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Tests of the antibiotic properties of the invasive vine *Vincetoxicum rossicum* against bacteria, fungi and insects

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Abstract

We tested extracts of *Vincetoxicum rossicum* for inhibition of bacterial and fungal growth and for anti-insect activities that may account for the invasive characteristics of this introduced species in North America. Bioassay-guided fractionation was used to identify (−)-antofine as the principle inhibitor of bacteria and fungi in root extracts. This compound had especially pronounced antifungal activity, inhibiting the growth of diverse taxa that include yeast-like and filamentous fungi and, notably, broad-host-range plant pathogens. A second compound(s), that is as yet uncharacterized but distinct from (−)-antofine, was detected as having antifeedant activity against a larval hymenopteran, rose sawfly (*Allantus cinctus*), and toxicity to two larval lepidopterans, the masked birch caterpillar (*Drepana arcuata*) and the European corn borer (*Ostrinia nubilalis*). That *V. rossicum* contains potent inhibitors of plant pathogenic fungi, diverse bacteria, and herbivorous insects likely contributes to its success as an invasive species.

Keywords: Antifungal; Antibacterial; Phenanthroindolizidine alkaloids; (−)-Antofine; Insect antifeedant; Insecticide

1. Introduction

The dog-strangling vine, or pale swallow-wort, *Vincetoxicum rossicum* (Kleopow) Barbar. [syn: *Cynanchum rossicum* (Kleopow) Borhidi], is an herbaceous perennial in the Asclepiadaceae that was introduced into North America from Russia and Ukraine in the late 1800s (Sheeley and Raynal, 1996). It has become a major pest of natural areas in the Great Lakes Basin, where it thrives in both sun and partial shade and tolerates a wide range of moisture conditions and soil types (Sheeley and Raynal, 1996; DiTommaso et al., 2005). Because of its ability to form dense monocultures, the species is of special concern to natural area managers of western New York, where rare alvar ecosystems with unique plant communities are being invaded (DiTommaso et al., 2005). Little evidence for insect herbivory has

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been observed on *V. rossicum* in Ontario (Ernst and Cappuccino, 2005) and this has obvious ecological importance for insectivorous mammals and birds in habitats invaded by *V. rossicum.* For example, declines in grassland bird abundance in large *V. rossicum* stands have been noted (The Nature Conservancy, Western New York Chapter, unpublished data). Monarch butterflies, whose native host is milkweed (*Asclepias* spp., Asclepiadaceae), will occasionally oviposit on *V. rossicum*, although the plant does not support larval growth (Mattila and Otis, 2003). In addition, we rarely observe plant pathogen-associated damage of *V. rossicum* in our study sites around Ottawa, Canada.

Recently, it was hypothesized that inhibitory activity by novel phytochemicals present in non-native species may partly account for an absence of herbivore and pathogen damage, and could contribute to the invasive nature of some introduced species (Cappuccino and Arnason, 2006). While the chemical ecology of *V. rossicum* is not well characterized, other species of *Vincetoxicum* as well as members of the related genus *Tylophora* are known to produce phenanthroindolizidine alkaloids (Staerk et al., 2005). These compounds are reported to have pronounced antibiotic activities towards insects (Kathuria and Kaushik, 2005; Verma et al., 1986), tobacco mosaic virus (An et al., 2001) and bacteria (Baumgartner et al., 1990; Rao et al., 1997). Specifically, *Vincetoxicum officinale* was noted in treatments of ''diseases of the pryvytes'' (Turner, 1551), which may have included diseases of microbial origin. These observations of diverse inhibitory activities by extracts of *Vincetoxicum* and related species prompted us to evaluate the antibiotic principle(s) in *V. rossicum* that might explain the ecologically invasive characteristics of this species in North America. Accordingly, we tested extracts of *V. rossicum* for growth inhibition of diverse bacterial and fungal species and for anti-insect activity.

2. Materials and methods

2.1. Plant material and extract preparations

Whole *V. rossicum* plants were harvested during the summers of 2002–2005 in Ottawa, Canada. Voucher specimens (#2005-10) are retained at Carleton University, Ottawa, Canada. Fresh leaves, mature fruits and roots were preserved separately in 95% ethanol until use. Plant material was blended in 95% ethanol, sonicated for 30 min and left overnight to shake at room temperature. The suspension was filtered, the solvent evaporated and the residue freeze-dried and stored in the dark at 4 °C until needed.

2.2. Antimicrobial assays

Fungal strains were selected to represent diverse taxonomic groups and growth forms (Table 1) and broad-host-range plant pathogens (Table 3). Fungi were grown at 30 °C in Sabouraud's dextrose or Potato Dextrose media (Difco, Detroit, MI), with or without 1.5% agar. Representative Gram positive and Gram negative bacterial strains used are also listed in Table 1. Bacteria were grown in Lennox medium (Invitrogen, Burlington, ON), with or without 1.5% agar, at 30 °C. All microbes were manipulated in a biohazard level II laminar flow hood (BioKlone 2, Microzone, Ottawa, ON).

Disk diffusion assays were done to test standard (dark) antimicrobial activity and phototoxicity of crude extracts from *V. rossicum* leaves, mature fruits and roots. Disk assays with fungi were done as described by Ficker et al. (2003) and with bacteria as in Omar et al. (2000). Sterile filter disks (6.5 mm diameter) were impregnated with the equivalent of 2 mg (10 μl, or as noted below) of crude extract suspended in 95% ethanol, air-dried and placed onto the surface of preinoculated agar medium. For phototoxicity assays, disc-assay plates were irradiated for 2 h (10 W/m^2, 4 × 20 W black light blue tubes, 300–400 nm range) prior to dark incubation. Negative and positive controls in disk diffusion assays employed ethanol and 2 mg berberine, respectively. Inhibition zones around filter disks were measured after incubation periods of 24–48 h and all tests were done in triplicate.

Broth dilution assays were used to determine the minimum inhibitory concentration (MIC) of extracts. For this, mid-log cultures were diluted to ca 1000 cells/ml and 100 μl was added to each well of a 96-well round-bottom microtitre plate. Extracts were serially diluted 2× or 3× across the microtitre plates and incubated stationary for 24–48 h at 30 °C in the dark. MICs were recorded as the concentration at which there was 80% reduction in growth in comparison to wells with no inhibitor present as determined by eye and optical density readings (OD_{600}, Spectra Max 340PC, Molecular Devices, Sunnyvale, CA).

Table 1
Strains used and mean inhibition zones (±sd) in disk diffusion assays with *V. rossicum* extracts (2 mg/disk)

Species	Phylum	Source[a]	Inhibition zone (mm)[b]					
			Root		Leaf		Fruit	
			+UV	−UV	+UV	−UV	+UV	−UV
Filamentous fungi								
Microsporum gypseum (Bodin) Guiart et Grigorakis	Ascomycota	OMH-FR2385	23.67 (2.08)	19.67 (2.52)	9.33 (0.58)	7.50 (1.32)	20.67 (1.53)	18.00 (1.00)
Pseudallescheria boydii (Shear) McGinnis et al.	Ascomycota	OMH-FR2625	22.33 (2.89)	24.00 (1.73)	7.83 (1.04)	6.83 (0.29)	19.67 (1.15)	21.67 (2.31)
Rhizopus sp.	Zygomycota	OMH-FR2874	15.67 (1.53)	8.17 (1.76)	12.67 (5.51)	6.83 (0.58)	17.33 (2.08)	17.00 (1.00)
Yeast-like fungi								
Saccharomyces cerevisiae Hansen	Ascomycota	OCI-S288C	12.00 (1.00)	10.33 (0.58)	8.67 (0.58)	7.00 (0.00)	9.67 (0.58)	8.33 (1.53)
Candida albicans (Robin) Berkhout	Ascomycota	OGH-308-1329	11.00 (1.00)	9.33 (1.15)	8.33 (1.53)	7.00 (0.87)	9.67 (0.58)	9.33 (0.58)
C. albicans D10	Ascomycota	N.D. Lees	11.33 (1.15)	9.67 (0.58)	10.00 (0.00)	7.00 (0.87)	9.00 (1.73)	8.00 (1.00)
C. albicans CN1A	Ascomycota	N.D. Lees	14.33 (0.58)	15.00 (1.00)	8.00 (1.00)	10.00 (2.00)	10.67 (0.58)	13.33 (1.15)
Cryptococcus neoformans (Sanfelice) Vuillemin	Basidiomycota	OMH-FR2704	14.00 (1.00)	12.67 (0.58)	7.17 (0.76)	6.67 (0.29)	10.67 (1.15)	10.67 (1.53)
Wangiella dermatitidis (Kano) McGinnis	Ascomycota	OMH-FR2236	8.50 (2.29)	11.33 (3.21)	n.i.	n.i.	6.67 (0.29)	10.00 (1.00)
Gram positive bacteria								
Bacillus subtilis (Ehrenberg) Cohn	Firmacutes	ATCC 23857	n.i.	6.67 (0.29)	n.i.	n.i.	n.i.	n.i.
Enterococcus faecalis (Andrews and Horder) Schleifer and Kilpper-Balz	Firmacutes	ATCC 49452	7.33 (0.58)	n.i.	7.67 (0.58)	7.50 (0.87)	n.i.	n.i.
Listeria innocua Seeliger	Firmacutes	ATCC 51742	8.67 (1.53)	8.00 (1.73)	n.i.	8.50 (0.50)	8.33 (0.58)	7.67 (0.29)
Gram negative bacteria								
Escherichia coli (Migula) Castellani and Chalmers	Proteobacteria	ATCC 29055	7.50 (0.87)	6.67 (0.58)	7.00 (1.00)	7.50 (0.50)	6.67 (0.29)	6.83 (0.29)
Neisseria perflava Bergey et al.	Proteobacteria	ATCC 14799	12.33 (1.53)	12.00 (0.00)	9.67 (1.15)	8.33 (0.58)	12.33 (2.52)	12.67 (1.53)
Pseudomonas putida (Trevisan) Migula	Proteobacteria	ATCC 12633	6.83 (0.29)	6.83 (0.29)	n.i.	9.67 (0.58)	6.83 (0.29)	6.83 (0.29)

[a] Strain sources as noted: OMH (Ontario Ministry of Health, Toronto, ON); OGH (Ottawa General Hospital, Ottawa, ON); OCI (Ontario Cancer Institute, Toronto, ON); ATCC (American Type Culture Collection, Manassas, VA).
[b] Mean diameter (±sd) based on three replicates observed after 48 h of incubation; n.i. = no appreciable inhibitory activity.

2.3. Bioassay-guided fractionation to identify antifungal compound(s)

Antifungal assays were done at each fractionation step using *Candida albicans* strain D10 and *Saccharomyces cerevisiae* strain S288C. Crude ethanol extract of *V. rossicum* roots was dissolved in 200 ml of 10% HCl (v/v) followed by three hexane washes to obtain the acidic fraction. The aqueous phase was removed and adjusted to pH 10 using ammonium hydroxide and extracted three times with dichloromethane to obtain the basic fraction. The remaining aqueous fraction and the acidic and basic fractions were separately evaporated to near-dryness, freeze-dried and weighed.

Based on relative mass recoveries, 0.5 mg of hexane (acidic) extract, 0.0015 mg dichloromethane (basic) extract and 3.5 mg of aqueous extract were separately used in disk diffusion assays with *C. albicans* D10. Significant antifungal activity was observed only with the basic fraction and the inhibition zones were of comparable size to those with 4 mg of crude extract. Two hundred milligrams of the basic extract was further separated by thin layer chromatography using a 60:40 ethyl acetate:methanol mixture as the mobile phase. Five bands were identified under short wave UV light,

individually dissolved in 125 ml of MeOH, filtered and tested for antifungal activity by broth-based assays with *S. cerevisiae*. A single band with antifungal activity was recovered and further purified by two additional TLC separations.

Characterization of the antifungal compound isolated through TLC was determined by HPLC-DAD-APCI/MS analyses (Bily et al., 2004) using a Hewlett–Packard ChemStation series 1100 LC–MSD with a APCI/MS detector equipped with a water YMC ODS-AM narrow bore column (100 × 2 mm i.d.: 3 μm particle size) at 50 °C. Elution conditions with a mobile phase system of MeOH (solvent A) and trifluoroacetic acid (0.05%) in water (pH 3.4; solvent B) were optimized for MS detection as follows: initial conditions 1:19 (A:B) for 5 min, changing to 1:4 for 7 min, then 1:1 for 3 min, then 9:1 for 10 min, then 100:1 for 5 min, then isocratic elution with 100:0 for 15 min, finally returning to the initial conditions for 5 min. The mobile phase flow rate was 0.4 ml/min and the total analysis time was 50 min. The sample injection volume was 5 μl and the elution profiles were also monitored on-line at 190–650 nm with the Diode Array Detector. For MS detection in the positive ionization mode, the optimized conditions were APCI conducted at 300 °C with the vaporizer at 350 °C: nebuliser pressure, 60 psi; nitrogen (drying gas) flow rate, 4 l/min; fragmentation voltage, 50 V; capillary voltage, 300 V; corona current 4.0 μA. The MS data were collected in the scan mode and pseudomolecular ion at *m/z* 364.2 was monitored on-line. NMR spectra were determined with a BrucherAMX spectrometer operated at 300 and 500 MHz in ^{1}H NMR and 150 MHz in ^{13}C NMR. $CDCl_3$ was used as solvent.

2.4. Insect antifeedant/toxicity assays

We tested fractions of *V. rossicum* root extracts [including (−)-antofine] for anti-insect activity. Whole leaflets of garden rose (*Rosa* sp.) and leaves of birch (*Betula papyrifera*) were selected and imaged on a flatbed scanner. Leaflets/leaves were then completely immersed in a selected extract that was diluted in water (treated), or immersed in dH_20 with carrier solvent without extract (untreated control), agitated for 30 s and allowed to air dry for 20 min. In total, four different treatments were used: 10 mg/ml of *V. rossicum* crude root extract, 1.3 mg/ml of acidic fraction, 0.1 mg/ml of basic fraction, 8.65 mg/ml of aqueous fraction and 0.004 mg/ml of purified (−)-antofine. The origin of each of these fractions is described above. For trials with rose, a late-instar, curled-rose sawfly larva (*Allantus cinctus*) was randomly selected, weighed, and placed in a Petri dish with one rose leaflet (treated or untreated). Similarly, a late-instar masked birch caterpillar (*Drepana arcuata*) was placed with a birch leaf (treated or untreated) in a Petri dish. After 24 h at room temperature and indirect natural light, leaflets/leaves were again scanned and the amount consumed was estimated by eye as a percent (±5%) using before and after images. Viability and notes on larvae were recorded for an additional 3 days.

3. Results

3.1. Growth inhibition of microbes by Vincetoxicum extracts

Disk diffusion assays with *V. rossicum* crude ethanol extracts are summarized in Table 1. Root and fruit extracts caused significant growth inhibition of all fungi, which included those with yeast-like and filamentous growth forms and which represented ascomycetes, basidiomycetes and zygomycetes. Root and fruit extracts were similar in degree of inhibition; both showed significantly greater activity than leaf extracts (*two-way ANOVA*, effect of plant part: $F_{2,48} = 7.16$, $P < 0.001$; *Tukey–Kramer HSD post-hoc test*; $\alpha = 0.05$). There was no significant effect of UV light on inhibition of fungi by the extracts (*two-way ANOVA*, $F_{1,48} = 0.58$, $P = 0.45$). These observations indicate that the active compound(s) is not photoactivated and is mainly in the root and fruits of the plant.

Overall, disk diffusion assays with *V. rossicum* extracts yielded very small or no inhibition zones with *Bacillus subtilis* but well-defined inhibition zones were observed with at least one extract with other bacterial strains tested. The inhibition zones in bacterial assays were noticeably less pronounced than in fungal trials. Neither plant part nor UV light significantly influenced the degree to which bacterial growth was inhibited (two-way *ANOVA*, effect of plant part: $F_{2,30} = 0.017$, $P = 0.983$; effect of UV light: $F_{1,30} = 0.048$, $P = 0.828$).

3.2. The antifungal compound in V. rossicum is (−)-antofine

Because of the marked antifungal activity, we carried out a bioassay-guided fractionation of the crude extract with yeast-like fungi to identify the inhibitory compound(s). The active TLC band was isolated and determined to be ≥95%

pure (−)-antofine (Fig. 1) based on HPLC–MS, and ^{1}H and ^{13}C NMR analyses. The HPLC chromatogram of the active TLC band had one major and one minor peak. The major peak had a molecular weight of 363.2, which is consistent with the molecular weight of (−)-antofine, and the smaller peak (<5%) had a weight of 379.2, which is consistent with (−)-antofine oxide (Staerk et al., 2000). The ^{1}H NMR and ^{13}C NMR spectra of the active TLC band yielded comparable values to those previously reported (Baumgartner et al., 1990) and confirmed (−)-antofine as the principle antifungal compound in the active fraction.

Overall, MIC values for (−)-antofine and crude extract corroborated earlier results from disk assays that suggested that fungi are more sensitive than the bacteria tested to the inhibitory compound(s) in *V. rossicum* (Tables 2 and 3). Furthermore, based on mass balance, the data indicated that (−)-antofine is the primary inhibitor in the crude extracts from *V. rossicum* roots of both fungi and bacteria. Significantly, (−)-antofine effectively inhibited growth of all the plant pathogenic fungi we tested, which were selected as ones having broad host ranges, whereas no antifungal activity was evident in disk assays with acidic or aqueous fractions (Table 3). This further demonstrates the efficacy of (−)-antofine as a chemical defense against fungi that are well characterized as plant pathogens.

3.3. Additional anti-insect compound(s) in *V. rossicum*

A clear indication that other bioactive compounds, in addition to (−)-antofine, occur in *V. rossicum* roots was provided through our insect bioassays (Fig. 2). Crude extract treatments of rose leaflets significantly reduced feeding by sawfly larvae (*two-tailed t-test* of arcsine square-root transformed values of percent leaf consumed, d.f. = 1, $P < 0.002$). On average, 1% of the treated leaflet was consumed after 24 h by a sawfly larva compared to 55% of untreated leaflet consumed. No mortality was observed for sawfly on treated leaflets ($n = 10$) in these experiments, suggesting that the crude extract is an antifeedant to sawfly larvae. We next tested the anti-insect activity of (−)-antofine given our finding that this was the main antifungal compound in *V. rossicum* roots, and that related alkaloids have been implicated as insect antifeedants/toxins in other members of the Asclepiadaceae (Kathuria and Kaushik, 2005; Verma et al., 1986). However, no difference was observed in sawfly larvae consumption of leaflets that were treated with the carrier solution (0.5% ethanol in water) to those treated with 0.004 mg/ml (−)-antofine (*t-test* as above, $P = 0.49$).

The same pattern was observed with masked birch caterpillars (Fig. 2; *ANOVA*: $F_{2,23} = 22.9$, $P < 0.001$) except that *V. rossicum* crude extract was toxic in these trials. Little or no feeding by the caterpillars was evident on birch leaves treated with *V. rossicum* crude extract and in all cases ($n = 15$) the caterpillars were dead within 1 day of exposure. No mortality of masked birch caterpillars ($n = 15$) was observed with carrier control treatments wherein 17% of the leaf was consumed. Likewise, an average of 19% of (−)-antofine-treated leaf was consumed by each caterpillar, and this did not cause death of any caterpillars ($n = 16$). We conclude that the insect antifeedant/toxin in *V. rossicum* is

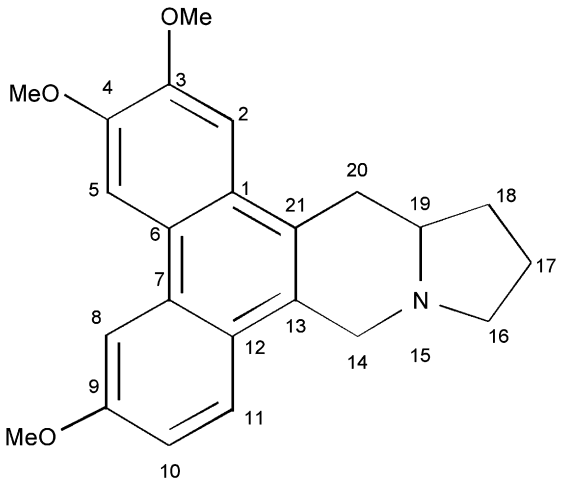

Fig. 1. Chemical structure of (−)-antofine.

Table 2
Comparison of minimum inhibitory concentration values with crude *V. rossicum* root extracts and (−)-antofine

	MIC (mg/ml)	
	Crude extract	(−)-Antofine
S. cerevisiae S288C	0.156	0.003
B. subtilis	12.5–25	0.34
E. faecalis	0.78	0.05
L. innocua	12.5–25	0.23
P. putida	12.5–25	0.60

not (−)-antofine. However, we noted that over the duration of these experiments (3 days), there was a tendency for both crude extract treatments (sawfly) and (−)-antofine treatments (sawfly larvae and masked birch caterpillars) to delay or prevent pupation. The proportion of sawfly larvae pupating in the water treatments (8/14) was significantly greater than for larvae exposed to either crude extract (0/10 pupated; *Fisher's exact test*, $P = 0.004$) or (−)-antofine (0/4 pupated, $P = 0.007$). A similar but non-significant trend was observed with masked birch caterpillars where 6/15 of water-controls and 3/16 of (−)-antofine-treated caterpillars pupated. It is unknown whether this apparent reduction in pupation frequency is a direct effect on larval development or a secondary effect through, for example, reduced food assimilation due to perturbation of gut microbes. Weights were not significantly different in control and extract treated trials for either sawfly larvae or masked birch caterpillars in our experiments (*two-tailed t-test*, $P \geq 0.48$) indicating that this apparent effect on pupation was not an artifact of differences in instar stage between treated and untreated experiments.

We subsequently determined that the toxic compound(s) to the masked birch caterpillar in *V. rossicum* root extracts was a polar compound in the acidic fraction that is not found in the basic or aqueous fractions. All caterpillars ($n = 5$) exposed to leaves treated with the acidic fraction were dead within 12 h, whereas no mortality was observed for caterpillars exposed to leaves treated with either of the basic or aqueous fractions ($n = 5$ for each). The acidic fraction extract was also toxic to larvae of the European corn borer, *Ostrinia nubilalis*. Exposure to food cubes dipped in a dilute acidic fraction extract resulted in death to all corn borer larvae ($n = 30$) within 12 h of exposure. Experiments are now underway to identify this insecticidal compound.

4. Discussion

We found that crude extracts of *V. rossicum* inhibit growth of Gram negative and Gram positive bacteria and have significant antifungal activities against diverse filamentous and yeast-like fungi, including broad-host-range plant

Table 3
Minimum inhibitory concentrations of (−)-antofine and inhibition zone diameters in disk assays with selected plant pathogenic fungi that have broad host ranges

Strain and source	Example host plants[a]	MIC (mg/ml)	Inhibition zone (mm)[b]		
			Ant	Acid	Aq
Fusarium graminearum Schwabe, NRRLK062[c]	Wheat, oat, corn, barley	0.006	14.33 (1.15)	n.i.	n.i.
F. graminearum, CBS 415.86[c]		0.003	25.00 (0.00)	n.i.	n.i.
Fusarium oxysporum Schlechtendahl, JB1D411[c]	Cucumber, garlic, potato, soybean	0.004	19.33 (0.58)	n.i.	n.i.
Verticillium dahliae Klebahn, 225753[c]	Eggplant, raspberry, potato, sunflower	0.003	19.67 (0.58)	n.i.	n.i.
Botrytis cinerea Persoon, CBS 156.71[c]	Tomato, blueberry, apple, strawberry	0.006	16.67 (1.53)	n.i.	n.i.
Alternaria alternata (Fries) Keissler, DAOM 234879[c]	Potato, canola, wheat, barley	0.006	16.33 (0.58)	n.i.	n.i.
Sclerotinia sclerotiorum (Libert) de Bary, Ss234[d]	Cabbage, alfalfa, bean, canola	0.003	19.33 (1.15)	n.i.	n.i.
S. sclerotiorum, 51(G)[d]		0.004	15.67 (1.15)	n.i.	n.i.

[a] From Morrall (2006).
[b] Mean diameter (±sd) based on three replicates after 48 h of incubation. Disks were impregnated with 6×10^{-4} mg (−)-antofine (Ant), 0.26 mg acidic fraction (Acid) or 1.73 mg aqueous fraction (Aq); n.i. = no appreciable inhibitory activity.
[c] A. Levesque, Agriculture and Agrifood Canada, Ottawa, ON, Canada.
[d] G. Boland, U. Guelph, Guelph, ON, Canada.

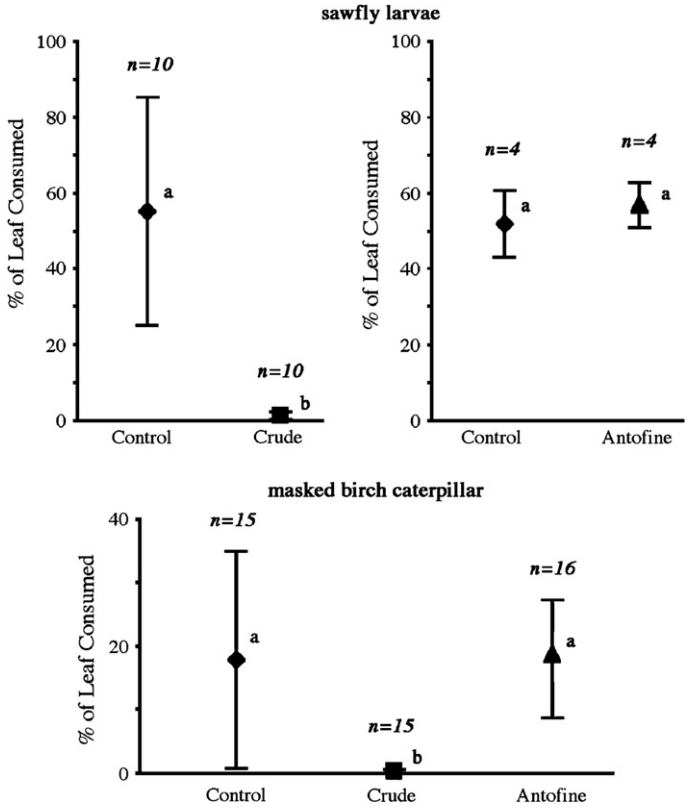

Fig. 2. Consumption of rose leaflets by sawfly larvae (top) and birch leaves by masked birch caterpillar (bottom) after treatment with carrier solvent (control), crude extract of *V. rossicum* root and (−)-antofine. Each trial consisted of a larva individually enclosed with treated or untreated leaflets/leaves. Means with the same lowercase letter were not significantly different according to a two-tailed *t*-test (top) or a Tukey–Kramer HSD post-hoc test; $\alpha = 0.05$ (bottom).

pathogens. The antifungal activity of *V. rossicum* is most pronounced in the roots and fruits suggesting a role in preservation of reproductive and perennating structures.

Phytochemical studies on *Vincetoxicum* and its sister genus *Tylophora* (Liede, 1996) have shown that these asclepiads are unusual in having phenanthroindolizidine alkaloids as their major cytotoxic principles (Lee et al., 2003; Staerk et al., 2002). Our data (Tables 1–3) indicate that, like *Vincetoxicum nigrum* (Capo and Saa, 1989), the principle antimicrobial agent in *V. rossicum* is the phenanthroindolizidine alkaloid (−)-antofine. It is estimated to be present at ca 0.04% in crude extracts of *V. rossicum* roots based on mass yields. Interestingly, (−)-antofine appears to be a more effective inhibitor of fungi than the bacteria tested in our study. Notably, crude extracts yielded large inhibition zones with a rapidly growing *Rhizopus* strain that is not inhibited by 2 mg/disk of berberine, another phytochemical with pronounced antifungal activity (Ficker et al., 2003). Furthermore, (−)-antofine is clearly inhibitory to fungi that are widely distributed and important pathogens on diverse plant species. This broad-range antifungal activity by *V. rossicum* extracts is significant since escape from fungal pathogens has been shown to be an important predictor of a plant's invasiveness in its introduced range (Mitchell and Power, 2003).

The pronounced antifungal activity by (−)-antofine may be due to a mode of action in common to the phenanthroindolizidine alkaloids pergularinine and tylophorinidine, both of which inhibit the enzymes dihydrofolate reductase (DHFR) and thymidylate synthetase (TS) in *Lactobacillus leichmannii* (Rao et al., 1997; Rao and Venkatachalam, 2000). These enzymes are both key components of the thymidine biosynthetic pathway and therefore essential for the *de novo* synthesis of DNA. Since many fungi lack thymidine kinase activity, and thus do not have a DNA salvage pathway (Sachs et al., 1997), inhibition of *de novo* thymidine biosynthesis by (−)-antofine may explain the pronounced sensitivity to *V. rossicum* extracts by all fungi tested in this study.

Phenanthroindolizidine alkaloids from extracts of *Tylophora* spp. have also been implicated in insect antifeedant activity (Verma et al., 1986; Kathuria and Kaushik, 2005). Specialist herbivores of these plants, however, are apparently adapted to metabolize or utilize these alkaloids. For example, the butterfly *Ideopsis similes* appears to derive phenanthroindolizidine alkaloids from its *Tylophora* host plants (Komatsu et al., 2001) and presumably sequesters these for chemical defense. However, (−)-antofine derived from *V. rossicum* did not act as an antifeedant or toxin in the insect tests done in the present study. Rather, a second compound(s) from *V. rossicum* had antifeedant (rose sawfly larvae) and toxic activity to two larval lepidopterans, the masked birch caterpillar and the European corn borer. Taken together, we show that *V. rossicum* contains a potent antimicrobial compound, (−)-antofine, and additional insecticidal/insect antifeedant compound(s). These antibiotic activities may account for the noted absence of pathogen damage and herbivory on the plant at our study sites, and thereby contribute to the invasive nature of the species in eastern North America.

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