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Bioactive Lignans from a Cultivar of *Helianthus annuus*

FRANCISCO A. MACÍAS,* ADRIANA LÓPEZ, ROSA M. VARELA, ASCENSIÓN TORRES, AND JOSÉ M. G. MOLINILLO

Grupo de Alelopatía, Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz, Apdo 40, 11510-Puerto Real, Cádiz, Spain

A bioassay-guided fractionation of water extracts from *Helianthus annuus* cv. SH-222 was carried out. Ten lignans and a phenylpropanoid were isolated from the polar bioactive fractions of *H. annuus*. This study is the first to report lignans as constituents of sunflower and is the first time that tanegool has been isolated as a natural aglycone. Additionally, we report biological activities of the isolated compounds. The general bioactivity has been evaluated using the wheat coleoptiles bioassay. The phytotoxic activities of compounds pinoresinol, lariciresinol, dihydro-dehydrodiconiferilic alcohol, and l-(4′-hydroxy-3′-methoxyphenyl)-2-[4″-(3hydroxypropyl)-2″-methoxyphenoxy]propane-l,3-diol were also evaluated in a bioassay on the standard target species. The structure−activity relationships are discussed.

KEYWORDS: ***Helianthus annuus*; lignan; tanegool; SAR**

INTRODUCTION

Lignans are secondary metabolites derived from the shikimic acid pathway. They are widely distributed in plants (*1*) and are formed by the union of two phenylpropane units. They constitute a complex family of skeletons and functionalizations. Lignans present a wide range of biological activities. Antitumor, antimitotic, antiviral, enzyme inhibitory, piscicidal, fungistatic, etc. properties have been reported (*2*−*4*). The phytotoxic activity of lignans isolated from *Leucophyllum frutescens* has been studied (*5*). They inhibited seed germination of *Agrostis stolonifera* and the development of *Lactuca sativa* seedlings. The inhibition produced by some aryltetralin lignans by affecting the formation of mitotic microtubular organizing centers has been described (*6*). Moreover, the presence of lignans has been related with the allelopathic activity of *Brassica fruticulosa* (*7*).

To study the potential use of residual leaves of sunflower as a source of natural herbicide models and/or bioactive compounds, we continue with our systematic allelopathic studies of cultivars. A bioassay-guided fractionation of water extracts from *Helianthus annuus* cv. SH-222 was carried out in order to isolate, identify, and characterize the allelopathic constituents and to establish their phytotoxic activities. Additionally, the biological activities of the isolated compounds and the structure−activity relationships (SAR) were investigated.

MATERIALS AND METHODS

General. IR spectra (KBr) were recorded on a Perkin-Elmer FT-IR Spectrum 1000, Matton 5020 spectrophotometer. NMR spectra were run on a Varian INOVA-400 spectrometer. Chemical shifts are given in parts per million with respect to residual $CHCl_3$ or $CDCl_3$ signals (δ 7.25 and 77.00, for ^1H and ^{13}C, respectively). Optical rotation was determined using a Perkin-Elmer polarimeter model 241 (on the sodium D line). High-resolution mass spectrometry was carried out on VG AUTOSPEC mass spectrometer at 70 eV.

Molecular Modeling and Estimation of log*P* and log*W* Values. Molecular geometric properties were calculated from PM3 in MOPAC 6.0. The following keywords were employed to control the computation and output: PM3, PRECISE, and GEO-OK. Briefly, the keywords have the following meaning: PM3 stipulates the use of PM3 Hamiltonian in the calculation; GEO-OK overrides the termination sequence in the geometry optimization procedure; and PRECISE increases the precision of the geometry optimization procedure.

Interactive Analysis, a program available on the Internet (*8*), was used for log*P* and log*W* predictions, naming the obtained values as IA log*P* and IA log*W*. Interactive Analysis employed electrotopological state indices using networks in order to predict log*P*. The on-line software SRC's logKow (*9*) and the on-line version of Daylight's ClogP program (*10*) were also used to calculate log*P*, and the obtained results were noted as logKow and C log*P*, respectively.

Plant Material. *H. annuus* cv. SH-222 (commercialized by Semillas Pacífico) was collected during the third plant development stage (*11*) (plants 1.2 m tall with flowers, 1 month before harvest) and were provided by Rancho de la Merced, Agricultural Research Station (CIFA), Junta de Andalucía, Jerez, Spain.

Extraction and Isolation. Fresh leaves of *H. annuus* cv. SH-222 (6 kg) were extracted in water (18 L) for 24 h at room temperature in the dark. The aqueous solution was extracted with CH_2Cl_2 and then with EtOAc at room temperature. The solvent was removed from the organic layer by reduced pressure evaporation yielding two extracts of 16 (DCM-A) and 6 g (EtOAc-A), respectively. EtOAc-A was chromatographed using hexanes−EtOAc mixtures of increasing polarity as the eluent over silica gel. Those fractions eluted between hexanes−EtOAc 6:4, 5:5, 4:6, and 3:7 yielded compounds **1** (50 mg), **3** (1 mg), **5** (16 mg), **6** (0.8 mg), **7** (5 mg), **8** (0.8 mg), and **9** (12 mg). DCM-A was chromatographed using hexanes−EtOAc mixtures of increasing polarity as the eluent over silica gel. Those fractions eluted between hexanes−EtOAc 5:5, 4:6, 2:8, and 1:9 yielded compounds **1** (14 mg),

* To whom correspondence should be addressed. Tel: +34(956)016370. Fax: +34(956)016193. E-mail: famacias@uca.es.

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3 (1 mg), **4** (14 mg), **5** (45 mg), **9** (25 mg), **10** (1.5 mg), and **11** (15 mg).

Tanegool. Colorless oil; $[\alpha]_D^{25}$ +12.0 (*c* 1.0, $CHCl_3$). IR ν_{max} (KBr) cm^{-1}: 3401 (OH), 1605 (C=C aromatic). ^1H NMR (399.952 MHz, $CDCl_3$): δ 2.01 (ddd, $J = 7.6, 5.9, 8.0$ Hz, H-8′), 2.60 (dddd, $J = 7.6, 7.4, 7.8, 4.9$ Hz, H-8), 3.41 (d, $J = 5.9$ Hz, H-9′), 3.84 (s, OCH_3), 3.85 (s, OCH_3), 4.01 (dd, $J = 9.2, 7.8$ Hz, H-9α), 4.20 (dd, $J = 9.2, 4.9$ Hz, H-9*β*), 4.48 (d, $J = 8.0$ Hz, H-7′), 4.65 (d, $J = 7.4$ Hz, H-7), 6.75 (dd, $J = 8.0, 1.9$ Hz, H-6′), 6.76 (d, $J = 1.9$ Hz, H-2′), 6.79 (dd, $J = 8.0, 2.0$ Hz, H-6), 6.84 (d, $J = 8.0$ Hz, H-5′), 6.86 (d, $J = 2.0$ Hz, H-2), 6.87 (d, $J = 8.0$ Hz, H-5). ^{13}C NMR data (100.23 MHz, $CDCl_3$): δ 50.3 (d, C-8), 51.6 (d, C-8′), 55.9 (s, OCH_3), 56.3 (s, OCH_3), 62.9 (t, C-9′), 70.3 (t, C-9), 75.6 (d, C-7), 83.9 (d, C-7′), 108.6 (d, C-2′), 109.1 (d, C-2), 114.1 (d, C-5′), 114.2 (d, C-5), 119.4 (d, C-6′), 119.8 (d, C-6), 133.3 (s, C-1), 134.2 (s, C-1′), 144.0 (s, C-4), 145.0 (s, C-4′), 146.5 (s, C-3′), 146.6 (s, C-3). HREIMS (*m*/*z*) [M^+] calcd for $C_{20}H_{24}O_7$, 376.1522; found, 376.1530.

Coleoptiles Bioassay. Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in 15 cm diameter Petri dishes moisturized with water and grown in the dark at 22 ± 1 °C for 3 days (*12*). The roots and caryopsis were removed from the shoots. The latter was placed in a Van der Weij guillotine, and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for bioassay. All manipulations were performed under a green safelight (*13*). Compounds were predissolved in dimethyl sulfoxide (DMSO) and diluted to the final bioassay concentration with a maximum of 0.1% DMSO. Parallel controls were also run.

Crude extracts, fractions, or pure compounds to be assayed for biological activity were added to test tubes. The assay was made in duplicate. Phosphate-citrate buffer (2 mL) containing 2% sucrose (*13*) at pH 5.6 was added to each test tube. Following the placement of five coleoptiles in each test tube (three tubes per dilution), the tubes were rotated at 0.25 rpm in a roller tube apparatus for 24 h at 22 °C in the dark. The coleoptiles were measured by digitalization of their images. Data were statistically analyzed using the Welch's test (*14*). Data are presented as percentage differences from control. Thus, zero represents the control; positive values represent stimulation of the studied parameter, and negative values represent inhibition.

Phytotoxic Bioassay. Bioassays used Petri dishes (90 mm diameter) with one sheet of Whatman no. 1 filter paper as support. Germination and growth were conducted in aqueous solutions at controlled pH by using 10 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) and 1 M NaOH (pH 5.6). A 5 μL/mL DMSO amount was added to the solutions in order to facilitate solubility of assayed compounds. After seeds and aqueous solutions were added, Petri dishes were sealed with Parafilm to ensure closed-system models. The seeds were further incubated in a Memmert ICE 700 (ANORSA, Seville, Spain) controlled environment growth chamber. Bioassays took 3 days for cress and 5 days for lettuce, tomato, and wheat. After growth, plants were frozen at −10 °C for 24 h to avoid subsequent growth during the measurement process. This helped the handling of the plants and allowed a more accurate measurement of root and shoot lengths.

The number of seeds in each Petri dish depended on the seed size. Twenty-five seeds were used for tomato, lettuce, cress, and onion (seed diameter < 8 mm), and 10 seeds were used for wheat (seed diameter > 8 mm). The commercial herbicide Logran was used as an internal reference, according to a comparison study previously described by us (*15*). It was used at the same concentrations and in the same conditions as the compounds in the study. Control samples (buffered aqueous solutions without any tested compound) were used for all of the vegetable species assayed. Five milliliters of treatment, control, or internal reference solution was added to each Petri dish. Treatment concentrations were 0.5, 0.1, 0.05, and 0.01 mM for each compound. The number of Petri dishes for each dilution was four for tomato, cress, onion, and lettuce (100 seeds), and 10 dishes (100 seeds) were used for wheat.

RESULT AND DISCUSSION

Isolation and Characterization. Fresh leaves of *H. annuus* cv. SH-222 were extracted with water at room temperature for 24 h. These aqueous extracts were re-extracted with methylene chloride and ethyl acetate. The different fractions obtained were fractionated and assayed. The polar bioactive fractions yielded compounds **1**−**11** (**Figure 1**). The spectroscopic data of **1**−**6** and **8**−**11** were identical to those previously reported for pinoresinol (**1**) (*16*), medioresinol (**2**) (*17*), syringaresinol (**3**) (*17*), buddlenol E (**4**) (*18*), lariciresinol (**5**) (*19*), 7-hydroxylariciresinol (**6**) (*20*), neo-olivil (**8**) (*21*), dihydro-dehydrodiconiferyl alcohol (**9**) (*22*), 3-(4-hydroxy-3,5-dimethoxyphenyl)-propan-l-ol (**10**) (*23*), and l-(4-hydroxy-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2-methoxyphenoxy]-propane-l,3-diol (**11**) (*24*).

Tanegool (**7**) was isolated from the EtOAc extract, as a resinous oil. The presence of hydroxyl groups in the molecule could be detected by a broad band in the IR spectrum at 3401 cm^{-1}. The ^1H NMR spectrum showed two aromatic methoxy groups at δ 3.84 and 3.85 (3H, s) and six aromatic protons as two groups of signals: the first one at δ 6.87 (1H, d, $J = 8.0$ Hz), δ 6.86 (1H, d, $J = 2.0$ Hz), and δ 6.79 (1H, dd, $J = 8.0, 2.0$ Hz) and the second group at δ 6.84 (1H, d, $J = 8.0$ Hz), δ 6.76 (1H, d, $J = 1.9$ Hz), and δ 6.75 (1H, d, $J = 8.0, 1.9$ Hz), indicating the presence of two trisubstituted benzenic rings. Other signals were assigned to two methylenes, one at δ 4.20 and 4.01 and the other at δ 3.41 and signals to four methines at δ 4.48, 2.01, 2.60, and 4.65. The comparison of the ^1H NMR spectrum of this compound with that for (−)-lariciresinol (**5**) established significant differences as the methylene at C-7 being modified as a methine due to the additional hydroxyl group located at this position (C-7 to δ 75.6).

The analysis of its ^{13}C NMR spectrum confirmed the proposed structure showing clearly, together with the 12 aromatic carbons and the two methoxyl groups, the presence of six other carbon atoms, four of them connected to oxygen. The ^1H NMR two-dimensional correlation spectroscopy spectrum allowed us to establish the correlations from the benzylic proton at δ 4.48 (H-7); with the signal at δ 2.01 (H-8′); and that of the H-8′ with the signal at δ 3.44 (H-9′); this methylene, according to its displacement in the ^{13}C NMR spectrum at δ 62.9, is a hydroxymethylene moiety.

The H-8′ signal was also correlated with a signal at δ 2.72 (H-8). This proton is coupled with a signal at δ 4.65 assigned to H-7 and two signals at δ 4.01 and 4.20 corresponding to H-9α and H-9*β*, respectively. The signal for H-7 integrated for one proton and it appeared deshielded with respect to that of H-7 in the spectrum of lariciresinol (**5**). A trans orientation of the H-7′ in relation with H-8′ should give a signal at around δ 4.7. The signal observed corresponding to H-7 appears at δ 4.65, in good agreement with this orientation. On the other hand, the relative configuration of the C-8 and C-8′ must be trans based on the nuclear Overhauser enhancement spectroscopy experiment and the nuclear Overhauser Effects (NOE) effects observed; irradiation of H-7′ produced an effect on H-8, and irradiation of H-9*β* produced an effect on H-8′, confirming the relative configuration of the centers C-7′, C-8′, and C-8 (**Figure 2**).

These data match the structure proposed for this compound in **Figure 1**. Previously, two glycosylated derivatives at C-7, named tanegosides A and B, have been described, with tanegool as the aglycone derivative (*25*). This is the first time that the isolation of the aglycone **7** has been described as a natural product. Recently, the isolation of **6** from *B. fruticulosa* has been reported, naming this compound as (−)-tanegol, instead of 7-hydroxylariciresinol (*7*).

Bioassay Results. The general bioactivity of isolated compounds has been evaluated as for the sunflower fractions and

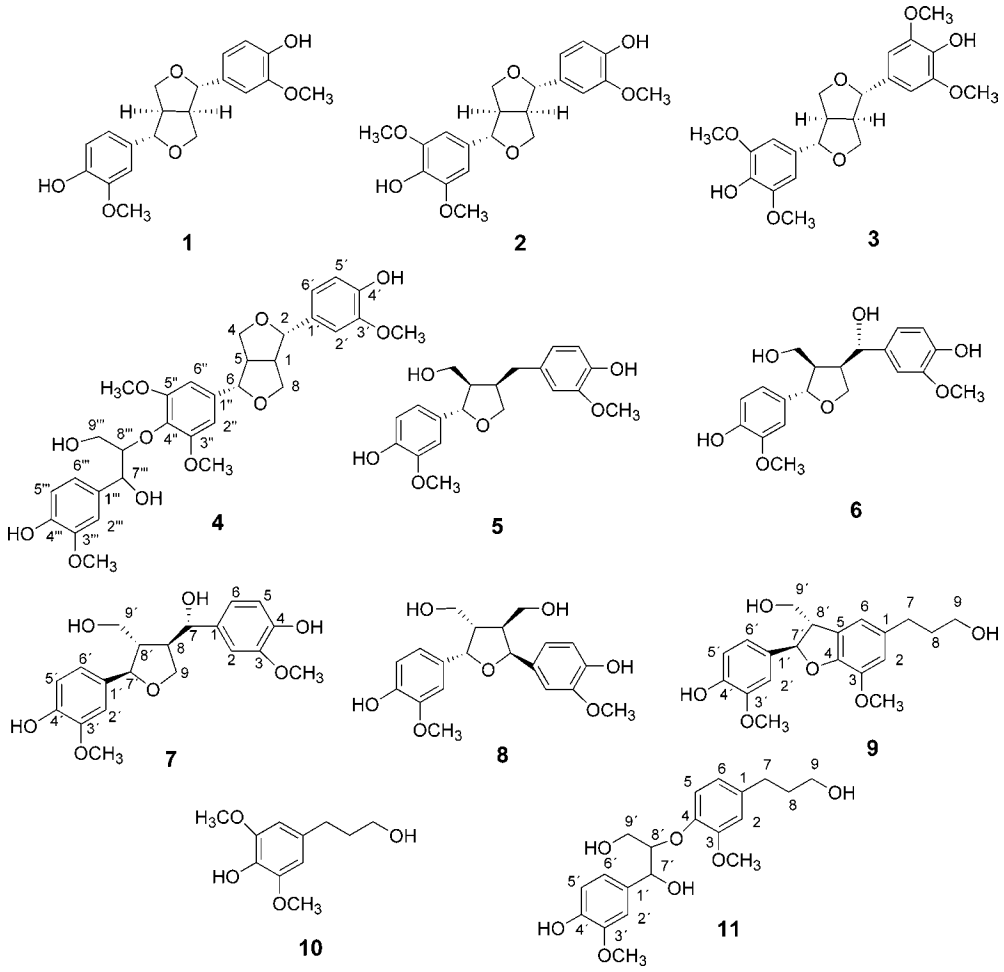

Figure 1. Biologically active lignans and phenylpropanol from *Helianthus annuus* L.

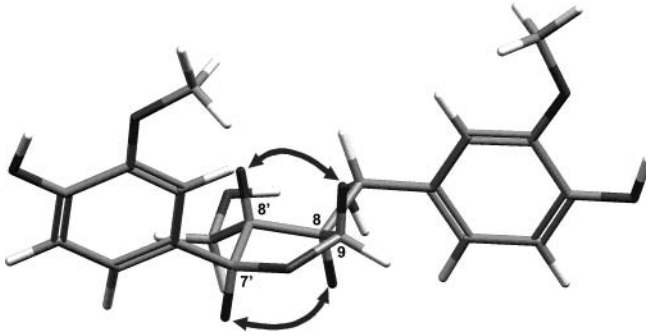

Figure 2. Selected NOE effects on the most stable conformer of tanegool (**7**) using PM3 calculations.

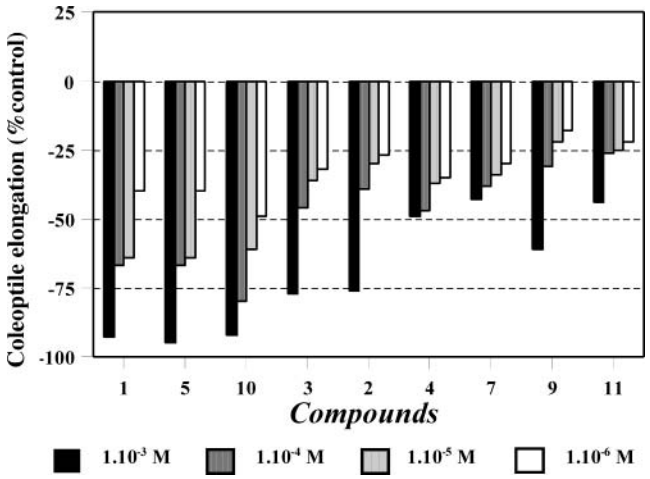

Figure 3. Bioactivities of compounds in the wheat coleoptiles bioassay.

extracts, with the exception of compounds **6** and **8**, because of the low amounts isolated. The phytotoxic activities of compounds **1**, **5**, **9**, and **10** were evaluated in a bioassay on the standard target species. The results obtained show that these compounds have good levels of activity (**Figure 3**). The bioassayed compounds present three different structural skeletons: tetrahydrofurofuranic (A), tetrahydrofuranic (B), and 3-phenylpropan-1-ol (C). The differences between each class, in most cases, are the presence or absence of hydrophilic moieties (hydroxyl or methoxyl groups). Thus, the different activities observed could be due to water solubility or hydrophilicity. To confirm this hypothesis, we calculated log*P* (logarithm of the octanol−water partition coefficient) using three different algorithms (IA log*P*, LogKow, and C log*P*) and log*W* (logarithm of water solubility constant, IA log*W*). We have noted in bold those values that were outside of the general trend. In the discussion, these data were disregarded. The solubility does not influence the activity significantly.

In tetrahydrofurofurans, the most active compound was **1** (−93%, 10^{-3} M), whereas **4** had activity levels around −40%. Compounds **2** (−76%, 10^{-3} M) and **3** (−77%, 10^{-3} M) had similar activities, and they are intermediate between **1** and **4**. This is in good agreement with the data of log*P* (LogKow and C log*P*) obtained for this compound, where a higher value means higher activity. In addition, the low activity observed for **4** is in accordance with the fact that this compound does not follow

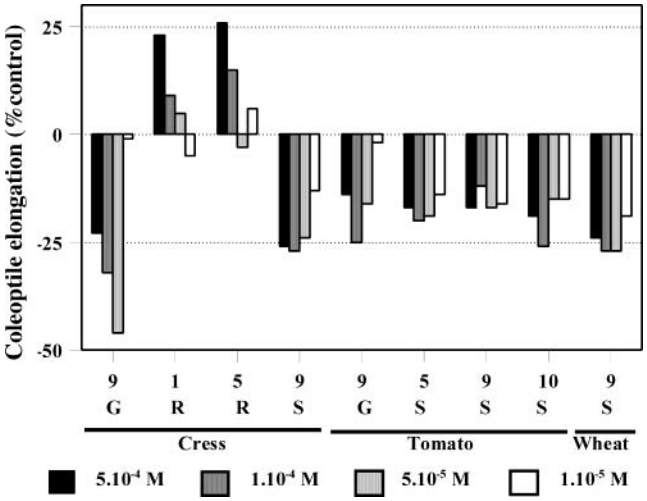

Figure 4. Selected effect on STS bioassay. The parameters affected are germination (G), root length (R), and shoot length (S).

the Lipinski's rule of five (*26*). Thus, the molecular weight is greater than 500 and the number of hydrogen bond acceptors is greater than 10. The tetrahydrofuranic lignans present a similar correlation. Thus, **5** (−95%, 10^{-3} M) presents a higher value of log*P* and a higher activity than **7** (−43%, 10^{-3} M).

The comparison of the activities of 3-phenylpropan-1-ol derivatives is not obvious. These compounds have a common substructure, but the rest of the molecule is very different. Nevertheless, it can be deduced that the introduction of the functionalizations present in **9** (−61%, 10^{-3} M) and **11** (−44%, 10^{-3} M) does not increase the bioactivity, as they are less active than **10** (−92%, 10^{-3} M). In conclusion, in addition to other structural factors, hydrophilicity seems to be an important aspect of the activity of all of these compounds.

To compare the activities of the different structural types bioassayed, we selected compounds with similar hydrophilic properties. The comparison of compounds **1** (IA log*P* = 1.9, LogKow = 2.02, C log*P* = 1.31, IA log*W* = −3.23; −93%, 10^{-3} M; −67%, 10^{-4} M), **5** (IA log*P* = 2.02, LogKow = 2.34, C log*P* = 1.43, IA log*W* = −3.11; −95%, 10^{-3} M; −67%, 10^{-4} M), and **9** (IA log*P* = 2.02, LogKow = 2.99, C log*P* = 1.63, IA log*W* = −3.11; −61%, 10^{-3} M; −31%, 10^{-4} M) that have similar log*P* and solubility values allows us to conclude that structures A and B have similar high activities, but C is the least active structural type. Therefore, the presence of tetrahydrofuran ring seems to increase the bioactivity of these lignans, whereas a second heterocyclic ring does not modify the activity substantially.

Regarding the phytotoxic activities of compounds **1**, **5**, **9**, and **10**, the bioassay was run using concentrations from $5 \times l0^{-4}$ to 10^{-5} M in STS, as it has already been described. The selected results obtained are summarized in **Figure 4**. The results are very different to those obtained on coleoptile bioassay. In general, the most active compound was **9**, which produces an inhibitory activity on cress (germination, −30% average; shoot growth, −27% at $l0^{-4}$ M), tomato (germination, −25% at 10^{-4} M; shoot growth, −17% at 5×10^{-4} M), and wheat (shoot growth, −27% at 10^{-4} and 5×10^{-5} M). On the other hand, the most sensitive species for this kind of compound is *Lycopersicum esculentum* Will, which is affected by **5** (shoot growth, −20% at 10^{-4} M, −19 at 5×10^{-5} M), **9**, and **10** (shoot growth, −26% at 10^{-4} M).

Ten lignans and a phenylpropanoid have been isolated from *H. annuus*. This study is the first to report lignans as constituents of sunflower and the first time that tanegool has been isolated as a natural aglycone. The general bioactivity of isolated compounds has been evaluated using the wheat coleoptiles bioassay. The phytotoxic activities of compounds **1**, **5**, **9**, and **10** were also evaluated in phytotoxic bioassay on the standard target species. We can conclude that lignans isolated from *H. annuus* present high levels of activity in the wheat coleoptiles bioassay. The lipophilicity of these compounds influences this activity. A tetrahydrofuran ring in the structure increases the bioactivity, whereas a second tetrahydrofuran ring does not modify the activity substantially. The most active compounds in the wheat coleoptiles bioassay (**1**, **5**, and **10**) have low levels of phytotoxicity, whereas the most phytotoxic compound is **9**.

We conclude that the isolated compounds can play a significant role in the allelopathic interactions of sunflower. On the other hand, the results confirm that the residual leaves of this crop are a source of bioactive compounds that can suppress the germination and/or growth of neighbor weeds.

Supporting Information Available: Structural types and QSAR properties. This material is available free of charge via the Internet at http://pubs.acs.org.

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