

Comparative Toxicity of Pokeweed (*Phytolacca americana*) Extracts to Invasive Snails (*Viviparus georgianus*) and Fathead Minnows (*Pimephales promelas*) and the Implications for Aquaculture

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Received: 29 October 2004/Accepted: 16 February 2005

Aquatic snails greatly impair the productivity and efficiency of growing and maintaining fish in aquaculture ponds, increasing the costs of operation. The snails compete with the fish for oxygen, food and space in the ponds, and contribute to the degradation of water quality by an increased input of nitrogenous waste. In central New York, many aquaculture ponds are invaded by the non-native European gastropod mollusk *Viviparus georgianus* to the extent that production can be severely impaired during the years with very large populations (D. Morehouse, pers. comm.). Since many snails are pest species in agricultural and residential settings or vectors for human disease (Pezzuto et al. 1984), pesticides have been developed to eradicate or control snail populations. However, the great majority of these molluscicides are either broad scope organically-based pesticides (pyrethroids, carbamates, organophosphates etc.) or special formulations of heavy metals. The most commonly used heavy metals are copper, aluminum and manganese (Oregon State University Extension Toxicology Network's Exttoxnet). Both of these types of molluscicides are extremely toxic to fish, birds and humans (Exttoxnet).

Lemma (1965, 1970) reported that a plant-derived compound (oleanolic acid glycoside, OAG – a saponin in the class of triterpene glycosides) selectively kills aquatic and semi-aquatic snails. The source of OAG in these studies was the berries of the Ethiopian plant Endod (*Phytolacca dodecandra* L.). Preliminary investigations showed that while extremely effective at killing snails, this chemical was harmless to commercial fish, wildlife, and the environment (Lemma 1970, 1983, Lemma et al. 1984). As a member of the *Phytolaccaceae* family, American pokeweed (*Phytolacca americana* L.) is closely related to Ethiopian Endod (*P. dodecandra*). American pokeweed is a poisonous plant because of its high content of saponins (Cornell University Poisonous Plant Database; Agronomy 1988). Some evidence exists that pokeweed contains high levels of triterpenoid glycosides (TG) (Kang and Woo 1987), which have been identified as compounds with important molluscicidal properties (Pezzuto et al. 1984; Marston and Hostettmann 1985). Although very sparse, existing data on pokeweed TG suggest that they do not have adverse environmental effects; for example extracts of the fruit and seed have no mutagenic properties (Pezzuto et al. 1984). We are

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currently unaware of any in vivo toxicity studies involving this plant, however, if proven effective against snails, pokeweed would offer several strong advantages for use as a molluscicide in aquaculture. The objective of this study was to investigate the toxicological properties of pokeweed (tissue extracts from berries and root) and assess its usability as a natural molluscicide both on a laboratory and a practical scale.

MATERIALS AND METHODS

Pokeweed (*Phytolacca americana* L.) seeds (Goodwin Creek Gardens, OR) were planted in wet potting soil and germinated in a growth chamber (16-hour photoperiod at 25°C and dark period at 20°C). The seedlings were repotted and moved to the greenhouse (March, 2002). After 6-8 weeks red (ripe) and green (unripe) berries were harvested and either dried to a constant weight in an oven (70°C) or flash frozen and stored in a deep freezer (-80°C) for later use. Additional berries were harvested from wild pokeweed bushes found in the field and processed identically to those grown in the greenhouse. The dried berries were ground to a fine powder (including seeds) and stored in a desiccator at room temperature. Root tissue was harvested from the fleshy taproot, homogenized with small amounts of water and stored at -80°C.

To determine the most effective pokeweed tissue extract, screening toxicity tests were performed using the different tissue preparations (root and berries) with five snails per treatment concentration and a negative control. The snails were exposed to concentrations of berry or root extracts ranging from 1 to 1000 mg/L (on a logarithmic scale) in static experiments and the mortality rate was assessed at 24 and 48 h. The preparations and concentrations that induced mortality rates higher than 50% were used in more detailed toxicity testing (as described below). We performed toxicity tests separately on the snails (*V. georgianus*) and larval fathead minnows (*Pimephales promelas*) as a model non-target organism.

For the target organism (*Viviparus georgianus*, obtained from Morehouse Bait Farms, Seneca, NY), toxicity testing was performed using a slightly modified method from the EPA guidelines for the testing of pesticides (OPPTS 870.4100, 870.1100 and 850.1010 referring to acute, chronic, and, respectively, aquatic invertebrate testing of pesticides; see References). Both the acute and chronic exposure tests followed a sustained exposure, static model (as detailed below) with three duplicated treatment levels, and were performed with the dry, ground, ripe pokeweed berries. The treatment levels, designated by the concentration of the applied berries, were 0 mg/L, 200 mg/L, and 500 mg/L. The tests were performed in a series of 20-L fish tanks (in 15 L of dechlorinated tap water) with 10 snails (*V. georgianus*, adult) per tank, in a growth room at constant temperature (23 ± 2°C) and photoperiod (16 hours). The tanks were aerated, but no food was provided for the duration of the experiment and there was no water change. Each tank also contained about 300 mL of active zeolite (Clinoptilolite, Aquatic Ecosystems, Inc., Apopka, FL) to trap ammonia waste from the water.

Before treatment, the snails were allowed to acclimate to the growth chamber for 48 h. Because triterpene glycosides (TG) are sensitive to hydrolysis by fermentation processes (Kang and Woo 1987), ground berries were applied to the water (3 g/tank for the 200 mg/L and 7.5 g/tank for the 500 mg/L) in the treated tanks three times (identical applications) at two-day intervals. Mortality was assessed at 7 and 30 days for the acute and the chronic toxicity data respectively. Counts of juvenile snails, “born” in the experimental tanks, were also noted. A snail was considered dead if the shell was empty, the body was suspended outside the shell or if there was no muscular tonus or response to tactile stimuli.

Toxicity tests on non-target organisms were performed separately using larval fathead minnows (*Pimephales promelas*, Chesapeake Cultures, Hayes, VA). Fathead minnow larvae (28 days of age) were allowed to acclimate to the growth room for 48 hours. The protocol was identical to the one used for the snails with the exception that the larvae were fed daily (Larval AP100, 150-250 μ m, Ziegler Bros., Inc., Gardners, PA) and there were 20 larvae per tank in duplicated treatments. The water contained 120-180 ppm sodium/calcium bicarbonate buffer (Buffer-UP, Mardel Laboratories, Inc., Glendale Heights, IL) to offset the decline in pH due to bacterial activity and high organic content of the water. Measurements were performed to ensure that the pH remained between 7.5 and 8.5 and that the oxygen concentration did not drop below 6 mg/L. *A priori* blocking was designed to offset the variability in growth chamber conditions due to the position of the tanks (complete random block design). There was no water change during the 8-day experiment; however, dechlorinated tap water was added to compensate for evaporative loss when needed.

We assayed the survival and mortality of the fathead minnow larvae at 8 days. We also determined the average length and weight of the fish for each treatment to look for any sublethal treatment effects. The weight was determined by weighing the live larvae in a known amount of water on a top-loading balance. The total length of each larva was measured with a ruler while the larva was partially immobilized in a thin plastic tube (a transfer pipette with its tip cut off).

Treatment effects were tested using pairwise multiple comparison procedures (Student-Newman-Keuls Method), versus control procedures (Holm-Sidak) and single and two-factor repeated measures analyses of variation (RM ANOVA), as appropriate (SigmaStat 8.0; SPSS Science, Chicago, IL). For the purpose of statistical analysis, data were log-transformed, where needed, to satisfy the conditions of normality and equal variance (Zar 1999). In our model, treatment concentration was the independent variable, day was the repeated factor, survival, size and weight were independent, and block was random. Statistical significance was considered at $P \leq 0.05$.

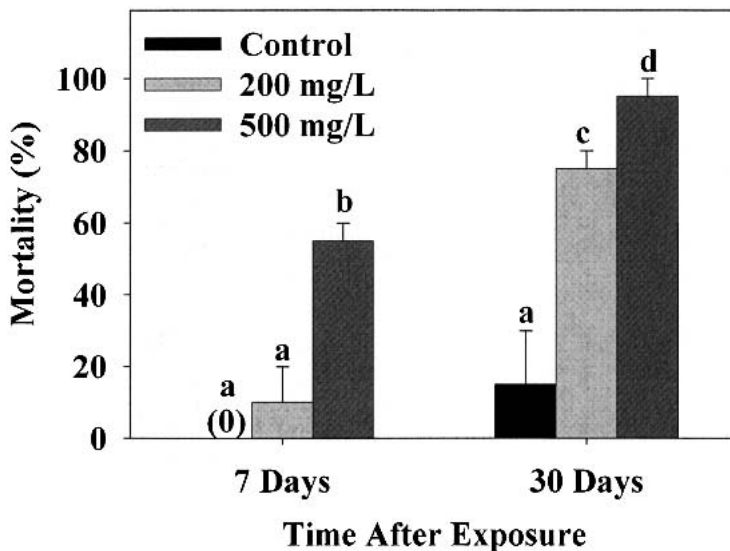


Figure 1. Snail mortality at 7 and 30 days after initial exposure to pokeweed berries, by treatment concentration. Snail mortality was significantly different for both treatment concentrations and both time points. Bars with different letters are statistically different at $P \leq 0.05$. All comparisons are versus a two-factor negative control = (no berries) \times at the beginning of experiment (day 0).

Using bicubic interpolation techniques and other modeling tools (SigmaPlot, SPSS Science, Chicago, IL) we obtained an exposure model for the snails to pokeweed berries. Bicubic interpolation is an image-rendering tool that creates polynomial regressions. The solutions of the regression are used to approximate values between the data points. Thus, we were able to extrapolate from it the recommended dose of treatment.

RESULTS AND DISCUSSION

The screening toxicity tests performed revealed that only ripe berries had detectable molluscicidal properties. There were no observable effects from the unripe berries or the root extracts. All subsequent tests were performed using the dried, ground, red pokeweed berries. For the sustained exposure model, the integrated (average) concentration across the first seven days was calculated to be 97 mg/L for the 200 mg/L treatment with a peak of 235 mg/L (233 mg/L average for the 500 mg/L treatment and peak of 541 mg/L). Having no means to measure these values, they were obtained by mathematical calculations from the half-lives reported in the literature (Lemma 1983), assuming first-order kinetics for all chemical transformations ([inactive stored TG] \rightarrow [TG active] \rightarrow [inactive decomposed TG]).

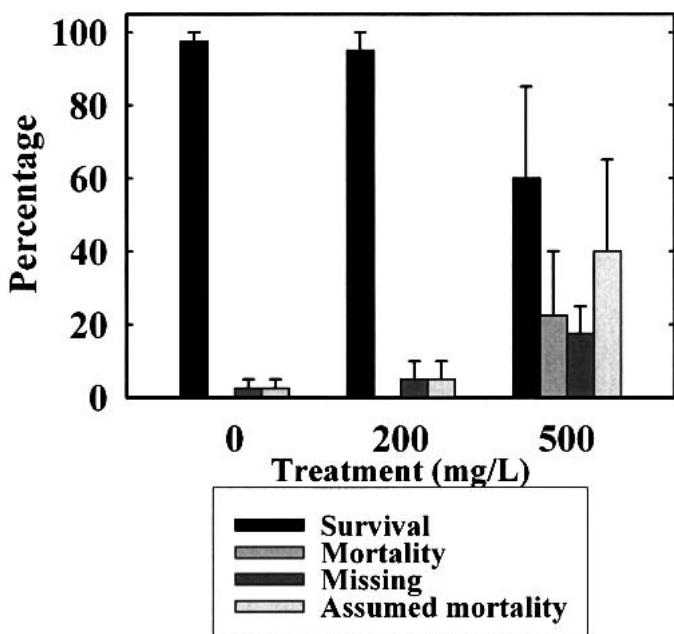


Figure 2. Fathead minnow larval survival at 8 days after initial exposure to pokeweed berries. Survival, mortality, missing and assumed mortality (sum of mortality and missing) show no statistically significant difference between treatments and control.

At 7 days past initial exposure, snail mortality was significantly higher for the 200 mg/L (10%) and the 500 mg/L (55%) treatments compared to the control – no mortalities ($P < 0.01$). The chronic exposure (30 days) also significantly reduced snail populations ($P < 0.01$) for the treatments: 95% at 500 mg/L and 75% at 200 mg/L with respect to the untreated control at 15% (Figure 1). Overall, there was a non-significant decrease ($P = 0.30$) in the number of juvenile snails born during the experiment from 20.5 for the control to 10.5 for the 200 mg/L and 4.5 for the 500 mg/L treatments. However, at 30 days past initial exposure, a Holm-Sidak test (vs. control method) shows a significant decrease in the number of juveniles between the control and 500 mg/L treatment ($P = 0.03$).

There was no significant decline in the survival ($P = 0.4$, $n=2$) as well as the average weight ($P = 0.2$) of the fish larvae due to the pokeweed berry treatment (Figs. 2 and 3). However, the larvae exposed to 500 mg/L pokeweed berries were significantly smaller (1.1 cm; $P = 0.02$) in size than the control (1.8 cm) (Fig. 3).

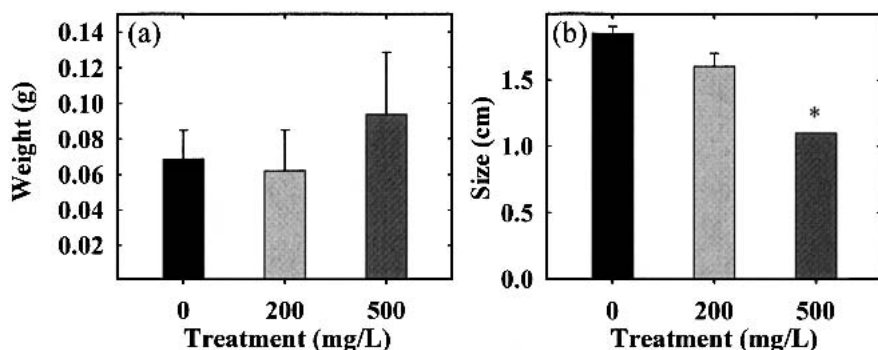


Figure 3. Sublethal effect in fathead minnow larvae at 8 days after initial exposure to pokeweed berries as a function of treatment concentration. Larvae exposed to 500 mg/L pokeweed powder were significantly shorter than the control. The asterisk indicates statistical significance for $P \leq 0.05$.

From our exposure model surface we extrapolated the minimum concentration of pokeweed berry powder necessary to significantly inflict losses to *V. georgianus* populations with maximum efficiency. This *recommended dose* for a 30-day program was 150 mg/L, which would be expected to reduce snail populations by 75% ($R^2 = 0.77$, $P = 0.045$). For a one-week program, the most efficient dose was 450 mg/L, with an expected mortality of 55% ($\pm 18\%$ estimate error).

Our results suggest that American pokeweed berries have significant molluscicidal properties and no effect on other species of importance in an aquaculture setting at the *recommended dose* levels of exposure. A treatment of 150 mg/L of pokeweed berry powder applied using our sustained exposure method will reduce *Viviparus georgianus* populations by 60-90% within a month. We observed no immediate lethal or sublethal toxicological effects on larval fathead minnows at exposure levels even 33% higher than the recommended dose. Larval fish are commonly more sensitive to pesticides than their adults and, in general, most adult fish, sometimes even by several orders of magnitude. We do not expect, thus, negative effects on other species of fish similar in environmental tolerances to fathead minnows.

The survival and development of an aquatic invertebrate were also unaffected by even the highest pokeweed-berry concentrations (qualitative data not shown). The larvae of this chironomid midge (*Orthoclad* subfamily) are entirely aquatic and require good water quality to survive (B. Smith pers. comm.) especially with respect to dissolved oxygen and factors influencing it. This suggests little or no impact of pokeweed berry extracts on the aquatic larvae of non-target invertebrates.

In terms of practical applicability in the ponds of an aquaculture farm, we predict that an application of 150 mg/L of pokeweed berry powder would dramatically reduce snail populations without affecting the standing fish crop or the environment. However, this exposure level requires a large quantity of pokeweed berries and may not be economically applicable for field conditions (except perhaps for small ponds). According to our estimates, an average aquaculture pond of 200,000 L (1 acre) would require 30 kg of dry pokeberry powder, which can be obtained from circa 100 kg of fresh berries, thus, from about 3-4 acres of mature pokeweed plants.

The sublethal effects (reduced growth) observed on the fish at the highest concentration of pokeberries may be of direct, toxicological, or indirect origin. Without more insight into the chemical composition of pokeweed berries, it is hard to describe these toxicological effects. Practically, applying 7.5 g of berries to 15 L of water is primarily a large addition of organic matter to a closed aquatic environment. From our observations, this adds enough organic carbon to the water to allow for the formation of large gel matrices (presumably supramolecular assemblies of organic polymers and particles, water and ions). Together with the intense purple color of pokeweed berries (water-soluble), this could potentially impair the ability of the small fish larvae to move and efficiently find food. The large amount of organic matter may also stimulate unforeseen bacterial blooms and transient but drastic reductions of the dissolved oxygen concentrations. In addition, bacterial blooms can add a new toxicological dimension to an already stressful environment, through the byproducts of their metabolism. Consequently, based on the data presented herein, we do not recommend the use of pokeweed berries in concentrations higher than 200 mg/L in the presence of fish. This is moreover justified by the fact that the increase in concentration to 500 mg/L pokeberry powder showed only a marginal increase in snail mortality compared to 200 mg/L.

Although saponins have been previously isolated and identified in pokeweed extracts (Kang and Woo 1987) and these results confirm previous finds that pokeweed has molluscicidal properties (Marston and Hostettmann 1985), the link between the two has not yet been documented. Following the identification of the molluscicidal compound, varieties of American pokeweed (or other plants) may be selected for higher concentrations of the active compound, rendering it more economically viable for large-scale applications. Nonetheless, these results provide a strong incentive for future investigations into the chemical identity and structure of the active molluscicidal compound in pokeweed berries, as well as a first empirical model for the use of pokeweed as a mollusk control agent in aquaculture applications.

Acknowledgments We acknowledge the valuable contributions of D. Morehouse (Morehouse Bait Farms); S. Greene and H. Mussell (IC Biology) for invaluable help with fish rearing, experimental setup and other logistic support; J. Hamilton and P. Melcher (IC Biology) for providing lab space and the HPLC for analytical

chemistry studies; B. Smith (IC Biology) for invertebrate identification. Funding for this project was provided through grants from Ithaca College's Dana Internship Program and Ithaca College Office of the Provost.

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