g, silica gel 60N, 70-230 mesh; ASTM, Kanto Chemical Co., Inc., Tokyo, Japan), eluting with a stepwise gradient of ethyl acetate (10% per step, v/v; 150 mL per step) and methanol (300 mL) in *n*-hexane, affording 11 fractions. The biological activity of the collected fractions was determined using the *L. sativum* germination bioassay according to the above procedure, and complete inhibition was found in fractions obtained by elution with 70-80% ethyl acetate in *n*-hexane. After evaporation, the concentrate was filtered through a column of Sephadex LH-20 (60 g, GE Healthcare Bio-Sciences AB SE-75184 Uppsala, Sweden), eluting with 20, 40, 60, and 80% (v/v) aqueous methanol (150 mL per step) and methanol (300 mL). The most active fraction was eluted with 60% aqueous methanol and subsequently evaporated until dryness. The concentrate was dissolved in 20% (v/v) aqueous methanol (2 mL) and loaded onto a reverse-phase C_{18} Sep-Pak cartridges (Waters Corporation, Milford, Massachusetts, USA) and purified with 20, 40, 60, 80% (v/v) aqueous methanol and methanol (15 mL per step).

The most active fraction was eluted with 20% aqueous methanol and evaporated until dryness. The concentrate was finally purified by C_{18} reversed-phase HPLC (COSMOSIL 5 C_{18}- AR-II; Nacalai Tesque, Inc., Kyoto, Japan), eluting at a flow rate of 3 mL/min with 50% aqueous methanol and detecting at 220 nm. Complete inhibition was detected for four peaks that eluted at 16.0, 19.0, 20.0 and 24.0 min as colorless substances. Mass Spectrometry with electro-spray ionization (ESI-MS) analysis was carried out on a Waters mass specttrometer. NMR spectra were measured in $CDCl_3$ on Bruker NMR spectrometers (500 MHz for ^{1}H and 125 MHz for ^{13}C). All chemical shifts were reported relative to tetramethylsilane (TMS). Optical rotation was measured in chloroform on a JASCO P-1010 polarimeter.

Bioassay of the isolated compounds

The isolated compounds were dissolved in methanol to prepare the concentrations of 1, 3, 5, 10, 30, 50, 100, 300, 500 and 1000 μM for each compound and 0.1, 0.3, 1, 3, 5, 10, 30, 50, 100 and 300 μM for a mixture of the compounds at a ratio of 1:1:1:1. The biological activity against *E. crus-galli* seedlings was examined using the above procedure.

Statistical analysis

To compare the results, the bioassays were carried out twice using a completely randomized design with three replicates. Significant differences between the treatments and controls were analyzed by Fisher's Protected Least Significant Difference test for each *L. sativum* and *E. crus-galli* species. The Type I error was set at 0.01 for all the statistical comparisons. The I_{50} (concentration of approximate 50% inhibition of the growth rate) value in the assay was analyzed from the regression equation of the concentration curves.

RESULTS AND DISCUSSION

The shoots of rice are the major site of the synthesis or accumulation of allelochemicals, while roots are a pathway of release (29); alternatively, allelochemicals are leached directly from leaves (9). Therefore, the whole rice plant was chosen as the source of allelochemicals in these experiments.

Phytotoxic effects of aqueous methanol and ethyl acetate extracts

Increased concentrations of the aqueous methanol extracts inhibited the germination and growth of the test species with very low (1 mg DW equivalent per mL) caused stimulation of test species (Figs. 1.A, B, 2.A, B). At 100 mg DW equivalent extract per mL *L. sativum* germination was completely inhibited, whereas *E. crus-galli* germination was 16.39% with respect to control (Fig. 1.A, B). At the same concentration, the root growth of *L. sativum* and *E. crus-galli* was 5.90 and 4.10% that of the control, respectively, while the shoot growth of both species was completely inhibited (Fig. 2.A, B). The fractionated ethyl acetate extract showed significant activity and the ethyl acetate extract treated plants showed severe root browning (data not shown). Germination and growth bioassays are primary tools for assessing phytotoxic activity (22,52) in which allelopathic effects can be observed under controlled laboratory conditions (46). In these studies, both stimulatory and inhibitory effects were found. Rice (47) reported how allelopathic activity that has a stimulatory effect at a lower concentration and an inhibitory

effect at a higher concentration can occur due to allelopathic compounds. Inderjit and Duke (20) also found different allelopathic responses for asymmetrical test plants due to the different selectivity of allelopathic substances.

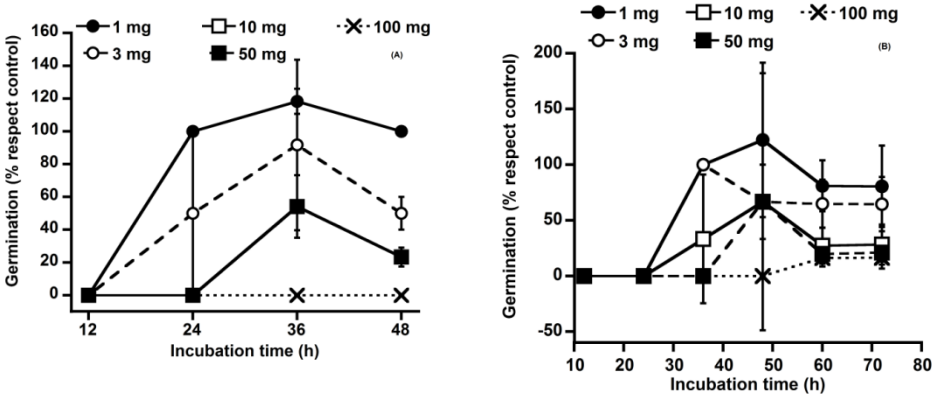

Figure 1. Effects of aqueous methanol extract of "Boterswar" rice plants on the germination of *L. sativum* (**A**) and *E. crus-galli* (**B**) at different concentration. *Bars* represent ±SD of values obtained from three biological replicates

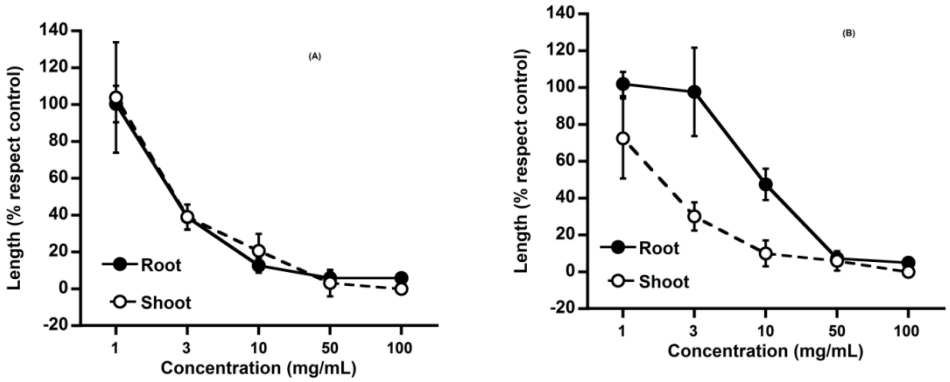

Figure 2. Effects of the aqueous methanol extracts of "Boterswar" rice plants on the shoot and root growth of *L. sativum* (**A**) and *E. crus-galli* (**B**) at different concentration. *Bars* represent ±SD of values obtained from three biological replicates

Structural elucidation of isolated compounds

Four biologically active compounds were obtained from the repeated column chromatography of the aqueous methanol extracts of 'Boterswar' indigenous rice.

Compound 1: It had a molecular formula of $C_9H_{10}O_4$ (LR-ESI-MS *m/z*183 $[M+H]^+$) and a specific rotation of $[\alpha]_D^{23}$+0.04 (*c*0.01, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$), δ 9.82 (^{1}H, s, H-7), 7.15 (2H, s, H-2 and 6), 3.97 (6H, s, 2×OCH_3); ^{13}C-NMR (125 MHz $CDCl_3$), δ 128.6 (s, C-1), 140.9 (s, C-2 and 4), 147.4(s, C-3 and 5), 106.8 (d, C-2 and 6), 190.7(d, C-7), 56.9 (OCH_3). The NMR data are consistent with those reported in Bo Yi *et al.* (2), and we

identified the substance as 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde) (Fig. 3.A).

Compound 2: It had a molecular formula of $C_{11}H_{16}O_3$ (LR-ESI-MS *m/z*197 $[M+H]^+$) and a specific rotation of $[\alpha]_D^{20}$ -65.7 (*c* 0.01, $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.27 (3H, s, H-9), 1.47 (3H, s, H-8), 1.52 (dd, *J* = 13.7 and 3.9 Hz, H-7), 1.76 (dd, *J* = 13.7 and 4.1 Hz, H-5), 1.78 (3H, s, H-10), 1.98 (dt, *J* = 2.4 and 14.0 Hz, H-7), 2.54 (dt, *J* = 2.8 and 14.0 Hz, H-5), 4.30 (m, H-6), 5.69 (s, H-3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 26.9 (C-10), 26.4 (C-9), 30.6 (C-8), 36.0 (C-4), 45.4 (C-7), 47.0 (C-5), 66.3 (C-6), 87.1 (C-7a), 112.4 (C-3), 172.2 (C-2), 183.0 (C-3a). The data were compared with the data reported by Park *et al.* (45), and the substance was identified as (-)-loliolide (Fig. 3.B).

Compound 3: It had a molecular formula of $C_{13}H_{20}O_3$ (LR-ESI-MS *m/z*225 $[M+H]^+$ and 247 $[M+Na]^+$) and a specific rotation of $[\alpha]_D^{25}$ -10.6 (*c*0.01, $CHCl_3$). The ^{1}H-NMR ($CDCl_3$, 500 MHz) spectrum of the compound showed two coupled olefinic protons [δ_H 7.03 and 6.29 (each 1H, d, *J*=15.6 Hz), an oxygenated methane proton [δ_H 3.91(1 H, m)], and four methyl groups [δ_H 2.28, 0.98 (each 3H, s) and 1.20 (6H, s)]. Its ^{13}C NMR spectrum revealed 13 carbon signals: a conjugated ketone (δ_C 197.5), one double bond [δ_C 142.4 and 132.7] and an oxygenated methine (δ_C 64.1). An extra four methyl groups, two methylenes, and three quaternary carbons were also found. Except for one double bond, a conjugated ketone, and four methyl groups, the isolated compound had a six-membered ring and was presumed to be a megastigmen derivative. In the HMBC spectrum, the proton signal at δ_H 6.29 (H-8) correlated with the carbon signals at δ_C 69.6 (C-6) and 197.5 (C-9), while the methyl proton signals at δ_H 0.98 (H_3-11) and 1.20 (H_3-13) could be correlated with the carbon signal at δ_C 69.6 (C-6). Thus, the 3-oxobutenyl group was located position C-6. Likewise, based on the HMBC and ^{1}H-^{1}H COSY spectra, the hydroxyl group was attached at C-3. Acetylation of 5 afforded the monoacetylate (5a), and the proton signal of H-3 (δ_H 4.90, in 5a) was downfield correlated with that of 5 (δ_H 3.91, in 5). Thus, the hydroxyl group was assigned to the C-3 position. Therefore, the structure was 3*β*-hydroxy-5*α*, 6*α*-epoxy-7-megastigmen-9-one (Fig. 3.C) and this corresponds to the data reported by Duan *et al.* (8).

Compound 4: It had the molecular formula of $C_{13}H_{20}O_2$ (LR-ESI-MS *m/z*209 $[M+H]^+$) and a specific rotation of $[\alpha]_D^{25}$ -5.4 (*c*0.01, $CHCl_3$). The ^{1}H NMR spectrum of the substance (500 MHz, $CDCl_3$, TMS as the internal standard) revealed δ values of 7.32 (1H, d, *J*=16.2 Hz, H-7), 6.13 (1H, d, *J*=16.2 Hz, H-8), 3.92 (1H, m, H-3), 2.40 (1H, dd, *J*=17.4 and 5.4 Hz, H-4a), 2.30 (3H, s, H-10), 2.06 (1H, dd, *J* =17.4 and 9.6 Hz, H-4b), 1.79 (3H, s, H-13), 1.77 (1H, dd, *J*=12.6 and 2.4 Hz, H-2a), 1.46 (1H, dd, *J* =12.6 and 12.0 Hz, H-2b), 1.14 (3H, s, H-11), and 1.11 (3H, s, H-12). The ^{13}C NMR spectrum of the substance (125 MHz, $CDCl_3$, TMS as the internal standard) contained δ values of 201.3 (C, C-9), 144.5 (CH, C-7), 136.9 (C, C-6), 134.3 (C, C-5), 133.3 (CH, C-8), 64.9 (CH, C-3), 49.6 (CH_2, C-2), 43.5 (CH_2, C-4), 37.8 (C, C-1), 30.8 (CH_3, C-11), 28.9 (CH_3, C-12), 27.3 (CH_3, C-13), and 21.8 (CH_3, C-10). These data are consistent with previous studies (6,16,26) and the substance was identified as 3-hydroxy-*β*-ionone (Fig. 3.D).

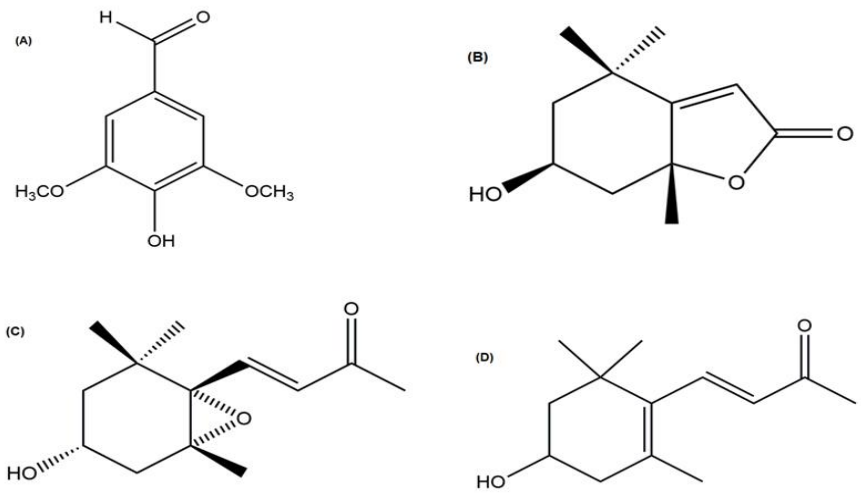

Figure 3. Structures of isolated allelochemicals viz., 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde) (**A**), (-)-loliolide (**B**), 3*β*-hydroxy-5*α*,6*α*-epoxy-7-megastigmen-9-one (**C**) and 3-hydroxy-*β*–ionone (**D**) from the Bangladesh indigenous rice var. 'Boterswar'

The endogenous concentrations of syringaldehyde, (-)-loliolide, 3*β*-hydroxy-5*α*, 6*α*-epoxy-7-megastigmen-9-one, and 3-hydroxy-*β*-ionone were at least 5.86, 8.16, 4.16 and 1.28 µmol/kg, respectively because 3.2, 4.8, 2.8 and 0.8 mg of the respective substances (MW 182, 196, 224 and 208, respectively) were isolated from 3 kg of fresh rice plants. These isolated and identified compounds were phenolic compounds which are one of the most common forms of allelochemicals (39) and have the potential to inhibit seed germination and seedling root and shoot elongation (37). Lin *et al*. (38) isolated syringaldehyde from *Ophiopogon japonicus* K**.** and described its allelopathic effect on *E. crus-galli*. Reigosa and Pazos-Malvido (46) also observed that the radicle length of *Arabidopsis thaliana* was inhibited by syringaldehyde both with and without nutrient bioassays. Grabarczyk *et al.* (15) noted that (-) loliolide provides defense against insect herbivory and affects the development of certain plants (allelopathic activity). Islam *et al.* (24) also found that loliolide inhibited the seedling growth of *E. crus-galli*. Duan *et al.* (8) isolated 3*β*-hydroxy-5*α*,6*α*-epoxy-7-megastigmen-9-one from *Saussurea medusa* as an immunosuppressive constituent. Kato-Noguchi *et al.* (28) isolated 3-hydroxy-*β*-ionone from the moss *Rhynchostegium pallidifolium* and described it as an allelochemical.

Biological activity of compounds

In the *E. crus-galli* seedling growth bioassay, the effects of four compounds were evaluated on the same basis but different indices were used for the same compounds. The results verified that the four compounds had diverse inhibitory effects on the root and shoot growth of *E. crus-galli* seedlings at the concentrations as low as 10 µM, and the effects increased with increasing concentrations of compounds (Fig. 4.A, B, C, D). The

concentrations causing approximately 50% growth inhibition in the assay (defined as I_{50}) were 27.23 and 35.99, 16.46 and 23.94, 16.03 and 25.50, 26.23 and 75.49 μM for compounds **1**, **2**, **3** and **4** for *E. crus-galli* roots and shoots, respectively (Table 1). The combined effects of the four compounds at concentrations as low as 3 μM showed significant inhibition of the root and shoot growth of *E. crus-galli* seedlings (Fig. 5). The I_{50} values of the compound mixture were 0.97 and 7.58 μM for *E. crus-galli* roots and shoots, respectively (Table 1). Concentration dependent inhibitory activities of the compounds were found on the seedling growth of *E. crus-galli*, which could be attributed to the allelopathic effects. Several studies also showed that growth inhibitory compounds released from rice inhibited the growth of *Cyperus difformis*, *C. iria*, *E. crusgalli*, *Eclipta prostrata* and *Leptochloa chinensis* weeds associated with rice (29,34,40,56). The mixture of the four compounds enhanced inhibition greatly than the individual of the four compounds, which implies that the four compounds may exert synergistic activity to strongly reduce the growth of *E. crus-galli*. The assumptions of Einhellig (10) appear to be realistic in that allelopathic growth inhibition is associated with the combined effects of several compounds. Similar consequences were also stated by many researchers (4,13,27).

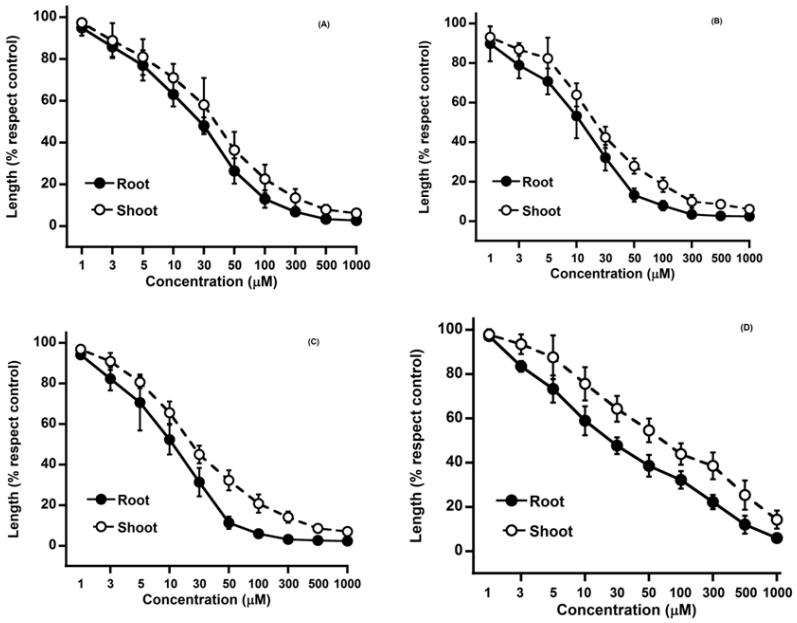

Figure 4. Inhibition of root and shoot growth of *E. crus-galli* s at different concentrations of **1 (A)**, **2 (B)**, **3 (C)** and **4 (D)** isolated compounds. *Bars* represent ±SD of values obtained from three biological replicates

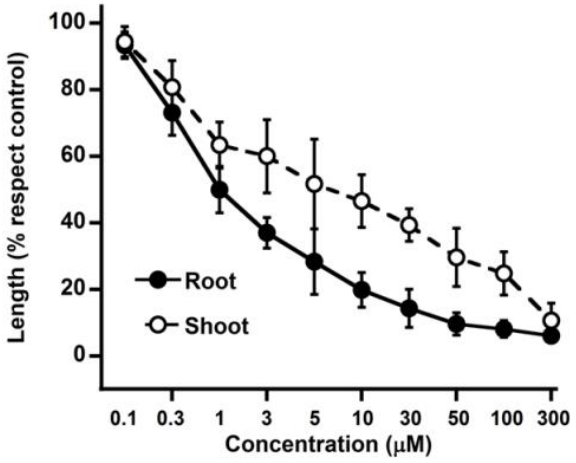

Figure 5. Effects of the mixture of four compounds on the root and shoot growth of *E. crus-galli*. The concentration of 100 μM represents 25 μM syringaldehyde, 25 μM (-)-loliolide, 25 μM 3*β*-hydroxy-5*α*, 6*α*-epoxy-7-megastigmen-9-one, and 25 μM 3-hydroxy-*β*–ionone. *Bars* represent ±SD of values obtained from three biological replicates

Table 1. Regression analyses of dose response curves for effects on *E. crus-galli* growth of different concentrations of the isolated compounds and their mixture

Compound	on root			on shoot		
	Regression Equation	r^2	I_{50} (μM)	Regression Equation	r^2	I_{50} (μM)
1	y = 1.039x + 21.71	0.906	27.23	y = 0.866x + 18.83	0.979	35.99
2	y = 1.403x + 26.91	0.924	16.46	y = 1.446x + 15.38	0.918	23.94
3	y = 1.420x + 27.24	0.916	16.03	y = 1.311x + 16.57	0.942	25.50
4	y = 0.978x + 24.35	0.806	26.23	y = 0.277x + 29.09	0.954	75.49
Mixture	y = 44.23x+ 7.200	0.927	0.97	y = 1.754x + 36.71	0.864	7.58

r^2 = Determination coefficient, I_{50}= concentration required to obtain 50% growth inhibition

The evidence from this study suggested that the syringaldehyde, (-)-loliolide, 3β-hydroxy-5α,6α-epoxy-7-megastigmen-9-one, and 3-hydroxy-β-ionone are allelopathic compounds isolated and identified initially in rice. With respect to the endogenous levels and inhibitory activity, these compounds may impart a competitive benefit to rice plants in the rhizosphere and may inhibit the growth of adjacent and successive weed species. The findings of this research explored the phytotoxic activity of the Bangladesh indigenous rice var. 'Boterswar' which may be useful for weed management under field conditions. Moreover, this variety may be used for the development of a commercially acceptable allelopathic rice variety. Further studies need to assess the dynamics of these compounds, in addition to evaluating their fate and activity under field conditions.

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