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Two novel phytotoxic substances from *Leucas aspera*

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ABSTRACT

Leucas aspera (Lamiaceae), an aromatic herbaceous plant, is well known for many medicinal properties and a number of bioactive compounds against animal cells have been isolated. However, phytotoxic substances from *L. aspera* have not yet been documented in the literature. Therefore, current research was conducted to explore the phytotoxic properties and substances in *L. aspera*. Aqueous methanol extracts of *L. aspera* inhibited the germination and growth of garden cress (*Lepidum sativum*) and barnyard grass (*Echinochloa crus-galli*), and the inhibitory activities were concentration dependent. These results suggest that the plant may have phytotoxic substances. The extracts were then purified by several chromatographic runs. The final purification was achieved by reversed-phase HPLC to give an equilibrium (or inseparable) 3:2 mixture of two labdane type diterpenes (compounds **1** and **2**). These compounds were characterized as (*rel* 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*S*,16*R*)-6-acetoxy-9,13;15,16-diepoxy-15-hydroxy-16-methoxylabdane (**1**) and (*rel* 5*S*,6*R*,8*R*,9*R*,10*S*,13*S*,15*R*,16*R*)-6-acetoxy-9,13;15,16-diepoxy-15-hydroxy-16-methoxylabdane (**2**) by spectroscopic analyses. A mixture of the two compounds inhibits the germination and seedling growth of garden cress and barnyard grass at concentrations greater than 30 and 3 μM, respectively. The concentration required for 50% growth inhibition (I_{50}) of the test species ranges from 31 to 80 μM, which suggests that the mixture of these compounds, are responsible for the phytotoxic activity of *L. aspera* plant extract.

Introduction

Phytotoxic substances are released into the surrounding environment from both above and below ground parts of phytotoxic plants through a number of processes (Rice, 1984; Weir et al., 2004). Upon release, these substances inhibited the germination and growth of other adjacent and/or succeeding plants, or even the secreting plant itself by affecting their many physiological properties (Weir et al., 2004; Yu et al., 2003). These phytotoxic substances also have the potential to modify rhizosphere soil properties including microbial biomass carbon and microbial community (Zhou et al., 2013), which may affect the properties of adjacent and/or succeeding plant species (Callaway and Aschehoug, 2000). Therefore, those phytotoxic plants especially their phytotoxic substances have received special attention due to their agricultural potential to develop natural herbicides for eco-friendly weed management strategies (Duke et al., 2000; Macías et al., 2007).

Leucas aspera is a medicinally important herbaceous plant belonging to Lamiaceae (Labiatae) family. It grows as a competitive weed in high land crop fields, homesteads, fallow lands, and along the roadsides of both tropical and temperate Asia, and Africa. The plant is also well-known to traditional healers due to its antioxidant, analgesic-antipyretic, anti-rheumatic, anti-inflammatory, anti-bacterial, anti-fungal, anti-venom, larvicidal and many other medicinal properties (Prajapati et al., 2010; Srinivasan et al., 2011). A number of chemical constituents have been isolated and characterized from the different parts of *L. aspera* (Prajapati et al., 2010, Srinivasan et al., 2011). Although the phytochemical study of *L. aspera* was started many years ago (Shirazi, 1947), no phytotoxic substance has been reported except for a few preliminary phytotoxicity bioassay studies (Islam and Kato-Noguchi, 2012, 2013a; Roy et al., 2006). In the present work, we isolated and characterized two novel phytotoxic compounds from the aqueous methanol extract of *L. aspera*.

Abbreviations: DW, dry weight; I_{50}, concentration required for 50% growth inhibition.

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Materials and methods

Plant materials

Plants of *Leucas aspera* (Willd.) Link were collected from the field laboratory of Bangladesh Agricultural University, Mymensingh-2202, Bangladesh during July–August 2011. After collection, plants were washed with tap water; sun dried and then kept in a refrigerator at 2 °C until extraction. Garden cress (*Lepidum sativum* L.) and barnyard grass (*Echinochloa crus-galli* (L.) Beauv.) seeds/seedlings were used for bioassays. Garden cress was chosen due to its known seedling growth pattern and higher sensitivity to phytotoxic substances (Xuan et al., 2005), and barnyard grass was chosen as it is the most common paddy weed throughout the world and has developed high resistance against many synthetic herbicides (Heap, 2013).

Extraction and bioassay

Whole plants (leaves, stem and roots) of dried *L. aspera* (1.6 kg) were cut into small pieces and extracted with 9 L of 70% (v/v) aqueous methanol for 48 h. After filtration using one layer of filter paper (No. 2; Advantec, Toyo Roshi Kaisha Ltd., Tokyo, Japan), the residue was re-extracted with same volume of methanol for another 48 h and filtered, and two filtrates were combined and evaporated.

An aliquot of the extract (final assay concentration was 3, 10, 30 and 100 mg dry weight [DW] equivalent extract/mL) was evaporated to dryness at 40 °C, dissolved in methanol and added to a sheet of filter paper (No. 2) in 28 mm Petri dishes. The methanol was evaporated in a draft chamber then the filter paper was moistened with 0.6 mL of 0.05% (v/v) aqueous solution of Tween 20 (polyoxyethylene sorbitan monolaurate; Nacalai Tesque, Inc., Kyoto, Japan). Ten seeds of garden cress or barnyard grass were placed on the filter paper in Petri dishes. The Petri dishes were then incubated in a dark chamber at 25 °C. Germination was considered when the radical emerge by rupturing the seed coat as per Faria et al. (2005), and was measured at every 12 h intervals up to 48 h (the time when germination became constant). The germination percentage to controls (without extracts) was determined as per Islam and Kato-Noguchi (2013b). For growth bioassay, 10 seeds of garden cress or 10 seedlings of barnyard grass (germinated in the darkness at 25 °C for 24 h) were sown in the Petri dishes. The shoot and root lengths of the seedlings were calculated at 48 h after incubation in darkness at 25 °C. The percentage length of seedlings was then determined by reference to the length of control (without extracts) seedlings.

Purification of active substance

Whole plants of dried *L. aspera* were extracted as stated above and evaporated with a rotary evaporator at 40 °C to produce an aqueous residue. The aqueous residue was then divided into two equal parts, and each part was adjusted to pH 7.0 with 1 M phosphate buffer and partitioned three times against an equal volume of ethyl acetate to yield aqueous and ethyl acetate fractions. The biological activity of these two fractions was measured by garden cress and barnyard grass growth bioassay.

The ethyl acetate fraction was then separated by silica gel column (60 g of silica gel 60, spherical, 70–230 mesh, Nacalai Tesque, Inc.), eluted stepwise with *n*-hexane containing increasing amounts of ethyl acetate (10% per step, v/v), ethyl acetate, acetone and methanol (300 mL per step). The biological activity of the fractions was determined using garden cress bioassay according to the aforesaid procedure, and inhibitory activity was found in the fraction obtained with 60% *n*-hexane in ethyl acetate. After evaporation, the active residue was applied to Sephadex LH-20 (50 g, GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and eluted with 20, 40, 50, 60, 70 and 80% (v/v) of aqueous methanol and methanol (300 mL per step). The most active fraction eluted at 50% aqueous methanol was dissolved in 20% (v/v) aqueous methanol (1.0 mL) and loaded onto reverse-phase C_{18} cartridges (YMC Co. Ltd., Kyoto, Japan). The cartridge was eluted with 20, 40, 50, 60, 70 and 80% (v/v) aqueous methanol and methanol (30 mL per step). The active fraction obtained from 70% aqueous methanol was finally purified by reverse phase HPLC (HP 3 μm, 4.6 × 250 mm I.D., Inertsil ODS-3, GL Science Inc., Tokyo, Japan) eluted at a flow rate of 0.5 mL/min with 60% (v/v) aqueous methanol and detected at 220 nm with 40 °C oven temperature. Inhibitory activity was found in a peak fraction eluted between 175 and 200 min as a colorless substance. The fraction gave an equilibrium (or inseparable) 3:2 mixture of compounds **1** and **2**. The compounds were characterized by high-resolution ESI mass data, and 1D and 2D NMR spectra.

Compounds **1** and **2**: HRESIMS *m/z* 433.2558 $[M+Na]^+$, Δ = −0.8 mmu (calcd for $C_{23}H_{38}O_6Na$, 433.2566); $[\alpha]_D^{18}$ −28.4° (*c* 0.2, acetone) for a mixture with the ratio of 3:2; ^{1}H NMR of **1** (600 MHz, CD_3OD) δ_H 5.39 (ddd, *J* = 5.5, 5.5, 2.8 Hz, H6), 5.29 (dd, *J* = 5.8, 6.9 Hz, H15), 4.26 (s, H16), 3.41 (s, 3H, 16OCH_3), 2.26 (dd, *J* = 6.9, 12.4 Hz, H14b), 2.19 (m, H11b), 2.14 (dd, *J* = 5.5, 12.4 Hz, H14a), 2.08 (m, H8), 2.02 (s, 3H, H2′), 1.91 (m, H12a), 1.91 (m, H12b), 1.73 (m, H11a), 1.72 (m, H2b), 1.71 (m, H1b), 1.70 (m, H5), 1.66 (m, H7b), 1.51 (m, H2a), 1.45 (ddd, *J* = 3.1, 6.2, 14.4 Hz, H7a), 1.37 (m, H1a), 1.35 (m, H3b), 1.25 (d, *J* = 3.4 Hz, 3H, H20), 1.20 (m, H3a), 1.00 (s, 3H, H19), 0.94 (s, 3H, H18), 0.86 (d, *J* = 6.5 Hz, 3H, H17); ^{13}C NMR of **1** (150 MHz, CD_3OD) δ_C 172.5 (C1′), 106.4 (C16), 97.8 (C15), 94.6 (C9), 90.6 (C13), 72.1 (C6), 54.9 (16OCH_3), 49.7 (C5), 45.2 (C3), 44.1 (C10), 44.0 (C14), 37.9 (C12), 37.7 (C7), 35.1 (C4), 33.6 (C1), 33.4 (C18), 32.9 (C8), 30.0 (C11), 24.2 (C19), 21.9 (C2′), 20.2 (C20), 20.0 (C2), 17.5 (C17); ^{1}H NMR of **2** (600 MHz, CD_3OD) δ_H 5.45 (d, *J* = 5.8 Hz, H15), 5.39 (ddd, *J* = 5.5, 5.5, 2.8 Hz, H6), 4.37 (s, H16), 3.37 (s, 3H, 16OCH_3), 2.54 (dd, *J* = 6.5, 12.7 Hz, H14b), 2.19 (m, H11b), 2.10 (m, H12a), 2.10 (m, H12b), 2.08 (m, H8), 2.02 (s, 3H, H2′), 1.76 (m, H14a), 1.73 (m, H11a), 1.72 (m, H2b), 1.71 (m, H1b), 1.70 (m, H5), 1.66 (m, H7b), 1.51 (m, H2a), 1.45 (ddd, *J* = 3.1, 6.2, 14.4 Hz, H7a), 1.37 (m, H1a), 1.35 (m, H3b), 1.25 (d, *J* = 3.4 Hz, 3H, H20), 1.20 (m, H3a), 1.00 (s, 3H, H19), 0.94 (s, 3H, H18), 0.86 (d, *J* = 6.5 Hz, 3H, H17); ^{13}C NMR of **2** (150 MHz, CD_3OD) δ_C 172.5 (C1′), 108.0 (C16), 97.3 (C15), 94.4 (C9), 89.7 (C13), 72.1 (C6), 54.8 (16OCH_3), 49.7 (C5), 45.2 (C3), 44.1 (C10), 44.1 (C14), 39.3 (C12), 37.7 (C7), 35.1 (C4), 33.6 (C1), 33.4 (C18), 32.9 (C8), 30.0 (C11), 24.2 (C19), 21.9 (C2′), 20.2 (C20), 20.0 (C2), 17.5 (C17).

Bioassay of the isolated compounds

The mixture of these compounds was dissolved in 0.2 mL of methanol to prepare assay concentrations, added to a sheet of filter paper (No. 2) in 28 mm Petri dishes. Ten seeds of garden cress, or 10 seeds (for germination bioassay) or seedlings (for growth bioassay) of barnyard grass were sown on the Petri dishes and biological activity was determined by the aforementioned procedure.

Statistical analysis

All the bioassays were conducted with three replications and repeated twice using a completely randomized design with 10 seeds/seedlings for each determination. Significant differences between treatment and controls were examined by Student's *t*-test for each test plant species. The concentration required for 50% growth inhibition (I_{50}) of the test species in the assay was determined from the regression equation of the concentration response curves.