

Research papers

Lignoid chemical defenses in the freshwater macrophyte *Saururus cernuus*

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Summary. Chemical defense against herbivores has rarely been investigated for freshwater plants, possibly due to the common misconception that herbivory on aquatic macrophytes is low and would not select for chemical defenses. In previous work, the freshwater angiosperm *Saururus cernuus* was shown to be a low preference food for omnivorous crayfish despite its high nutrient value and relatively soft texture. We used feeding by the crayfish *Procambarus clarkii* to guide fractionation of the deterrent lipid-soluble extract of this plant, leading to the identification of seven deterrent lignoid metabolites, (–)-licarin A, (+)-saucer-netin, (–)-dihydroguaiaretic acid, (–)-sauriols A and B, (–)-saucerneol, and (–)-saucerneol methyl ether. Lignans have been implicated in terrestrial plant chemical defenses as insect growth inhibitors, insect toxins, nematocides, antibacterial, and antifungal agents. However, these activities have rarely been demonstrated using ecologically relevant methodologies in terrestrial systems, and never before in freshwater systems. The widespread nature of lignans amongst very distantly related plants, along with their rich diversity of molecular structure, suggests that they could play a large role in mediating plant-herbivore interactions. In addition to the lignoid compounds we identified, there were other compounds present in low concentration or unstable compounds that were deterrent, that did not appear to be lignans, but that we were unable to identify. This plant thus appears to be defended by a complex mixture of natural products.

Key words. Plant-herbivore interactions – chemical defense – lignan – freshwater macrophyte – crayfish – *Saururus cernuus* – *Procambarus clarkii*

Introduction

Two basic, but contested, assumptions have shaped the study of plant-herbivore interactions in freshwater systems. These are 1) that rates of herbivory on live

aquatic macrophytes are low and have minimal effects on the structure of freshwater communities (Shelford 1918; Wetzel 1983; Gregory 1983), and 2) that these low rates of feeding are due to the toughness and poor nutritive value of freshwater macrophytes relative to benthic algae and decomposing macrophytes (Hutchinson 1975; Gregory 1983; Lamberti & Moore 1984). Some researchers have thus concluded that freshwater macrophytes have little need for chemical defenses and, indeed, appear to contain few toxic or unpalatable secondary metabolites (McClure 1970; Hutchinson 1975). Recent experiments, as well as more rigorous reviews of older data, question the validity of these premises (Lodge 1991; Newman 1991; Cyr and Pace 1993; Lodge *et al.* 1998; Cronin 1998; Bolser *et al.* 1998). Cyr & Pace (1993) compared herbivory in terrestrial, freshwater, and marine systems and found that herbivory in freshwater systems was about three times higher than in terrestrial systems. They concluded that herbivory on freshwater macrophytes was lower than on marine seaweeds, but considerably higher than on terrestrial plants. Overviews of previous studies also support the idea that grazers significantly affect the distribution and abundance of macrophytes in freshwater systems (Lodge 1991; Newman 1991; Lodge *et al.* 1998). In particular, herbivorous macroinvertebrates such as crayfish (Feminella & Resh 1989; Lodge 1991; Cronin 1998) and vertebrates such as fishes, mammals, and waterfowl (*e.g.*, Fassett 1940) can have dramatic effects on aquatic macrophytes (Carpenter & Lodge 1986; Feminella & Resh 1989; Lodge 1991; Lodge *et al.* 1998).

The differential susceptibility of freshwater macrophytes to relevant herbivores has been tested in relatively few systems (*e.g.*, Lodge 1991; Bolser & Hay 1998; Bolser *et al.* 1998; Cronin 1998). In these studies, plant traits such as food quality, morphology (size, shape, toughness), and total phenolic content were often inadequate predictors of plant palatability, suggesting that secondary metabolites other than tannins play important roles in determining the susceptibility of macrophytes to herbivory (Smock & Stoneburner 1980; Otto & Svensson 1981; Ostrofsky & Zettler 1986; Suren 1989; Lodge 1991; Bolser *et al.* 1998). However, to our

knowledge, there are only two systems for which rigorous demonstrations of chemical deterrence were made by isolating, identifying, and assaying the compounds responsible. First, Newman *et al.* (1990, 1992, 1996) used feeding assays with several herbivores to show that watercress (*Nasturtium officinale*) was protected by 2-phenylethyl isothiocyanate, which was produced by myrosinase activity on the biological precursor 2-phenylethyl glucosinolate. Second, a hydroxybenzylsuccinic acid derivative, habenariol, was found to deter the crayfish *Procambarus clarkii* from feeding on the aquatic orchid *Habenaria repens* (Bolser *et al.* 1998; Wilson *et al.* 1999).

In a survey of plant palatability to the omnivorous crayfish *Procambarus clarkii*, the macrophyte *Saururus cernuus* ranked low relative to other freshwater macrophytes (Bolser *et al.* 1998; Hay *et al.* unpublished). Bolser *et al.* (1998) reported that amongst four co-occurring species, *S. cernuus* was the least palatable, despite its high relative nutritive value and low tissue toughness. Both lipophilic and water-soluble extracts from this plant significantly deterred crayfish feeding, but the active compounds were not identified. The goal in this study was to identify the defensive compounds from the lipophilic extracts of *Saururus cernuus*. We used feeding by the crayfish *Procambarus clarkii* as a bioassay to guide our chemical investigations, viewing this research as an additional test of the recent suggestions that chemical defenses could be important, and common, among freshwater macrophytes.

Methods and materials

The plant

Saururus cernuus (Saururaceae) is an emergent freshwater angiosperm commonly found throughout the southeastern United States. Also known as lizard's tail and breast weed, it has long been used as a folk remedy for inflammation of breasts, kidneys, and bladder (Phares 1867), as a poultice for tumors (Hartwell 1971), and as a sedative (Phares 1867). The chemical constituents responsible for these properties have never been identified, although lignoid natural products with some notable biological activities have been isolated from this plant (Rao & Alvarez 1982, 1983; Rao *et al.* 1987; Rao & Chattopadhyay 1990; Rao & Reddy 1990; Rao & Rao 1990; Rao & Oruganty 1997). No ecological functions for these compounds in *S. cernuus* have been postulated or tested.

Emergent portions of *Saururus cernuus* were collected from the pond at the entrance to White Sands subdivision in Newport, North Carolina. Tissue toughness, dry mass per volume, ash-free dry mass per volume, total phenolics, and carbon, nitrogen, and soluble protein content were measured for this collection and are reported in Bolser *et al.* (1998). Tissues from this same collection were frozen at -70°C , freeze-dried, and used to generate the extracts for the data reported here.

Feeding assays

Laboratory feeding assays with the omnivorous Louisiana red crayfish *Procambarus clarkii* were used to follow and isolate the chemical deterrence of plant extracts and purified fractions. *P. clarkii* was used because (1) crayfishes are generalist feeders that include a wide variety of freshwater plants in their diets (Gaeveskaya 1969; Hobbs 1993), (2) they can have dramatic effects on the distribution and abundance of freshwater macrophytes (Lodge & Lorman 1987,

Creed 1994; Lodge *et al.* 1994), and (3) they feed well in laboratory assays (Chambers *et al.* 1991; Lodge 1991). Adult crayfish were acquired from a Louisiana hatchery and maintained on a diet of commercial trout food. Each of 60 crayfish was held separately in a 2-L plastic tub with re-circulating freshwater. Artificial foods used in assays were prepared according to the methods in Bolser *et al.* (1998) and generalized in Hay *et al.* (1994, 1998). The treatment assay food consisted of chemical extracts or purified fractions of *Saururus cernuus* coated onto finely powdered freeze-dried broccoli and lettuce (1:1 mixture) mixed with molten agar. This mixture was then poured onto plastic window screening material to create an artificial food over a grid matrix. Feeding was quantified as the number of grid squares from which the food was removed. Concentrations of extracts and pure compounds matched those isolated from *S. cernuus* on the basis of food dry mass. Control foods were made in the same manner as treatment foods but without the addition of extracts or purified chemicals. For each assay, 30 replicate animals were offered a window-screen strip containing both a control and a treatment food square of the same size. Assays were monitored every 30 minutes and terminated when approximately 50% of either the treatment or control food had been eaten or when three hours had passed, whichever occurred sooner. Replicates in which greater than 90% or less than 10% of total food was consumed were discarded because feeding may have been so extensive that preferences were obscured or because there may have been so little feeding that preferences were not clearly expressed. Feeding on control versus treatment foods was analyzed using one-tailed, paired t-tests (Zar 1984).

Isolation of plant antifeedants

Frozen plants were freeze-dried, ground to a powder, weighed, and then extracted with methanol/dichloromethane (1:2) six times (6×600 mL extraction solvent for 40 g freeze-dried plant). Extracts were filtered, concentrated *in vacuo*, and then suspended in deionized water and repeatedly extracted with dichloromethane. The dichloromethane extract (7.54 g oil from 40 g dry plant) was fractionated using Sephadex LH-20 with methanol/dichloromethane (1:1) as eluent. Fractions were pooled according to TLC characteristics and assayed. Deterrent fractions were subjected to further fractionation using silica gel flash column chromatography with a gradient eluent system of hexanes to diethyl ether and then to diethyl ether/methanol (1:1). The most deterrent fractions were purified by HPLC using a C-18 silica semi-preparative column with isocratic eluent systems methanol/water (9:1) or acetonitrile/water (7:3). Fractions and pure compounds were protected from oxidation by the addition of 0.1–1.0 mg of ascorbic acid (vitamin C). Preliminary assays with ascorbic acid demonstrated that it had no detectable effect on crayfish feeding.

Characterization of plant antifeedants

Pure compounds that deterred crayfish feeding were analyzed by spectroscopic methods in order to elucidate their molecular structures. ^1H , ^{13}C , DEPT, and two-dimensional inverse-detected nuclear magnetic resonance spectral data (COSY, HMQC, HMBC) were acquired on Varian Inova 300 and Gemini 400 MHz NMR spectrometers. Electron impact mass spectra were run on a Hewlett-Packard 5988A mass spectrometer. Pure compounds were also analyzed by FAB, ESI, or MALDI mass spectrometry. Infrared spectra were run as thin films on a Perkin Elmer 1600 Series FTIR system. UV measurements were made on a Perkin Elmer Lambda 3B UV/VIS spectrophotometer. Optical rotations were measured on a Rudolph Research Autopol III polarimeter. Data from these analyses are reported elsewhere (Kubanek *et al.* 2000).

Results

Bioassay-guided fractionation of lipophilic extracts from *Saururus cernuus* led to the identification of seven deterrent lignoid metabolites (Fig. 1). Five of these were known to occur in *S. cernuus*: the two lignans

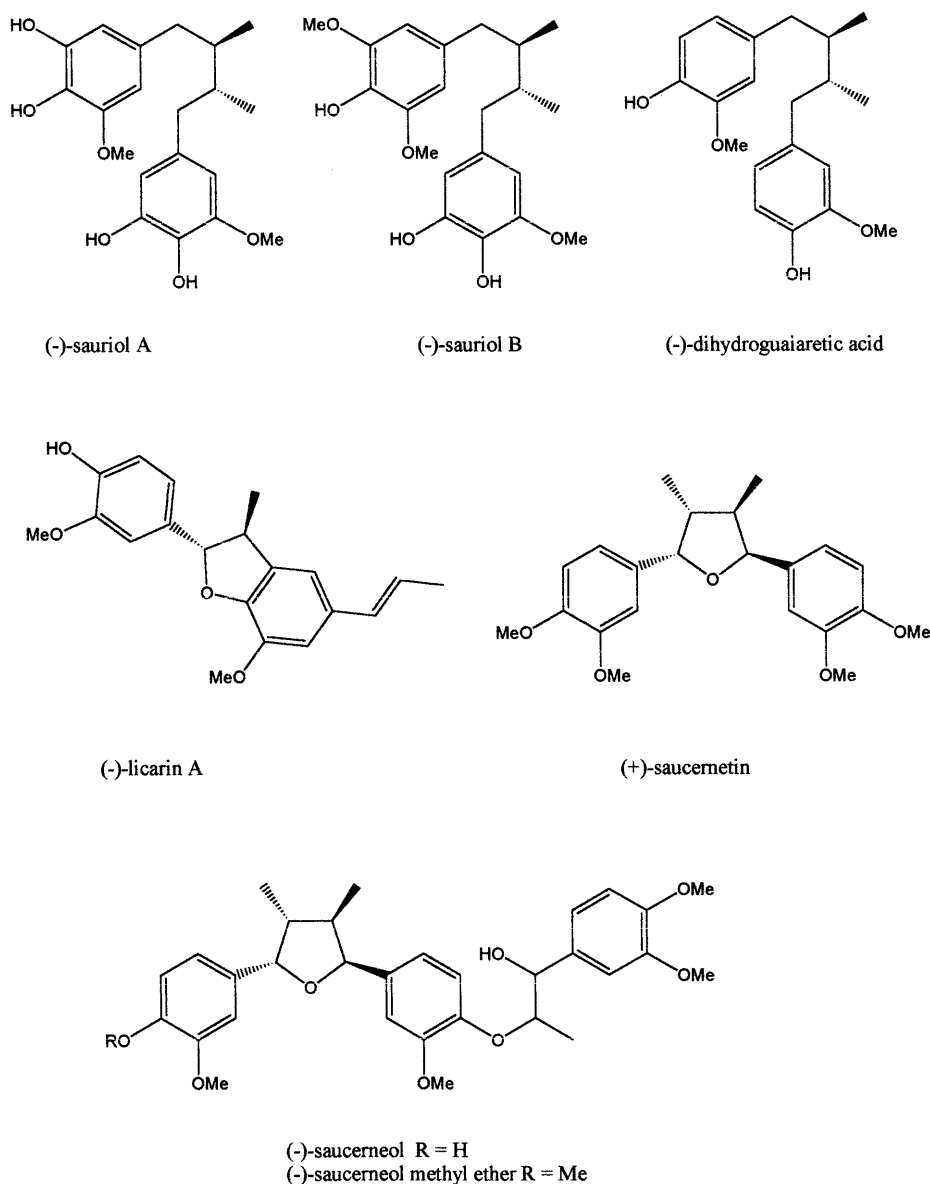


Fig. 1 Antifeedant compounds of *Saururus cernuus*

(-)-dihydroguaiaretic acid (Rao & Chattopadhyay 1990) and (+)-saucermetin (Rao & Alvarez 1982), the neolignan (-)-licarin A (Rao & Oruganty 1997), and the sesquieneolignans (-)-saucerneol (Rao & Alvarez 1983) and (-)-saucerneol methyl ether (Rao & Oruganty 1997). Two novel lignoid natural products, (-)-sauriols A and B, were also identified and were found to share the carbon skeleton of guaiaretic acid, but with different oxidation and methylation patterns. Their structures were elucidated by spectroscopic analysis (Kubaneck *et al.* 2000). Six of these compounds were shown to significantly deter feeding by *Procambarus clarkii* when tested at natural concentrations in agar-based artificial diets. The seventh, saucerneol, was not deterrent at its isolated concentration (0.020% w/w), but was deterrent at three times this concentration (Table 1, Fig. 2). Because of small losses of material at each step of the purification process, it is possible that three times the isolated concentration is within the

range of natural concentrations in whole plant tissue. The small quantities of metabolites isolated prevented assaying compounds at several different concentrations to determine minimum inhibitory concentrations.

Table 1 Isolated yields of deterrent compounds from *Saururus cernuus*

Compound	Isolated yield (% by dry mass of plant)
(-)-licarin A	0.55
(-)-dihydroguaiaretic acid	0.11
(+)-saucermetin	0.84
(-)-sauriol A	0.006
(-)-sauriol B	0.038
(-)-saucerneol	0.020
(-)-saucerneol methyl ether	0.012

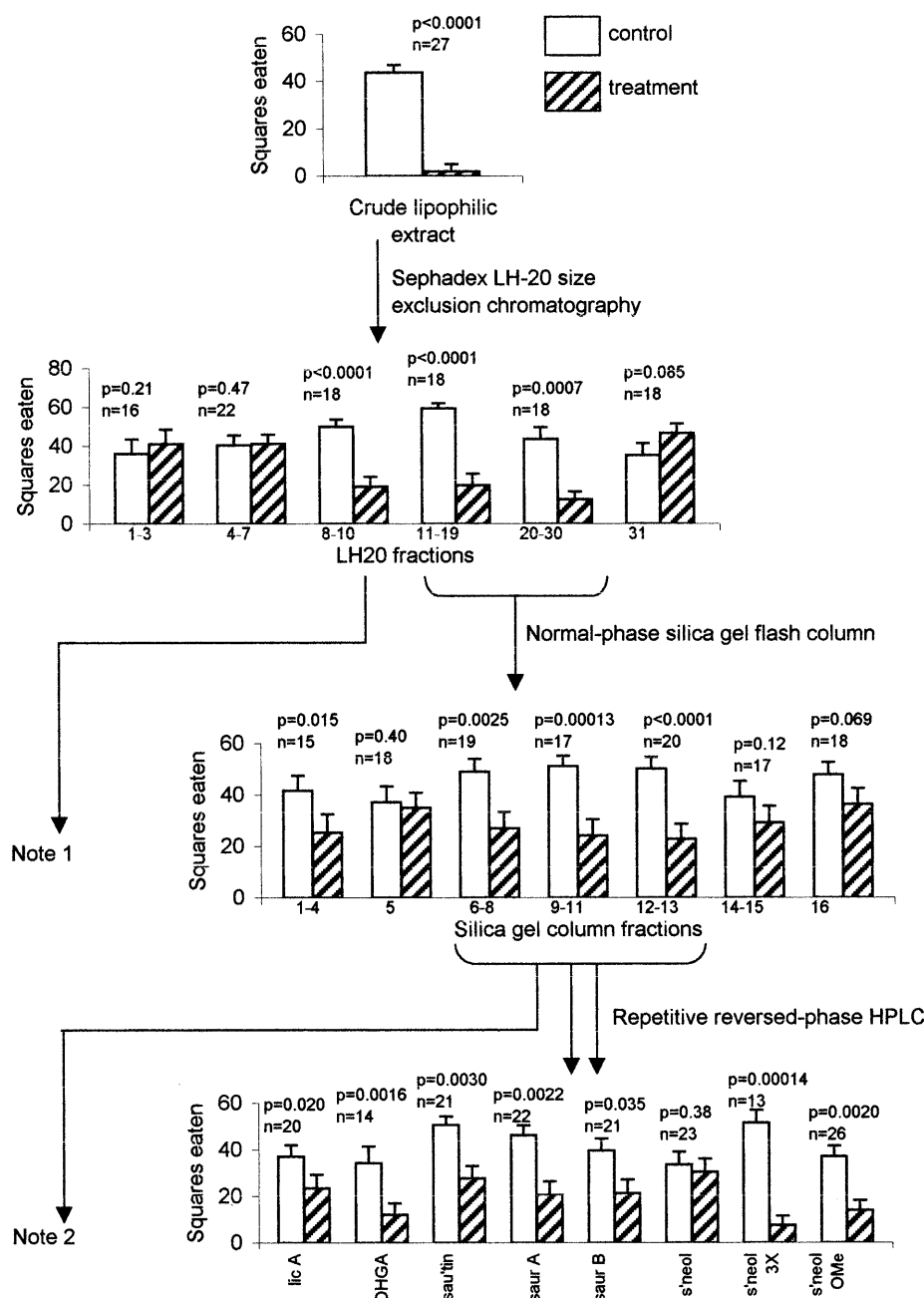


Fig. 2 Bioassay-guided fractionation of the lipophilic extract of *Saururus cernuus*. Bars show means + one standard error. Number of paired replicates used in crayfish assays is indicated above each bar pair. P-values are from 1-tailed paired t-tests. All fractions and compounds were tested in artificial foods at the concentration (by dry mass) at which they were isolated from *S. cernuus*, except saucerneol which was also tested at three times its isolated concentration. Repetitive HPLC separation of deterrent fractions yielded many more fractions than shown; all were assayed, but only deterrent pure compounds that could be identified are shown in the last chart. Lic A = licarin A; DHGA = dihydroguaiaretic acid; sau'tin = saucerneol; saur A = sauriol A; saur B = sauriol B; s'neol = saucerneol; s'neol OMe = saucerneol methyl ether. For yields of compounds, see Table 1. *Note 1* – Further fractionation and bioassays from this LH-20 fraction led to a deterrent compound that decomposed during purification and to a deterrent fraction that contained primarily the vitamin C we added, to prevent compound oxidation, along with a complex mixture of trace compounds, none of which possessed any aromatic ^1H NMR spectral signals characteristic of lignans. The low concentrations of these metabolites mandated that we leave them unresolved. *Note 2* – In addition to the data shown, we also produced (1) a deterrent fraction that represented less than 0.001% of plant dry mass and had no visible ^1H NMR spectral signals – probably representing a deterrent metabolite that functioned at concentrations too low to detect with these methods, (2) a deterrent fraction consisting of the “wash” from our column – this wash fraction contained small traces of the compounds shown in the figure, and (3) a deterrent fraction composed of vitamin C (added as an antioxidant) and many low concentration compounds, none of which produced ^1H NMR spectral signals characteristic of lignans.

The deterrent metabolites (Fig. 1) represented the majority by dry weight of lipid-soluble antifeedant components in *Saururus cernuus*. However, in addition to the seven compounds purified and identified, several other fractions also contained deterrent components that remain unidentified (Fig. 2). Some fractions represented less than 0.001% of plant dry mass and had no visible ^1H NMR signals, yet were significantly deterrent. Other deterrent fractions consisted of complex intractable mixtures of many compounds, none of which possessed any aromatic signals by ^1H NMR spectroscopy that are characteristic of lignoids. One non-lignoid deterrent compound decomposed before its molecular structure was determined. The fractionation of extracts of *S. cernuus* also led to many non-deterrent

portions, representing the bulk of the extract. The fractions that did not deter crayfish feeding were not subjected to further purification or spectral analysis, and therefore the identities of compounds within these fractions remain unknown.

Discussion

Lignoid metabolites from *Saururus cernuus* deterred feeding by the crayfish *Procambarus clarkii*. Seven antifeedant compounds (four classical lignans, one neolignan, and two sesquiolignans; Fig. 1) were identified by bioassay-guided fractionation of lipophilic extracts of *S. cernuus* (Fig. 2). Five of these compounds were

known from this plant species, whereas two others were novel natural products (Kubaneck *et al.* 2000).

The unpalatable nature of these metabolites helps explain why *Saururus cernuus* is a low preference food to the crayfish despite average protein, high nitrogen, and low toughness when compared to other co-occurring plants (Bolser *et al.* 1998). In addition to the seven compounds identified, other metabolites that were lower in concentration, or that decomposed during the purification procedures, also deterred crayfish feeding. The small quantities of these metabolites, along with their similar physical properties, made their purification and identification problematic, although spectral analysis indicated that lignans other than the seven found (Fig. 1) were not present amongst these deterrent fractions. Bolser *et al.* (1998) found that water-soluble components of *S. cernuus* were also significantly deterrent to this crayfish. It thus appears that *S. cernuus* is defended by numerous different types of metabolites. Although the lignans identified in this study function as defenses against consumers, these are not the plant's only feeding deterrents.

It is unlikely that all lignans of *Saururus cernuus* function as feeding deterrents. More than 20 such compounds have been identified from *Saururus cernuus* (Rao & Alvarez 1982, 1983; Rao *et al.* 1987; Rao & Chattopadhyay 1990; Rao & Reddy 1990; Rao & Rao 1990; Rao & Oruganty 1997), but only seven deterrent lignans were detected during this study. We did not identify the metabolites in the large number of non-deterrent fractions. Therefore, it is possible that other secondary metabolites, including previously characterized lignoids of *S. cernuus*, were present but not deterrent. However, because of potential geographic or seasonal variation in secondary metabolite content and concentration, we cannot be certain that these other lignans occurred in the extract we tested.

The deterrent compounds of *Saururus cernuus* represent three lignoid structural types. Sauriols A, B, dihydroguaiaretic acid, and saucermetin are classical lignans, oxygenated products of the 8-8' coupling of two phenylpropanoid residues (Ayres & Loike 1990; Lewis & Davin 1999). The other deterrent metabolites are an 8-5' linked neolignan, licarin A, and two sesquiolignans, saucerneol and saucerneol methyl ether. Despite the structural diversity of this group, five out of seven identified antifeedants (*i.e.*, except saucermetin and saucerneol methyl ether) possess at least one free phenolic hydroxyl group. Sauriols A and B were isolated in the lowest concentrations of the classical lignans (0.006 and 0.038% by dry mass, respectively, Table 1) yet still showed potent antifeedant properties at these concentrations. These two compounds also possess the greatest number of free phenolic hydroxyl groups. It is possible that antiherbivory effects are related to this structural feature of lignoids. None of the antifeedant compounds isolated contains any methylenedioxy functional groups, known to be important for inhibition of mixed function oxidases, which insects possess to detoxify plant chemical defenses (Casida 1970).

Licarin A, a major feeding deterrent from this study, was previously isolated from the laurel tree *Machilus japonica* and found to inhibit larval growth of the insect *Spodoptera litura* when incorporated into artificial foods at a concentration of 0.1% of food dry mass (Gonzalez-Coloma *et al.* 1994). However, it is unclear whether the concentrations tested were related to those found in the plant, and the ecological importance of deterring this insect was not discussed. Dihydroguaiaretic acid, another deterrent compound from our study, is a prominent component of the creosote bush *Larrea tridentata*, and displays potent activity in laboratory assays against soil-inhabiting fungi that negatively affect seedlings in the field (Fernandez *et al.* 1979).

Several thousand lignans and related compounds are known from diverse representatives of the plant kingdom (Ayres & Loike 1990; Ward 1999; Lewis & Davin 1999 and references cited therein). Although these compounds have been shown to be biologically active (MacRae & Towers 1984; Lewis & Davin 1999), most interest has been focused on biomedical and agricultural applications rather than on the ecological roles of these compounds. Defensive functions for plant lignans often have been inferred from agricultural studies that report insecticidal, nematocidal, and antifungal properties (Haller *et al.* 1942a,b; Matsui *et al.* 1976; Fernandez *et al.* 1979; Belmares *et al.* 1979; Hamartha & Nawrot 1984; Taniguchi *et al.* 1989; Nitao *et al.* 1991; Yamauchi & Taniguchi 1991; Bernard *et al.* 1995; Lajide *et al.* 1995). However, ecologically rigorous and realistic tests of these hypothetical roles have rarely been conducted. A chemical defense role was invoked for lignans isolated from *Bupleurum salicifolium* (Umbelliferae) because of observed toxic effects against nematodes and brine shrimp (Gonzalez *et al.* 1995). In this study, the authors extended their suggestion of chemical defense to potato plants because the nematodes commonly infect potato plants, even though the lignans tested were not isolated from a potato plant, and the nematodes used in the assay were not found near the plant that contained the lignans. Reports of these kinds are valuable because of the potential for commercial agricultural applications. However, extrapolation to natural ecological functions is potentially misleading.

Two areas of previous focus have produced valuable insights into lignan functions. The first of these is the inhibition of PMSO/MFO activity in insects (Casida 1970; Bernard *et al.* 1989). Plant lignans containing at least one methylenedioxy functional group can bind to insects' detoxifying enzymes called polysubstrate monooxygenases or mixed function oxidases (PSMO/MFO), suggesting a synergistic relationship between lignans and other plant toxins (MacRae & Towers 1984). The second rich area of study has been examination of lignan specificity against generalist and specialist insects in host selection. Lignans of the creosote bush *Larrea tridentata*, particularly nordihydroguaiaretic acid, are responsible for stimulating feeding of a *Larrea*-specializing grasshopper and for

inhibiting feeding by generalist species (Chapman *et al.* 1988). Similar findings were reported for butterflies and silkmths (Nitao *et al.* 1992; Johnson *et al.* 1996). Toxic effects of magnolia neolignans were tested against butterflies and silkmths that were generalist feeders, specialists to the magnolia species studied, or specialists to another host species. Toxicity and growth-inhibitory effects of magnolia neolignans on non-user species were observed at a range of concentrations including natural plant tissue concentration. The authors convincingly argued for natural selection towards host specialization by resistant insects and avoidance by those that remain susceptible to the effects of these metabolites. Detoxification of neolignans in consumers as a mechanism driving this specialization was inferred from crude gut enzyme activity (Johnson 1999).

In terrestrial studies of plant-herbivore interactions, deterrence caused by lignans could mistakenly be attributed to tannins, despite the distinct biogenetic origins and structural features of these two molecular classes. Most lignans possess some free phenolic hydroxyls and hydrogens *alpha* to aryl oxygens, making these compounds reactive to general tannin-measuring reagents. Thus, in studies in which crude phenolic levels have been measured in whole plants or crude extracts (and assumed to be equivalent to tannin concentrations), correlations between tannins and herbivory may have been confounded by lignans. This could occur in freshwater chemical defense studies. Lodge (1991), in a synthesis of available data, presented evidence for a correlation between tannin content and antiherbivore defenses in which tannins would be indistinguishable from lignans. Similarly, Kerfoot (1989) measured total phenolics in several macrophytes and concluded that tannins were significantly correlated with allelopathy. He also described an assay in which the shikimate pathway can be blocked (stopping synthesis of tannins) and changed susceptibility to herbivores can then be evaluated on these altered plants. Both tannin measurement and biosynthetic blockage according to his methodology could be confounded by lignans. In contrast to the previous studies (although with the same methodological limitations), Bolser & Hay (1998) found that total phenolic content in the water lily *Nuphar luteum* was negatively associated with feeding deterrence of the crayfish used in our assays. To date, no freshwater plants have been demonstrated to be chemically defended by tannins that have been isolated and identified; whereas several non-tannin phenolics (seven lignans [this study] and one hydroxybenzylsuccinate [Bolser *et al.* 1998]) have been shown to deter herbivory. Therefore, until more evidence is available on the role of tannins in freshwater systems, we recommend an end to associating feeding preferences on freshwater macrophytes with tannin measurements that potentially confound tannins and other hydroxyl-bearing phenolics.

Freshwater macrophytes are subject to significant herbivory (Lodge 1991; Newman 1991; Cyr & Pace 1993; Lodge *et al.* 1998) and they can defend themselves using secondary metabolites (Newman *et al.*

1990, 1992, 1996; Bolser *et al.* 1998, this study). Freshwater herbivores show strong feeding preferences that often mirror the feeding deterrence of extractable organic compounds (Lodge 1991; Newman 1991; Bolser *et al.* 1998; Cronin 1998). The diverse nature of the chemical deterrents, including 2-phenylethyl isothiocyanate (Newman *et al.* 1992, 1996), habenariol (Bolser *et al.* 1998), four classical lignans, one neolignan, and two sesqueneolignans (this study), suggests that mechanisms of chemical defenses in freshwater plants are likely to be widespread and complex.

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