

Fractionation of Allelochemicals from Oilseed Sunflowers and Jerusalem Artichokes

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The phenolic and related components present in stems and leaves of sunflower, *Helianthus annuus* L., and Jerusalem artichoke, *Helianthus tuberosus* L., were extracted sequentially and their activity as phytotoxic agents evaluated. Total acids and neutral compounds were isolated by extraction with methanol, acetone, and water. The free acids and neutral compounds were partitioned into the organic phase, whereas the acids, present as esters and aglycones, were liberated by subsequent alkaline hydrolysis of the aqueous phase. This procedure was compared with sequential extractive techniques employing alkaline hydrolysis of dried plant tissue followed by extraction of the acidified mixture with ethyl acetate. Fractions were individually evaluated for phytotoxic properties. Selected fractions from those showing a positive response were analyzed by gas-liquid chromatography. Structural identification and characterization of the individual components in these selected fractions were accomplished by gas chromatography-mass spectrometry.

The term allelopathy, when first proposed by Molisch (1), referred to either the beneficial or detrimental interaction between all types of plants and microorganisms. As presently used, this definition is generally accepted. Since 1970 a concerted effort has been made to understand the phenomenon of allelopathic interaction. The many interpretations resulting from these studies are well documented in the literature (2-4). An area currently receiving considerable attention is the allelopathic effect resulting from weed-crop and weed-weed interactions (2, 5-7). One study conducted by Wilson and Rice (7) showed that the common sunflower, *Helianthus annuus* L., possessed allelopathic properties. Realizing the inherent potential

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that these findings had for natural weed control, Leather (8) undertook a systematic study to determine if the allelopathic properties noted for the "wild sunflower" were exhibited by cultivated varieties. He found that cultivated sunflowers also contained weed-suppressing allelochemicals. Earlier, several researchers reported on the various structures of some of the secondary natural compounds that are responsible for the inhibitory effect of the sunflower on the growth rate of other plants (9-11). Inhibitory effects noted for other plant species were attributed to the presence of compounds which were subsequently identified as belonging to several classes which included simple phenolic acids, coumarins, terpenoids, flavanoids, alkaloids, cyanogenics, glycosides, and glucosinolates (12-15). Although there are many reports in the literature that describe the isolation and identification of these major classes of compounds from a variety of plant species and plant parts, such as seeds and roots, there is comparatively little information concerning their isolation from the leaves and stems of the sunflower and related species. Furthermore, many of these studies have concentrated primarily on the major components present, with little attention to the minor components. The phytotoxic properties of some of the minor components have been reported only recently (16-18). The identification and characterization of these compounds also was described. The investigation reported here was prompted by the findings of Leather (8). The purpose of this initial investigation was to extract and fractionate components from the leaves and stems of the sunflower and Jerusalem artichoke, to evaluate the phytotoxic activity of the crude fraction by bioassay, and to separate and identify major components comprising the active fractions.

Experimental

Plant Material. Dried and fresh tissue from the leaf and stem of sunflower (*H. annuus* L.) and the dried ground tissue from the leaf and stem of the Jerusalem artichoke (*H. tuberosus* L.) were used as source materials for the investigation reported here. The fresh tissues were harvested from plants grown in pots under illumination provided by 1000-watt, metal halide lamps for a photoperiod of 12 hr in a greenhouse maintained at 75°F and 80-85% RH. The plants were approximately 4 months old at time of harvesting. Immediately after collection, the fresh material was stored at -60°C. Sufficient material was removed for the extractions and either lyophilized or used directly, depending on the extraction procedure. Dried material was ground in a Wiley mill to pass through a No. 40 mesh screen. Except for the fresh leaves and stems of the sunflower grown in our greenhouse, all of the other dried tissues were obtained from plants grown in a greenhouse with supplemental light from full-spectrum metal halide lamps at the USDA Weed Science Research Laboratory, Frederick, MD. Leather (8) found no difference in allelopathic potential between sunflower plants grown under these conditions and field-grown plants. (Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.)

Extraction. A variety of extraction procedures were evaluated from a simple leaching of the macerated tissue with warm water or dilute alcohol to the more harsh procedures employing alkaline hydrolysis. Except for a small number of the hydroxylated benzoic acid derivatives, leaching under mild conditions did not effectively extract many of the potentially allelopathic chemicals which were covalently bound as esters and in other forms. Many of these higher molecular weight compounds remained insoluble but contained the allelopathic constituent acids which are released slowly during natural biodegradation of the plant debris in the soil (3). In order to isolate these bound acids within the tissue, procedures were employed which would effectively hydrolyze these chemical entities and thereby release many of the acids from their bound form. Solvents used in the extraction procedures described below were all HPLC grade and residue free.

Extraction Procedure A. Based on a method reported by Krygier et al. (19), fractions containing the free, esterified, and insoluble-bound organic acids were obtained. One or two grams of either the fresh or dried sunflower plant material was homogenized in a Polytron with 20 to 40 ml of 70% methanol:70% acetone (1:1, v:v) for 5 min and then centrifuged. This step was repeated five times. The supernatants were combined and reduced to one-fifth of the original volume with a rotary evaporator at 40°C and 20 Torr vacuum. The resulting solution was acidified to pH 2 with 6N HCl and filtered to remove a small amount of precipitate. The filtrate was extracted five times with 25 ml hexane to remove the lipids, and then extracted five additional times with 20 to 40-ml portions of ethyl acetate-ethyl ether (1:1, v:v). The extracts were combined, dehydrated with anhydrous sodium sulfate, and filtered. The filtrate was evaporated to dryness to give a residue containing the free uncombined acids. The aqueous solution remaining after the above extraction was hydrolyzed with 20 to 40 ml of 4N NaOH for 4 hr under nitrogen at room temperature. The hydrolysate was acidified to pH 2 with 6N HCl and extracted as above—hexane followed by ethyl ether-ethyl acetate (1:1, v:v). This gave a residue containing the byproducts from the hydrolysate from the ester-bound compounds. The insoluble-bound compounds, which were contained in the residue remaining from the original Polytron extraction with 70% methanol/ H₂O:70% acetone/H₂O (v:v), also were hydrolyzed with 20-40 ml of 4N NaOH at room temperature under a nitrogen atmosphere to release the acids from the compounds in which they were bound. After 4 hr the mixture was acidified as above and centrifuged. The supernatant was extracted as above with hexane, followed by ethyl acetate-ethyl ether (1:1, v:v). The organic extracts were combined and dehydrated over sodium sulfate. After filtering, the solvent was removed and the residue dried to constant weight at 40°C and 20 Torr vacuum. Each of these residues was used for the bioassay described below and in subsequent analysis.

Extraction Procedure B. Figure 1 gives a flow diagram for this fractionation procedure, which was based on a modification of the simplified methods described by Serve et al. (20) and Hartley and Buchan (21). Two grams of ground dried sunflower leaves were added

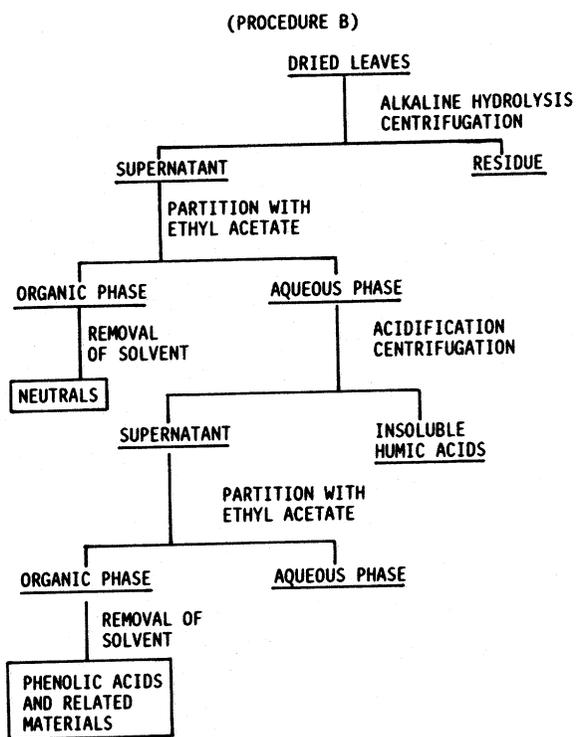


Figure 1. Fractionation of plant material.

to 200 ml of 1N NaOH. The mixture was stirred at room temperature for 24 hr under a nitrogen atmosphere and then centrifuged. The supernatant was decanted and the residue was washed with two 100-ml portions of distilled water. The combined wash water and the supernatant were extracted four times with 100-ml portions of ethyl acetate. The extracts were combined and dried with anhydrous sodium sulfate, filtered, and the solvent removed by rotary evaporation at 40°C and 20 Torr vacuum. The residue, containing neutral components, was dried under vacuum at 20 Torr to constant weight. The aqueous fraction was acidified to pH 1 with 6N HCl, and the small amount of humic acids which precipitated was removed by filtration. The filtrate was extracted three times with 100-ml portions of ethyl acetate. The organic extracts were combined, dried over anhydrous sodium sulfate, and filtered. The solvent was removed by rotary evaporation and the residue contained the freed byproducts from the hydrolyzed esterified and insoluble-bound compounds.

In addition to the above, a variety of other extractive methods were evaluated (21, 22), but those described here were judged most satisfactory for our purposes.

Analytical Methods

The extracted fractions were esterified with either BF₃-MeOH reagent or diazomethane and analyzed by GLC. Gas liquid chromatography (GLC) was conducted with a Perkin-Elmer Sigma 3 equipped with flame ionization detector. Separations were obtained on a Hewlett Packard 12 m x 0.2 mm i.d. capillary column coated with methyl silicon fluid (OV-101). The temperature was maintained at 80°C for 2 min then programmed from 80 to 220°C at 8°C/min. The injector temperature was 250°C. Mass spectra were obtained on a Hewlett Packard model 5995 GC-MS mass spectrometer, equipped with a 15 m fused silica capillary column coated with 5% phenyl methyl silicone fluid. Spectra were obtained for major peaks in the sample and compared with a library of spectra of authentic compounds.

Bioassay for Phytotoxic Properties

The phytotoxicity of the crude residues obtained in the various fractions was assayed with the aquatic macrophyte plant, Lemna minor L. (23). The plants, each a rosette of three fronds (a mother and two daughters), were placed in 24-well tissue culture cluster plates with 1.5 ml of medium containing mineral additives (24, 25). Except where noted, all test samples were dissolved in 50 µl of absolute ethanol. Einhellig et al. (23) have shown that ethanol amendments amounting to 0.3% (v/v) did not adversely effect the growth of L. minor in the bioassay. The test was replicated 6X with one control for each three test treatments. A 5-µl aliquot of test sample was added to each well and the system cultured for 5 to 7 days under constant light at 28°C after which the effect on growth rate of L. minor was noted by a count of the final number of fronds, and the weight of the dried fronds was determined and compared with the dried weight of the controls. The data were analyzed by analysis of variance with Duncan's multiple-range test.

Discussion

Tables I and II show the response noted for the samples obtained from the various sources of sunflower and Jerusalem artichoke. The two extractive procedures, the sources, and the samples are compared. The tables show that all fractions, regardless of extractive method or source, were phytotoxic to various degrees. The bioassay was designed to evaluate the response of *L. minor* at the highest concentrations that the solubility of the extracted material in the substrate would permit. In the case of the sunflower, the amount of extracted material per assay varied from 28 ppm to 620 ppm; for the Jerusalem artichoke, 286 ppm to 686 ppm. Where an effect was noted, a second assay was performed with one-third the original concentration of extracted material. This is shown in the last column of the tables. Reducing the concentration did not, in all instances, proportionately reduce the phytotoxic effect. As shown in Table I, fractions C and G, and Table II, fractions B, I, and K, the inhibitory effect was equal to or more pronounced at the diluted concentration than at the original higher concentration, but this was only true for the fractions where the initial concentration was the highest of the fractions tested. This observation is not uncommon for crude plant extracts which may sometimes stimulate growth at higher concentrations. As a rule, however, growth is inhibited at the higher concentrations and stimulated at the lower. In this study, except for instances noted, the effect was concentration dependent and the degree of growth inhibition varied with the concentration. Generally, there is little difference in phytotoxicity between the fractions obtained from the sunflower when compared with Jerusalem artichoke. Further, little difference is shown in phytotoxicity among the fractions regardless of source or procedure used. What was surprising was that all fractions were phytotoxic, and most to a great degree when compared to control. Still more surprising, as shown later, was the finding that even fractions which did not appear to contain any phenolic acid were equally phytotoxic. Since the acidified hydrolysate fractions containing the ester and insoluble-bound components gave phytotoxic responses comparable to those noted for the unhydrolyzed fraction (neutral compounds and free acids), the question arises, does hydrolysis release from the complex the compounds found in the unhydrolyzed fraction, and are these the same or related compounds or are they completely different? Preliminary analysis using high performance liquid and gas chromatography indicates that the fractions all contain, among other things, similar and related chemical species. The major components have been identified tentatively as phenolic and fatty acids. At this time, seven phenolics have been identified in only four of the fractions. These are shown in Table III. A measure of the magnitude of the confidence level (cc) with a spectrum of standards is given. The first three entries are from the sunflower; the last, from the Jerusalem artichoke. In all fractions isolated, both from the sunflower and the Jerusalem artichoke, a homologous series of fatty acids ranging from C₁₀ to C₁₈ have been identified also by GC-MS. Even-chain, C₁₆ to C₁₈ saturated and C₁₈ mono- and di-unsaturated, predominated. This is not surprising, since fatty acids are major constituents of plant

Table I. Helianthus annuus. Growth Inhibition of L. minor. The Effect of the Fractions of Fronds as Percentage of Control after 5 Days Exposure

Sample	Source	Procedure	Fraction	Application Conc. (ppm)	% of Control at Original Conc.	% of Control at 1/3 Dilution
A	Lyophilized leaves	B	1 ^a	66	22*	87
B			2 ^b	98	8*	68*
C	Frozen fresh leaves	A	1 ^c	620	12*	8*
D			2 ^d	520	7*	22*
E			3 ^e	120	12*	38*
F	Lyophilized leaves	A	1 ^c	28	88*	79*
G			2 ^d	780	10*	6*
H			3 ^e	514	9*	21*
I	Lyophilized stems	A	1 ^c	392	8*	29*
J			2 ^d	256	7*	15*
K			3 ^e	100	13*	79*

^a Unbound neutral components and lipids.

^b Hydrolyzed compounds from ester and insoluble-bound compounds.

^c Free acids and unbound simple compounds.

^d Hydrolyzed products from ester-bound compounds.

^e Hydrolyzed products from insoluble-bound compounds.

* Significantly different from controls, $P \leq 0.05$.

Table II. Helianthus tuberosus. The Effect of the Fractions on Growth Inhibition of L. minor. Dry Weight as a Percentage of the Control after 7 Days Exposure

Sample	Source	Procedure	Fraction	Application Conc. (ppm)	% of Control at Original Conc.	% of Control at 1/3 Dilution
A	Dried leaves	A	1 ^c	392	4*	109*
B			2 ^d	-	6*	3*
C			3 ^e	200	6*	-
D	Dried leaves	A	1 ^c	484	4*	6*
E			2 ^d	-	-	-
F			3 ^e	350	4*	6*
G	Dried leaves	B	1 ^a	340	6*	10*
H			2 ^b	200	122	-
I	Dried stems	A	1 ^c	664	4*	3*
J			2 ^d	286	5*	15*
K			3 ^e	686	4*	3*

^a Unbound neutral components and lipids.

^b Hydrolyzed compounds from ester and insoluble-bound compounds.

^c Free acids and unbound simple compounds.

^d Hydrolyzed products from ester-bound compounds.

^e Hydrolyzed products from insoluble-bound compounds.

* Significantly different from controls, $P \leq 0.05$.

Table III. Phenolics Identified by GC-MS

Phenolic	Sample			
	D ^a	K ^a	J ^a	I ^b
Gallic acid		+++ ^c		
Protocatechuic acid		+++ ^c		++ ^d
P-hydroxybenzoic acid		+++ ^c	++ ^d	
Benzoic acid	+++ ^c		++ ^d	
Vanillic acid		++ ^d		
Syringic acid		+++ ^c		
Salicyclic acid	+ ^e	++ ^d	+ ^e	++ ^d

^a Sunflower.

^b Jerusalem artichoke.

^c +++ ($cc_f^f > 0.95$).

^d ++ ($cc_f^f > 0.85$).

^e + ($cc_f^f < 0.85$).

^f Confidence level for match of mass spectrum of known phenolic acids.

membranes as phospholipids, glycolipids, waxes, and triglycerides and are readily released during the hydrolysis and extraction procedures.

Tables IV and VII give the percentage of the final frond number noted compared to the control for the same fractions as given in Tables I and II. The magnitude of the response in all cases is proportional to that noted on the basis of dried weight of fronds. The difference, where present, may be due to the size of the fronds since the effect of the phytochemical may be to limit frond size but not necessarily the number of fronds. Tables V, VI, and VIII give a description of the visual appearance of the fronds treated. Tables V and VI give the observations after 5 and 7 days, respectively, for the fraction from the sunflower, and Table VIII, for those from Jerusalem artichoke after 7 days.

The tables show that the fronds treated with extracted materials exhibited bleaching, chlorosis, and other morphological changes. The phytotoxic effect may be due to interference with chlorophyll production or other metabolic processes. The observations noted in Table VI suggest that there is some recovery at 7 days from the conditions described in Table V for 5 days. However, this is not the case, since the same chlorotic effect is noted for the new fronds which were the first to emerge early in the observation period. This indicates that the phenomenon is due to the growth of new fronds which have not yet been exposed to the test media.

Those fractions showing activity were equal to or greater in toxicity to that noted for the crude water extracts of sunflower. Although the techniques employed to extract the fractions described are not the same as those which prevail in nature, the purpose of these investigations was to isolate and test the compounds indigenous

Table IV. *Helianthus annuus*. Growth Inhibition of *L. minor*. The Effect of the Fractions on Final Frond Number as Percentage of Control after 5 Days Exposure

Sample	Source	Procedure	Fraction	Application Conc. (ppm)	% of Control at Original Conc.	% of Control at 1/3 Dilution
A	Lyophilized leaves	B	1 ^a	66	35*	88*
B			2 ^b	98	8*	78*
C	Frozen fresh leaves	A	1 ^c	620	10*	5*
D			2 ^d	520	8*	19*
E			3 ^e	120	9*	36*
F	Lyophilized leaves	A	1 ^c	28	78	79*
G			2 ^d	780	10*	5*
H			3 ^e	514	10*	13*
I	Lyophilized stems	A	1 ^c	392	9*	32*
J			2 ^d	256	9*	20*
K			3 ^e	100	21*	81*

^a Unbound neutral components and lipids.

^b Hydrolyzed compounds from ester and insoluble-bound compounds.

^c Free acids and unbound simple compounds.

^d Hydrolyzed products from ester-bound compounds.

^e Hydrolyzed products from insoluble-bound compounds.

* Significantly different from controls, $P \leq 0.05$.

Table V. Helianthus annuus. Description of Fronds after 5 Days Exposure

Sample	Procedure	Observation
A ^a B ^b	B	Fronds light green; some white, small in size Fronds all pure white
C ^c D ^d E ^e	A	Fronds appear beige; roots beige Fronds off-white Fronds pure white
F ^c G ^d H ^e	A	One to two white fronds (total 3.7) Fronds brown; roots black; veins darkened Fronds off-white; roots brown

^a Unbound neutral components and lipids.

^b Hydrolyzed compounds from ester and insoluble-bound compounds.

^c Free acids and unbound simple compounds.

^d Hydrolyzed products from ester-bound compounds.

^e Hydrolyzed products from insoluble-bound compounds.

Table VI. Helianthus annuus. Description of Fronds after 7 Days Exposure

Sample	Procedure	Observation
A ^a B ^b	B	Fronds and roots green Fronds and roots lighter shade of green
C ^c D ^d E ^e	A	Fronds and roots beige in color, veins visible Fronds part green, yellow, and white; daughter fronds green; fronds smaller than control; roots white Fronds green but light shade, some light markings; roots white
F ^c G ^d H ^e	A	Fronds green in color, some fronds with white markings; roots green Fronds brown with veins visible (brown); roots dark brown Fronds green, yellow, and white; daughter fronds same as adults; fronds clumped together, roots white

^a Unbound neutral components and lipids.

^b Hydrolyzed compounds from ester and insoluble-bound compounds.

^c Free acids and unbound simple compounds.

^d Hydrolyzed products from ester-bound compounds.

^e Hydrolyzed products from insoluble-bound compounds.

Table VII. *Helianthus tuberosus*. The Effect of the Fractions on Growth Inhibition of *L. minor*. Final Frond Number as a Percentage of Control after 7 Days Exposure

Sample	Source	Procedure	Fraction	Application Conc. (ppm)	% of Control at Original Conc.	% of Control at 1/3 Dilution
A	Dried leaves	A	1 ^c	392	6*	112*
B			2 ^d	-	6*	6*
C			3 ^e	200	10*	-
D	Dried leaves	A	1 ^c	484	6*	9*
E			2 ^d	-	-	-
F			3 ^e	350	6*	19*
G	Dried leaves	B	1 ^a	340	6*	15*
H			2 ^b	200	114	-
I	Dried stems	A	1 ^c	664	6*	6*
J			2 ^d	286	6*	22*
K			3 ^e	686	6*	7*

^a Unbound neutral components and lipids.

^b Hydrolyzed compounds from ester and insoluble-bound compounds.

^c Free acids and unbound simple compounds.

^d Hydrolyzed products from ester-bound compounds.

^e Hydrolyzed products from insoluble-bound compounds.

* Significantly different from controls, $P \leq 0.05$.

Table VIII. Helianthus tuberosus. Description of Fronds after 7 Days Exposure

Fraction	Observation	
	Original Concentration	1/3 Dilution
A ^c	Fronds and roots white	Fronds and roots green
B ^d	Fronds and roots beige	Fronds and roots beige
C ^e	Most fronds white; younger fronds green; roots beige	-
D ^c	Most of frond white; daughter frond part green; roots white	Larger frond mostly white; daughter fronds green and yellow
E ^d	-	-
F ^e	Fronds and roots white	Fronds pale green and roots white
G ^a	Fronds and roots white	Fronds are whitening (larger fronds); daughter fronds green
H ^b	Fronds and roots green	-
I ^c	Fronds and roots white	Fronds and roots beige
J ^d	Fronds and roots beige	Some fronds pale green; some beige; roots beige
K ^e	Fronds and roots white	Fronds and roots white

^a Unbound neutral components and lipids.

^b Hydrolyzed compounds from ester and insoluble-bound compounds.

^c Free acids and unbound simple compounds.

^d Hydrolyzed products from ester-bound compounds.

^e Hydrolyzed products from insoluble-bound compounds.

to the plant tissues studied. Since some of them are conjugated and released slowly in nature, the harsh alkaline hydrolysis was employed to liberate the potential allelochemical moieties from the compounds in which they were covalently bound and which would not be readily extracted under milder conditions. All fractions showing a phytotoxic effect are being further characterized and their phytotoxicity evaluated.

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