Plant Compounds in *Pachysandra terminalis* That Act as Feeding Deterrents to Prairie Voles

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Abstract. Many plants have mechanisms of physical or chemical resistance that protect them from herbivores in their environment. The ornamental plant *Pachysandra terminalis* Sieb. and Zucc is highly unpalatable to voles, but the nature of this resistance is not fully understood. Extracts of *P. terminalis* were prepared to determine the extent to which chemical constituents could account for its avoidance by voles. A bioassay in which samples were mixed with applesauce showed that ethanolic extracts were highly deterrent to captive prairie voles (*Microtus ochrogaster* Wagner, 1842). Bioassay-guided fractionation of ethanol extracts showed that antifeedant activity was present in both polar and non-polar fractions. Further separation of each fraction by open column chromatography and high pressure liquid chromatography revealed that combinations of compounds were responsible for the deterrent activity. Preliminary ultraviolet and mass spectroscopic analyses indicated that steroidal alkaloids that are characteristic of this plant are likely to be involved.

Wild mammals that forage on agricultural or ornamental crops can cause significant financial losses for both growers and homeowners (Conover et al., 1995; Lemieux et al., 2000a). One common method for reducing damage is the use of chemical repellents (Mason, 1998). Under certain conditions, some repellents can prevent severe damage for 14–18 weeks (Lemieux et al., 2000b; Nolte, 1998; Wagner and Nolte, 2001), but they do not always provide adequate protection (Conover, 1984; Palmer et al., 1983). Furthermore, the cost of repellents is prohibitive for crops covering large areas (Consumer Reports, 1998). Therefore, there is a need for repellents that are more effective, provide longer-term protection, and are less expensive than those currently available.

Several different types of active ingredients are used in repellents, including naturally occurring plant compounds (Wagner and Nolte, 2001). Many plants produce secondary compounds that are bitter-tasting, cause irritation, hinder digestion, or are toxic, and thus afford the plant with some measure of protection from herbivory (Harborne, 1991). The plant compound that has been most frequently used

in commercial repellents is the irritant capsaicin (from *Capsicum* plants) (Wagner and Nolte, 2001). The search continues for other plant compounds that could be used to produce a more effective repellent. For example, pine oil (Wager-Page et al., 1997) and quebracho (Swihart, 1990) were tested on voles (*Microtus* sp.), and DeNicola et al. (1991) tested eight secondary plant compounds on deer (*Odocoileus virginianus* Zimmermann, 1780). However, no commercial repellent has resulted from these studies. More recently, Ries et al. (2001) tested whole extracts from six plant species for their repellency against deer and other herbivores. Four of these showed promising results. In addition, they isolated several of the active ingredients in daffodil (*Narcissus pseudonarcissus* L.) and catnip (*Nepeta cataria* L.).

The long-term goal of our research is to develop a vole repellent based on plant compounds (Curtis et al., 2002). We selected voles as our study subjects because they cause serious damage in orchards (Byers, 1984), coniferous plantations (Sullivan et al., 2001), and nurseries (Hjalten and Palo, 1992) through their feeding activities. Furthermore, voles provide a bioassay for potential deer repellents, because the digestive system (Kudo and Oki, 1981; Kudo et al., 1986) and dietary preferences of voles appear similar to those of large ruminants (Kendall and Sherwood, 1975). Voles are also less expensive and easier to maintain in captivity than ruminants.

In the first stage of our research, we identified four plant species that were highly unpalatable to voles, and therefore likely to contain potent defensive compounds (Curtis et al., 2002). The objective of the present study was to identify the plant compounds that deter feeding by voles in one of these

plants. We selected the herbaceous landscape plant *Pachysandra terminalis* (Buxaceae), also known as Japanese spurge. About 30 steroidal alkaloids have been isolated from *Pachysandra* sp. (Qiu et al., 1994), and some are known to be toxic (Harborne et al., 1999). In this study, we used a no-choice feeding assay to monitor the fractionation and isolation of the active constituents (Bryant et al., 1983; Ries et al., 2001) with captive prairie voles. Our hypothesis was that after each fractionation stage, the sub-fractions obtained would differ in their ability to deter voles from feeding on a preferred food item.

Materials and Methods

Vole selection and maintenance. Voles were obtained from an outbred colony of laboratory-reared animals that had been maintained at Cornell Univ. since 1969. The origin and maintenance of this colony is described elsewhere (Richmond and Conaway, 1969). *Microtus ochrogaster* is a social species (Getz and Carter, 1996), so we housed the study animals in pairs to reduce stress. We used single-sex pairs to prevent reproduction. Details of housing and maintenance are given in Curtis et al. (2002). Briefly, each pair was given a unique identity number and was housed in a clear polycarbonate cage in a controlled environment room at 21 to 24 °C with a 14/10 light-dark cycle. Water and commercial food pellets were provided ad libitum, including during feeding trials. The voles were naïve to *pachysandra* until we conducted preliminary studies (May to Aug. 2000). We used a total of 25 vole pairs (12 female, 13 male). Of these, most (64%) were used in 10 or more of the 22 feeding trials we conducted, and only five pairs were used in less than six trials.

Materials. Silica Gel, 32 to 63 μ m (Selecto Scientifics, Ga.) was used for normal phase low performance column chromatography. Bakerbond Octadecyl (reverse phase C₁₈) (J.T. Baker, Phillipsburg, N.J.) was used for reverse phase low performance chromatography. Luna 5 μ m C₁₈, 250 $\times$ 10.00 mm (Phenomenex, Torrance, Calif.) was used for high pressure liquid chromatography (HPLC). Whatman 5 $\times$ 10 cm (layer thickness 250 μ m) with fluorescent indicator thin layer chromatography plates were used for thin layer chromatography (TLC). Compounds on TLC plates were visualized using a short and long wave ultraviolet (UV) detector and by exposure to iodine vapor.

Instrumentation. A Waters Alliance 2690 HPLC module in combination with a Waters 996 Photodiode Array detector was used for HPLC separations. The eluant was monitored at 218 and 254 nm. Mass spectra were recorded on a Waters Platform LC-MS instrument by direct infusion.

Extraction and purification of plant secondary metabolites. Fresh, healthy foliage (1 kg) of *P. terminalis* was collected from a 10 $\times$ 10 m area on Cornell Univ. campus, Ithaca, N.Y., on 7 Sept. 2000. Only the top whorl of leaves were harvested because the concentration of defensive compounds tends to be higher in younger leaves (McKey, 1974; Menkovic et al., 2000).

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The leaves were immediately boiled with about four times their weight of 100% ethanol for 10 min, then homogenized in a blender. After filtering to remove the insoluble material (primarily cellulose), the excess solvent was reduced to a smaller volume under reduced pressure. The resulting ethanolic solution (Step 1, Fig. 1) was subjected to sequential fractionation, where separation was based on polarity. First, it was partitioned between water and methylene dichloride to provide a non-polar methylene dichloride fraction (Fraction I), and a polar, aqueous fraction (Fraction II) (Step 2, Fig. 1). Because both these fractions showed activity (see results), they were subjected to further separation steps (Steps 3–6, Fig. 1).

Separation of each fraction (I and II) yielded 2 to 10 sub-fractions, based on volume. Combinations of fractions were tested to allow for possible synergistic or additive interactions of compounds (Howe and Westley, 1988). Combinations of sequential fractions were based on TLC patterns.

At each stage, the fractions or fraction combinations were bioassayed (see feeding trials) for feeding deterrent activity. Also, additional TLC data were used to determine whether compounds present in the least palatable fraction were also present in an adjacent fraction. The bioassay and TLC data were used to decide which fractions to select for the next separation. This bioassay-guided fractionation approach (Bryant et al., 1983; Ries et al., 2001) was more efficient than conducting randomized bioassays of all possible fractions of the ethanol extract, because the total number of possible fractions was prohibitively high, and many of these would have likely been inactive.

Initial chromatography of Fraction I used column chromatography with normal phase (NP) silica gel using hexane, methylene dichloride, and ethanol mixtures of increasing polarity (Step 3, Fig. 1). The second step used a reverse phase (RP) C_{18} column (Step 4, Fig. 1). The column was eluted with a gradient of 1% Hac to methanol.

Fraction II was first separated into a butanol-soluble fraction and a post-butanol aqueous fraction (Step 5, Fig. 1). Then the most active fraction (butanol) was chromatographed on the HPLC system using LUNA RP C_{18} column (Step 6, Fig. 1), using a similar solvent gradient as for Fraction I. Fractions containing prominent UV absorbing peaks were collected for bioassays. Compounds that were associated with the highest activity were characterized by HPLC, mass spectrometry, and UV analysis.

Sample preparation for bioassays. Following Curtis et al. (2002), we evaluated the ability of each fraction (or fraction combination) to deter voles from feeding by mixing it with unsweetened applesauce, which is a favored food item. Mixes of fresh pachysandra with applesauce are unpalatable to voles at concentrations as low as 14% pachysandra by weight (Curtis et al., 2002). We offered fraction/applesauce samples in which the concentration of pachysandra compounds would be equal to that found in a mix of 20% fresh pachysandra and 80% applesauce (by weight), so that an aversive response would be likely if

the compounds were active. Hence, for each fraction, we added 20 gram leaf equivalents (gle; the amount of extract derived from 20 g of fresh material) to 80 g of unsweetened applesauce (Food Club, Topco Associates, Skokie, Ill.). The 20 gle were first added to a solvent (methylene dichloride or a mixture of methylene dichloride and ethanol); then this solution was slowly added to 50 g of applesauce in a glass jar held in a water bath at 45 °C, and stirred continuously for 5 min to evaporate the solvent. After removing the jar from the water bath, additional applesauce was stirred into the mix to give a total of 100 g of sample. This was kept in a sealed jar at 4 °C until use (maximum of 24 h). We confirmed that fresh pachysandra from the collection used for the extraction was repellent to voles by offering a mix of fresh pachysandra (20%) and applesauce, homogenized to a paste in a blender. We confirmed that any solvents that may have remained in mixes would not affect consumption of applesauce by voles by offering voles unaltered applesauce and applesauce/solvent mixes in preliminary feeding trials following the method described here.

Feeding trials. Previous studies with voles found that no-choice feeding trials were more effective than choice trials in discriminating among test diets (Curtis et al., 2002; Kendall and Sherwood, 1975) because if a preferred food is provided, voles tend to consume only this during their initial feeding period. Therefore, we conducted no-choice feeding experiments. However, laboratory food was provided ad libitum during trials for two reasons. The voles cache food (Whitaker, 1996) and buried feed pellets in the cage bedding. Subjecting voles to a starvation plan would have necessitated transferring them to other, pellet-free cages, which would have stressed the voles. Because voles are social animals (Getz and Carter, 1996), they would have spent time exploring and constructing tunnels in the new bedding rather than exhibiting normal feeding behavior. Trials were carried out in the mornings, starting 3 to 6 h after the lights came on, between 8 Sept. 2000 and 3 June 2001, with a maximum of one trial per 2 d. Following the bioassay guided fraction approach (Bryant et al., 1983; Ries et al., 2001), each sample was offered once (with one exception; see results) to 20 pairs at the same time, constituting one feeding trial.

We used an equal number of male/male and female/female pairs in all trials except for Step 3 (fraction combinations 5+6 and 9+10 at 40%). Following Curtis et al. (2002), each vole pair was offered 4 g of the sample for 15 min, and the weight consumed over this time was recorded. To reduce the likelihood of voles developing either an aversion to all samples offered, or a detoxification system for pachysandra compounds, each pair was offered 4 g of applesauce for 15 min on days between consecutive trials.

Statistical analysis. Our hypothesis was that after each fractionation step, the sub-fractions obtained would differ ($P \leq 0.05$) in their ability to deter voles from consuming applesauce. Hence, for each fractionation a

General Linear Model (GLM) Analysis of Variance (MINITAB, Statistical Software Release 13.1; State College, Pa.) with Tukey's multiple comparisons was used to compare consumption among samples made from the sub-fractions, the fraction from which they were obtained, and—in the case of Steps 4 and 6—the ethanol fraction. The model included cage number, to control for effects of vole pairs, and fraction number. We also used the GLM procedure to examine the effect of trial date on consumption of unaltered applesauce, the effect of addition of solvents to the applesauce on consumption (cage was included in both these models), and the effect of sex on consumption [including the nested factor cage (sex) in the model].

Results

Activity of ethanolic extract (Step 1). Mean consumption of the fresh homogenized pachysandra sample (0.39 g) was similar to that of the ethanol extract (0.65 g), and both these were less palatable than applesauce alone (3.35 g) ($P < 0.0001$, Fig 2A). The ethanol extract reduced consumption of applesauce by 81%.

Partitioning of the ethanol fraction (Step 2). Fraction I (methylene dichloride soluble, non-polar) and Fraction II (insoluble aqueous fraction, polar) were equally unpalatable when mixed with applesauce (mean consumption = 1.33 and 0.90 g, for Fractions I and II, respectively) (Fig 2B). Consumption of both the Fraction I and Fraction II mixes was similar to that of the ethanol fraction (0.65 g) (Fig. 2 A and B). Based on these results, Fractions I and II were both subjected to further separation by chromatographic techniques.

Fraction I

Normal phase silica gel fractionation (Step 3). Ten fractions were obtained from the non-polar Fraction I. Consecutive pairs of fractions (e.g., 1 and 2, 3 and 4, etc.) were combined to give five fractions for bioassays. No single sub-fraction combination was significantly more effective as a feeding deterrent than all the others (Fig. 2C). Also, two of these combinations were less effective than the total Fraction I (1+2; $P = 0.046$, and 7+8; $P = 0.0002$). This indicated that multiple compounds in Fraction I acted together to produce the deterrent effect.

The least palatable mixes were those with fraction combinations 5+6, and 9+10, as consumption was significantly lower than with combinations 1+2 and 7+8 ($P = 0.0001$), and slightly lower than with combination 3+4. TLC data indicated that some compounds present in these fractions were also present in fractions 7 and 8. To determine which of fractions 5 to 10 contained the most active compounds, we repeated trials with fraction combinations 5+6, 7+8, and 9+10 at 40% concentration to compensate for possible divisions of active compounds between adjacent fractions.

There were no significant differences among these three fraction combinations, but consumption was slightly lower with the 9+10 combination (Fig 2D). TLC data revealed that fractions 8, 9, and 10 had compounds in

Fig. 1. Flow chart of bioassay-guided fractionation of extracts from *Pachysandra terminalis* for compounds that deter feeding by voles. TLC = thin layer chromatography. Bioassay results for steps 1–6 are given in Fig. 2 (also labeled Steps 1–6).

common, so we combined these three for the RPC_{18} fractionation.

Reverse phase C_{18} fractionation (Step 4). Eight fractions were obtained from the separation, which we combined in four groups for bioassay: 1–3, 4+5, 6+7, and 8. Of these four, mean consumption was lowest with fraction 8 (1.5 g, $P < 0.0001$) (Fig. 2E). TLC showed that fraction 8 consisted of a mixture of many compounds that could have contributed to activity. All of the sub-fraction combinations were less effective deterrents than the total ethanol extract ($P < 0.01$).

HPLC and mass spectroscopy of fractions revealed recognizable molecular ions. Based on their UV absorptions, some of the compounds appear to be glycoalkaloids. One representative alkaloid had a molecular weight (MW) of 462.71, which matches the MW of

a known alkaloid of pachysandra, spiropachysine ($C_{31}H_{46}N_2O$; Kikuchi et al., 1968).

Fraction II

Partitioning of Fraction II (Step 5). Fraction II was divided into two fractions. Activity was evidently concentrated in the butanol sub-fraction. Mean consumption of this was the same as that of Fraction II (0.89 g and 0.90 g respectively, $P = 0.8055$), and lower than that of the post-butanol aqueous sample (1.6 g; $P < 0.0001$) (Fig. 2F). The latter sub-fraction was more palatable than Fraction II ($P = 0.0014$). We therefore subjected the butanol sample to further fractionation.

HPLC fractionation on C_{18} LUNA (Step 6). This yielded three fractions, but we had only sufficient extract to offer each vole pair

2 g of the fraction mix. All voles consumed >1.9 g of each fraction (Fig. 2G). The mean consumption for each of these was greater than consumption of the butanol sample ($P < 0.0001$), indicating that the repellency of the butanol fraction was dependent on synergistic or additive effects among compounds. Also, each of these three fractions was less effective as a deterrent than the total ethanol fraction ($P < 0.0001$).

Effect of trial date, solvents used, and vole sex on consumption. There was little variation in consumption of plain applesauce over the study period (mean weight consumed 3.46–3.94 g, $SE = 0.02$ – 0.20). Mean consumption of applesauce/solvent mixes (3.34–3.71 g; $SE = 0.07$ – 0.09) was the same as that of unaltered applesauce (3.4 g; $SE = 0.09$) ($P = 0.098$). Sex also had minimal effect on consumption; mean consumption of samples depicted in Fig. 2 was 1.74 g ($SE = 0.09$) for males and 2.05 g ($SE = 0.09$) for females ($P = 0.07$).

Discussion

Herbivores are usually capable of recognizing plants that produce harmful compounds without actually feeding on them (Provenza, 1995). The mechanisms used are not fully understood, but innate recognition and learning (by conditioned aversion, or from parents and siblings) are probably involved (Provenza, 1995). Mammals may also use gustatory, odor, and visual cues (Provenza, 1995). Some of the compounds that serve as gustatory or odor cues may not in themselves produce harmful effects. However, unpleasant tastes or smells are rarely effective deterrents unless associated with negative effects (Villalba and Provenza, 2000). Therefore, the plant compounds that would be most useful for developing a commercial repellent would be those that do produce a conditioned aversion.

In many plants, chemical defense is dependent on several interacting compounds (Howe and Westley, 1988). For example, defense of mature balsam poplar (*Populus balsamifera* L.) twigs against snowshoe hares (*Lepus americanus* Erxleben, 1777) appears to be dependent on the synergistic effect of two compounds present in the twigs (Reichardt et al., 1990). Daffodil and catnip both contain more than one repellent compound (Ries et al., 2001). Plants that produce several defensive chemicals may be better protected against a variety of herbivores than those that rely on few compounds (Howe and Westley, 1988).

We found that the ethanol extract and homogenized fresh leaves of *P. terminalis* were equally effective in deterring voles from consuming applesauce. This indicated that avoidance of pachysandra by voles can be explained by the presence of chemical constituents, and that our extraction process did not destroy or remove the active compounds. Several compounds of differing polarities must be involved in pachysandra's chemical defense because activity was distributed among several fractions. After the initial separation, the non-polar (I) and polar (II) fractions were equally unpalatable to voles. Following the

Step 1: Ethanol extraction

Step 2: Ethanol extract partitioned between CH₂Cl₂ (I), and H₂O (II)

Step 3: Normal phase silica gel fractionation

Fraction combinations 5+6, 7+8, 9+10 tested at 40% concentration

Step 4: Reverse phase C₁₈ fractionation of combined fractions 8-10

Step 5: Partitioned between H₂O and n-butanol

Step 6: HPLC on C₁₈ LUNA

Fig. 2. Grams of applesauce consumed by voles when this was offered unaltered (A), or mixed with fresh *Pachysandra terminalis* foliage (20% by weight), an ethanol extract of this plant (A), or different fractions of the ethanol extract (B–G). For the ethanol extract (A) and in all sub-fractions except those in (D), we used 20 gram leaf equivalents of the extract or sub-fraction to 80 g applesauce. Sub-fractions in (D) were offered at double this concentration. The y axis on each graph is mean consumption (grams $\pm$ SE) of sample by vole pairs. Numbers of vole pairs are given above bars. Values with different letters above bars are significantly different. ($P < 0.05$).

final separation (RPC₁₈) of Fraction I, activity was mainly concentrated in one fraction, but TLC data indicated that this still contained several potentially active compounds. The last fractionation (HPLC) of Fraction II resulted in three fractions of equal potency. None of these was as effective a deterrent as the butanol fraction from which they were derived, suggesting that the latter contained several compounds that interacted to produce the repellent effect. The fact that the final fractions we obtained (from the original Fractions I and II) were less effective feeding deterrents to voles than the total ethanol extract further supports the conclusion that several compounds are involved

in pachysandra's chemical defense. In this respect, *P. terminalis* is similar to many other plant species (Howe and Westley, 1988).

Because of the complex nature of pachysandra's chemical defense and time-consuming nature of the fractionation process, we were unable to identify the specific repellent compounds involved. These included steroidal glycosides and other steroidal alkaloids. Steroidal alkaloids are found in four plant families, including Buxaceae, each family having a different set (Harborne et al., 1999). Some of the Buxaceae alkaloids are known to be purgative or toxic (Harborne et al., 1999). Over 30 steroidal alkaloids have been identified from

Pachysandra species (Qiu et al., 1994) and *P. terminalis* contains at least 20 steroidal alkaloids and four triterpenes (Funayama et al., 2000). There appear to be few data on the physiological effect of *Pachysandra* alkaloids on herbivores. Some *P. terminalis* alkaloids have cytotoxic effects against leukemia cells (Funayama et al., 2000) or anti-ulcer effects (Watanabe et al., 1986).

Volatile compounds may play some role in herbivore defense in *P. terminalis*. However, they are obviously not essential, because the ethanol extract was as unpalatable to voles as the fresh leaves, even though most volatile compounds would have been removed from the ethanol extract during evaporation of the solvent.

Further studies would be needed to ascertain how effective the repellent fractions we identified might be against free-ranging voles when food is scarce. To do this, feeding trials should be conducted on voles that have been starved for a period immediately prior to the trial. Additional studies would also be needed to determine whether our conclusions about the active compounds in our sample of *P. terminalis* apply to this species generally. Because this species tends to reproduce vegetatively and can be easily propagated from cuttings (Still, 1994), the plant material we used in our study probably belonged to one clone. Further investigations should test extracts made from several genetically different *P. terminalis* plants from different locations.

No clear conclusion can be drawn from comparison of our results with those from similar studies because of differences in methodology. The most appropriate comparisons are with studies that tested whole plant extracts. Ries et al. (2001) conducted many field trials using extracts of six plant species, which they tested on deer, rabbits (*Sylvilagus* sp.), raccoons (*Procyon lotor* Linnaeus, 1758), and squirrels (*Sciurus* sp.). Our whole pachysandra extract at 20% concentration reduced consumption by 81%. This was similar to the repellency recorded by Ries et al. (2001) for daffodil extracts (at 100% concentration), and other combinations [e.g., hot pepper (*Capsicum frutescens* L.) with catnip, and a hot pepper/catnip/daffodil mix]. Our reductions with pachysandra extracts were greater than those with iris (*Iris* sp.) at 50% concentration (Ries et al., 2001). Harris et al. (2000) tested 10 plant extracts as repellents against deer, and the most promising were yucca (*Yucca* sp.) and peppermint (*Mentha piperita* L.). Our pachysandra extract appeared to be more effective against voles than either of these for deer.

Some workers have tested individual plant compounds for their potential use in repellents. For example, Swihart (1990) found that a 3% concentration of quebracho (a condensed tannin) reduced vole consumption by 92%. DeNicola et al. (1991) reported that lupinine and quinine were the most effective deer repellents of those they tested, although efficacy was low (31% and 66% reductions in consumption, respectively). Ries et al. (2001) also tested four compounds isolated from daffodil. These were less effective than the whole daffodil extract,

indicating additive effects.

Our results confirm that the sample of *P. terminalis* we tested was chemically defended against herbivory, and show that several compounds of different polarities are involved. Some of the active compounds are previously identified steroidal alkaloids and their glycosides.

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