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Germination inhibitory constituents from *Erigeron annuus*

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Abstract

(5-Butyl-3-oxo-2,3-dihydrofuran-2-yl)-acetic acid was isolated from the flowers of *Erigeron annuus* as one of four germination inhibitory constituents. Its structure was determined by analysis of MS and NMR spectroscopic data. Three known compounds, 3-hydroxy-pyran-4-one, 4-hydroxycinnamic acid, and 3,4-dihydroxycinnamic acid methyl ester were also identified as active constituents. These compounds showed 50% inhibitory effects (IC_{50}) on the germination of lettuce seed at concentrations of 2.13 ± 0.03, 12.85 ± 0.56, 4.97 ± 0.24, and 4.87 ± 0.25 mM, respectively. 4-Hydroxybenzoic acid was used as a positive control, displaying an IC_{50} value of 4.02 ± 0.39 mM.

Keywords: *Erigeron annuus*; Compositae; Germination inhibitors; (5-Butyl-3-oxo-2,3-dihydro-furan-2-yl)-acetic acid

1. Introduction

Plants are known to produce secondary metabolites that affect germination and growth of other plants. This is one of a variety of ways in which certain plants can reduce interspecies competition in their natural habitats. Allelopathy is the term used to describe such interactions (Harborne, 1988). In many cases, such allelochemicals are known to possess selective toxicity toward target species (Baruah et al., 1994). Although conclusive proof of their ecological involvement under natural conditions could only be drawn from carefully designed and detailed investigations, some allelochemicals could have the potential to be developed as effective and environmentally safe agrochemicals (Duke and Abbas, 1995; Rice, 1995).

The genus *Erigeron* is a member of the Compositae (Asteraceae) family and contains more than 400 species. *Erigeron annuus* (L.) Pers., commonly named as annual fleabane, is an indigenous weed from eastern North America widely found in early-successional communities in fields (Bennington and Stratton, 1998). This species is also commonly found all over Korea. In an effort to explore the potential value of secondary metabolites from *E. annuus* as agriculturally useful products, germination inhibitory effects of the extracts from the aerial parts of *E. annuus* were investigated. Bioassay-directed fractionation and further purification of the crude extracts by various chromatographic methods afforded four active compounds **1**–**4**. Details of the isolation, structure elucidation, and biological activities of these compounds will be discussed.

2. Results and discussion

The dried flowers of *E. annuus* were extracted with MeOH, and the resulting organic extract was suspended in H_2O, and sequentially partitioned with *n*-hexane, EtOAc, and *n*-BuOH, which afforded four fractions. Germination inhibitory effects of these fractions were determined with lettuce seeds. The results showed that the EtOAc-soluble fraction from the flowers of *E. annuus* had significant inhibitory effects at the 6000 ppm level. Subsequent bioassay-guided fractionation (normal-phase) on the EtOAc-soluble fraction from the flowers of *E. annuus* yielded an active compound (**2**) and one

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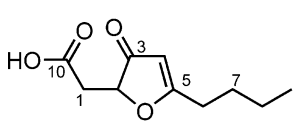

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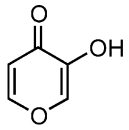

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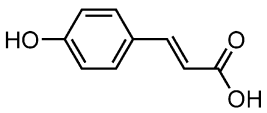

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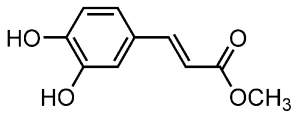

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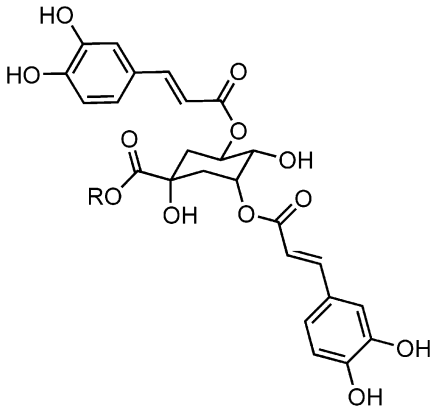

5 R = H
6 R = CH_3

active sub-fraction. Further fractionation of the active sub-fraction by reversed-phase column chromatography, followed by reversed-phase HPLC afforded another active compound (**1**), along with two inactive compounds (**5** and **6**). The EtOAc-soluble fraction from stems and leaves of *E. annuus* was also investigated since the extract also showed significant inhibitory activity. Active compounds **3** and **4** were isolated from this extract by utilizing normal-phase and reversed-phase column chromatography, followed by reversed-phase HPLC.

Compound **1** has the molecular formula $C_{10}H_{14}O_4$, as deduced from the ^{13}C NMR and HRFABMS data, which indicated four degrees of unsaturation. The IR absorption bands at 1655, and 1635 cm^{-1} were suggestive of the presence of two carbonyl groups. DEPT data indicated that 13 of the protons are bound to carbon atoms. Comparison of DEPT results and the molecular formula indicated the presence of a hydroxyl group. The ^1H, ^{13}C, and DEPT data for **1** included signals corresponding to four methylenes, a methyl, an oxymethine, and a carboxylic functionality. The remaining carbon resonances (δ 206.4, 195.8, and 102.7) were strongly suggestive of the presence of a β-oxy-α,β-unsaturated enone moiety based on their chemical shift considerations (Kuster et al., 1994; Terreaux et al., 1995). These features accounted for all but one unsaturation equivalent, which must then represent a ring in the molecule. The structure of **1** was unambiguously deduced by analysis of 2D NMR spectroscopic data (COSY, HMQC, and HMBC). Analysis of the data from COSY experiment enabled the establishment of two isolated spin systems; an *n*-butyl moiety (i.e. C-6 to C-9) and $–CH_2–$CH–O– moiety. The structures of these two units were confirmed by analysis of long-range $^1H–^{13}C$ correlations observed in an HMBC experiment, and HMQC data allowed assignment of all of the individual signals to the corresponding carbon signals. Further connections of these two units to remaining elements were revealed by HMBC data (Table 1). Connection of the *n*-butyl moiety to C-5, thus locating the *n*-butyl unit at the β-position of the β-oxy-α,β-unsaturated enone moiety, was evidenced by the observation of HMBC correlations of H-6 to C-5 and C-4, along with correlation of H-4 to C-6. Confirmation of the presence of the β-oxy-α,β-unsaturated enone moiety, and attachment of another spin system to this moiety was also obtained by analysis of HMBC data. Signals for H_2-1 and H-2 were correlated with to C-3. These correlations, together with HMBC correlations of H-4 to C-2 and C-3, provided the connection sequence from C-1 to C-5. HMBC correlation of H-2 to C-5 and chemical shift considerations of C-2 and C-5 (82.4 and 195.8, respectively) enabled connection of C-2 and C-5 via an oxygen atom. At this point, carboxylic carbon C-10 and a hydroxyl group remained to be assigned. The carboxylic acid moiety must then be connected to C-1 based on HMBC cross peaks of H_2-1 and H-2 to C-10. Thus, the complete structure of the molecule was assigned as (5-butyl-3-oxo-2,3-dihydrofuran-2-yl)-acetic acid (**1**). The configuration at C-2 was not determined.

Another active component from the flowers of *E. annuus* was identified as 3-hydroxy-pyran-4-one (**2**) by analysis of its MS and NMR spectroscopic data. In addition to active compounds **1** and **2**, two inactive

Table 1
NMR spectral data for compound **1**[a]

Position	^{1}H NMR δ_H (integral, *mult*., *J* in Hz)	^{13}C NMR δ_H (*mult*.)	HMBC (^{1}H to ^{13}C)
1	2.90 (1H, *dd*, 16.9, 3.2) 2.61 (1H, *dd*, 16.9, 8.3)	36.9 (*t*)	2, 3, 10 2, 3, 10
2	4.83 (1H, *m*)	82.4 (*d*)	1, 3, 5, 10
3	–	206.4 (*s*)	–
4	5.54 (1H, *s*)	102.7 (*d*)	2, 3, 5, 6
5	–	195.8 (*s*)	–
6	2.56 (2H, *t*, 8.3)	31.3 (*t*)	4, 5, 7, 8
7	1.65 (2H, *quintet*, 7.4)	29.2 (*t*)	5, 6, 8, 9
8	1.42 (2H, *sextet*, 7.4)	23.2 (*t*)	6, 7, 9
9	0.95 (3H, *t*, 7.4)	14.0 (*q*)	7, 8
10	–	171.6 (*s*)	–

[a] Recorded in MeOH-d_4 at 500 MHz for ^{1}H and 125 MHz for ^{13}C.

compounds, **5** and **6** were also isolated from the flowers of *E. annuus*. These compounds were identified as 3,5-di-*O*-caffeoylquinic acid (**5**) and methyl 3,5-di-*O*-caffeoylquinate (**6**) by analysis of MS, ^{1}H NMR, and ^{13}C NMR data, together with ^{1}H NMR, and ^{13}C NMR chemical shift comparison to the relevant values for 3,4-di-*O*-caffeoylquinic acid and its methyl ester reported in the literature (Timmermann et al., 1983).

In a similar fashion, bioassay-guided fractionation and purification using normal and reversed-phase column chromatography and HPLC on the EtOAc-soluble fraction from the stems and leaves of *E. annuus* led to the isolation of two active cinnamic acid derivatives, **3** and **4**. These compounds were identified as 4-hydroxycinnamic acid and 3,4-dihydroxycinnamic acid methyl ester, respectively, by analysis of their MS and NMR spectroscopic data. Compound **4** may be an artifact since MeOH was used as an extraction solvent.

The anti-germination activity of compounds **1**–**6** was examined using lettuce seeds. Initially, compounds **1**–**4** completely inhibited the germination of lettuce seeds at the 2000 ppm level, while compounds **5** and **6** did not show any activity. To evaluate the potency of active compounds **1**–**4**, the 50% inhibitory concentrations (IC_{50}) were obtained, and compared with the value for 4-hydroxybenzoic acid, which is known to be an effective germination inhibitor (Sebeson et al., 1969; Mizutani, 1999). 4-Hydroxybenzoic acid was employed as a positive control, and its IC_{50} value was determined as 4.02±0.39 mM. As shown in Table 2, compound **1** was found to be the most active of those isolated, showing stronger lettuce seed germination inhibitory activity than 4-hydroxybenzoic acid. Cinnamic acid derivatives, **3** and **4** showed comparable IC_{50} values with that of 4-hydroxybenzoic acid, while compound **2** showed the weakest inhibitory activity among the compounds tested.

This work represents the first report of the anti-gemination compound **1**. In addition, three known metabolites were also isolated as active constituents, including two phenolic compounds. While phenolics have been described as a group of secondary metabolites implicated in allelopathy (Mizutani, 1999), the structure of compound **1** represents a unique natural alkyl furanone possessing carboxylic acid and *n*-butyl substituents. The carbon skeleton of **1** seems to share the same biosynthetic origin with C_{10} acetylenic compounds (e.g. 2,8-decadiene-4,6-diynoic acid methyl ester), which were previously described from the genus *Erigeron* (Jakupovic et al., 1986).

Table 2
Anti-germination activity of compounds against lettuce seeds[a]

Compound	IC_{50} (mM)[b]
1	2.13±0.03d
2	12.85±0.56a
3	4.97±0.24b
4	4.87±0.25b
4-Hydroxybenzoic acid	4.02±0.39c

[a] Means within a column sharing the same letter are not significantly different at the 0.05 probability level according to DMRT.
[b] Means of five observations with±S.D.

3. Experimental

3.1. General

The optical rotation was recorded on an Optical Activity AA-10 Automatic Polarimeter. The infrared (IR) spectrum was recorded as dry film on a JASCO FT/IR-5300. The ultraviolet (UV) spectrum was recorded on a Hewlett Packard HP 8453 spectrophotometer in MeOH. FABMS data were obtained on a JEOL JMS HX-110 spectrometer using 3-nitrobenzyl alcohol as a matrix. ESIMS data were obtained on a Macro Mass Quatro LC with electro spray ionization. NMR spectra (1D and 2D) were recorded in acetone-d_6 or MeOH-d_4 using a JEOL Eclipse-500 MHz spectrometer (500 MHz for ^{1}H and 150 MHz for ^{13}C), and chemical shifts were referenced relative to the corresponding residual solvents signals. HMQC and HMBC data were optimized for $^1J_{CH} = 140$ Hz and $^nJ_{CH} = 8$ Hz, respectively. Solvents

for extractions and open column chromatography were reagent grade and used without further purification. Solvents used for HPLC were analytical grade. Flash column chromatography was carried out using Merck Kieselgel 60 or Aldrich octadecyl-functionalized silica gel (C-18). HPLC separations were performed on an Alltech HS Hyperprep 100 BDS C_{18} column (1.0×25 cm; 8-μm particle size) with a flow rate of 2 ml/min. Compounds were detected by UV absorption both at 210 and 254 nm.

3.2. Plant material

Fresh aerial parts of *E. annuus* were collected in the Botanical Garden of Wonkwang University, Iksan, Korea, in July 2000. The plant was identified and authenticated by one of the co-authors (T.-O. Kwon). Collected aerial parts of the plant were divided into flowers and the rest of the plant (i.e. leaves and stems), and both parts were dried in a well-ventilated darkroom and a voucher specimen (No. KTO-54) was deposited in the Herbarium of the Medicinal Resources Research Center, Wonkwang University.

3.3. Extraction and isolation

The dried flowers of *E. annuus* (600 g) were crushed and extracted with MeOH (5 l) for 24 h at room temperature. The MeOH extract (138 g) was concentrated, suspended in H_2O, and sequentially partitioned with *n*-hexane, EtOAc, and BuOH. The EtOAc-soluble fraction (12.6 g) showed an 82% inhibitory effect on germination at a concentration of 6000 ppm. Thus a portion (4.5 g) of the EtOAc-soluble fraction was subjected to silica gel (Merck Kieselgel 60; 0.063–0.2 mm particle size; 2.5×40 cm) column chromatography. The column was eluted with a gradient consisting of mixtures of *n*-hexane–CH_2Cl_2–MeOH in order of increasing polarity. The fraction eluted with 100% CH_2Cl_2 was identified as compound **2** (251 mg). The fraction (831 mg) eluted with 10% MeOH in CH_2Cl_2 was further subjected to C-18 column chromatography (MeOH–H_2O, stepwise gradient) to afford 10 fractions. The active fraction (103 mg) eluted with 20% MeOH in H_2O was finally purified by HPLC using a gradient from 20 to 30% CH_3CN in H_2O over 40 min to yield compound **1** (R_t 27.7 min; 5.8 mg), **5** (R_t 18.3 min; 19 mg), and **6** (R_t 24.5 min; 15 mg).

A portion (5.0 g) of the EtOAc-soluble fraction (28.7 g) from the leaves and stems of the plant (dry wt; 3 kg), obtained using the same method as used for the flower extract, showed a 97% inhibitory effect on germination at 6000 ppm. Using purification steps similar to those described for the EtOAc extract from the flowers, normal-phase (active fraction eluted with 7% MeOH in CH_2Cl_2), reversed-phase (active fraction eluted with 20% MeOH in H_2O), and finally HPLC using a gradient from 20 to 30% CH_3CN in H_2O over 60 min, afforded compound **3** (R_t 20.8 min; 10.8 mg) and **4** (R_t 33.3 min; 2.3 mg).

3.4. (5-Butyl-3-oxo-2,3-dihydrofuran-2-yl)-acetic acid (*1*)

White oily gum; $[\alpha]_D^{25}$ +29° (*c* 0.07, MeOH); UV λ_{max} nm (log ε): 207 (3.3), 261 (3.4); IR ν_{max} cm^{-1}: 3447, 1655, 1635, 1604 cm^{-1}; ^1H, ^{13}C, and 2D NMR spectroscopic data, Table 1; ESIMS *m*/*z*: $[M\text{-}H]^-$ 197; LRFABMS *m*/*z*: $[M+H]^+$ 199; HRFABMS *m*/*z*: $[M+H]^+$ 199.0969 (calcd for $C_{10}H_{15}O_4$, 198.0970).

3.5. Lettuce seed germination test

The lettuce seed germination bioassay was performed on filter papers contained in plastic Petri-dishes of 35 mm radius. Methanolic solution of fractions and pure compounds at various concentrations were prepared. Each solution was placed on the filter paper and allowed to dry under reduced pressure, and distilled water (0.2 ml) was added. Ten lettuce seeds were placed in each Petri-dish and all the tested samples were kept in the dark at 25 °C. The number of germinated seeds was counted after 72 h. The same procedure was applied for the control (blank). The 50% inhibitory concentrations (IC_{50}) were determined for compounds **1**–**4** and 4-hydroxybenzoic acid. The germination inhibitory data was subjected to Duncan's multiple range test (DMRT) to determine significant differences among mean values at the 0.05 probability level. At least five separate determinations were conducted for each compound.

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