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Allelopathy of small everlasting (*Antennaria microphylla*): Identification of constituents phytotoxic to leafy spurge (*Euphorbia esula*)¹

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Abstract:

The ether extract of small everlasting (*Antennaria microphylla* Rydb.) was phytotoxic to lettuce seed root growth and leafy spurge (*Euphorbia esula* L. #² EPHEs) root elongation and cell culture growth. Three phenolic compounds (hydroquinone, arbutin, and caffeic acid) were chromatographically isolated and characterized from the ether extract. Arbutin and caffeic acid were moderately phytotoxic to leafy spurge root growth at 300 ppm (w/v), and hydroquinone was strongly phytotoxic to leafy spurge root growth at 50 ppm (w/v). The observed phytotoxicity of hydroquinone and the high-yield natural occurrence of arbutin, a water soluble, easily hydrolyzed monoglucoside of hydroquinone, in small everlasting is consistent with the participation of these two compounds in the observed allelopathy of small everlasting against leafy spurge.

Additional index words:

Hydroquinone, arbutin, caffeic acid, benzoquinone, phenolics, EPHEs.

Introduction

The rapid expansion of leafy spurge in the open rangeland of the upper Great Plains of the United States represents one of the major noxious weed problems in North Amer-

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² Letters following this symbol are a WSSA-approved computer code from Composite List of Weeds, Weed Sci. 32, Suppl. 2. Available from WSSA, 309 West Clark St., Champaign, IL 61820.

ica (4, 10). While rigorous chemical control programs have been effective in controlling this weed (8), the cost is high and continuous. No chemical control programs have been successful in eradicating leafy spurge.

Biological control utilizing insects and pathogens has been successful in controlling leafy spurge infestations in Europe (12), but when applied to North America spurge, these control methods have proven ineffective. The lack of success of insect biocontrol methods has been attributed to nonhost specificity (1), which may be the result of environmental factors and/or genetic diversification of the domestic spurge.

New methods for the control and eradication of leafy spurge are needed. The observed natural susceptibility of spurge to competing plants may be an important factor in developing improved control methods. One plant, small everlasting, has previously been reported to be allelopathic to leafy spurge (11). Experimental evidence supported the suggestion that small everlasting produced a chemical substance that inhibited leafy spurge seed germination and seedling development (13). It was noted that the biologically active chemical constituents of this plant could be potentially important model compounds that might be useful in developing chemical control systems for leafy spurge.

We report the isolation and characterization (guided by bioassay) of specific chemical compounds from small everlasting that are biologically active against leafy spurge. These compounds may be important in the observed allelopathy of small everlasting against leafy spurge.

Materials and methods

Plant collection and extraction

Small everlasting plant material was obtained from a plot measuring approximately 2 m² in the Sand Hills area near Fargo, ND, in July 1983. Whole plant material was collected, air dried (1.9 kg), and hammermilled (3 mm mesh), and the plant material was sequentially extracted (for 1-week periods) in a soxhlet extractor following a nonpolar to polar solvent extraction scheme to minimize fat and wax constituent effect on polar component extraction. The solvent sequence was *n*-hexane, ethyl ether, acetone, and methanol. The resulting extracts were concentrated to dryness (in vacuo, 50 C), and stored (10 C).

Lettuce root growth bioassay

Lettuce (*Lactuca sativa*) root growth bioassay was used to determine the extracts, chromatographic fractions, or individual chemical compounds that possessed significant biological activity. Lettuce seeds (Ferry Morse, variety "Black Seedless Simpson") were germinated (radical length > 2 mm, 24-hour illumination, 28 C, overnight) on 40-ml lay-

ers of agar gel (Bacto-Agar)³(0.5% w/v) in covered petri dishes to insure viable seedlings for every assay.

Test extracts, chromatographic fractions, or individual commercially available compounds⁴ were dissolved in methanol or accurately weighed for introduction into the 40-ml solutions of hot agar in petri dishes. The agar layers were cooled and twelve germinated lettuce seeds were placed in each dish. The dishes were covered and kept in the dark for 72 hours (room temp.). The root length for each seedling was measured to the nearest millimeter and the highest and lowest lengths were discarded. A mean root length was determined for 10 roots (three replications). A control, containing solvent only (no extract or fraction), was included in each determination.

The test concentrations used in the bioassays of this investigation represent reasonable biological test levels for the comparison or identification of naturally occurring phytotoxic chemicals or chemical complexes. The minimal test concentration (50 ppm) used in this investigation was considered a phytotoxic "threshold" level consistent with an acute evaluation of a specific naturally occurring chemical acting in the allelopathic complex of a native plant colony without clearly defined physical, chemical, and microbiological parameters.

Figure 1. Chemical constituents of small everlasting I, II, III) and benzoquinone (IV).

Ether extract fractionation

A portion (10 g) of the total ether extract was dissolved in methanol (30 ml) and applied to a Sephadex LH-20 chromatographic column (5 cm × 45 cm). Macromolecular and polymeric material (including chlorophyll) was removed from the extract by elution with chloroform (2 L), and this biologically inactive fraction was discarded. The remain-

³ Reference to a company and/or product named by the U.S. Dep. Agric. is only for purpose of information and does not imply approval or recommendation of the product to the exclusion of others that may be suitable.

⁴ Sigma Chemical Co., St. Louis, MO.

ing extract was stripped from the column with CHCl_3 :MeOH (4:1, v/v) (2 L). The stripped extract was concentrated (in vacuo, 50 C) and reapplied to LH-20 (CHCl_3 :MeOH (8:1, v/v) and ten 400-ml fractions were again collected. The fractions were evaporated (in vacuo, 50 C) to dryness and tested in the lettuce root growth bioassay. Fractions 7 and 8 were phytotoxic in the lettuce root growth bioassay [23 and 42% of control, respectively, at 250 ppm (w/v)] .

Fraction 7 was redissolved in MeOH and applied to a semipreparative high-performance liquid chromatography (HPLC) column (silica, 7.8 mm $\times$ 30 cm). The column was eluted (isocratic, 2 ml/min, RI detection) with CHCl_3 : MeOH (9:1, v/v) and 30 two-ml fractions were collected. Fractions 8 and 9 from the HPLC chromatography appeared crystalline upon concentration (in vacuo, 50 C) and were recrystallized (water) to yield compound I (light buff-colored needles, 27 mg), site yield⁵ ($23.4 \times 10^{-2} \text{ g/m}^2$, $2.1 \times 10^{-4} \text{ moles/m}^2$), mp 138 C. M.S.: $\text{C}_6\text{H}_6\text{O}_2$ m^+ m/z requires 110.03678; found 110.03677. The compound was identical [melting point (mp), mixed melting point (mmp), spectral, chromatographic] with authentic 1,4-dihydroxybenzene (hydroquinone) (I, Figure 1).

Fraction 8 from the LH-20 chromatography of the stripped ether extract was semipreparatively chromatographed on the silica HPLC column (isocratic, 2 ml/min, RI detection, CHCl_3 : MeOH (8:1, v/v) and 30 four-ml fractions were collected. HPLC fractions 5, 6, 7, and 8 crystallized upon concentration (in vacuo, 50 C). Recrystallization of fractions 5 and 6 (water) yielded compound II (buff-colored needles, 136 mg), site yield⁵ ($11.8 \times 10^{-2} \text{ g/m}^2$, $6.6 \times 10^{-4} \text{ moles/m}^2$), mp 225 C (d). M.S.: $\text{C}_9\text{H}_8\text{O}_4$ requires m^+ m/z 180.04226; found 180.04228. The compound was identical (mp, mmp, spectral, chromatographic) with authentic caffeic acid (II, Figure 1).

Crystalline fractions 7 and 8 from the HPLC chromatography of LH-20 fraction 8 were recrystallized (water) to yield compound III (colorless needles, 383 mg), site yield (0.331 g/m^2 , $1.2 \times 10^{-3} \text{ moles/m}^2$), mp 143 C. M.S.: $\text{C}_{12}\text{H}_{16}\text{O}_7$ requires m^+ m/z 272.08960; found 272.08961. The compound was identical (mp, mmp, spectral, chromatographic) with authentic arbutin (III, Figure 1).

Leafy spurge root elongation bioassay

A root elongation bioassay was used to assess the biological activity of various extracts or specific chemical compounds obtained from small everlasting against leafy spurge. Field-collected (summer 1983) leafy spurge seeds were germinated by placing 20 seeds between two layers of Whatman No. 1 filter paper in a 9-cm-diam covered petri dish. Approximately 10 ml distilled water was added to each dish, which was placed in a growth chamber at 28/10 C, day/night temperatures on a 16-hour day/8-hour night photoperiod for 3 days. Test extracts, single compounds were dissolved in water or methanol and a given aliquot was introduced to each petri dish to achieve the desired final concentration in a 40-ml solution of warm agar [0.5% (w/v)]. The mean root length (to the nearest millimeter) of six uniformly germinated spurge seeds was determined, and the seedlings were then placed in the petri dishes (3 replications). Controls containing solvent

⁵ Calculated yield of chemical constituent occurring in plant material from sample plot.

only were included. After 72 hours under the same conditions as for germination, final mean root lengths were determined.

Leafy spurge cell culture bioassay

Leafy spurge cell suspension cultures (Accession No. 1979 ND 001) established from callus tissue in the Botany Department, North Dakota State University, Fargo, were used to evaluate the biological activity of small everlasting against leafy spurge. Cultures were maintained on a basic Gamborg's (14) B5 medium containing 1 ppm (w/v) (2,4-dichlorophenoxy)acetic acid (B5+1). Subcultures were established by inoculating 25-ml aliquots of B5+1 medium contained in 125-ml erlenmeyer flasks with 5-ml portions of a mature culture (about 0.25 mg spurge cells). Test compounds were dissolved as for spurge root elongation bioassays and appropriate volumes injected into the culture medium to establish desired concentrations from 200 to 500 ppm (w/v) (3 replications). Controls containing no compounds were included. Test samples were filtered through a sterilizing filter (0.45 μ m). Subcultures were incubated in the dark at 24 C on a gyratory shaker (100 rpm) for 7 days. Cultures were harvested by vacuum filtration on miracloth (Calbiochem), washed with distilled water, and oven dried (25 C, 72 hours), and dry weights were determined.

Statistical analysis

Mean root length and cell culture weight measurements of individual determinations were subjected to analyses of variance to estimate pooled variances based upon three replications per treatment $\times$ concentration combinations. Standard errors of means, 5% least significant difference, and F-values were calculated to determine the significance of treatment $\times$ concentration interaction in each bioassay. All bioassays included three replications per treatment, and twelve lettuce seeds or six leafy spurge seeds were used in each replication. Extracts and fractions were analyzed in a single bioassay. Bioassay of individual chemical constituents' effect on lettuce seedlings and leafy spurge seedlings and cell cultures was duplicated.

Results and discussion

The phytotoxicity tests of the four extracts of small everlasting (assessed by a lettuce root growth bioassay) established that the ether extract of small everlasting possessed the highest biological activity (Table 1). The activity of the ether extract is of particular significance, considering its limited solubility in the aqueous bioassay system. This extract was also the most phytotoxic to leafy spurge root growth.

On the basis of bioassay results, the ether extract was selected for closer examination to identify specific components toxic to leafy spurge. Gel filtration/absorption chromatography and subsequent silica gel absorption chromatography of the extract, guided by lettuce root growth bioassay, resulted in the isolation and characterization of three crystalline compounds: hydroquinone [I, 0.002% (w/w) of dry plant wt], caffeic acid [II,

0.012% (w/w) of dry plant wt], and arbutin [III, 0.034% (w/w) of dry plant wt] (Figure 1). A lettuce root growth bioassay of pure commercial samples of these compounds showed that hydroquinone and caffeic acid reduced lettuce seed root growth by 81 and 49%, respectively, at 50 ppm (w/v), whereas arbutin showed mild auxin effects, i.e., increased lengths by 15% at 50 ppm (w/v) (Table 2). Benzoquinone (the oxidation product of hydroquinone (IV, Figure 1) was also evaluated in this bioassay and was more toxic to root growth (89% reduction) than hydroquinone.

Table 1. The effect of small everlasting extracts on the growth of lettuce roots and the elongation of leafy spurge roots.

Extract	Lettuce			Leafy spurge		Solubility factor ^a
	Conc. [ppm (w/V)]			Cone. [ppm (w/v)]		
	100	300	500	300	500	
	————— (%) ^b —————			————— (%) ^c —————		
Hexane	91	100	97	78	82	0
Ether	77 ^d	41 ^d	26 ^d	36	24	2
Acetone	92 ^d	84 ^d	63 ^d	62	58	1
Methanol	81 ^d	69 ^d	66 ^d	65	60	3

^a Empirical solubility of extract: O=insoluble, 1=mostly insoluble, 2=mostly soluble, 3=totally soluble in aqueous test system.

^b Percent of control, average of averaged extract/concentration mean lengths (three replications) vs. average of averaged control mean lengths (three replications). Standard error of means (treatment × concentration) = 0.49,

^c All values significantly different from control, 5% LSD = 0.84, and standard error of means (treatment × concentration) = 0.28.

^d Significantly different from control, 5% LSD = 1.43.

The assessment of phytotoxicity of compounds I, II, III, and IV toward leafy spurge was achieved through a leafy spurge root elongation bioassay and a leafy spurge cell culture inhibition test (Table 2). Benzoquinone was again included in these two tests to consider the effect of in vivo oxidation on the phytotoxicity of hydroquinone. The data in Table 2 shows the toxicity of hydroquinone and benzoquinone toward leafy spurge in the two test systems. Arbutin was observed to be phytotoxic to spurge seedlings with a 51% reduction in spurge root length observed at 300 ppm (w/v). This level of toxicity is in contrast to the observed auxin effect of arbutin in the lettuce root growth bioassay. The spurge root elongation bioassay of caffeic acid showed lower inhibition of root elongation than was observed in the lettuce root growth bioassay with some mild auxin effect appearing at low concentrations. The cell culture bioassay (Table 2) supported the root growth bioassay results.

The phenolic compounds (hydroquinone, arbutin, and caffeic acid) isolated and characterized from biologically active chromatographic fractions of the ether extract of small everlasting have been found to occur in several plant families. However, the identification of arbutin and hydroquinone in small everlasting represents only the second reported occurrence of these compounds in the Compositae family. The two compounds were previously reported to occur in three species of *Serratula* (Compositae) (16).

Hydroquinone and arbutin have been considered as allelopathic agents from the aqueous leachates of species of manzanita (*Arctostaphylos*) (2, 7) and chaparral (*Adenostoma*) (9). Because of the low toxicity of arbutin, there has been speculation (2, 9) that hydroquinone and benzoquinone, originating from a large arbutin pool by hydrolysis and oxidation, were the chemical agents responsible for the observed allelopathy of manzanita and chaparral. Testing of hydroquinone with wild oats (*Avena fatua*) and bromegrass (*Bromus rigidus*) (2) showed significant radical growth suppression at 50 ppm (w/v).

The relatively high yield of arbutin in small everlasting and its high water solubility provide favorable characteristics for leaching from plant tissues and the subsequent transport and absorption by other plants. The ultimate chemical state (arbutin vs. hydroquinone) available for absorption would be dependent upon decomposition mechanisms (i.e., hydrolysis, oxidation) affecting arbutin during transport.

Both arbutin and hydroquinone are readily and continuously absorbed by barley roots (6), and hydroquinone is glycosylated to form arbutin. This suggests that hydroquinone is detoxified by glucosylation unless an oversupply of arbutin could trigger a hydrolysis/equilibrium mechanism producing phytotoxic hydroquinone from the excess arbutin. It is clear from our cell culture bioassay results that hydroquinone is not significantly detoxified *in vivo*, therefore questioning the ability of leafy spurge to glucosylate and detoxify hydroquinone as observed in barley. The observed toxicity of benzoquinone in the cell cultures and seed bioassays also indicates that oxidation processes affecting hydroquinone will not detoxify the compound *in vivo*.

Phenolic compounds and their derivatives have been frequently cited as chemical sources of allelopathy (11). Some investigations of the effects of phenolic on seed germination have shown: a) inhibition only at concentrations higher than usually occur in the soils (approx. 10^{-3} M) (15), b) additive effects but no synergism among common phenolics (3, 15), and c) antagonism toward phenolic inhibition by caffeic acid (3). However, synergistic effects have been reported for four cinnamic acids on grain sorghum (*Sorghum bicolor*) seed germination, radical elongation, and seedling growth, with seedling growth the most sensitive to phenolic combinations at concentrations above 1×10^{-4} M (5).

Our investigation clearly shows hydroquinone to be a potent phytotoxin to developing leafy spurge seedlings in an acute test system at concentrations near 5×10^{-4} M. The calculated availability of hydroquinone (as hydroquinone and hydrolyzed arbutin) in the surface layer (1 cm) of the small everlasting sample plot was 3.5 times less (1.4×10^{-4} M) than the acute hydroquinone test concentration. This concentration is above the observed threshold level for inhibition seedling growth in grain sorghum (5) and is consistent with the effective chronic phytotoxic exposure of developing leafy spurge seedlings to hydroquinone in the natural biosphere of established stands of small everlasting. It is therefore probable that hydroquinone functions with arbutin in the observed allelopathy of small everlasting to leafy spurge.

Table 2. The effect of chemical constituents occurring in small everlasting on the growth of lettuce roots, root elongation, and cell culture growth of leafy spurge.

Compound	Lettuce roots				Spurge rows			Spurge cell culture		
	Conc. [ppm (w/v)]				Conc. [ppm (w/v)]			Conc. [ppm (w/v)]		
	50	100	300	500	50	100	300	200	300	500
	(%) ^a				(%) ^a			(%) ^a		
Hydroquinone (I)	19	12	9	0	36	7	0	79	20	24
Caffeic acid (II)	51	48	18	13	110	81	36	106	105	103
Arbutin (III)	115	114	113	119	81	70	49	87	86	74
Benzoquinone (IV)	11	9	6		34	10	0	87	36	19

^a Percent of control, average of averaged compound/concentration mean lengths (three replic:16011S) vs. average of averaged control mean (three replications).

^b All values significantly different from control, 5% LSD = 1.25 and standard error of means (treatment × concentration) = 0.43.

^c All values significantly different from control, 5% LSD = 0.79 and standard error of means (treatment × concentration) 0.27.

^d All values significantly different from control, 5% LSD = 0.86 and standard error of means (treatment × concentration) 0.29.

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