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Neurobehavioral Evaluation of Mice Dosed With Water Hemlock Green Seeds and Tubers

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Neurobehavioral Evaluation of Mice Dosed With Water Hemlock Green Seeds and Tubers

Abstract

Water hemlock are plants of the genus *Cicuta* and are toxic to animals and humans. The primary toxin is cicutoxin, which is abundant in the tubers, but less abundant in other parts of the plant. Other cicutoxin-like compounds, such as cicutols, which may also contribute to the toxicity of water hemlock, are more abundant in non-tuber plant parts. The objective of this study was to determine the toxicity of different parts of water hemlock and characterize their effects on motor function/coordination in mice. An aqueous extract of green seeds, dry seeds, tubers, flowers and stems of water hemlock was dosed orally to mice to determine their acute toxicity. The results indicated that only the green seeds and tubers were sufficiently toxic to animals to induce seizures and death. The LD₅₀ for tubers and green seeds was 17 mg/kg and 1320 mg/kg, respectively. Several tests were used to evaluate motor function and behavior in treated mice including rotarod, tremor monitor, and open field. The animals were evaluated before dosing and 30, 90, 120, 150, 180, 240, and 300 min after dosing. Water hemlock affected muscle function of mice, including their balance and motility on a rotarod, motor activity, and exploratory and anxiety-related (i.e., thigmotaxis) behaviors in an open field. Seizures interspersed with central nervous system (CNS) motor depression were observed in animals poisoned by water hemlock. Extracts from tubers were especially potent in causing a decrease in motor activity and resultant depression, while periodically provoking seizures. Further research is needed to identify, quantitate, and purify cicutoxin and the other polyacetylene compounds from the various water hemlock plant parts to evaluate their toxicity and effects on motor function.

Keywords

nervous system, neurotoxicity, poisonous plants, seizures, water hemlock, cicutoxin, cicutol

Cover Page Footnote

All correspondence should be addressed to (kevin.welch@ars.usda.gov). At the time of this research, Camila and Daniel Goulart were PhD candidates at the Federal University of Goiás, and Visiting Scientists at the USDA-ARS facility in Logan, UT.

Authors

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Introduction

Cicuta douglasii, *C. maculata*, *C. virosa* and *C. bulbifera* (Apiaceae family) are perennial forbs generally known as water hemlock (Mulligan, 1980; Burrows and Tyrl, 2013). These plants are characterized by a thickened tuber with many parsnip-like roots (Fig. 1). The middle of the tuber contains a pale yellow oil which is highly toxic (Anet *et al.*, 1953). All species of *Cicuta* grow directly in water, marshy areas, or on the banks of shallow streams, creeks, ditches or lakes (Kingsbury, 1964; Panter *et al.*, 1988; Panter *et al.*, 2011; Burrows and Tyrl, 2013).



Fig. 1. Photograph of water hemlock plants. Note the parsnip like tubers.

All *Cicuta* spp. are highly toxic to livestock and humans. The water hemlocks are considered economically important to livestock because the plants are often found in pastures of North America and Europe and the poisoning of cattle leads most often to death (Mulligan, 1980; Panter *et al.*, 1988; Panter *et al.*, 1996). Some reports suggest that as little as 2 g of tuber / kg BW can be lethal (Kingsbury, 1964; Panter *et al.*, 1996). The primary toxin in water hemlock is cicutoxin, a conjugated polyacetylene with the chemical formula $C_{17}H_{22}O_2$ (238.35g/mol) which causes severe effects in the central nervous system (CNS). However, there are several cicutoxin-like compounds also found in water hemlock, including cicutol, cicudiol, isocicutoxin, and isocicutol (Wittstock *et al.*, 1995). Studies suggest that cicutoxin is a competitive antagonist of GABA_A receptors, resulting in blocked chloride channels, neural

depolarization, hyperstimulation, and seizures (Schep *et al.*, 2009; Panter *et al.*, 2011; Green *et al.*, 2015). The clearance time of cicutoxin in ruminants appears to be relatively long (i.e., several days), as complete recovery after non-lethal poisoning may take a week (Panter *et al.*, 1996).

Most field cases of poisoning by *Cicuta* spp. occur after intake of the tuber (Panter *et al.*, 1996). However, studies suggest that the green seeds are also toxic (Panter *et al.*, 2011; Panter *et al.*, 2012). The relative toxicity of other plant parts and the influence of plant age on toxicity warrant investigation. Moreover, there is no information about how the toxin might interfere with motor activity or other aspects of behavior in poisoned animals. The objectives of this study were to determine the toxicity of different parts of water hemlock, and to investigate behavioral disorders and motor incoordination in poisoned animals using a mouse model.

Materials and Methods

Plant extracts. Western water hemlock (*Cicuta douglasii*) was collected in August from a small mountain stream near Naf, ID (N41.97073 W113.42539; elevation 1825 m) and separated into green seeds, tubers, flowers and stems and stored at -80°C. Approximately three months later, dried seeds were collected from water hemlock plants at the same location. Voucher specimens from this location were placed in the USDA-ARS Poisonous Plant herbarium. The frozen plant material was homogenized in a Waring blender with two parts distilled water (30g of plant to 60 mL of water). The plant/water slurry was filtered through two layers of cheesecloth. The extract was divided into 12 mL aliquots, which were stored at -80°C until use. Each day one aliquot was thawed and the mice were dosed via oral gavage.

Animals. All tests used male Swiss Webster mice weighing an average of 23g (Simonsen Laboratories, Gilroy, CA, USA). Mice were housed at 21°C in a humidity-controlled room on a 12-h light/12-h dark cycle with free access to water and food (2014 Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Laboratories Inc., Indianapolis, IN, USA). All the procedures were conducted under veterinary supervision and with approval of the Utah State University Animal Care and Use Committee.

Median lethal dose determination. A water extract (1 part plant : 2 parts water) of each plant part (i.e., green seeds, dry seeds, tubers, flowers and stems) was dosed to mice (n = 5/dose) via oral gavage at doses of 5, 10, 20, 30, 40, and 50 mL/kg. The mice were observed for two hours post-dosing, and clinical signs and mortality were recorded. The median lethal dose (LD₅₀) of each extract was calculated using SAS Proc Probit in a logistic regression (SAS V. 9, SAS Inst. Inc., Cary, NC). Following this extraction protocol, and at these doses, only tubers and green seeds were found to provoke clinical signs of seizure and muscle incoordination as well as lethality. Therefore only tubers and green seeds were used in tests of motor function and behavior.

Motor coordination and behavior tests. Motor coordination and behavior were evaluated using the following tests for tubers and green seeds:

rotarod, tremor monitor, and open field. For these tests the mice were dosed via oral gavage immediately after a baseline evaluation (time zero). For each of the three tests, three groups of 12 mice were evaluated; a) saline for controls (GC), b) 40% of the LD₅₀ (G40) and c) 85% of the LD₅₀ (G85). Mice were evaluated at 30, 90, 120, 150, 180, 240, and 300 min after dosing to monitor intoxication and recovery.

Rotarod. The rotarod test evaluated motor coordination and resistance to fatigue using an accelerating rotarod apparatus (IITC Life Science Inc., Woodland Hills, CA, USA). The apparatus had five lanes and the rod diameter used was 9.5 cm. Mice were trained to walk on the rotarod the day before the experiment began until their performance was stable (Rustay *et al.*, 2003; Welch *et al.*, 2013a). The apparatus was set to gradually accelerate from 1 to 20 rpm in 300 s. The mice were positioned on the rod in their respective lanes while the rods were not moving. Once all the mice were positioned, the rods began accelerating. The maximum rpm achieved, and the latency (s) to fall from the rod was recorded automatically. After each test the rotarod was cleaned with 70% ethanol.

Tremor Monitor. This test allows differentiation of normal movement from tremors (San Diego Instruments, San Diego, CA, USA; (Welch *et al.*, 2013a; Welch *et al.*, 2013b)). The monitor uses an ultra-sensitive movement sensor to record continuous movement waveforms from 1 to 64 Hz. Mice were placed into the tremor monitor for 512 s at each time point. A video feed from inside the tremor chamber allowed unobtrusive observations. The chamber was cleaned between each trial with 70% ethanol. The software provided data for two variables, first the magnitude (i.e., movement energy in mV at each frequency) expressed as the Fast Fourier Transform (FFT) for the magnitude at each of the 64 frequencies. The second variable, and the one used in the statistical analysis, was the percentage of the total FFT magnitude at each frequency; the total percent FFT magnitude always summed to 100% over all frequencies for each subject. The percent of magnitude was summed over two subsets of frequencies (10-20 Hz and 42-64 Hz) that were selected based on animal responses in the tremor monitor. These two subsets of frequencies were analyzed for treatment effects.

Open Field. The open field test was used to evaluate the locomotor activity and spatial orientation of the animals (Krežel *et al.*, 1998; Welch *et al.*, 2013b). Mice were placed in the center of an open field (50cm x 50cm and 38cm wall height) and monitored for 10 minute sessions at baseline and after dosing as detailed above. ANY-MAZE software (Stoelting Co., Wood Dale, IL, USA) was used to monitor locomotor activity via an overhead camera. The software automatically tracked the movement of each animal in the open field. The software was used to digitally divide each open field into a start zone (8 x 8 cm) at the center, a 2nd level zone around the start box (28 x 28 cm at outer border), and a 3rd level outer zone from the outer edge of the 2nd level zone to the edge of the walls. A 50 dB noise generator was used to mask noise during testing.

Data Analysis. Rotarod variables, magnitude of tremors, and open field measurements were analyzed using a repeated measures analysis (baseline and 8 subsequent periods) in a linear mixed model (Proc Mixed in SAS, Cary, NC, USA) with animals as a random factor within the treatment groups. Mean comparisons were made on least square means using the PDIFF (probability of difference) option in SAS. The lethal dose value was calculated using SAS Proc Probit in a logistic regression (SAS V. 9, SAS Inst. Inc., Cary, NC). The results were considered significant when $P < 0.05$.

Results

LD₅₀ determination. Lethality in mice was not observed after oral administration of the extracts of dried seeds, flowers or stems of water hemlock at any doses tested (up to 50 mL/kg). Dry matter conversions were only determined in extracts that showed toxicity. The dry matter of tuber extract doses corresponded to 3 mg/kg, 6 mg/kg, 12 mg/kg, 18 mg/kg, 24 mg/kg and 30 mg/kg (dry matter basis) with a lethal dose of 17 mg/kg. For green seeds the doses were 165 mg/kg, 330 mg/kg, 660 mg/kg, 990 mg/kg, 1320 mg/kg, and 1650 mg/kg (dry matter basis) with a lethal dose of 1320 mg/kg.

Due to the lack of clinical signs observed in mice dosed with extracts of dried seeds, flowers, or stems, only green seeds and tubers extracts were used for subsequent motor function/behavioral tests. The clinical signs observed in poisoned mice were reluctance to move, piloerection and motor depression followed by seizures in 100% of the animals; however, in some cases these seizures resulted in the death of the animal. Of the 144 animals used in the motor activity tests with tubers, 14 (9.7%) of the animals died during the experimental period; all were in the G85 treatment group. Six (4.16%) animals dosed with green seed extract died, two from the G40 group and four from the G85 group.

Rotarod. The rotarod test evaluates coordination, motor function, and resistance to fatigue. Mice in both the G40 and G85 groups showed a marked decrease in performance compared to controls (Fig. 2). For tubers (Fig. 2A) there were no treatment differences at time 0 (baseline; $P > 0.30$), however for all subsequent periods, the controls differed ($P < 0.002$) from both the G40 and G85 groups. In all periods, with one exception (90 min; $P < 0.03$), the G40 and G85 groups did not differ ($P > 0.15$). The G40 group only differed from the G85 group at 90 min.

Again baseline performance of mice on the rotarod was not different ($P > 0.6$) among the treatment groups (Fig. 2B) for mice dosed with green seed extract. Mice in the G40 group differed ($P < 0.05$) or tended to differ from control mice in their latency to fall at 30 ($P = 0.07$) and 90 min ($P = 0.004$) post-dosing, but not at the other times ($P > 0.10$). The G85 group differed ($P < 0.05$) from controls at all time periods except for 60 ($P = 0.11$), 240 ($P = 0.89$), and 300 min ($P = 0.12$). The G40 group differed from the G85 group at 150 ($P = 0.01$) and 180 min ($P = 0.03$), but did not differ at the other time periods ($P > 0.20$).

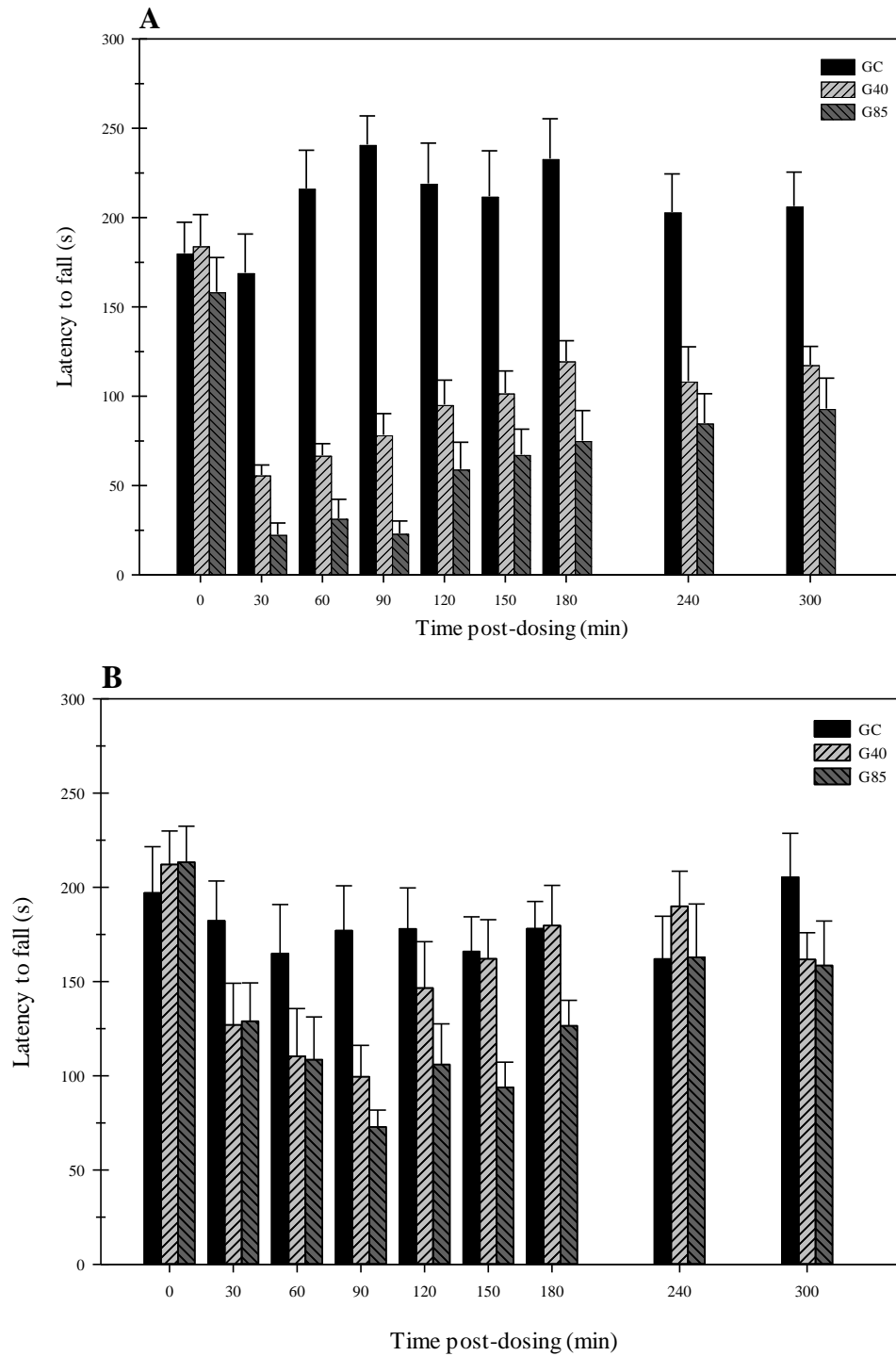


Fig. 2. The effect of water hemlock treatment on the latency (s) for mice to fall from an accelerating rotarod. Data represent the mean \pm SE after a tuber (A) or green seed (B) extract of water hemlock was

dosed at either 40% of the LD₅₀ (G40), 85% of the LD₅₀ (G85), or saline (GC).

Tremor monitor. The effect of tubers and green seeds extracts on movement energy of the mice was similar, although tubers elicited greater immobility (i.e., motor depression) interspersed with occasional visible seizures and tremors (Fig. 3), whereas green seed extract did not cause extreme visible seizures as noted for the tuber extract. The sum of the percent of magnitude for the frequencies from 42-64 Hz showed increased movement energy reflecting periodic water hemlock-induced tremors, whereas the equivalent measurement for frequencies from 10-20 Hz showed less movement energy. For both tuber (Tables 1 and 2) and green seed extract (Tables 3 and 4), there was a treatment x time interaction ($P < 0.003$) for the percent of magnitude with frequencies from 10-20 Hz and from frequencies from 42-64 Hz ($P < 0.005$). Mice recovered at about 150 to 180 minutes from the green seed extract (Table 3 and 4), whereas recovery even at 300 minutes was incomplete and erratic from dosing tuber extract (Table 1 and 2), especially for G85 mice.

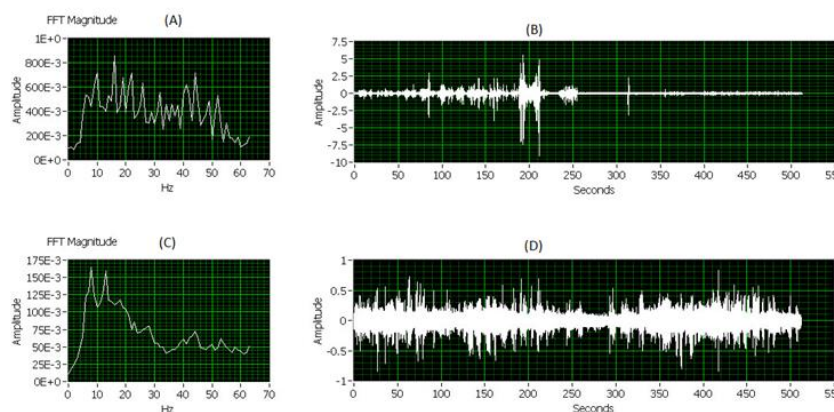


Fig. 3. The effect of water hemlock treatment on muscle tremor. Output from the tremor monitor software showing: (A) Fast Fourier Transform (FFT) magnitude of movement energy (mV or 10^{-3} volts) at 1 to 64 Hz of a mouse showing seizures when tested 30 min post-dosing after being dosed with a water hemlock tuber extract at 85% of the LD₅₀. The FFT magnitude plot shows movement energy during the 190 to 225 sec segment of the 512 sec trial. (B) Relative amplitude of movement energy over a 512 sec trial for the same mouse treated with a water hemlock tuber extract at 85% of the LD₅₀. Visual observations via video feed noted seizures at approximately 195 to 210 sec during the trial. (C) Same as panel A except movement energy is shown for a control mouse treated with saline. (D) Same as part B above except showing movement energy of the same control mouse over the 512 sec trial. Note the

differences in scale between part A and C, and also part B and D, indicating the severity of the water hemlock-induced seizure.

Table 1 Effect of a water hemlock tuber extract on muscle tremors from 10-20 Hz.

Time (min)	GC ²		G40		G85	
	Mean ¹	SE	Mean	SE	Mean	SE
(0)	29.1 ^a	0.8	26.5 ^a	1.1	26.9 ^a	0.9
30	30.7 ^a	0.7	21.8 ^b	1.5	23.1 ^b	1.4
60	28.4 ^a	0.9	22.5 ^b	1.6	16.7 ^c	3.2
90	27.7 ^a	1.2	20.3 ^b	1.5	12.8 ^c	2.9
120	27.8 ^a	1.3	20.9 ^b	1.6	13.9 ^c	3.1
150	28.4 ^a	0.8	22.4 ^b	1.3	12.6 ^c	2.9
180	25.7 ^a	1.3	23.3 ^a	1.1	14.3 ^b	3.2
240	26.9 ^a	0.8	23.1 ^a	1.0	14.9 ^b	3.3
300	27.9 ^a	0.9	22.1 ^b	1.1	15.0 ^c	3.3

¹The percentage of magnitude (i.e., % of total movement energy in mV at each frequency) is the mean percentage of frequencies summed from 10-20 Hz; for each subject the total percent magnitude always summed to 100% over all frequencies.

²Treatment groups were GC = controls dosed orally with saline; G40 = treated mice dosed orally with 40% of the LD₅₀ of water hemlock extract from tubers; G85 = treated mice dosed orally with 85% of the LD₅₀ of water hemlock extract from tubers. Different superscript letters in the same row indicate differences (P < 0.05) between treatment groups at that time period.

Table 2 Effect of a water hemlock tuber extract on muscle tremors from 42-64 Hz.

Time (min)	GC ²		G40		G85	
	Mean ¹	SE	Mean	SE	Mean	SE
(0)	27.3 ^a	0.8	28.9 ^a	0.9	28.8 ^a	0.9
30	25.6 ^a	0.8	37.6 ^b	2.0	36.4 ^b	2.2
60	29.8 ^a	1.0	36.0 ^b	2.2	35.9 ^b	2.4
90	29.8 ^a	1.7	38.8 ^b	1.9	39.4 ^b	2.4
120	30.1 ^a	1.8	35.9 ^b	1.8	35.8 ^b	2.3
150	28.8 ^a	1.0	34.2 ^b	1.7	40.9 ^c	2.5
180	32.3 ^a	2.0	35.1 ^a	1.6	36.5 ^a	2.4
240	29.9 ^a	0.9	33.2 ^a	1.2	34.7 ^a	1.9
300	29.4 ^a	1.1	34.8 ^b	1.3	34.9 ^b	1.4

¹The percentage of magnitude (i.e., % of total movement energy in mV at each frequency) is the mean percentage of frequencies summed from 42-64 Hz; for each subject the total percent magnitude always summed to 100% over all frequencies.

²Treatment groups were GC = controls dosed orally with saline; G40 = treated mice dosed orally with 40% of the LD₅₀ of water hemlock extract from tubers; G85 = treated mice dosed orally with 85% of the LD₅₀ of water hemlock extract

from tubers. Different superscript letters in the same row indicate differences ($P < 0.05$) between treatment groups at that time period.

Table 3 Effect of a water hemlock green seed extract on muscle tremors from 10-20 Hz.

Time (min)	GC ²		G40		G85	
	Mean ¹	SE	Mean	SE	Mean	SE
(0)	27.4 ^a	0.9	27.4 ^a	0.5	27.2 ^a	0.4
30	29.4 ^a	0.9	25.8 ^b	1.0	24.9 ^b	1.1
60	27.1 ^a	1.2	23.3 ^b	1.4	20.3 ^b	1.7
90	25.3 ^a	0.8	21.5 ^b	1.3	18.9 ^b	0.9
120	23.6 ^a	1.2	23.2 ^a	1.5	18.2 ^b	1.3
150	21.7 ^a	1.4	23.3 ^a	1.8	19.9 ^b	1.9
180	23.0 ^a	1.3	24.0 ^a	1.3	22.2 ^a	1.7
240	26.6 ^a	0.9	25.2 ^a	1.1	25.4 ^a	0.8
300	25.4 ^a	0.9	23.9 ^a	1.2	25.6 ^a	1.5

¹The percentage of magnitude (i.e., % of total movement energy in mV at each frequency) is the mean percentage of frequencies summed from 10-20 Hz; for each subject the total percent magnitude always summed to 100% over all frequencies.

²Treatment groups were GC = controls dosed orally with saline; G40 = treated mice dosed orally with 40% of the LD₅₀ of water hemlock extract from green seeds; G85 = treated mice dosed orally with 85% of the LD₅₀ of water hemlock extract from green seeds. Different superscript letters in the same row indicate differences ($P < 0.05$) between treatment groups at that time period.

Table 4 Effect of a water hemlock green seed extract on muscle tremors from 42-64 Hz.

Time (min)	GC ²		G40		G85	
	Mean ¹	SE	Mean	SE	Mean	SE
(0)	28.3 ^a	0.4	28.4 ^a	0.5	27.9 ^a	0.4
30	26.9 ^a	0.8	31.7 ^b	1.0	33.3 ^b	0.8
60	30.0 ^a	1.4	34.3 ^b	1.4	38.3 ^b	1.4
90	31.6 ^a	0.9	37.2 ^b	1.3	40.3 ^b	0.9
120	34.6 ^a	1.5	34.9 ^a	1.5	41.4 ^b	1.5
150	36.4 ^a	1.7	34.7 ^a	1.8	38.5 ^a	1.7
180	34.7 ^a	1.8	33.3 ^a	1.3	35.3 ^a	1.8
240	31.0 ^a	1.0	33.2 ^a	1.1	32.0 ^a	1.0
300	31.0 ^a	1.1	34.3 ^a	1.2	32.4 ^a	1.0

¹The percentage of magnitude (i.e., % of total movement energy in mV at each frequency) is the mean percentage of frequencies summed from 42-64 Hz; for each subject the total percent magnitude always summed to 100% over all frequencies.

²Treatment groups were GC = controls dosed orally with saline; G40 = treated mice dosed orally with 40% of the LD₅₀ of water hemlock extract from green seeds; G85 = treated mice dosed orally with 85% of the LD₅₀ of water hemlock

extract from green seeds. Different superscript letters in the same row indicate differences ($P < 0.05$) between treatment groups at that time period.

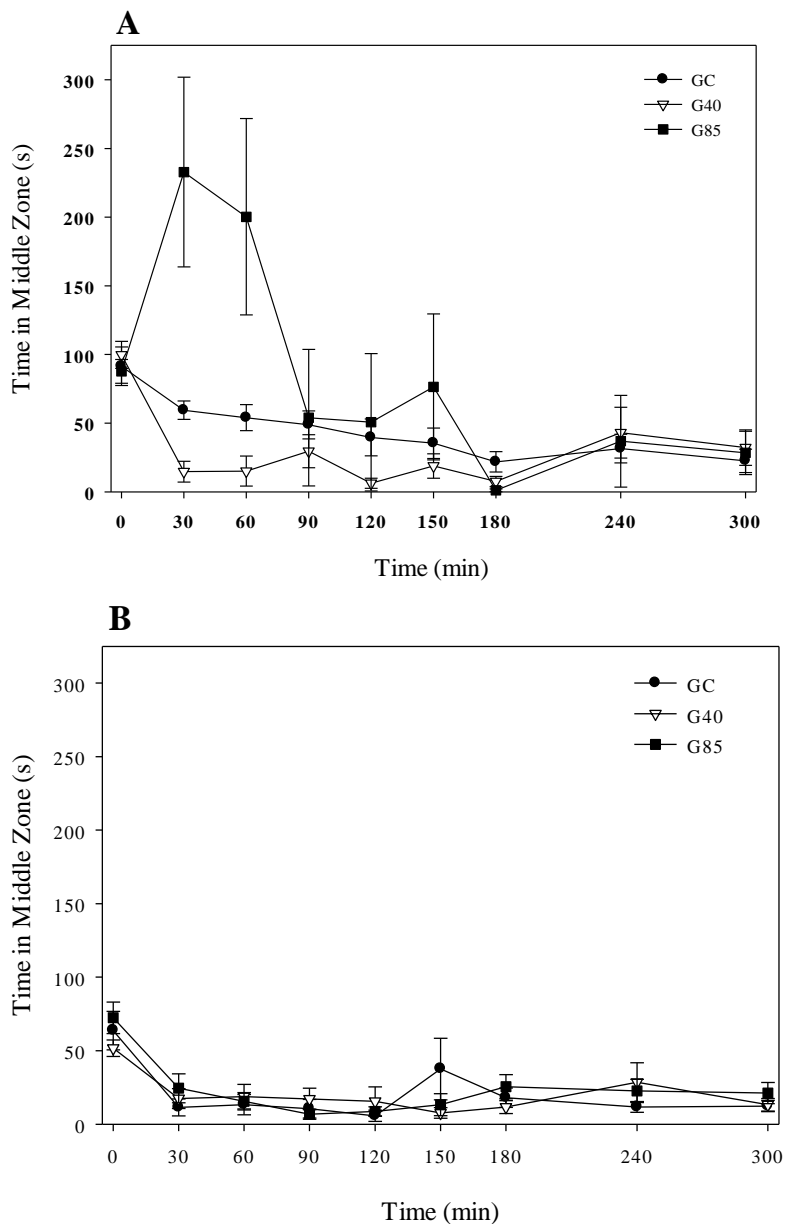


Fig. 4. Effect of water hemlock treatment on open field movement. Data represent the means \pm SE for mice tested in an open field apparatus for 10 min at indicated time points. A) Mice were treated with an extract of water hemlock tubers dosed at either 40% of the LD₅₀ (G40), 85% of the LD₅₀ (G85), or saline (GC). B) Mice were treated with an extract of water hemlock green seeds dosed at either 40% of the LD₅₀ (G40), 85% of the LD₅₀ (G85), or saline (GC).

Open field. The open field test was used to measure the motor activity and behavior of mice poisoned by water hemlock. There was a time x

treatment interaction ($P < 0.01$) for time spent (s) in the middle zone of the open field for animals dosed with tuber extract (Fig. 4A) but not green seed extract (Fig. 4B). There were no differences between the controls, G40, and G85 at baseline. The G85 treatment using a tuber extract differed ($P < 0.001$) from controls and from the G40 treatment for the time spent by animals in the middle zone of the apparatus at 30 and 60 min post-dosing (Fig. 4D), with the G85 animals greatly increasing their time spent in this zone. The G85 treatment animals did not differ ($P > 0.30$) from the G40 animals or from the controls from 90 to 300 min post-dosing. The G40 treatment did not differ ($P > 0.25$) from controls at any time post-dosing. Green seed extract did not elicit any treatment responses that differed from controls in the open field apparatus (Fig. 4B). However, as expected, there were time effects over the 10 min sessions, as animals adapted to the novel environment, and reduced movement over time.

Discussion

The results of this study indicated that only the water extract of green seeds and the tubers caused intoxication with induction of seizures, which can be fatal, in mice. The other parts of the plant were found to be non-toxic to mice, under the parameters used in the study. The LD_{50} measurement demonstrated the greater toxicity of the tubers compared to green seeds, especially considering that the amount of dry matter present in the lethal dose of tuber extract was considerably lower than the dry matter present in the lethal dose of green seeds. The greater toxicity of the tubers compared to green seeds was also described previously (Panter *et al.*, 1996; Panter *et al.*, 2011). Cattle were poisoned from ingestion of immature seeds of water hemlock showing that parts of the water hemlocks, other than tuber, could also be lethal to cattle (Panter *et al.*, 2011). This study confirmed that tubers and green seeds of water hemlock were toxic to animals.

The extracts of tuber and green seeds significantly altered the motor activity and coordination of the mice when evaluated by their ability to stay on a rotarod. In both treatments we observed significant reductions in the animals' ability to remain on the rotating apparatus, with a tendency to recover over time. The reduction of motor activity and coordination of the animals can be explained by episodes of seizures and ataxia observed in intoxicated animals. These findings are characteristic of water hemlock intoxications. Cicutoxin, one of the known toxic principles of water hemlock, acts directly on the CNS causing seizures and respiratory paralysis (Anet *et al.*, 1953; Starreveld and Hope, 1975). However, signs such as muscle weakness and ataxia have been described in studies involving animals intoxicated by the plant (Panter *et al.*, 1988; CDC, 1994; Panter *et al.*, 1996). This incoordination provoked by the intoxication explains the rotarod results. According to Dunham and Mya (Dunham and Miya, 1957) and Brooks and Dunnnett (Brooks and Dunnnett, 2009)

this test was specifically developed to measure neurological deficits in rodents and is a commonly used test to evaluate motor function in mice (Crabbe *et al.*, 2003).

Both the clinical signs of motor depression and seizures were noted in the tremor monitor results. The animals' reluctance to move and apparent motor depression was shown by reduced movement energy in lower frequencies; in contrast, at higher frequencies the mice displayed additional movement energy from the water hemlock treatment, likely from periodic tremors and also from occasional seizures. Recovery was more rapid and complete when mice were dosed with the less-toxic green seed extract compared to extract from tubers. Researchers (Welch *et al.*, 2008) examined the effects of dosing a diterpene alkaloid from *Delphinium* (methyllycaconitine, MLA) in mice whose clinical signs are similar to those found in water hemlock intoxication, such as reluctance to move, followed by muscle tremors and seizures. Mice poisoned by neuromuscular toxins, such as MLA and nicotine, had a significant decrease in the percentage of magnitude of movement when compared to the control group, similar to what is described in this study.

The open field test showed that the tubers cause a greater change of locomotion and behavior when compared to the effect of green seed poisoning of water hemlock. Throughout the experiment it was observed that the animals were more active in the first moments of the test when placed in a novel environment, but with the passage of time they became accustomed to the open field as the novelty decreased, and reduced their overall movements. The tendency of tuber-treated animals to remain in the middle zone within the first hour after intoxication demonstrates a behavioral change likely to be caused by the reluctance to move. When placed in the center of the field the animals typically run to the external region near the lateral wall and explore this region, always remaining close to the wall (i.e., thigmotaxis). Behavioral changes may lead these animals to stop exploring the periphery and remain more in the middle zone of the field (Bhatnagar *et al.*, 2004; Brooks and Dunnett, 2009), as observed in this study.

The difference between intoxication from tubers and green seeds can likely be explained by the composition of the toxins in the plant parts. There is evidence that there are different toxins involved in water hemlock intoxication, in addition to cicutoxin. Uwai *et al.* (Uwai *et al.*, 2001) and Panter *et al.* (Panter *et al.*, 2011) have reported evidence of other polyacetylene compounds in green seeds. The green seeds have only 1% of the total cicutoxin present in the tubers (Panter *et al.*, 2011). Therefore, cicutoxin likely plays a minor role in the toxicity of green seeds, and thus other polyacetylenes, such as cicutols, cicudiols or compounds that have not been identified, are likely responsible for the toxicity of green seeds.

In conclusion, among the parts of water hemlock studied, only the green seeds and the tubers were toxic when dosed orally in mice, and tubers were more toxic than green seeds. Tuber extracts were especially potent in causing a decrease in motor activity and resultant depression, while periodically

provoking seizures. Further research will be required to identify, quantitate, and purify cicutoxin and the other polyacetylene compounds from the various water hemlock plant parts for future studies on toxicity and effects on motor function. Additionally, the results from this study increase the understanding of water hemlock toxicity and will thus aid in the design of future studies to study the toxicity of water hemlock in livestock species in order to develop management recommendations for livestock producers to prevent livestock losses.

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