An Evaluation of the Toxicity of White Snakeroot (*Ageratina altissima*) and Rayless Goldenrod (*Isocoma pluriflora*) in a Lactating Mouse Model

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Abstract

Rayless goldenrod (*Isocoma pluriflora*) and white snakeroot (*Ageratina altissima*) have been implicated in the poisoning of various livestock species including cattle, sheep, goats, and horses, as well as humans. It has also been observed that nursing young can be poisoned when their mothers consume these plants, indicating that the toxins are excreted in the milk. Both plants contain similar benzofuran ketones, which have been suggested to be the toxins. However, recent research suggests that another compound (or other compounds) may be responsible. Consequently, additional research needs to be done to definitively identify and characterize the toxic component(s) of these plants. The objective of this study was to determine if a lactating mouse model can serve as a good small-animal model to study the toxicity of white snakeroot and rayless goldenrod, with the end goal of identifying the toxin(s) in these plants. White snakeroot and rayless goldenrod were dosed orally, via the chow, to lactating dams from post-natal days (PND) 1-21. The pups were evaluated for locomotor activity as well as motor coordination and function on PND 14, 21, and 28. There was no indication of muscle weakness or tremors in any of the dams or their pups. The results from this study suggest that neither white snakeroot nor rayless goldenrod is toxic to lactating mice or their pups. Consequently, mice do not appear to be a viable small-animal model to study the toxicity of these plants.

Abbreviations: BFK, benzofuran ketones; PND, post-natal day; RGR, rayless goldenrod; WSR, white snakeroot.

Keywords: *Ageratina altissima*, benzofuran ketones, BFK, *Isocoma pluriflora*, mice, rayless goldenrod, white snakeroot

Introduction

Rayless goldenrod (RGR, *Isocoma pluriflora*) is a toxic range plant found in the southwestern United States and northern Mexico commonly growing in dry, open sites especially in alkaline areas (Burrows and Tyrl 2001, 2013). White snakeroot (WSR, *Ageratina altissima*) is found throughout the eastern half of North America, usually in low, moist, partially shaded sites such as wooded areas (Burrows and Tyrl 2001, 2013). Both plants have been implicated in the poisoning of various livestock species including cattle, sheep, goats, and horses, as well as humans (Beier and Norman 1990). The consumption of milk from cows grazing WSR caused many deaths among Midwestern settlers during the 1800s, at times forcing entire settlements to be abandoned (Couch 1927). In 1917, WSR was shown to be responsible for “trembles” and “milk sickness” (Couch 1927, Moseley 1941). Cases of

milk sickness in humans who have consumed milk from cows grazing WSR have been reported as recently as 1963 (Hartmann et al. 1963). In the early 1900s, a disease with nearly identical clinical signs broke out in the southwestern United States, and it was quickly established that the southwestern "milk sickness" was due to ingestion of RGR (Marsh 1926). Both WSR and RGR have been shown experimentally to poison nursing livestock (Wolf et al. 1918, Marsh 1926).

Both WSR and RGR contain a substance historically referred to as "tremetol," which causes "trembles" and "milk sickness" in livestock and humans, respectively (Couch 1927). Tremetol has been shown to be a complex mixture of sterols and derivatives of methyl ketone benzofuran that includes, but is not limited to, tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyl-tremetone **3**, and 6-hydroxytremetone **4** (figure 1) (Bonner et al. 1961, Bonner and DeGraw 1962, Lee et al. 2009). The toxicity of RGR and WSR is due to myoskeletal and myocardial degeneration and necrosis (Stegelmeier et al. 2010, Davis et al. 2013a,b). Consequently, the most characteristic clinical sign of poisoned animals is muscle tremors, which is accentuated by exercise. Toxicity occurs after consumption of 0.5-1.5% of the animal's body weight during a 1-3 week period (Kingsbury 1964; Davis et al. 2013a,b, 2015).

Although the benzofuran ketones (BFK) have traditionally been thought to be the toxic components of WSR and RGR, recent research by our group suggests that another compound (or other compounds) may be at least partially responsible. In a recent study using yearling goats, the BFK were extracted from WSR, adsorbed onto both alfalfa and the extracted residue at the same concentrations as in the WSR plant material, and dosed to goats. The goats dosed with ground WSR plant were poisoned; however, the goats dosed with BFK adsorbed onto alfalfa and BFK adsorbed on to the previously extracted WSR plant residue were not poisoned, suggesting that another compound(s) responsible for the poisoning was lost or modified in the extraction process (Davis et al. 2015). Consequently, additional research needs to be done to definitively identify and characterize the toxic component(s) of these plants. The fact that nursing young can be poisoned when their mothers eat these plants, or when humans drink milk from cows that have consumed toxic WSR, indicates that the toxic components are passed through the milk. Therefore, the objective of this study was to determine if a lactating mouse model can serve as a good small-animal model to study the toxicity of WSR and RGR, with the end goal of identifying the toxin(s) in these plants.

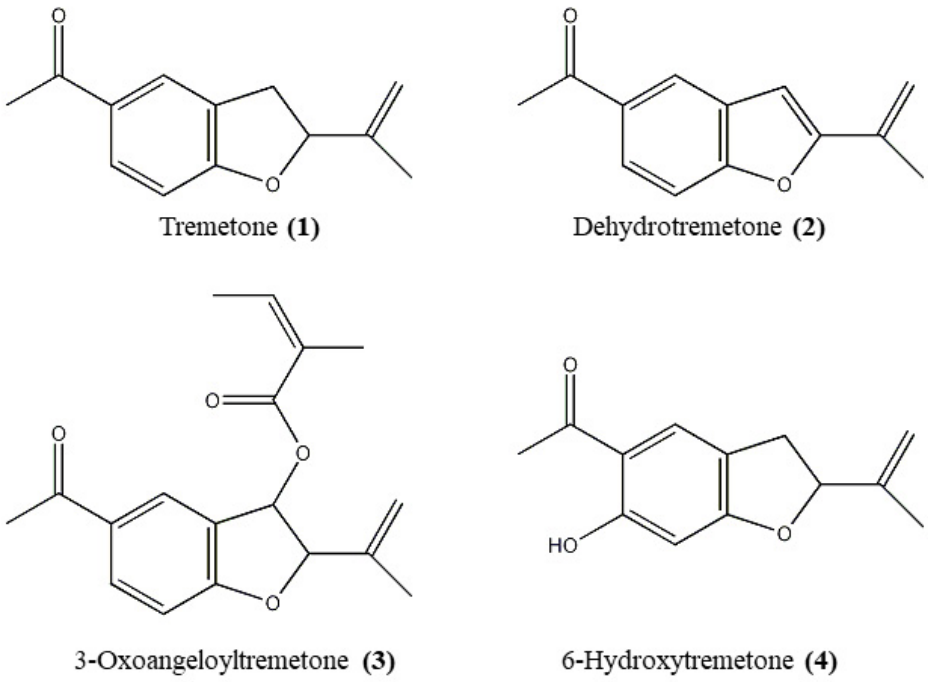

Figure 1. Structure of the benzofuran ketones identified in white snakeroot and rayless goldenrod.

Materials and Methods

Plant Material

White snakeroot (WSR, *Ageratina altissima*) was collected in the early to mid-flowering stage during September 2010 near Champaign, IL (N40 05.54 W87 49.68, at an elevation of 181 m). Rayless goldenrod (RGR, *Isocoma pluriflora*) was collected in the pre-flowering stage during May 2010 near Pecos, TX (N31 23.56 W103 29.58, at an elevation of 788 m). Both plants were air dried and stored intact in a protected enclosure. Immediately prior to preparing rodent chow, the plants were ground to pass through a 2 mm screen using a Wiley Mill. Alfalfa (*Medicago sativa*) was harvested in June 2014 near Logan, UT (N41 54.82 W111 48.57, at an elevation of 1425 m). The alfalfa was harvested using normal agriculture practices of swathing, drying, and baling the hay. The dried alfalfa hay was ground to pass a 2 mm screen using a Gehl Mix-All model 55 (Gehl Company, West Bend, WI, USA).

Animals

All procedures were conducted under veterinary supervision and were approved by the Utah State University Institutional Animal Care and Use Committee. Male and female Swiss Webster mice (8 weeks old) were purchased from Simonsen Laboratories Inc., Gilroy, CA. Mice were acclimated for 3 to 4 d with free access to a commercially pelleted rodent chow (Harlan Teklad rodent diet (w) 8604) and tap water before beginning experiments. Mice were housed under controlled temperature (20-22 °C) in a 12:12 h light:dark cycle. Mice were hand mated (3 females and 1 male per cage) for 12 h each night. Once the females were visibly pregnant, they were housed individually for the remainder of the study. The mice were dosed as outlined below beginning on post-natal day (PND) 1 through PND 21. The mice were weaned on PND 21, with each litter of pups housed as a group for the remainder of the study.

Diets were prepared using the same commercially pelleted rodent chow (Harlan Teklad rodent diet (w) 8604), which was ground and mixed with 10% corn starch and either ground WSR or RGR in hot water to obtain chow containing 75%, 50%, 25% WSR and 40%, 20%, 10% RGR. Pellets, approximately 1 x 3 cm in size, were formed and air dried overnight at room temperature (~ 21 °C). The chow for the control group was prepared in the same manner but with 90% commercial rodent chow and 10% corn starch. Additionally, a plant control pellet was prepared using 50% alfalfa, 10% corn starch, and 40% commercial rodent chow. Chow consumption, maternal body weight, and the number of pups in each litter were measured every 2 days beginning on PND 1 and through PND 21.

HPLC Analysis

Using an HPLC method developed for the quantitation of BFK in WSR and RGR (Lee et al 2009), the concentrations of **1**-**4** were determined in dry, ground plant material and pelleted rodent chow containing WSR or RGR. Ground plant or pelleted material was weighed (100 mg) into a screw-top glass test tube (16 mL). The plant and pellet material was extracted (16 h) by mechanical rotation with 8 mL hexane:ethyl acetate (70:30 v:v) at ambient temperature. HPLC was performed using a Shimadzu LC-20AT (Shimadzu Co., Kyoto, Japan) equipped with an autosampler and PDA detector from the same vendor and a 100 mm x 2 mm i.d., 5 µm, Betasil C_{18} column (Thermo Hypersil-Keystone, Bellefonte, PA). Samples (10 µL) in hexane:ethyl acetate (70:30 v:v) were injected onto the column and eluted with a 20 mM ammonium acetate-acetonitrile mobile phase at a flow rate of 0.4 mL/min. The mobile phase program was 20 mM ammonium acetate-acetonitrile, 65:35 v:v for 4 min followed by a linear gradient to a composition of 65% acetonitrile at 20 min. At 21 min, the composition was increased to 100% acetonitrile for 5 min. The eluant was monitored at λ=280 nm. Under these conditions, tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyltremetone **3**, and 6-hydroxytremetone **4** eluted at 9.8, 14.2, 17.4, and 12.9 min, respectively. The concentrations of **1**-**4** in plant material and pellet material were quantified against a seven-point calibration curve using previously isolated tremetone **1**, dehydrotremetone **2**, 3-oxyangeloyltremetone **3**, and 6-hydroxytremetone **4** (Lee et al. 2009). The standard solutions were prepared by serial dilution with hexane:ethyl acetate (70:30) over the range of 0.39 µg/mL-200.0 µg/mL. The calibration curves had R^2 < 0.9997.

Rotarod

An accelerating rotarod apparatus (IITC Life Science Inc.) was used to assess motor function (Jones and Roberts 1968, Crawley 1999). The apparatus had 5 lanes, and rod diameter was 3.2 cm. Mice were tested on PND 21 and 28 in a balanced

order across treatments. Animals were briefly trained to walk on the rotarod immediately prior to the PND 21 test. The apparatus was set to accelerating mode from 3 to 40 rpm in 300 sec, reaching a maximum at 180 sec. The mice were positioned in their respective lanes while the rods were not moving. Once all the mice were positioned, the rods began accelerating. The latency to fall was recorded automatically for each lane. The individual test of any animal was terminated if it exhibited a passive rotation whereby it was hanging by its forelimbs from the rod during a complete rotation. Each animal was given two consecutive trials on the rotarod at each time period, with an inter-trial interval of 30 sec after the last mouse fell.

Open Field Analysis

The open field apparatus is a common measure of exploratory and anxiety behavior in mice. In the context of this study, our interest centered on evaluating motor impairment or changes in locomotor activity (Kręz˙el et al. 1998). Any-maze® software (San Diego Instruments) was used in conjunction with four open fields (50 x 50 cm, 38 cm wall height) to test four pups simultaneously. Pups were tested in the open field for 10 minutes on PND 14, 21, and 28. An overhead camera and the software automatically tracked the movement of each animal in the open field.

Statistical Analysis

Statistical comparisons were performed using ANOVA with a Bonferroni posthoc test of significance between individual groups using SigmaPlot for Windows (version 12.5, SPSS Inc., Richmond, CA). Correlations were determined with a Spearman Rank Order Correlation analysis using SigmaPlot for Windows (version 12.5, SPSS Inc., Richmond, CA). Differences were considered statistically significant at $P < 0.05$. Four pups per litter were evaluated on each apparatus, unless there were fewer than four pups in the litter. In those instances, all of the remaining pups (1-3) were evaluated. The values for the litter were averaged, and the litter was considered the experimental unit for statistical analyses.

Results and Discussion

Both RGR and WSR have been known to be poisonous to livestock and humans for many years. They both cause very similar clinical signs of poisoning, including muscle weakness and tremors. Additionally, they both contain BFK. Even though it has been suggested that the BFK are the toxic components of these plants, this has not been demonstrated in a mammalian model. To this end, a number of small-animal models have been previously evaluated, including several rodent species (Bowen et al. 1963, Beier and Norman 1990). All research to date suggests that adult rodents, with guinea pigs possibly an exception, are not a good model to study the toxicity of these plants (Bowen et al. 1963, Davis unpublished data and personal communications). It is, however, quite common for neonatal animals to be more sensitive to toxins than mature, adult animals (Whitney et al. 1995, Zimmerman 1999, Piñeiro-Carrero and Piñeiro 2004). Therefore, the objective of this study was to determine if a lactating mouse model can be used to study the toxicity of RGR and WSR.

The BFK profiles of the RGR and WSR used for this study are shown in figure 2. The RGR contained tremetone **1**, dehydrotremetone **2**, and 3-oxyangeloyltremetone **3**, with **3** accounting for the majority of the BFK. The WSR contained tremetone, dehydrotremetone, and 6-hydroxytremetone, with similar amounts of tremetone and 6-hydroxytremetone. The plants were dosed orally to lactating dams via their chow. Commercial rodent chow was mixed with the plants to make chow that consisted of 75%, 50%, and 25% WSR and 40%, 20%, and 10% RGR. Higher percentages of WSR were used because preliminary experiments indicated that mice will more readily eat rodent chow containing WSR than RGR (data not shown). The concentration of BFK in the various batches of chow is shown in table 1.

Mice were fed treated lab chow from PND 1 through PND 21. During this period, chow consumption, maternal weight, and the number of pups in each litter were recorded. There was significantly less chow consumed in all treatment groups compared to controls, except for the alfalfa control chow (figure 3). Similarly, there was significantly less chow consumed in all other groups compared to alfalfa control chow, except for the chow containing 20% RGR. In general, there was a trend for less chow consumption with increased percentage of plant in the chow for both WSR and RGR. The decreased chow consumption translated to a daily weight loss in the 75% WSR, 50% WSR, and 40% RGR groups (figure 4). Finally, all of the mice dosed with WSR chow received a much higher dose of BFK than the RGR-dosed mice (figure 5).

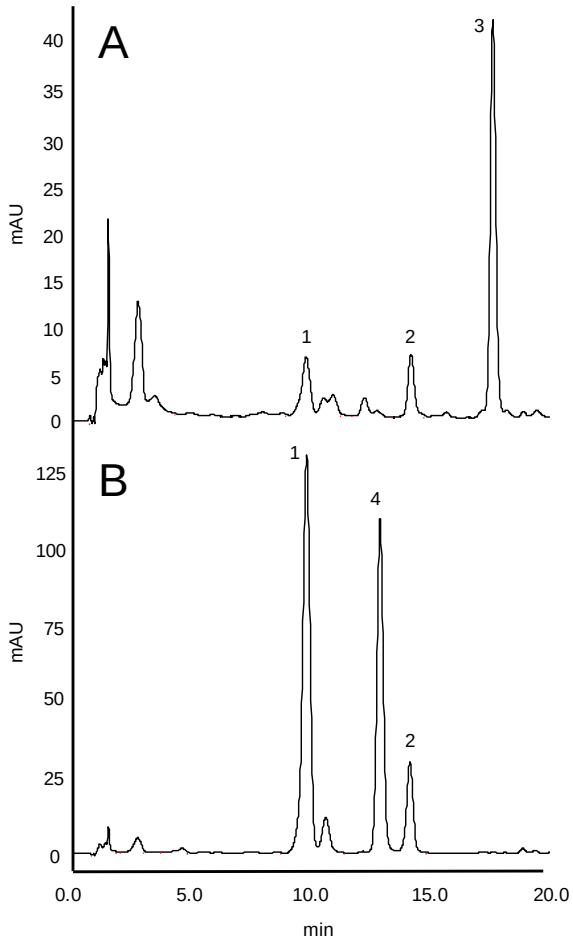

Figure 2. HPLC chromatograms of the benzofuran ketones in rayless goldenrod (A) and white snakeroot (B). mAU, milli-absorbance units.

The number of pups in each litter was counted every other day from PND 1 to PND 21 to determine if the treatments affected the survival rate of the pups. There was a significant decrease in the survival of pups in all treatment groups compared to the controls, including the alfalfa control group (figure 6A). Most notable were the 75% WSR, 50% WSR, and 40% RGR groups, which lost all of their pups within 4 to 7 days on treatment. There was also a high mortality rate observed in the 25% WSR group, with 95% of the pups dying. Interestingly, the pups in the alfalfa control group also saw a significant mortality with 75% of the pups dying. Only the 75% WSR, 50% WSR, and 40% RGR groups had a significantly higher mortality rate than the alfalfa control group. The mortality rate in the dams was also noted (figure 6B). None of the dams in the control, alfalfa control, 40% RGR, 20% RGR, or 10% RGR groups died, whereas there was 100%, 83%, and 29% mortality in the dams from the 75% WSR, 50% WSR, and 25% WSR groups, respectively. It is interesting to note that while all of the pups in the 40% RGR group died, none of the dams died. These data indicate that the WSR is either more toxic to the dams than RGR or that RGR is more nutritious to mice than WSR.

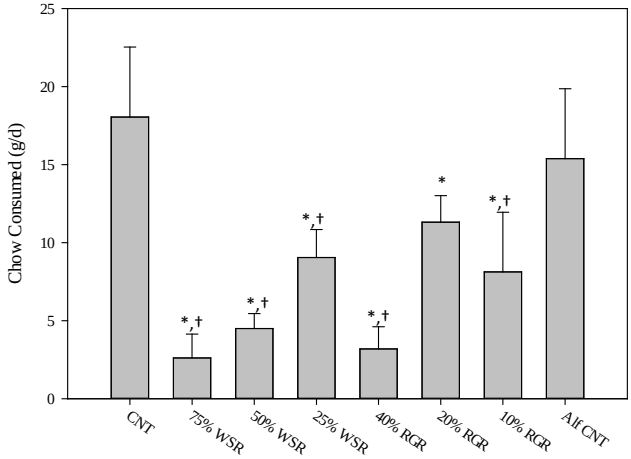

Figure 3. Daily chow consumption. Data represent the mean ± SD for the daily chow consumption from post-natal day (PND) 1-21 (n = 6-7 dams per group). *indicates statistical significance compared to control group. †indicates statistical significance compared to alfalfa control group. WSR is white snakeroot, and RGR is rayless goldenrod.

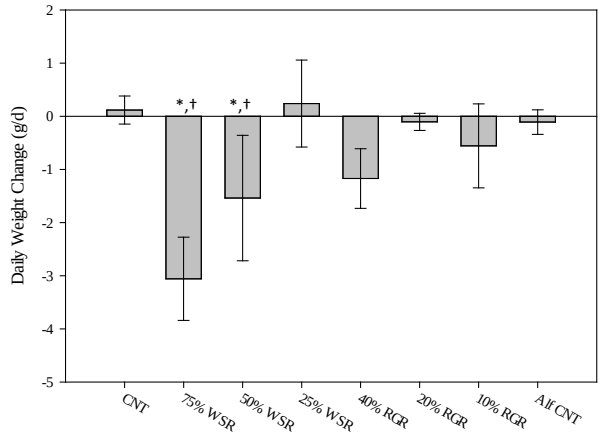

Figure 4. Maternal daily weight change. Data represent the mean ± SD for the maternal daily weight change from post-natal day (PND) 1-21; n = 6-7 dams per group. *indicates statistical significance compared to control group. †indicates statistical significance compared to alfalfa control group. WSR is white snakeroot, and RGR is rayless goldenrod.

In order to assess any muscle weakness that may have occurred in the pups during the treatment period, we analyzed the pups using rotarod and open field analyses. Of important note, no overt visual signs of muscle weakness including muscle tremors were observed in any of the pups from any of the

Table 1. The concentration of benzofuran ketones in rodent chow

Plant	% Plant[a]	mg benzofuran ketone[b] / g chow				
		Tremetone	Dehydro tremetone	6-Hydroxy tremetone	3-Oxyangeloyl tremetone	Total[c]
White snakeroot	75	1.35	0.14	1.29	n.d.[d]	2.78
	50	0.76	0.10	0.74	n.d.	1.59
	25	0.43	0.06	0.40	n.d.	0.89
Rayless goldenrod	40	0.03	0.03	n.d.	0.17	0.22
	20	0.02	0.02	n.d.	0.13	0.16
	10	0.01	0.01	n.d.	0.07	0.09

[a]Percentage of plant material in the rodent chow.
[b]The benzofuran ketones analyzed included tremetone, dehydrotremetone, 6-hydroxytremetone, and 3-oxyangeloyltremetone.
[c]The total is a sum of the three individual benzofuran ketones detected in each plant.
[d]n.d. = not detected.

treatment groups. The pups from 75% WSR, 50% WSR, and 40% RGR groups were not evaluated on the rotarod nor open field due to the fact that they all died within 7 days of the beginning of the treatment. We evaluated the pups from the control, alfalfa control, 25% WSR, 20% RGR, and 10% RGR groups in the open field on PND 14, 21, and 28. The primary aim of the open field evaluation was to determine if any of the treatments affected the pups' ability to move normally in terms of distance traveled, average speed of movement, maximum speed of movement, and total time mobile. There was no difference in these four measurements at PND 14 or 28 in the pups from any of the groups (figure 7). There was however, a significant decrease in the distance traveled, average speed, and total time mobile in the pups from the 25% WSR group on PND 21. The pups were evaluated for muscle coordination and function using a rotarod. The pups were not evaluated on PND 14 as they were not able to adequately walk on the rotating rod at this age. Consequently, evaluations were only made on PND 21 and 28. Again, the pups from the 25% WSR group were not able to walk on the rotating rod for as long as the controls at both periods of evaluation (figure 8). There were no differences in the ability of the pups from any of the other treatment groups to walk on the rotarod compared to the control or alfalfa control groups.

There were only three pups in the 25% WSR group from six litters that survived (95% mortality rate). All three mice were lethargic and did not move around normally (figures 8 and 9). Although the pups from the 25% WSR group did not perform as well as the pups from the other treatment groups, there was no indication that this was due to muscle weakness, and there were no signs of muscle tremors in these pups.

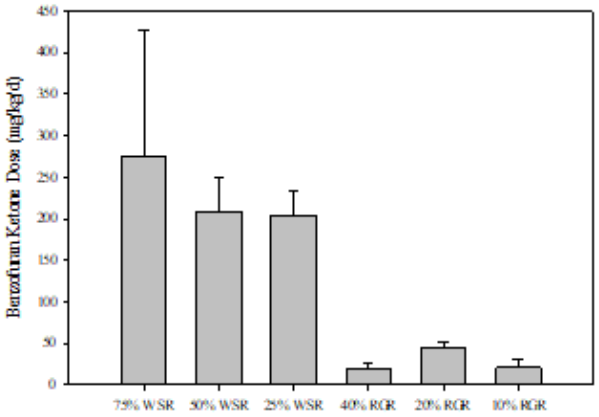

Figure 5. Daily dose of benzofuran ketones. Data represent the mean ± SD for the daily dose of all benzofuran ketones (mg/kg/d), n = 6-7 dams per group. WSR is white snakeroot, and RGR is rayless goldenrod.

Although we did not see any evidence of muscle damage or weakness/trembling in the mice, we did observe that the chow containing high amounts of the plants had a negative effect on the survival of the pups (figure 6). We found a significant positive correlation between the survival rate of the pups and both the amount of chow the dams consumed ($r = 0.854$, $p = 0.002$) as well as the weight change in the dams ($r = 0.810$, $p = 0.01$) (figure 9). This suggests that the dams that ate more chow were able to maintain their body weight better and thus were able to provide the proper nourishment for their pups to survive. There was no correlation between pup survival and the total BFKs dose ($r = -0.479$, $p =$

0.207), indicating that the BFKs did not contribute to the pup mortality. Therefore we suggest that the adverse effects observed such as increased mortality were due to poor nutrition in the lactating dams fed chow containing large portions of either plant and not toxicity of the BFKs.

In conclusion, the data from this study suggest that neither WSR nor RGR is toxic to lactating mice or their pups. Given the fact that these plants are known to be toxic to nursing livestock, mice do not appear to be a viable small-animal model to study the toxicity of RGR or WSR. The results of this study suggest that all adverse effects observed such as increased mortality were due to poor nutrition in the lactating dams fed chow containing large portions of either plant. Future work will continue to try and identify a suitable small-animal model that can be used to identify and characterize the toxic compounds in WSR and RGR.

Acknowledgments

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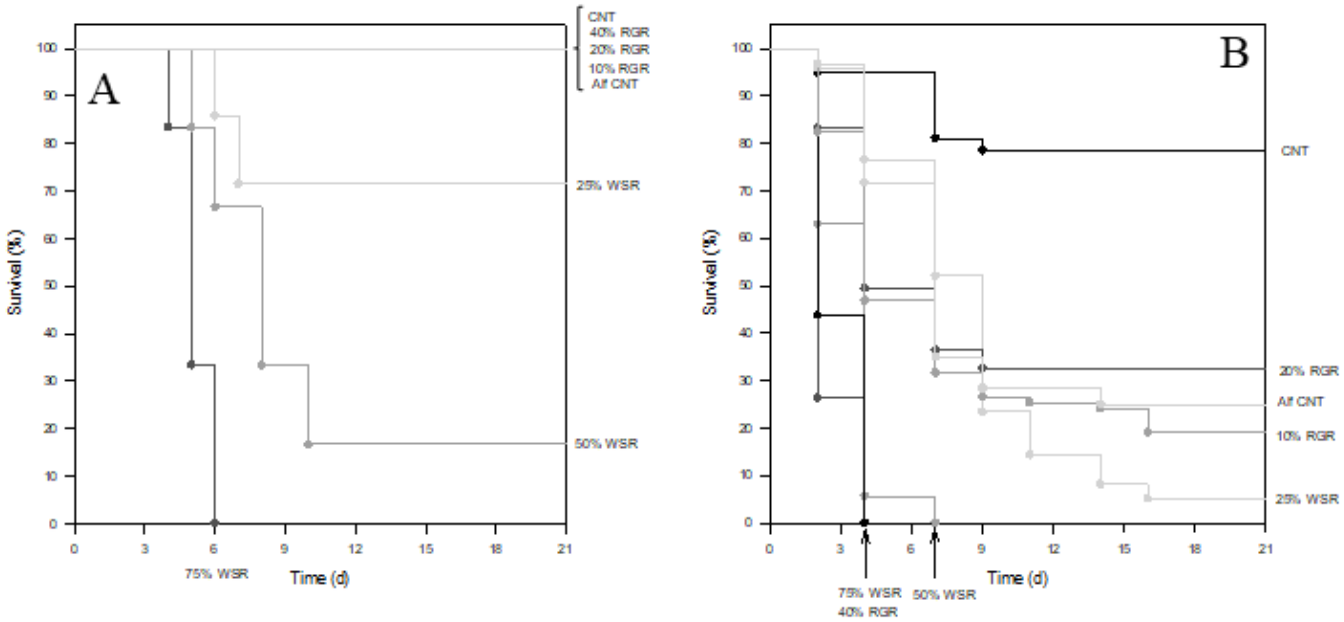

Figure 6. Effect of rayless goldenrod (RGR) and white snakeroot (WSR) treatment on pup and dam survival. Data represent the percent of surviving animals over time. At the beginning of the experiment, there were 60-98 pups per group and 6-7 dams per group.

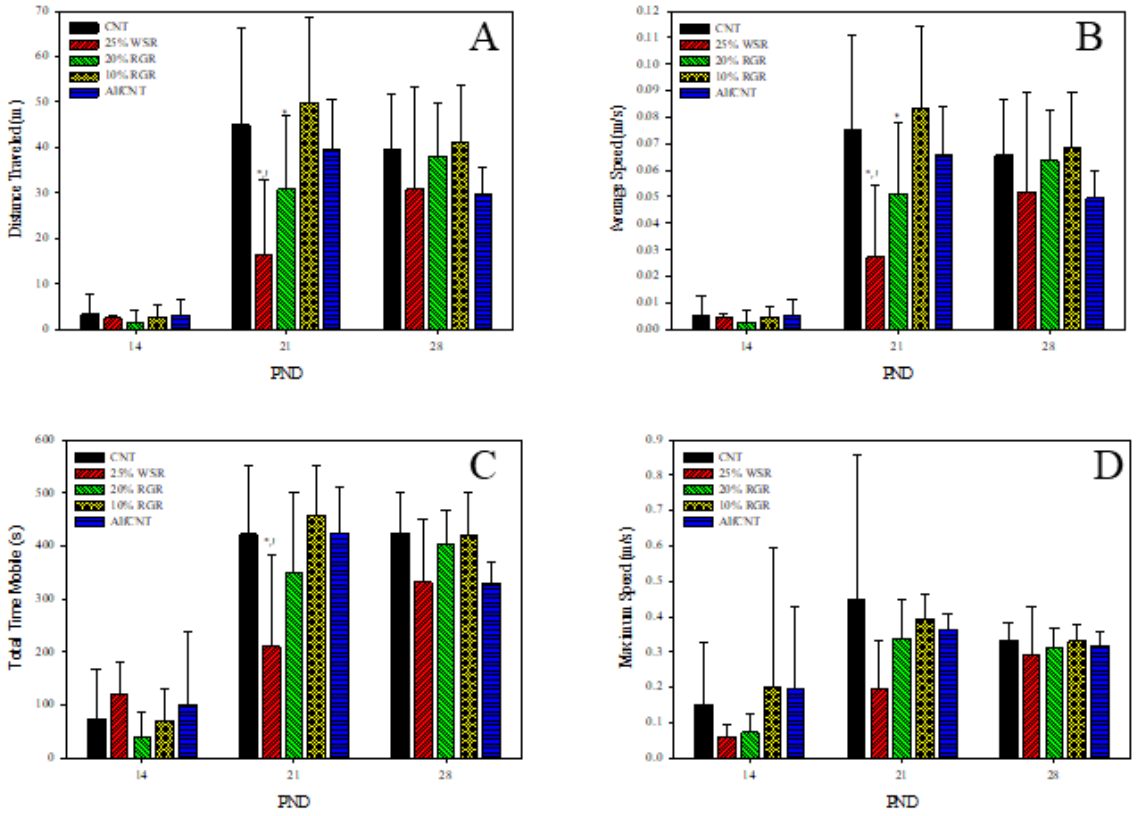

Figure 7. Effect of rayless goldenrod (RGR) and white snakeroot (WSR) treatment on locomotor activity. Locomotor activity of the pups was assessed via open field analysis of distance traveled (A), average speed of movement (B), total time mobile (C), and maximum speed of movement (D) on post-natal day (PND) 14, 21, and 28. Data represent the mean ± SD, n of 6-7 dams per group. *indicates statistical significance compared to control group. †indicates statistical significance compared to alfalfa control group.

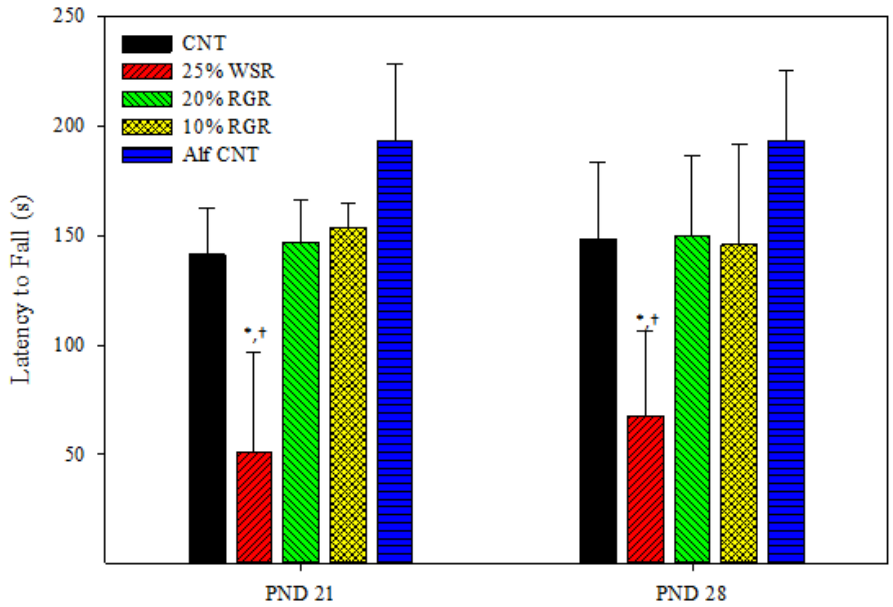

Figure 8. Effect of rayless goldenrod (RGR) and white snakeroot (WSR) treatment on motor coordination and function on post-natal day (PND) 21 and 28. Motor coordination and function were assessed by evaluating the ability of pups to walk on a rotating rod. Data represent the mean ± SD for the latency to fall from the rod, n of 6-7 dams per group. *indicates statistical significance compared to control group. †indicates statistical significance compared to alfalfa control group.

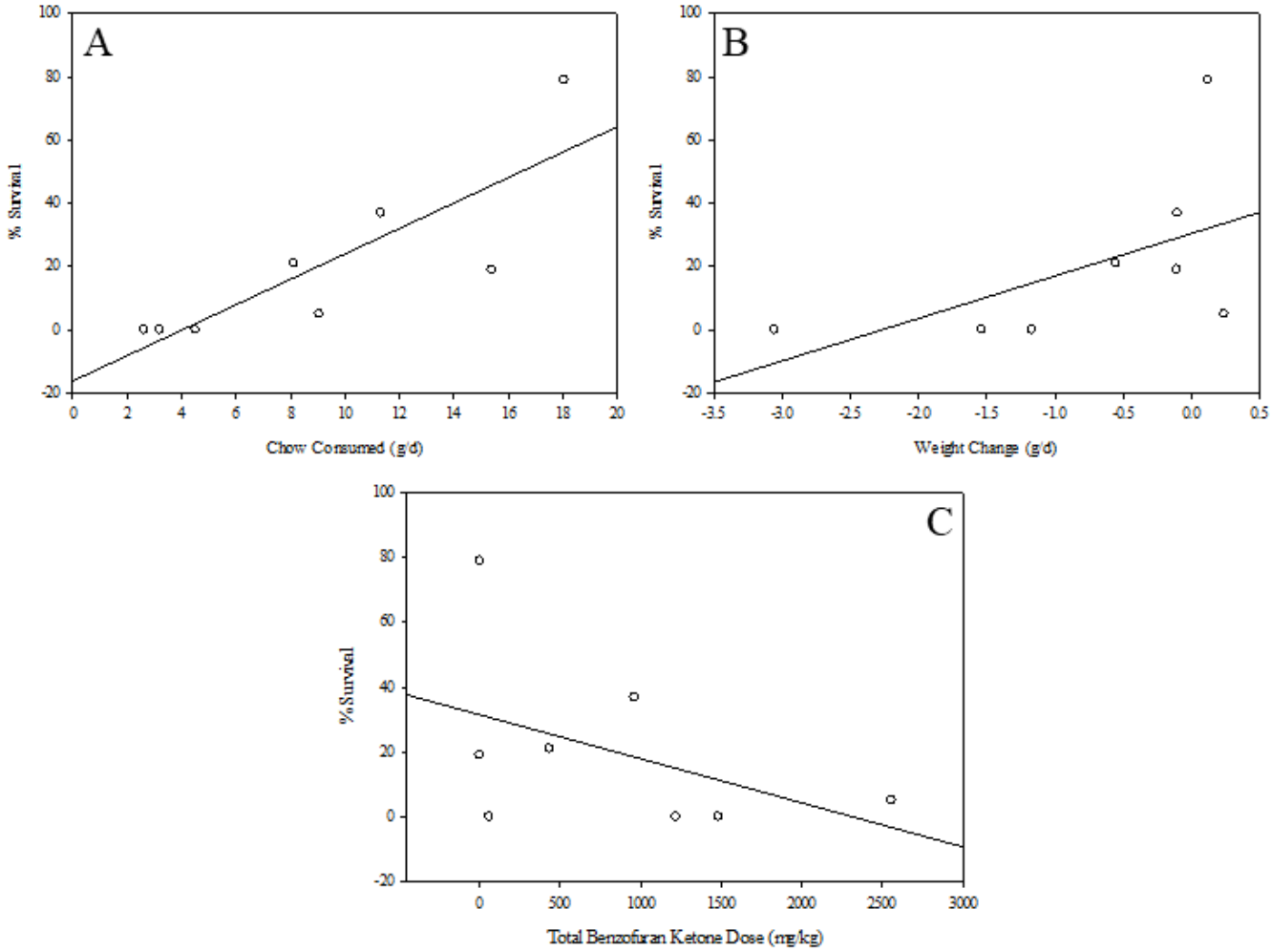

Figure 9. Correlation of pup survival with maternal chow consumption, maternal weight change, and total benzofuran ketone dose.

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