Original Research Paper

Bioactivity of the Alkaloids, Norharman and L-N-Methylcrotonosine/Linearisine

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Abstract: The toxic effects of the alkaloids, Norharman and a mixture of L-N-Methylcrotonosine/Linearisine (M/L mixture), on two organisms, *Gambusia puncticulata* and *Veronicella sloanei* were determined. The effect of the alkaloids on the fecundity of *Thiara granifera* was also investigated. Norharman was harmless to *G. puncticulata* but the M/L mixture inflicted 55.5% mortality on fish populations. The alkaloids had narcotizing, repellent and anti-feedant effects on *V. sloanei*. The M/L mixture suppressed the development of hatchlings in the brood pouches of *T. granifera*. Norharman and the M/L mixture reduced the number of hatchlings released from the brood pouches of *T. granifera* by 60 and 40%, respectively.

Keywords: Norharman, L-N-Methylcrotonosine, Linearisine, *Gambusia puncticulata, Veronicella sloanei*

Introduction

The bioactivity of secondary metabolites such as saponins, alkaloids, steroids, flavonoids and tannins is well documented (Evans and Evans, 2002; Mott, 1987). They are believed to be responsible for defence as well as the pesticidal, medicinal and allelopathic properties of plants (dos Santos *et al*., 2003; Hafiza *et al*., 2002; Amusan *et al*., 2000; Hostettmann and Marston, 1987; Kloos and McCullough, 1987; Youdeowei and Service, 1983). Alkaloids comprise the largest class of secondary plant substances (Harborne, 2012). They are believed to function as growth regulators and insect repellents or attractants (Harborne, 2012). Norharman is an indole alkaloid (Jitendra and Singh, 2009) and L-N-methylcrotonosine and linearisine are isoquinoline alkaloids (Stuart and Cava, 1968). Norharman is present in some members of the family, Zygophyllaceae (Jitendra and Singh, 2009) while methylcrotonosine and linearisine are found in some *Croton* species (Stuart and Cava, 1968). Norharman and a mixture of L-N-methylcroronosine/linearisine were isolated from the aqueous extracts of the plants *Guaiacum officinale* (family: Zygophyllaceae) and *Croton linearis* (family: Euphorbiaceae), respectively (Ellis-Tabanor, 2010). To evaluate the bioactivity of Norharman and an L-N-methylcrotonosine/linearisine mixture, three test organisms, *Thiara granifera, Gambusia puncticulata* and *Veronicella sloanei* were exposed to solutions of the alkaloids.

The Test Organisms

It is imperative to know the toxicity to fish of any substance that may be proposed for use in the aquatic environment. *Gambusia puncticulata* (Caribbean gambusia) is non-endemic to Jamaica but commonly occurs in most Jamaican freshwaters (Bennett and Hyslop, 2005). *Veronicella sloanei*, the terrestrial Pancake Slug is found throughout the Greater and Lesser Antilles (Fields and Robinson, 2004). It is described as an aggressive, phytophagous and opportunistic pest (Fields and Robinson, 2004; Stange and Deisler, 2004).

Thiara granifera is a small, invasive gastropod commonly found in Jamaican freshwater habitats. The young are carried in a brood pouch behind the head prior to release (Fretter and Graham, 1994). This process is under parental control (Berry and Kadri, 1974). In Jamaica, specimens of *T. granifera* breed throughout the year and have at least two periods of juvenile recruitment (Ellis-Tabanor and Hyslop, 2005). One way to control the numbers of snails of this invasive species may be by disrupting reproduction as their reproductive characteristics allow them to rapidly colonize and dominate freshwater habitats (Dudgeon, 1986; Berry and Kadri, 1974).

The objectives of this study then were to determine the toxicities of the alkaloids Norharman and L-N-methylcrotonosine/linearisine to *Gambusia puncticulata* and *Veronicella sloanei*. The effects of the alkaloids on the fecundity (number and size of hatchlings) in *Thiara granifera* were also investigated.

Materials and Methods

Bio-assays of Norharman and L-N-methylcrotonosine/Linearisine Mixture against Gambusia puncticulata

Specimens of *Gambusia puncticulata* were collected from the Mona Reservoir near to the University of the West Indies, Mona Campus, Jamaica and housed until required for use in aerated aquaria (45×23×23 cm) containing de- chlorinated tap water treated with Nutrafin Aqua Plus tap water conditioner. They were fed standard fish flakes. Female fish of body length of 3.0-3.5 cm were selected for testing. The fish were exposed to Norharman (11.91 mg L^{-1}) and the M/L mixture (40.55 mg L^{-1}) and percent mortality was recorded after 24 h. The concentrations represent the 24 h LC_{50} values of the alkaloids against *T. granifera*. For each toxicant, four treatments including one control (de-chlorinated tap water only) were prepared in 2000 mL glass beakers, each containing 1000 mL of treatment. The treatments were set up in three replicates. Three specimens of *G. puncticulata* were added to each treatment. Wire mesh covers prevented fish from jumping out. Fish which floated on their backs and did not respond to prodding by a blunt seeker were recorded as dead.

Exposure of Veronicella sloanei to Treated Soil

Specimens were collected from the Botany Garden of the Department of Life Sciences of the Mona Campus of the University of the West Indies and used immediately. Four slug cages were made by using large plastic containers (27×17×7 cm) covered with lids. Each lid was modified to allow aeration of the cage. This was done by cutting a hole (24×14 cm) into the lid, then stapling a portion of wire mesh of pore size 1.0×1.0 mm over the hole. Soil (loam type) collected from the habitat of the slugs was placed on the base of the cages to a height of 1.5 cm (weight of soil = 365 g). The soil in three cages was saturated with 150 mL of the toxicant, equivalent to the 24 h LC_{90} of Norharman (123.75 mg L^{-1}) and the M/L mixture (218.22 mg L^{-1}) against *T. granifera*. The fourth cage was a control containing soil saturated with de-chlorinated tap water only. The cages were set up in three replicates. Five slugs (4.0-4.5 cm body length) were placed on the saturated soil in each cage. The behaviour of the slugs was observed for the first hour of exposure. Escape indexes were derived from the percent of slugs that climbed away from the treated soil after the first 60min of exposure. They were prevented from avoiding contact with the soil for at least six hours by putting them back on the soil if they attempted to climb up. After a 24 h period, the slugs were observed for mortality.

Feeding Experiment: Veronicella sloanei

Slugs were kept for two days without food in cages containing loam soil moistened with de-chlorinated tap water. Lettuce leaves (*Lactuca sativa*) were soaked for 12 h in a solution equivalent to the 24 h LC_{90} of Norharman (123.75 mg L^{-1}) and the M/L mixture (218.22 mg L^{-1}) against *T. granifera*. Soaked leaves were cut in square pieces (4×4 cm) and three square pieces were placed in each of three cages containing five slugs (4.0-4.5 cm in body length). A similar cage was set up as control except that it contained three pieces of lettuce leaf that were not soaked in extract. The experimental set up was replicated thrice. After 24 and 48 h, evidence of feeding and the effects of feeding on the slugs were noted. During the experiment, the soil in the cages was kept moist with de-chlorinated tap water.

Fecundity Test

Specimens of *Thiara granifera* were collected from the Mona Reservoir. The snails were reared in glass aquaria (60×30×37.5 cm) containing de-chlorinated tap water aerated by conventional air pumps with plastic tubing and standard air-stones. They were fed shredded lettuce leaves (*Lactuca sativa*) *ad libitum*. The water temperature and pH were measured at least once per week. The mean temperature was 27±1°C and pH varied between 7.5-7.9. The water in the aquaria was changed once per week and dead snails were removed and hatchlings transferred to other aquaria.

Six snails (12-14 mm shell height) were selected from the rearing aquaria and added to each treatment. Snails were exposed to a 12 h light: 12 h dark photoperiod. Over a six week period, treatments of 0.115 mg L^{-1} of Norharman and 0.753 mg L^{-1} of L-N-methylcrotonosine/linearisine mixture, representing 10% of the LC_{10} values of the substances against *Thiara granifera,* were administered. The treatments, including a control of de-chlorinated tap water, were prepared in glass aquaria measuring 30×15×15 cm in three replicates. Each aquarium contained 1000 mL of solution. During the experiment the treatments were renewed every three days.

Hatchlings were removed from the treatments every three days, counted and shell heights measured. At the end of the experiment the adult snails were removed from the treatments and preserved in 70% ethanol. The brood pouch was dissected by crushing the shell in the region of the body whorl. The broken pieces of shell were removed to expose the viscera consisting of the head-foot with the operculum attached. The viscera were cut behind the head and placed on a microscope slide. The brood pouch was opened, water was added and seeker was used to dislodge the young snails onto a glass microscope slide. These were viewed with a compound microscope fitted with an ocular micrometer (1 µm =

0.04 mm). The shell height of each offspring was recorded. All young snails in the brood pouch from one shell whorl in size upwards were counted and measured. The brood count data were added to the number of hatchlings produced in each replicate by the snails, during the experiment. The number of hatchlings per snail was calculated.

Data Analysis

The data were arranged into size-frequency distributions. Kruskal-Wallis, Mann-Whitney and Chi-squared tests were done by hand on the data using documented procedures (Zar, 1999).

Results

Norharman (11.91 mg L^{-1}) was not toxic (0% mortality) to *G. puncticulata* for an exposure period of 24 h. However the L-N-methylcrotonosine/linearisine mixture (40.55 mg L^{-1}) inflicted 55.5% mortality on *G. puncticulata.* Norharman (123.75 mg L^{-1}) and the M/L mixture (218.22 mg L^{-1}) were not lethal to *V. sloanei* for an exposure period of 24 h. Upon exposure to both substances, the slugs became immobile initially for about 60 sec, after which most of them started to move rapidly from the treated soil and up the sides of the cages. The slugs in the control cages did not show the initial immobility or the rapid movement. Those slugs that remained on the treated soil either curled the body into a ball and /or secreted excess mucus. The escape indexes of slugs exposed to Norharman and L-N-methylcrotonosine/linearisine mixture were 87 and 80%

respectively. The escape index of slugs in the controls for both substances was 40%.

Table 1 shows that the lettuce leaves treated with Norharman and L-N- methylcrotonosine/linearisine mixture were not eaten by slugs during the first 24 h. Only after approximately 36h was the food consumed, but there was no resulting mortality. Feeding was significantly less in the treatments (p<0.05) compared to controls indicating feeding inhibition by the toxicants. Also, the percent of leaves (soaked in Norharman) eaten by the slugs within 48 h (16.75%) was significantly less than the percent of leaves (soaked in the mixture of L-N- methylcrotonosine/linearisine), (50%) eaten by the slugs (p<0.05).

From Table 2, significantly (p<0.05), fewer hatchlings were produced by snails exposed to Norharman (4.0±2.0) than by those in the L-N-methylcrotonosine/linearisine mixture (6.0±4.0) and in the control (8.0±4.0). Norharman reduced the number of hatchlings in the brood pouches as well. The number of hatchlings released into the aquaria was significantly less in snails exposed to L-N-methylcrotonosine/linearisine mixture than in the control (p<0.05). Table 3 reveals that when counts were combined the 1.1-2.0 mm size class is dominant.

Table 1. Feeding of slugs (% leaves eaten) for exposure periods of 24 and 48 h

Treatment	24 h	48 h
Norharman	-	16.7
L-N-methylcrotonosine/linearisine	-	50.0
De-chlorinated tap water	33.0	70.0

Table 2. Mean number of hatchlings per parent snail, *Thiara granifera* (± standard error) obtained from the aquaria, the brood pouches and the aquaria and brood pouches combined

Treatment	Mean number of hatchlings/snail		
	Brood pouch	Aquaria	Combined
Norharman (0.115 mg L^{-1})	2.0±1.0	2.0±1.0	4.0±2.0
L-N-methylcrotonosine/linearisine (0.753 mg L^{-1})	3.0±2.0	3.0±2.0	6.0±3.0
De-chlorinated tap water	3.0±1.0	5.0±3.0	8.0±4.0

Table 3. Class/Frequency distribution of hatchlings of *Thiara granifera*

Treatment	Size class		
	0.1-1.0 mm	1.1-2.0 mm	2.1-3.0 mm
From aquaria and brood pouches			
Norharman (0.115 mg L^{-1})	19 (18.3%)	74(71.2%)	11 (10.5%)
L-N-methylcrotonosine/linearisine (0.753 mg L^{-1})	56 (30.3%)	111 (60.0%)	18 (9.7%)
De-chlorinated tap water	39 (17.6%)	165 (74.3%)	18 (8.1%)
From brood pouches only			
Norharman (0.115 mg L^{-1})	9 (19.0%)	36 (77.0%)	2 (4.0%)
L-N-methylcrotonosine/linearisine (0.753 mg L^{-1})	46 (53.5%)	37 (43.0%)	3 (3.5%)
De-chlorinated tap water	27 (33.3%)	48 (59.2%)	6 (7.4%)
From aquaria only			
Norharman (0.115 mg L^{-1})	10 (17.5%)	38 (66.7%)	9 (15.8%)
L-N-methylcrotonosine/linearisine (0.753 mg L^{-1})	10 (10.0%)	74 (74.8%)	15 (15.2%)
De-chlorinated tap water	12 (8.3%)	117 (81.3%)	12 (8.5%)

Table 4. Mean number of hatchlings per snail per day (± standard error) produced by *T. granifera* in each treatment

	Brood pouches	Aquaria	Combined
Norharman	0.05±0.02	0.05±0.02	0.10±0.04
M/L mixture	0.07±0.05	0.07±0.05	0.14±0.10
De-chlorinated tap water	0.07±0.02	0.12±0.07	0.14±0.10

Table 5. Mean number of hatchlings per treatment (± standard error) produced by 30 specimens of *T. granifera*

	Brood pouches	Aquaria	Combined
Norharman	16±3	9±5	35±8
M/L mixture	29±4	33±3	62±7
De-chlorinated tap water	26±0	48±0	74±0

The higher percentage of young snails in the smallest size class (0.1-1.0 mm) for snails exposed to the M/L mixture may indicate slower growth rate of hatchlings in this treatment. Chi-squared tests performed on the raw frequency data reveal that the frequencies of the 0.1-1.0 mm size class were significantly different between Norharman and the M/L mixture ($p<0.05$) and between the M/L mixture and the control ($p<0.05$); but not between Norharman and the control. Snails exposed to Norharman released no hatchlings from the brood pouch after approximately four weeks, while those exposed to the M/L mixture released hatchlings throughout the six week exposure period. Table 4 reveals consistency in the mean number of hatchlings produced per day by snails in all treatments. Overall hatchling numbers were significantly lowers ($p<0.05$) in snails exposed to the alkaloid treatments, with the lowest values found for Norharman-exposed individuals (Table 5).

Discussion

The bio-assays of Norharman and L-N-methylcrotonosine/Linearisine mixture against *Gambusia puncticulata* reveal that whereas *Guaiacum officinale* should be safe for field application, *Croton linearis* should be used with caution in the aquatic environment. The production of excess mucus is an indicator of poisoning in slugs but they are known to be well equipped to detoxify poisons (Aguiar and Wink, 2005). In this study, it is possible that the slugs detoxified the initial quantity of toxicant absorbed and then secreted mucus to prevent further contact with the toxicant or to nullify its effects. The escape indexes probably indicate that the slugs were irritated by both substances and were trying to escape from an environment detrimental to their survival. The results of the feeding experiment (Table 1) support the findings of Birkett *et al.* (2004) that alkaloids exhibit antifeedant properties towards slugs. The effects of both Norharman and the L-N-methylcrotonosine/linearisine mixture point favourably to their use in agriculture/plant protection as repellents or for lowering damage caused by slugs.

T. granifera is known to retain hatchlings in the brood pouch in the presence of toxicants (Ellis-Tabanor and Hyslop, 2005). It is possible that the release of hatchlings was reduced by the parent snails because the alkaloids posed some degree of risk to the survival of the hatchlings (Table 2). The mode of action of Norharman involves its accumulation in tryptamine-associated neurons of the central nervous system and irreversible interaction with a specific gene DNA sequence (Louis *et al.*, 2008). If Norharman exhibits this mode of action in *T. granifera*, then the damaged nervous system will adversely affect development and release of hatchlings from the brood pouches. Prentice (1983) reports that young *T. granifera* are released when they are approximately 2 mm in length. This implies that the M/L mixture either induced the transfer of embryo from the ovary to the brood pouch leading to an accumulation of the 0.1-1.0 mm size in the brood pouch or suppressed the development of the embryos from this size to the larger sizes, 1.1-2.0 and 2.1-3.0 mm (Table 3).

The brood pouches of snails exposed to both alkaloids contained hatchlings of the three class sizes. Hence different size hatchlings were released from the brood pouches (Table 3). Prolonged exposure to Norharman may cause snails to stop releasing hatchlings from the brood pouch after a time. Similar interference with reproductive processes has been demonstrated by alkaloids from the plants, *Peganum harmala* (Zygophyllaceae) (Abbassi *et al.*, 2003) and *Catharanthus roseus* (Nalina Sundari, 1998). Since Norharman and the M/L mixture exhibited antifeedant activities in *Veronicella sloanei* perhaps feeding in *T. granifera* was also adversely affected thereby contributing to a lowering of its reproductive performance.

Isoquinoline alkaloids are known to inhibit respiratory enzymes for example monooxygenase, to destroy cytochrome P-450 and to interfere with the biosynthesis of amino acids and proteins in cells (Singh and Singh, 1999). It is possible that the development of hatchlings may have been impeded by the improper functioning of the respiratory and metabolic enzymes and by interference with the biosynthesis of proteins as a result of exposure to the alkaloids.

Conclusion

The results indicate that Norharman is safer than L-N-methylcrotonosine/linearisine for use in the aquatic environment. Prolific reproduction in *T. granifera* enables rapid colonization and dominance in freshwater habitats. Norharman and the M/L mixture allow population control of the species through their negative effect on fecundity. The data substantiate the potential of these compounds for use as molluscicides both for invasive aquatic species and in agriculture and pest management for slug control.

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Author's Contributions

Margelette Ellis-Tabanor: Designed and conducted experiments. Drafted, corrected and gave final approval of the manuscript.

Dwight Robinson and Eric Hyslop: Gave technical support and advice; corrected and gave final approval of manuscript.

Ethics

All authors have read and approved the manuscript and declare that there is no conflict of interest.

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