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Acute toxicity study of stone-breaker (*Phyllanthus tenellus* Roxb.)

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ABSTRACT

***Phyllanthus tenellus* Roxb. is a herbaceous plant native to Brazil and appears frequently in humid environments. This plant is used to treat urolithiasis, inflammatory bowel disease, diabetes and hepatitis B. The acute toxicity and LD_{50} of an aqueous extract of *P. tenellus* were determined in laboratory mice and their behavior was analyzed. The intraperitoneal LD_{50} was calculated by the Karber & Behrens (1964) method, for which a 96% alcoholic extract was concentrated in a rotary evaporator. Male albino mice *(Mus musculus)* were divided into three batches of six animals and observed for 24 hours after administration of the extract, diluted in 0.9% saline, at doses of 500, 1000, 1500, 2000 and 2500 mg / kg. Short-term studies have demonstrated this plant to be non-toxic; however, we found that this species induced agitation in animals, with stereotyped movements, spasms and increased respiratory frequency, as well as signs of depression, such as sleepiness, prostration, dyspnea and a reduction in respiratory frequency.**

Keywords: Acute toxicity. LD_{50}. Stone-breaker. Euphorbiaceae

INTRODUCTION

In Brazil, the medicinal plants of the native flora are consumed with little or no knowledge of their pharmacological properties. These plants are commonly used for medical purposes other than those of indigenous communities. Moreover, it is estimated that 25,000 plant species are destined for the production of medicines worldwide, including those synthesized from natural products and those commercialized as herbal medicines (Reston & Lima, 2002).

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The toxicity of medicinal plants and the medicines derived from them may seem of little importance compared to that of conventional treatments. This, however, is not the case. The toxicity of medicinal plants is a serious problem for public health (Veiga Jr. et al., 2005). The adverse effects of plant medicines, due to possible adulteration and toxicity of individual products or synergistic action (i.e. interaction with other drugs), are currently unknown (Oga, 2003). Research on the safe use of plant medicines and derived drugs is still at an early stage, and the control of their commercialization by official agencies is not well maintained in free fairs, public markets or stores for natural products. Today, there are approximately 120 clinical products used in allopathic medicine that originate from plants used by indigenous groups. These plant drugs are extensively commercialized in France, Italy, the United Kingdom, Asian countries and in the USA (Lapa et al., 2001).

The *Phyllanthus* species known locally as "stone breaker" (believed to break up kidney stones), belong to Euphorbiaceae, one of the largest and commonest plant families in the world (326 genera, 7750 species). These plants are used traditionally to treat urolithiasis (eliminating the renal calculi), inflammatory bowel disease, diabetes and hepatitis B. One of the species of "stone breaker" (quebra-pedra), *Phyllanthus tenellus* Roxb., is native to Brazilian tropical and sub-tropical regions and a common weed in humid environments (Silva & Sales, 2004). It is found in cultivated areas, land strips, garden seedbeds and sidewalk edges. According to the literature, *Phyllanthus tenellus* Roxb. possesses immunomodulatory (Ignácio et al., 2001), analgesic (Santos et al., 1994) and anti-hepatitis activity (Shead et al., 1992; Venkateswaran et al., 1987). Regarding the chemical composition of *Phyllanthus tenellus* Roxb. aerial parts, the fragmentation of phenolic deposits was observed in the cell vacuole by electronic and optical microscopy (Vickery & Vickery, 1981). It was found that the deposits accumulated in cell compartments after impregnation of aerial parts with copper sulphate (Santiago, 2000). Furthermore, the presence of many known metabolites, such as nirantin, nirtetralin, hinoquinine and geranin (pertaining to the alkaloid group), was detected

(Huang et al., 2003; 1998). In previous studies by Silva et al. (2010), evaluating the biological activity of three species of the genus *Phyllanthus*, we observed a strong antimicrobial and antifungal activity in the species tested, with best results in the crude extracts of *P. tenellus*, which showed strongest activity against *S. aureus* (MIC 0.10 mg / ml) and *B. subtilis* (MIC 0.30 mg / mL).

The macroscopic characteristics of *Phyllanthus tenellus* Roxb. allow it to be distinguished from other *Phyllanthus* species, although many tend to confuse the species because of its anatomical structure. In the literature, no acute toxicity data exist yet for *Phyllanthus tenellus* Roxb. (Nascimento et al., 2008; Silva & Sales 2004; Yeh et al., 1993).

MATERIALS AND METHODS

Plant Material

The collected specimens had no signs of fungus or damage and their leaves and stems were green and healthy. *P. tenellus* is a plant of easy dissemination that is always found in abundance at the collection sites described above. Samples were collected in the University City district and identified by Dr. Ulysses Paulino de Albuquerque and Marcos Jose da Silva of the Federal Rural University of Pernambuco (UFRPE).

Plant processing and preparation of crude extract

Aerial parts of the collected plants were dried under laboratory conditions, protected from sunlight. The plant was then finely ground and samples of about 350g were weighed on a semi-analytical balance and extracted until their exhaustion - approximately six litres of cold commercial ethanol (96%). The extracts were subsequently filtered and evaporated in a rotary evaporator.

Animal selection

Nine groups of approximately six 60-day-old male albino Swiss mice (*Mus musculus*) each were used, the animals weighing from 25 to 35 g. Animals were acquired from the animal facility of the Department of Pharmaceutical Sciences of the Federal University of Pernambuco. All experiments were supervised by Dr. Ivone Antonia de Souza of the Department of Antibiotics. All animals were properly sacrificed after the experiment. This study was approved by the Committee of Ethics in Research with Animals (resolution 009440/2006-48).

Acute toxicity and LD_{50}

An assay was carried out, by the method of Karber & Behrens (1964), to assess the general toxicity of the crude alcohol extract. Mice were marked and weighed and kept in polypropylene cages under controlled conditions (12/12 h light/dark cycle and 25ºC ± 2ºC). Animals were fasted for a period of 12 hours, with free access to water, before administration of the extract. The crude extract was dissolved at 0.9% (w/v) in physiological saline and 0.2% Tween 80 (v/v) and administered by intraperitoneal (ip) injection. This route was chosen since ip enables a lower dose to be used and the systemic effects are seen quickly, thus optimizing the study.

Assays were carried out in two stages. In the first (preliminary) stage, the extract was administered in increasing doses, in order to determine the dose closest to the lethal dose (D1), as well as the dose capable of inducing death in 100% of the animals (definitive phase, or D2). Mice were then monitored and the following parameters recorded: toxicity, ambulatory effects, changes in respiratory frequency and death rate. This last was also followed in the preliminary phase, to determine the DL_{50} (lethal dose for 50% of the animals).

The increasing doses of extract ranged from 500-2500 mg per kg body weight (bw). The criteria for toxicity classification shown in Table 1 were adopted, in accordance with COBEA (Brazilian College of Animal Experimentation).

Table 1. Toxicity classification, according to COBEA (Brazilian College of Animal Experimentation).

Category	DL50 for rats (mg/kg body weight)
Very toxic	Less that 25
Toxic	From 25 to 200
Harmful	From 200 to 2500

RESULTS

The largest dose (2500 mg/kg bw) failed to result in death after 48 hours. Hence the species was considered non-toxic. Table 2 lists the doses and how they related to the behavioral tendencies observed.

The results indicate that the plant has effects on the central and peripheral nervous system, such as agitation, stereotyped and circular movements, spasms and increased respiratory frequency. Dose dependence was not observed for the stimulant and depressant effects, except for respiratory frequency, for which it was very evident. For both types of effect, changes were observed in accordance with the dose administered. However, the stimulant effects occurred immediately and the depressor effects occurred approximately 10-15 minutes after extract administration. These symptoms became exacerbated as the dose increased.

As the initial doses were increased, we also observed that diuresis fell and fecal production increased. During this decrease in urine output, the formation of edema of the snout became evident, showing a correlation between the three events.

In the doses ranging from 1000-2500 mg/kg bw, we also observed constant episodes of spasms (Fig. 1). A reduced interval between spasms was observed with a dose of 2000 mg/kg bw. This dose also produced the largest spasms in the animals.

Between the doses of 1000 and 1500 mg/kg, There has a sharp reduction in the occurrence of spasms and an increase in the number of abdominal contraction episodes occurred, suggesting a nociceptive consequence in the animals at these doses (Fig. 2).

Table 2. Toxicological effects of the doses administered for preliminary/definitive acute toxicity assessment.

ACTION/MEASURE	DOSES (mg.kg-1)				
	500	1000	1500	2000	2500
Stimulant					
Respiratory frequency increase	+++	++	+	-	-
Agitation	+++	++	+	++	+++
Coat erection	+++	+++	+++	++	+++
Exophthalmia	-	+	-	+	++
Stereotyped movements	+++	+++	+++	+++	++
Jaw movements	+++	+++	+++	+++	++
Movement of Vibrissae	++	-	+	++	++
Clonic convulsion	-	-	-	-	-
Convulsion	-	-	-	-	-
Coarse/fine tremors	++	+	-	+	+
Tail erection	++	++	++	++	++
Attack position	++	++	++	++	++
Jumps	++	-	-	-	+
Irritability	+	+	+	++	+++
Hindlimb suspended	-	+	-	++	++
Hindlimb dragging	-	-	-	-	-
Depressor					
Hindlimb decaying	+	-	+	-	++
Static position	+++	+++	+++	+++	+++
Dyspnea	+++	+++	+++	+++	+++
Sleepiness	+++	++	++	++	++
Respiratory frequency reduction	-	-	+	++	+++
Prostration	++	++	++	+++	+++
Altered stride	-	++	-	++	+++
Others					
Fecal production	+	+	++	+++	+++
Abdominal contractions	+++	+++	+++	++	++
Escape reaction	+++	+++	+++	+++	+++
Spasms	++	+++	+++	+++	+++
Diarrhea	-	-	-	-	+
Regurgitation	++	++	+	+++	+++
Pallor	+++	+++	+	++	+++
Abdominal distention	-	+	+	+	++
Aggressiveness	-	+	++	+++	++
Diuresis	+++	+++	++	+	-
Irritation of conjunctiva	-	-	-	-	-
Spasticity	-	-	-	-	-
Cyanosis	-	+	+	++	++
Testicular hypertrophy	++	+	+	-	-
Edema of snout	++	+	++	+++	+++
Altered libido	-	+	++	-	-
Hemorrhagic spots	-	-	-	-	-

(-) No effect, (+) light effect, (++) moderate effect, (+++) accentuated effect.

It was found that, during diuresis reduction, the animals exhibited a nociceptive reflex characterized by abdominal contractions. When diuresis ceased completely, however, there was an abrupt reduction in the contractions over time, until they were completely absent at the maximum dose.

It can be suggested that the LD_{50} was never reached because of the rapid excretion, either fecal or urinary, that occurred during the experiment, accompanied by strong spasmodic activity, preventing the retention of the extract at high levels. However, each administration of the extract provoked at least one undesirable effect, stimulant or depressant.

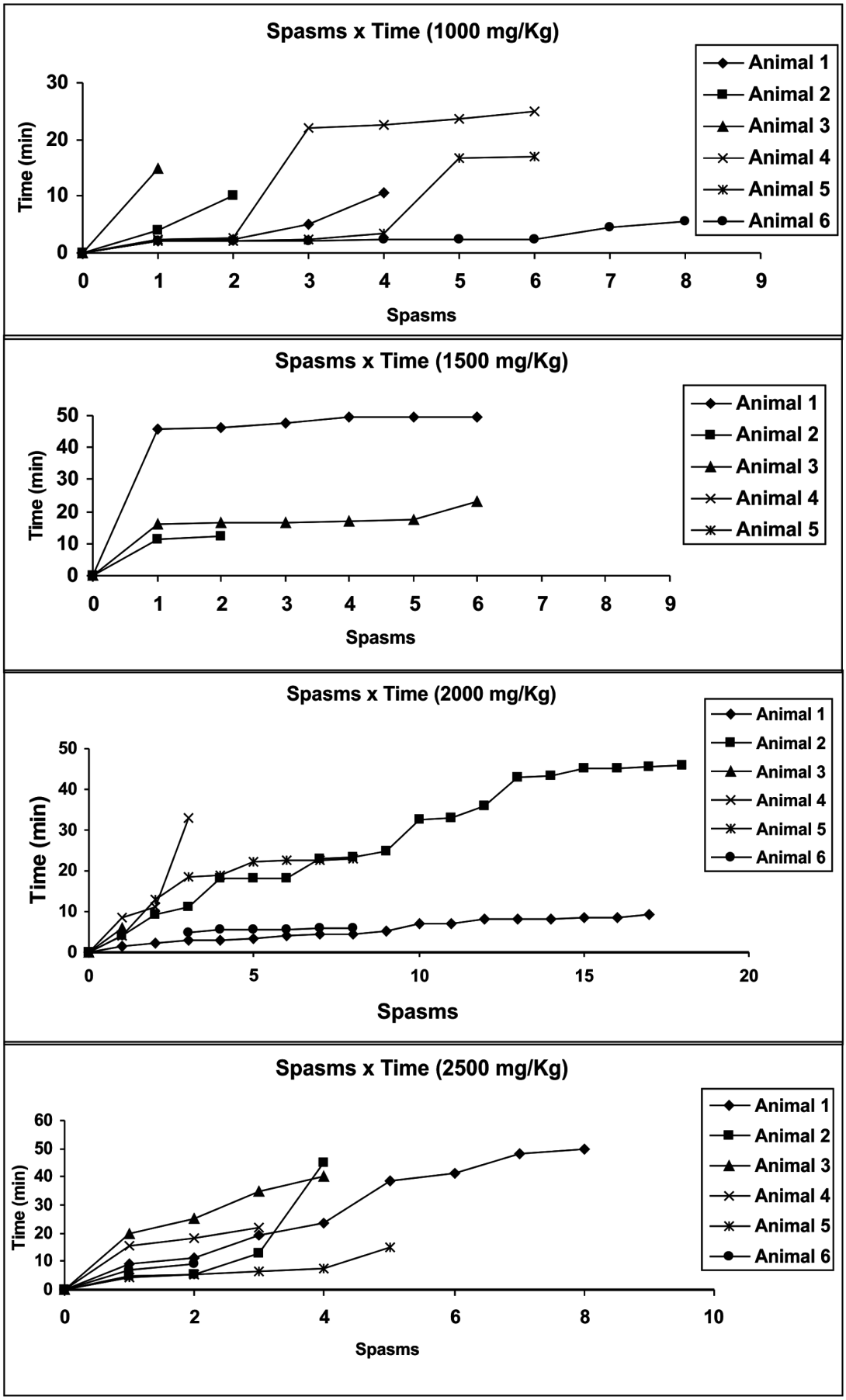

Figure 1. Number of spasms versus time after ip injection of crude alcoholic extract of *Phyllanthus tenellus* Roxb. (a- 1000 mg/kg; b- 1500 mg/kg; c- 2000 mg/kg; d- 2500 mg/kg)

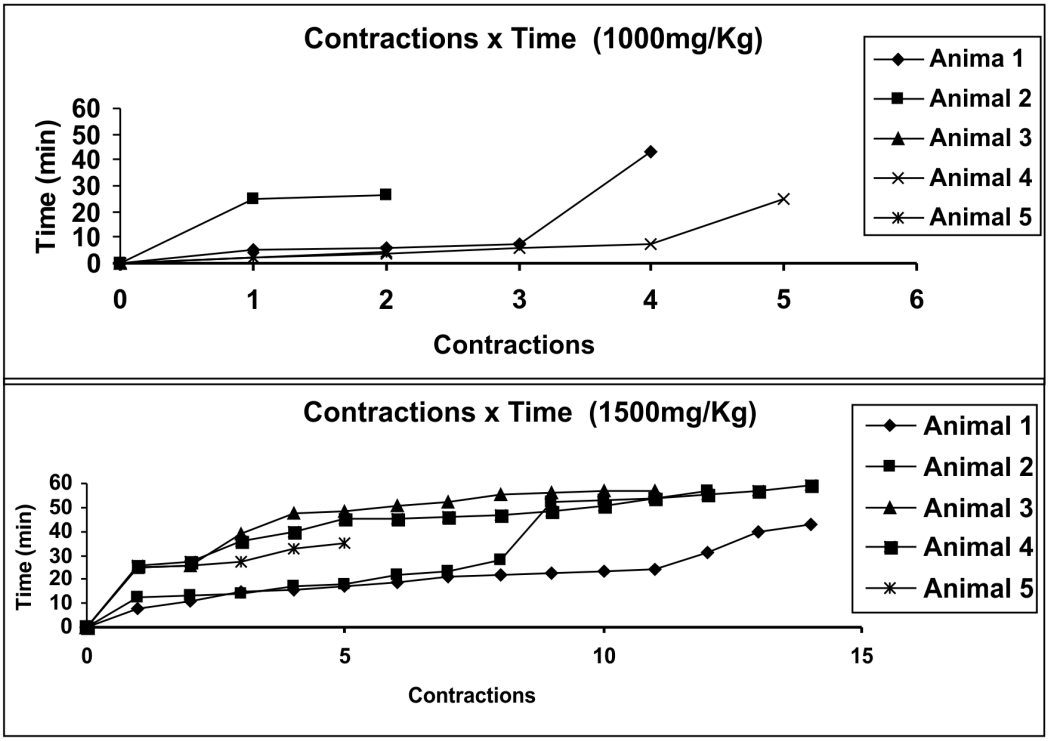

Figure 2. Number of abdominal contractions versus time after ip injection of crude alcoholic extract of *Phyllanthus tenellus* Roxb. (a- 1000 mg/kg; b- 1500 mg/kg)

DISCUSSION

To date, the acute toxicity of the *Phyllanthus* species has not been described in detail. To our knowledge, only one study of this topic exists, which describes the lack of toxic effects of *P. amarus* and *P. niruri* (Matos, 2002).

In preliminary studies on *Phyllanthus* species collected in various cities in the state of Pernambuco by Nascimento et al. (2008), with brine shrimp larvae as toxicity indicator, the plant was considered non-toxic when collected from the city of Garanhuns, exhibiting a 50% lethal concentration (LC_{50}) of about 1003.62 ± 65.15 µg/mL, and toxic from two other cities (Paulista and Recife) in the conurbation of the state capital, with LC_{50} of 534.60 ± 46.83 µg/mL and 642.91 ± 39.02 µg/mL, respectively.

Barros (2002) found that *Phyllanthus niruri* (another species of "stone breaker") had an effect on calcium oxalate crystallization, but was not toxic *in vitro*. The crystallization of phyllanthin in colorless needles, however, is most likely responsible for an increase in the toxicity found in this species (Reutter, 1923).

Given the lack of a definitive DL_{50}, the species *Phyllanthus tenellus* cannot be considered toxic by the COBEA criteria. The species did, however, have harmful effects on animals during the acute toxicity test. Animals showed stereotyped reactions of agitation, jaw movements, spasms and raised respiratory frequency, as well as depressant behavior (e.g. sleepiness, prostration and reduced respiratory frequency), suggesting that, albeit considered non-toxic, this plant extract can lead to behavioral changes (Silva et al., 2008).

We observed that depressant behavior was exacerbated as the dosage increased. Medicinal and phytotherapeutic plants are generally considered harmless and many people believe there is no risk of toxicity. The use of these plants for medicinal purposes can, however, have serious side-effects and interactions and even become poisonous at certain concentrations. This makes it necessary to educate the public about the rational use of medicinal plants and their derivatives (Silva et al., 2008).

One should beware of the indiscriminate use of this species, because of possible toxic components. Depending on the concentration used, the user may feel abdominal and breathing discomfort, or experience changes in the gastrointestinal system, leading to irritability or even prostration by respiratory problems. Furthermore, when an inadequate dose is used, the opposite to the medicinal purpose of the plant can occur; for example, instead of obtaining a diuretic effect, the retention of fluids may result.

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RESUMO

Estudo da toxicidade aguda de quebra-pedra (Phyllanthus tenellus Roxb.)

***Phyllanthus tenellus* Roxb. é nativa do Brasil, mais frequentemente em ambientes úmidos, e usada para**

o tratamento de litíase urinária, doença inflamatória intestinal, diabetes e hepatite B. Neste trabalho objetiva-se determinar a toxicidade aguda e DL_{50} do extrato aquoso de *P. tenellus* **em animais de laboratório e avaliar o seu comportamento. A DL_{50} por via intraperitoneal foi calculada pelo método de Karber e Behrens (1964), em que o extrato alcoólico a 96% foi concentrado em evaporador rotativo. Utilizou-se camundongos albinos (***Mus musculus***) machos, divididos em 3 lotes de seis animais. Eles foram observados por 24 horas a partir da administração do extrato diluído em solução fisiológica a 0,9% nas dosagens de 500; 1000; 1500; 2000; 2500 mg/kg. Estudos de curto prazo têm demonstrado que esta planta não é considerada tóxica, porém, constatamos que esta espécie provoca agitação nos animais por movimentos estereotipados, espasmos, e um aumento da frequência respiratória, bem como ações de depressão, tais como: sonolência, prostração, dispneia e diminuição da frequência respiratória.**

Palavras-chave: Toxicidade aguda. DL_{50}. Quebra-pedra. Euphorbiaceae.

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