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Full Length Research Paper

Acute mammalian toxicity of four pesticidal plants

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Many plant species show potential as alternatives to synthetic pesticides but little is known about their acute mammalian toxicity. The single-dose acute oral toxicities of crude aqueous extracts of *Strychnos spinosa* and *Bobgunnia madagascariensis* fruits and the foliage of *Vernonia amygdalina* and *Cissus quadrangularis* (plant species that are commonly used as pesticides in Southern Africa) were evaluated in BALB/c mice. Plant extracts (up to 75% *w/v* or *v/v*) were orally administered to sexually mature mice. Behavioural changes, clinical signs and mortality were monitored for 4 days. Mice that received *S. spinosa* had generalized tonic muscle spasms and a high mortality of 83%. Those that received *B. madagascariensis* exhibited signs of buccal and nasal irritation with occasional sneezes during administration and a high mortality of 75% was recorded. *C. quadrangularis* consumption resulted in much lower mortality of 21% while no clinical signs of toxicity were evident on mice administered *V. amygdalina.* Chemical analysis showed the presence of secoiridoids in the seedless pulp of *S. spinosa.* These results indicate that aqueous extracts of *S. spinosa* and *B. madagascariensis* fruits and foliage of *C. quadrangularis* may have deleterious health implications on humans and animals; hence, advice on their safety should accompany promotion of their use.

Key words: Pesticidal plants, mammalian toxicity, BALB/c mice, aqueous plant extracts, *Strychnos spinosa*, *Bobgunnia madagascariensis*, *Vernonia amygdalina*, *Cissus quadrangularis.*

INTRODUCTION

Developed countries continue to reduce chemical inputs in agriculture while using more effective products that are less persistent or hazardous in the environment (Carvalho, 2006). Unfortunately these products are unavailable or unaffordable in developing countries, particularly for small-scale farmers who continue to rely on the cheaper but more toxic synthetic products or seek alternatives such as pesticidal plants. These natural products have been used for generations for pest management on livestock and are acceptable alternatives (Belmain and Stevenson, 2001; Wanzala et al., 2005). There is also an increasing global interest in their use to control agricultural pests (Duke et al., 2010) but they have the greatest potential benefits in developing countries (Isman, 2008), particularly for the resource-constrained, smallholder farmers. The received wisdom is that plants are safe because they are natural but some plants used as pesticides have known toxicity (Isman, 2006) while some plant compounds like aconitine are highly toxic even in very small quantities (Kolev et al., 1996). Previous studies of pesticidal plants have focused more on establishing their efficacy at the expense of safety issues associated with their use (Belmain et al., 2001). However, potential health risks to users exist, especially during the preparation and application of the plant material; hence, before materials can be developed

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Table 1. Plant extracts and the dosages used in the experiments.

Plant species	Dosage (%)				Sample size
*S. spinosa**	0	25	50	75	34
*S. spinosa**	0	0.1	1	5	24
*B. adagascariensis***	0	2	5	10	24
*V. amygdalina***	0	15	25	75	24
*C. quadrangularis***	0	2	5	10	24

*, Dosage on a v/v basis; **, dosage on a w/v basis.

or promoted, their potential toxicity should be considered. This is conducted routinely for plant materials that have therapeutic or medicinal properties (Antonelli-Ushirobira et al., 2010) but rarely for plants used as pesticides. In Southern Africa, *Strychnos spinosa* Lam. (Loganiaceae) is used by smallholder farmers as a tick control remedy, while *Bobgunnia madagascariensis* (Desv.) (J.H Kirkbr and Wiersma) (Leguminosae) is used as a post-harvest grain protectant and tick control (Stevenson et al., 2010a; Madzimure et al., 2011). *Vernonia amygdalina* Del. (Asteraceae) and *Cissus quadrangularis* Linn. (Vitaceae) are used to control post-harvest grain insect pests, while *B*. *madagascariensis* and *V. amygdalina* are also used for controlling vegetable pests (Kamanula et al., 2011; Nyirenda et al., 2011).

In order to optimise pesticidal plant material use in the management of pests (Kestenholz et al., 2007; Stevenson et al., 2009), this study determined acute oral toxicity, in mice, of four plant species used in southern Africa: *S. spinosa, B. madagascariensis*, *V. amygdalina* and *C. quadrangularis.*

MATERIALS AND METHODS

Study site

The experiments were conducted in the Paraclinical Department's experimental animal rooms in the Faculty of Veterinary Science at the University of Zimbabwe.

Collection and preparation of plant extracts

Fruits of *S. spinosa* were collected from Mazowe at Henderson Research Institute (17º35'S, 30º58'E) located about 32 km North of Harare. The vegetation mainly consists of tree savanna or bush clump savanna with tall perennial grasses such as *Hyparrhenia filipendula* on red clay soils. Rainfall is confined to summer (November through to March) and is moderately high (750 to 1000 mm). *B. madagascariensis* pods were collected from Nyamaropa village in Nyanga district (17º52'S, 32º53'E) along the eastern border with Mozambique. The area receives an annual rainfall of between 600 and 800 mm. Mean annual temperatures are between 20 and 22℃ and vegetation is predominantly miombo woodlands. *C. quadrangularis* and *V. amygdalina* were collected from the Southern African Pesticidal Plants (www.nri.org/projects/sapp) project sites in Southern Zambia and Northern Malawi, respectively. For *S. spinosa*, two different experiments at high (25 to 75%) and low (0.1 to 5%) inclusion levels were conducted. The inner soft pulp and seeds were removed from the hard outer shell of unripe *S. spinosa* fruits and crushed at speed 1 for 60 s in a 3-Speed Ultra Power Electric blender (KitchenAid® Appliances, Michigan, USA) to form a viscous homogenous fluid. The homogenate was first passed through a muslin cloth and then through a tea strainer to make a stock extract that was used to prepare treatment dilutions of 25, 50 and 75% *v/v* in tap water for the first experiment and then dilutions of 0.1, 1 and 5% v/v in the second. All the preparations were stored in a refrigerator at 4ºC before use.

The foliage of *V. amygdalina was shade-dried* while that of *C. quadrangularis* was sun-dried, as practised by farmers, because it is fleshy and succulent, and dries too slowly in the shade. The powder was then mixed with distilled water to form *w/v* dilutions of 2, 5 and 10% for *C. quadrangularis* and 15, 25 and 75% for *V. amygdalina*. Dry pods of *B. madagascariensis* were also ground to a powder which was mixed with distilled water to produce *w/v* dilutions of 2, 5 and 10%. The treatment dilutions selected were based on the ranges of dosage that farmers normally prepare and use. Tap water was used as a control for all plant species.

Animals and experimental design

Acute oral toxicity of each of the 4 plants was studied in separate completely randomised design experiments. Sexually mature BALB/c mice, kept individually in conventional mice cages, and given commercial feed (SAFCO® Pvt. Ltd, Harare, Zimbabwe) and water *ad libitum*, were used. BALB/c mice were used because they are readily available, easy to handle, and manage. An adaptation period of 2 weeks was allowed before the commencement of each experiment. Thirty-four unsexed mice, aged 6 weeks and weighing 22±2 g, randomly divided into four groups, were used for the *S. spinosa* trial. The groups were the control (placebo) which received water only (0%) and the 3 treatments of 25, 50 and 75% plant extract. Replication was 10 times for the three treatment groups and four times for the placebo. The same design was used for the other plant species (Table 1), but individual treatments were assigned an equal number of mice (six per treatment), and hence replicated six times. In a separate experiment, the effect of lower concentrations (0 to 5% w/v) of *S. spinosa* in the mice, was tested.

Oral administration of plant extracts

The mice were deprived of feed and water overnight prior to administration of the treatments across the experiments. A 10 ml plastic syringe with a 16 mm long 22-gauge gavage needle was used to administer the aqueous plant extracts suspension *per os*. The needle was held horizontally, in a position parallel with the head of the mouse, and inserted at the back of the animal's throat. Once the needle was in place, a single dose of 4 ml of the extract was carefully introduced into the oesophageal opening. The extract

Table 2. The effect of oral administration of aqueous extracts of whole unripe fruit of *Strychnos spinosa* at high dose (25-75% v/v) on BALB/c mice.

Sample size (n) and hours post-treatment	Mortality of mice after exposure to different concentrations of whole *S. spinosa* fruit				Pooled mortality (%)/ post-treatment period
	Dosage (%)				
	0	25	50	75	
N	4	10	10	10	30
0 - 12	0	1	3	1	5(16.7)
13 - 24	0	4	3	5	12(40.0)
25 - 36	0	1	1	3	5(16.7)
37 - 48	0	0	0	0	0(0.0)
49 - 60	0	0	1	0	1(3.3)
61 - 72	0	1	1	0	2(6.7)
Total mortality (%)	0	7 (70)	9 (90)	9 (90)	25 (83.3)

volume was adjusted according to Bachmanov et al. (2002) who found that a 30 g mouse can consume 6 to 11 ml. In these experiments, the mice were force-fed because they did not feed voluntarily during preliminary experiments. The control group received 4 ml of tap water in the same manner.

Chemical analysis

Chemical analysis was carried out on *S. spinosa* only to identify compounds that might be responsible for toxicity of this species to mice. A recent comprehensive chemical analysis of the other species which in the present study affected behavior and caused mortality in mice, *B. madagascariensis* (Stevenson et al., 2010b) was used to inform discussion about potential compounds responsible for these toxic effects. Dried plant material of *S. spinosa* was milled in a coffee grinder. A sample (1 g) was extracted in methanol (10 ml) for 24 h and filtered through Whatman Grade 1 filter paper and refiltered through an acrodisc with a 45 µm pore size.

The sample was diluted 10 times before analysis. The LC-MS of the extract was carried out using a Waters ZQ LC-MS system and a Thermo LTQ-Orbitrap XL instrument. For chromatographic separation, a 150 mm × 3.0 mm i.d., 3 µm, Phenomenex Luna C18 (2) column was used with a linear mobile phase gradient, A = H_2O; B = MeOH; C = 1% HCO_2H in MeCN with A = 90% and C = 10% at t = 0 min; B = 90% and C = 10% at t = 20 to 25 min at 400 µL/min flow rate and 30ºC.

Injection volumes were 5 µl and data analysis was performed using Xcalibur 2.0.7 software. MS data for individual peaks were compared to the natural products library database at the Royal Botanic Gardens, Kew, UK. Where compounds could not be identified by MS, they were isolated by semi-preparative HPLC. MeOH extracts from fruits of *S. spinosa* were taken to dryness using rotary evaporation, redissolved in 10 ml MeOH, and passed through 0.45 µm nylon acrodisc filters prior to HPLC. Isolation was carried out on a Waters system (600E pump and 996 PDA detector) using a Spherisorb ODS2 column (250 mm × 10 mm i.d., 5 µm particle size) with a gradient elution program based on A = MeOH and B = H_2O; A = 25% at t = 0 min, A = 100% at t = 20 min, and A = 100% at t = 40 min; column temperature 30℃ and flow rate 4.7 ml/min. NMR spectra were acquired in MeOH-d_4 at 30℃ on a Bruker Avance 400 MHz instrument. Standard pulse sequences and parameters were used to obtain spectra. Chemical shift referencing was carried out with respect to internal TMS at 0.00 ppm.

Monitoring of animals

Clinical observations were carried out daily for 4 days with the assistance of a veterinarian during which behavioural change and mortality were noted. The time of onset and duration of clinical signs were also recorded. Gross post-mortem examination of the liver, heart and kidney was carried out on dead mice from all the treatments. Ethical guidelines of the University of Zimbabwe for the handling of experimental animals in research trials were followed.

Statistical analysis

A Chi-square test was conducted using the PROCFREQ procedure of SAS (2004) to determine association between the treatments and mortalities within each experiment.

RESULTS

No mortalities, behavioural changes and clinical signs were observed in all the control groups which received tap water. In the higher *S. spinosa* concentration experiment, 17 mice died within the first 24 h post-administration. Five mice, across the treatments, died within 2 h of oral administration (Table 2). In this experiment, the *S. spinosa* treatments had higher (χ^2 = 5.60, p = 0.003) mortality than the control. However, there were no differences (χ^2 = 1.92, p = 0.38) in mortality among the treatment groups. Some of the mice exhibited powerful convulsions accompanied by signs of tonic muscle spasms involving all muscles leading to, almost, sudden death. During the convulsions, there was typical opisthotonos where a severe spasm causes the head to arch and the head to bend backwards and the heels flexing towards the back. By the end of 24 h, 22 of the 30 mice that received the *S. spinosa* extract had shown signs of poisoning and 17 had died. In others, the symptoms were more gradual with a prolonged period of apnoea/dyspnoea prior to death or recovery. Only five of the 30 mice, which received the *S. spinosa* treatments in the high concentration experiment recovered completely.

Table 3. The effect of oral administration of aqueous extracts of whole fresh unripe *S. spinosa* fruit at high dose (0.1 to 5% v/v) on BALB/c mice.

Sample size (n) and hours post-treatment	Mortality of mice after exposure to different concentrations of whole *S. spinosa* fruit				Pooled mortality (%)/post-treatment period
	Dosage (%)				
	0	0.1	1	5	
n	6	6	6	6	24
0 - 12	0	0	0	0	
13 - 24	0	0	0	0	0
25 - 36	0	0	0	0	0
37 - 48	0	0	0	0	0
49 - 60	0	0	0	1	1(4.2)
61 - 72	0	0	0	0	0
Total mortality (%)	0	0	0	1(17)	1(4.2)

On post mortem examination, there were not many gross changes other than cyanotic congestion and haemorrhages on serosal surfaces of the liver, heart and kidney. However, mortality was low for the low *S. spinosa* concentration treatments with only one death in the 5% treatment (Table 3).

The mice that received *B. madagascariensis* exhibited signs of buccal and nasal irritation with occasional sneezes during administration. Once the latter had ceased, the mice displayed a lethargic stance prior to death or recovery. Of all the mice under *B. madagascariensis* treatment, 18 died (64.3%) with 100% mortality in the 10% treatment group. There was an equal percentage mortality of 62.5% in the 2 and 5% treatment groups (Table 4). The treatments had higher (χ^2 =9.82, p = 0.002) mortality than the control. However, there were no differences (χ^2 = 6.02, p = 1.18) in mortality among the *B. madagascariensis* treatment groups. Most mortality occurred within 48 h post-treatment. After 3 days post - administration, all the surviving mice had completely recovered with no clinical developments throughout the observation period. There appeared to be a time-related potency with the highest concentration of 10% killing the mice within 12 h whereas the lowest dose of 2% killed the mice after 25 to 36 h.

There were no observed behavioural changes and no mortality in mice on the *V. amygdalina* treatment for all the administered dosages. In the *C. quadrangularis* experiment, there were no observed behavioural changes and the treatment had no effect on mortality (p > 0.05) even though some deaths were recorded (Table 5).

Analysis of the methanol extracts of the fruit pulp of *S. spinosa* revealed the presence of two major polar Components 1 and 2 eluting at 4.52 and 6.57 min, respectively. Both components were characterized by UV spectra with an absorbance maximum of 237 nm. Component 1 showed mass spectra characterized by sodiated and protonated molecules at *m/z* 427 $[M+Na]^+$ and 405 $[M+H]^+$ indicating a molecular weight of 404 (1). Both components were isolated by semi-preparative HPLC recovering 19.75 mg of 1 and 2 mg of 2, subjected to NMR and identified as kingiside (1) and loganin (2), respectively by comparison of their NMR spectra with published data (Msonthi et al., 1985). Specific searches using extracted ion chromatograms for strychnine, brucine and related compounds revealed the presence of no terpene indole alkaloids in the pulp of this species. The chemical analysis of *B. madagascariensis* dried fruits was reported elsewhere recently (Stevenson et al., 2010b). The major compounds in the powdered dry fruits were quercetin and kaempferol pentaglycosides comprising up to 20% of the total dry weight of the fruit. Other compounds recorded in the species include oleanolic acid saponins (Stevenson et al., 2010b; Marston et al., 1993) such as putranoside C which could have been responsible for the toxic effects. No other group of secondary metabolites that could explain the toxicity reported from the fruit of *B. madagascariensis*. *C. quadrangularis* and *V. amygdalina* were not analysed since they did not cause any acute toxicity in the present study.

DISCUSSION

The clinical signs exhibited by the mice exposed to high dosages of *S. spinosa* were similar to those reported for strychnine poisoning (Makarovsky et al., 2008). Strychnine is a toxic indole alkaloid which occurs in the seeds of *S. nux vomica* and other *Strychnos* species (Johnson, 1999) but has not been reported from *S. spinosa*. Where it does occur, the alkaloid exerts its effects on the central nervous system almost immediately after ingestion (Phillipe et al., 2004; Makarovsky et al., 2008), causing over-stimulation of the medulla, spinal cord and other parts of central nervous system which increase motor (muscular) and mental activity. However, *S. spinosa* is not regarded as a toxic species considering

Table 4. The effect of oral administration of aqueous extracts of *B. madagascariensis* fruits (2 to 10% w/v) on BALB/c mice.

Sample size (n) and hours post-treatment	Mortality of mice after exposure to different concentrations of *B. madagascariensis* fruit				Pooled mortality (%)/post-treatment period
	Dosage (%)				
	0	2	5	10	
N	8	8	8	8	24
0 - 12	0	0	0	2	2(7.1)
13 - 24	0	0	4	3	7(25)
25 - 36	0	1	1	2	4(14.3)
37 - 48	0	3	0	1	4(14.3)
49 - 60	0	1	0	0	1(3.6)
61 - 72	0	0	0	0	0(0)
Total mortality (%)	0	5 (62.5)	5 (62.5)	8 (100)	18 (64.3)

Table 5. The effect of oral administration of aqueous extracts of *C. quadrangularis* fruits (2 to 10% w/v) on BALB/c mice.

Sample size (n) and hours post-treatment	Mortality of mice after exposure to different concentrations of *C. quadrangularis*				Pooled mortality (%)/post-treatment period
	Dosage (%)				
	0	2	5	10	
N	6	6	6	6	18
0 - 12	0	1	0	2	3 (12.5)
13 - 24	0	0	1	1	2 (8.3)
25 - 36	0	0	0	0	(0)
37 - 48	0	0	0	0	(0)
49 - 60	0	0	0	0	(0)
61 - 72	0	0	0	0	(0)
Total mortality (%)	0	1 (16.7)	1 (16.7)	3 (50)	5 (20.8)

that the fruit pulp without the seeds did not contain strychnine or any related compounds and anecdotal reports that the ripe fruit is consumed by monkeys and baboons. The secoiridoids, kingside and loganin, that were present in *S. spinosa* pulp have been reported previously in this species (Msonthi et al., 1985). These secoiridoids are not known to induce the symptoms exhibited by the mice. However, in the absence of alkaloids in the toxic extracts, it is possible that these iridoids present some toxicity previously unreported and further work will be required to establish if this is the case. It is possible that the seeds, which were excluded from chemically analysed samples, contain strychnine or other terpene indole alkaloids that cause symptoms exhibited by mice in this study. Farmers in the study sites normally use whole crushed raw fruit without removing the seed (Stevenson et al., 2010a), as was used in the current study. Future studies should include chemical analysis of both the fruit pulp and the seed. The low mortality in the low dosages suggests a dose-dependent toxicity where the 0.1 to 5% levels is insufficient to elicit a physiological response that would cause significant mortality. Thus typical use of the plant species is not likely to expose farmers to high risk of acute toxicity. The high mortality observed in the *B. madagascariensis* treatments indicates that the aqueous plant extract is toxic to mice. This is not surprising since *B. madagascariensis* is used as fish poison and to poison arrow heads used for hunting in some parts of Africa (Neuwinger, 2004). In a survey of toxic plants on the market in Bamako, Mali, traditional healers considered *B. madagascariensis* to be a plant which is toxic (Maiga et al., 2004). The toxicity of *B. madagascariensis* could be attributed to saponins found in the pods (Marston et al., 1993; Stevenson et al., 2010b). Borel and Hostettmann (1987) and Marston et al. (1993) report saponins from *B. madagascariensis* to be toxic to aquatic snails while elsewhere in literature, saponins are reported to be toxic to several insect pests, including termites and coleopteran stored product pests (Malaya and Banda, 1995; Stevenson et al., 2009). The irritation exhibited by the mice during oral administration could have been caused by the bitterness of the pod contents or saponin foams blocking air passages. Stevenson et al. (2010b)

also report the occurrence of extraordinarily high quantities of flavonoid pentaglycosides in the pods of *B. madagascariensis,* as much as 20% of dry pod weight. Ordinarily, these flavonoids (quercetin and kaempferol glycosides) that are ubiquitous in plants are not toxic. However, their occurrence in the plant at such high levels and high doses may enhance the apparent toxicity of the pods.

The low mortality of mice across *C. quadrangularis* treatments reported in the present study concurs with the work of Udupa and Guru (1964) that reported an LD_{50} value of 2000 mg/kg for extracts of *C. quadrangularis* in rats. In sub-chronic toxicity trials, rats had no signs of adverse effects after oral administration of *C. quadrangularis* water extracts for three months (Aimmanas et al., 2002; Kothari et al., 2011).

The lack of mortality in mice that received *V. amygdalina* extracts concurs with the findings of Ojiako and Nwanjo (2006) in which *V. amygdalina* had low toxicity in rats. Numerous toxicology studies carried out on various subjects showed that *V. amygdalina* only induced mild toxic side effect when administered at very high concentration (Yeap et al., 2010). *V. amygdalina* is used as a vegetable and to make a soup in some parts of Nigeria suggesting further that this plant is non-toxic to mammals (Ologunde et al., 1992). Phytochemical analysis showed the presence of anthracene, glycosides, steroids, flavonoids, proteins, carbohydrates, saponins and tannins which are responsible for the astringent taste and odour (Butler and Bailey, 1973; Ologunde et al., 1992).

In all mortality cases across the experiments, deaths occurred within 3 days post-treatment and the mice exhibited minimal gross post-mortem lesions. These lesions are characteristic of any acute or sub-acute pathological condition which usually manifests itself more as a biochemical or functional imbalance rather than gross or microscopic architecture changes of organ systems (Gad, 2006). A longer exposure time would be needed to observe more of the organ system changes. Body mechanisms swiftly respond to correct the anomaly in the processes of elimination and detoxification where the animal recovers in a short period of time or dies if the body systems fail to cope with the toxin (Polson et al., 1983).

Despite the toxic effects observed in this study, in reality, the human health risks associated with these plant materials are largely mitigated through the use of preparations in which the quantities that might be consumed accidentally are typically very low (Isman, 2008). Ideally, local production of plant extracts should be standardized and regulated to ensure product safety and efficacy, but this may be an unrealistic expectation in many parts of Africa where these plants are used. The fact remains that these plant materials are used widely by farmers and will continue to be used, thus efforts need to be invested in the promotion of safe preparation and application. There is no question that these materials continue to have an important role to play in pest management as environmentally benign and cheaper alternatives to synthetic products.

Conclusion

This study demonstrated that some of the acaricidal and insecticidal plants used by smallholder farmers are potentially toxic to mammals. Despite a long history of use by farmers, fruits of *S. spinosa* fruits and *B. madagascariensis* contain substances that could harm users if consumed at high concentrations and in large quantities; hence, they should be handled with care and kept out of reach of children. However, when administered at the dosages of 10% or less (w/v), *V. amygdalina* and *C. quadrangularis* were not toxic to mice, and are therefore likely, at this dosage level, to be safe to humans and other mammals. Future studies should investigate LD_{50} dosages of the pesticidal plants tested in this study and further identify the toxic substances to minimize potential hazards from their use.

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