A Strategy for Detecting Natural Anthelmintic Constituents of the Grassland Species *Plantago lanceolata*

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A STRATEGY FOR DETECTING NATURAL ANTHELMINTIC CONSTITUENTS OF THE GRASSLAND SPECIES PLANTAGO LANCEOLATA.

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Abstract

A strategy to detect anthelmintic constituents in plantain (*Plantago lanceolata*) using a bioassay-driven purification approach was tested. Plantain consumed by cattle may control or reduce internal parasite titers, possibly due to the iridoid glucoside aucubin. Lyophilized, ground leaves of wild *P. lanceolata* were extracted with 95 % ethanol or boiling water containing calcium carbonate. Partially purified extracts (0 to 250 mg ml^{-1}), 5 µg ml^{-1} of the anthelmintic levamisole, or 5 mg ml^{-1} of aucubin were tested with sheathed bovine parasites (*Ostertagia ostertagi*). The percent moving worms was unchanged for water controls and reduced to 0 % for anthelmintic levamisole. Aucubin significantly reduced the number of swimming worms at day 2, but they returned to starting values at day 3. Extracts from tall fescue and white clover foliage did not show anthelmintic effects. Treatment of extract or aucubin with ß-glucosidase did not alter their activity. Beneficial anthelmintic action of ingested plantain is not due to aucubin.

Keywords: *Ostertagia ostertagi*, brown stomach worm, bioassay, aucubin.

Introduction

Reports in the literature suggest that narrow-leaf plantain (*P. lanceolata*) can control or reduce internal parasite (worm) infestations in cattle and sheep (Grieve, 1977; Stewart, 1996). Plantain is palatable to sheep and cattle and is nutritious (Cupahina, 1963; Deaker et al., 1994; Derrick et al., 1993; Ivins, 1952). Animals consuming plantain may have improved health and increased productivity due to reduced parasite load (Stewart, 1996). Aucubin, catalpol, and verbascoside occur in plantain, and are among those iridoid constituents suggested to possess anthelmintic activity. We have developed a laboratory bioassay that tests the effect of plantain extracts on the swimming activity of the brown stomach worm (*Ostertagia ostertagi*), an internal parasite of cattle. We show here that the iridoid glycoside aucubin reversibly inhibited swimming of *O. ostertagi* at concentrations of 14 mM. This bioassay driven strategy for identifying anthelmintic constituents can be applied to other grassland species as well.

Material and Methods

Plant extracts. Leaves of wild *P. lanceolata* were harvested from local fields and frozen the same day. Lyophilized plant material was ground in a Wiley mill (0.5 mm sieve, Thomas Scientific, Swedesboro, NJ). Freeze-dried plant powder was extracted with 95 % ethanol or boiling water (10 ml g^{-1}) containing 12.5 mg calcium carbonate (to prevent iridoid glucosides from decomposing and polymerizing to insoluble black pigments (Trim and Hill, 1952)).

Ethanol extract. Following filtration and evaporation of the ethanol extract, the resulting aqueous extract was partitioned against methylene dichloride to produce aqueous and organic fractions.

Hot water extract. The extraction procedure was based on that of Trim and Hill (1952). The hot water extract was filtered through kieselguhr, and the filtrate was treated with activated

charcoal (2 g g^{-1} plant tissue) and filtered through kieselguhr. The charcoal was washed several times with water to elute carbohydrates and salts. The charcoal filter cake was then washed and filtered several times with 50 % aqueous ethanol to elute catalpol and aucubin (in the early washes), and verbascoside (in the later washes).

Bioassay. Sheathed bovine parasites (*Ostertagia ostertagi*) were obtained through Dr. Louis C. Gasbarre, USDA-ARS, Beltsville, MD. The parasites were suspended in chlorine-free tap water, transferred to several petri dishes to promote gas exchange, and stored at 4°C. Wells of 24-well tissue culture plates (Falcon Plastics, Inc., Washington, PA) contained multiple concentrations of plantain test extracts, 5 mg ml^{-1} of the anthelmintic levamisole (Sigma-Aldrich, St. Louis, MO), 0.005 to 5 mg ml^{-1} of aucubin, and no treatment in a final volume 1 ml of 0.01 mM sodium acetate buffer, pH 5.0. Treatments and the control were replicated three times. *Ostertagia ostertagi* (30 to 80 worms) were added to each well, counted, and classified with the aid of a binocular microscope before and after addition of treatments, and after 48 and 72 hours of incubation at room temperature in the dark. Worms classified as swimming or coiled were considered viable, and worms classified as straight were considered dead. Coiled worms were immobile, but they sometimes uncoiled and resumed swimming activity. Decline in numbers of moving worms was indicative of anthelmintic activity, because either worms coiled up and became inactive, or they died and became straight. Data analysis was based on the percent of moving worms normalized at 100 % before the addition of treatments.

Bioassay with aucubin and ß-glucosidase. Several preparations of ß-glucosidase were used in experiments: 2.2 and 22 units of activity mg^{-1} enzyme (Sigma-Aldrich); and 1270 and 3811 units of activity mg^{-1} (Biozyme Laboratories, San Diego, CA). Bioassays were conducted

with 25 units of enzyme activity (10, 1.1, 0.02, and 0.01 mg enzyme ml^{-1}) and 78 mg ml^{-1} of the aqueous fraction of the 95% ethanol extract containing aucubin.

Results and Discussion

Ethanol extract. In the presence of the water-soluble portion of the ethanol extract (about 150 mg), *O. ostertagi* swimming activity was reduced to 10 to 30 % of the control values (data not shown). When incubated with up to 150 mg of the organic-soluble portion, *O. ostertagi* were not affected (data not shown). Similar amounts of the water-soluble and the organic-soluble fractions from a mixture tall fescue and white clover reduced the swimming activity of the parasites transiently, but the percentage of worms dying after 3 days of treatment or after 3 days of incubation in buffer did not differ (data not shown).

Hot water extract. The lyophilized early 50 % ethanol washes that contained primarily aucubin produced anthelmintic activity in the bioassay (Fig. 1). Addition of each concentration of extract resulted in immediate reduction of swimming activity on day 0. The 100 mg extract ml^{-1} treatment; coiled worms resumed normal swimming activity by day 2. However, by day 3 swimming activity were less than 50 % of the control wells and the percent of straight worms increased. The treatments with the three higher concentrations of extract resulted in essentially no swimming activity at days 2 and 3. The affected worms were straight and presumed dead. The increased swimming activity in the control treatment was not statistically significant. Swimming activity following addition of anthelmintic levamisole to wells was at zero on day 0, all worms were coiled, and no recovery was observed.

Since aucubin was the primary chemical constituent in the preparation tested, we tested authentic aucubin from Indofine Chemical Co. (Sommerville, NJ, USA) at concentrations up to 5 mg ml^{-1}. At the highest concentration, we found significantly lower swimming activity 2 days

after treatment, with recovery to normal levels at day 3 (data not shown). Concentrations of 0.005 to 0.5 mg ml^{-1} did not affect swimming activity of worms.

Aucubin and verbascoside levels can be as high as 6 and 9 %, respectively, of dry matter in *P. lanceolata* (Stewart, 1996). If a 630 kg animal consumes 100 g dry wt. (about 1 kg fresh wt) of plantain in a short period, that animal would ingest 6 g of aucubin. This would provide a dose of 0.01 mg aucubin kg^{-1} body wt, a factor approximately 1000 less than would be needed to affect *O. ostertagi* in vitro.

To test whether the aglycone of aucubin possesses more anthelmintic activity than the corresponding glycoside, we conducted bioassays with aucubin hydrolyzed with ß-glucosidase. Aucubin or aqueous 50 % ethanol extract (78 mg ml^{-1}) in the presence or absence of ß-glucosidase had no effect on swimming activity of *O. ostertagi* by day 3 in the bioassay. However, at the two highest enzyme concentrations (1270 and 3811 units ml^{-1}), a black precipitate formed in the bioassay wells that prevented accurate assessment of moving worms. Bioassay well contents were examined for aucubin content by thin-layer chromatography (silica gel plate developed in butanol-glacial acetic acid-water (4:1:1; v, v, v)). Aucubin content before addition of enzyme was high, as indicated by large distinct spots on the plate under ultra violet illumination. After 3 days in the bioassay wells, aucubin spots were much smaller, but still detectable.

These results indicate that if plantain fed to cattle has beneficial anthelmintic action, it is due to something other than aucubin or its aglycone. Further testing for other active constituents in *P. lanceolata* is necessary to identify anthelmintic compounds.

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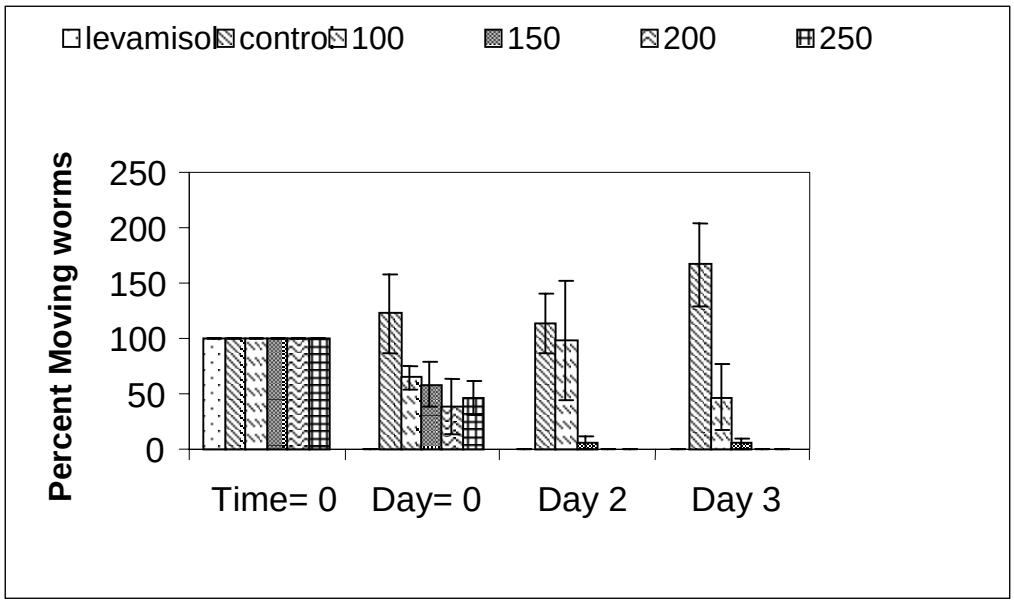

Figure 1 – Active O. ostertagi worms counted during bioassay of plantain extract and levamisole. Data columns are the mean of three determinations and vertical bars represent the standard error.