Área de Farmacologia[1], Departamento de Ciências Básicas em Saúde, Faculdade de Ciências Médicas, Universidade Federal do Mato Grosso, Cuiabá; Núcleo de Investigações Químico-Farmacêuticas (NIQFAR)[2], Universidade do Vale do Itajaí (UNIVALI), Brasil; Centro Chimica dei Recettori del C.N.R.[3], Istituto di Chimica, U.C.S.C., Roma Italy

Toxicological and phytochemical studies of *Aspidosperma subincanum* Mart. stem bark (Guatambu)

S.R. Santos[1], E.T. Rangel[1], J.C.S. Lima[1], R.M.Silva[1], L. Lopes[1], V.F. Noldin[2], V. Cechinel Filho[2], F. Delle Monache[3], D.T.O. Martins[1]

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Prof. Dr. Domingos Tabajara de Oliveira Martins, Rua Sírio Libanesa n° 165 Apt° 602 Bairro Popular, Cep: 78045-390 Cuiabá, MT, Brasil
taba@terra.com.br

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Aspidosperma subincanum Mart. is widely used in Brazilian folk medicine to treat digestive disorders. In this study, acute and subchronic toxicity and cytotoxicity of stem bark ethanolic extract of *Aspidosperma subincanum* (EE*As*) have been evaluated. In addition, phytochemical analysis was performed. The EE*As* had low acute toxicity in mice with $LD_{50} = 1129 \pm 154$ mg/kg p.o. and 397 ± 15 mg/kg i.p. The LC_{50} was 1340 ± 428 μg/mL in the brine shrimp assay. There was no relevance of serious changes in behavioral, hematological and biochemical parameters and no deleterious effect on vital organs of rats that resulted after 30 days daily exposure to 5 and 100 mg/kg of EE*As*. Phytochemical analysis of stem bark of *A. subincanum* revealed the presence of indole alkaloids, saponins, terpenoids, steroids and tannins and resulted in the isolation of oleic acid and guatambuine as major constituents. Using the method of the dose by factor approach, the human safe dose was 210 mg/70 kg/day. The EE*As* appears to be safe and non-toxic in low doses in rodents and domestic preparations used by population have relatively security.

1. Introduction

Aspidosperma subincanum Mart (Apocynaceae), popularly known as "guatambu", is a tree that grows in the Cerrado regions of Brazil and the infusion or decoction of its stem bark is used by the population for the treatment of diabetes, hypercholesterolenemia, gastric problems and for loosing weight (Correa 1926) Oliveira et al. (2009) presented a review of main reports involving chemical and biological studies on the *Aspidosperma* genus. The plants of the *Aspidosperma* genus are known to contain indole alkaloids, several of which possess important pharmacological properties (Obitz et al. 1997; Kutney 1981).
In a preliminary toxicological study, Goloni et al. (2005), showed extremely low oral acute toxicity in mice of the methanolic extract obtained from stem bark of this plant.
The objective of the present study was to evaluate the safety of the ethanolic extract from the stem bark of *A. subincanum*, focusing on its acute and subchronic toxicity. In addition, we have performed a phytochemical study with this plant extract.

2. Investigations and results

2.1. Phytochemical analysis

n-Hexane and dichloromethane fractions from *A. subincanum* stem bark yielded two pure compounds, identified as oleic acid, an unsaturated fatty acid, and a tetrahydropyridine alkaloid known as guatambuine.

2.2. Acute toxicity

Oral administration of doses up to 300 mg/kg of EE*As* did not lead to any sign of toxicity or change in general behavior in mice. However, doses from 500 to 2500 mg/kg were followed by signs and symptoms typical of central nervous system stimulant effects like piloerection, tremors, convulsions, cyanosis and death. These effects of extract were dose-related and the estimated oral LD_{50} was 1129 ± 154 mg/kg. Intraperitoneal administration of EE*As* produced piloerection and tremors beginning with 300 mg/kg dose. At doses of 350 to 475 mg/kg, were observed piloerection, tremors, convulsions, cyanosis and mortality of 100% of mice with the highest dose. The intraperitoneal LD_{50} value of extract was 397 ± 15 mg/kg (Table 1).

2.3. Subchronic toxicity

None of the animals treated with EE*As* (5 and 100 mg/kg) exhibited any behavioral changes, and no toxic signs or symptoms were observed. As shown in Table 2, mean weights, food and water intake of animals that received repeated doses of EE*As* did not differ statistically from the control group.
There were no significant differences in the relative weights of stomach, liver, heart, lungs, kidneys and spleen and at necropsy, no gastrointestinal lesions over the experimental period between treatment groups and control were observed in the rats treated with EE*As*.
The effect of subchronic oral administration of EE*As* in hematological parameters is presented in Table 3. All the hematological parameters (white blood cells, red blood cells,

Table 1: Acute toxicity of ethanolic extract from *Aspidosperma subincanum* Mart. stem bark (EE*As*) after oral and intraperitoneal administration in mice

Dose mg/kg	Dead/alive	Toxic signs and symptoms	
		oral	intraperitoneal
100	0/10	None	None
300	0/10	None	Piloerection, tremors
350	1/10	–	Piloerection, tremors, convulsions, cyanosis and death
400	4/10	–	
425	7/10	–	
450	9/10	–	
475	10/10	–	
500	1/10	Piloerection, tremors, convulsions, cyanosis and death	–
850	3/10		–
1000	5/10		–
1250	7/10		–
1500	9/10		–
2500	10/10		–

LD_{50} = 1129 ± 154 and 397 ± 15 mg/kg oral and intraperitoneal administration, respectively

hemoglobin, hematocrit, platelets, absolute lymphocytes and relative lymphocyte) remained within normal limits throughout the treatment period and no significant differences were observed when compared with control group. Subchronic oral administration of EE*As* did not cause significant changes in serum glucose, urea, asparagine transaminase, alanine transaminase, total cholesterol, triglycerides, albumin and uric acid.

2.4. Brine Shrimp Lethality Bioassay

The EE*As* did not show significant lethality against brine shrimp until 0.1 μg/mL. Lethality of nauplii beginning with 1 μg/mL of EE*As* (6%) culminating with 100% of death with 5000 μg/mL. The LC_{50} calculated for EE*As* was 1340 ± 428 μg/mL, while for quinidine sulfate was 130 ± 33 μg/mL.

Table 2: Body and organ weights (g) of Wistar rats after subchronic oral treatment with of ethanolic extract from *Aspidosperma subincanum* Mart stem bark (EE*As*) for 30 days

Parameter	Control group	EE*As* (mg/kg)	
		5	100
Body weight gain (g)			
Initial	20.78 ± 3.12	20.60 ± 3.36	19.36 ± 2.29
Final	3.15 ± 3.48	−3.15 ± 1.42	−1.51 ± 1.31
Food Intake (g)			
Initial	88.36 ± 4.64	88.36 ± 4.64	88.36 ± 4.64
Final	127.25 ± 4.14	126.70 ± 2.54	124.97 ± 1.33
Water consumption (mL)			
	133.33 ± 7.14	131.67 ± 7.60	123.33 ± 5.57
	177.00 ± 6.26	178.67 ± 6.71	192.00 ± 4.08
Organ weight (g% body wt)			
Heart	1.88 ± 0.23	1.68 ± 0.25	1.46 ± 0.11
Liver	15.73 ± 2.02	14.32 ± 1.73	12.21 ± 0.85
Lungs	2.63 ± 0.28	2.57 ± 0.32	2.16 ± 0.14
Kidneys	3.76 ± 0.60	3.64 ± 0.50	3.16 ± 0.30
Spleen	1.01 ± 0.13	0.85 ± 0.12	0.69 ± 0.026

One way ANOVA

3. Discussion

The toxicological evaluation of oral administration of EE*As* has shown that the dose up to 300 mg/kg did not cause any signs of toxicity in mice. The toxic effects were observed from 500 mg/kg and the severity was dose-dependent culminating with 100% of death with 2500 mg/kg dose with LD_{50} = 1129 ± 154 mg/kg p.o. Toxic effects elapsed after intraperitoneal administration of EE*As* were observed earlies than after administration oral with LD_{50} = 397 ± 15 mg/kg i.p. These results show that the EE*As* are better absorbed by the intraperitoneal route than orally.

The subchronic toxicity study in rats receiving EE*As* orally at doses of 5 and 100 mg/kg caused no change in animal body weight gains, behavior and food and water intake. Since the changes in body weight have been used as an indicator of adverse effects of drugs and chemicals (Desmarchelier et al. 1996), the present results suggest that oral doses of EE*As* do not show toxicity in rats.

There were no significant alterations in the biochemical parameters of rats treated with EE*As*. Plasma cholesterol levels, an indirect indicator of liver function (Hilaly et al. 2004), were not affected by EE*As* administration in rats. This was confirmed by the fact that no alterations in the transaminase levels (ALT and AST) were observed, which are good indicators of liver function, thus it is reasonable to deduce that the EE*As* did not induce any damage to the liver. The rats remained within physiological limits throughout the treatment period in the serum levels of glucose, albumin, uric acid and urea suggesting that the subchronic administration of EE*As* (5 and 100 mg/kg p.o.) did not interfere with the metabolism of rats. Since there was no effect on the levels of albumin and urea, we can deduce that EE*As* did not induce any damage to the kidney. There were no significant changes in the uric acid limits suggesting that there were no deleterious effects of EE*As* on the functions of any organs (Chavalittumrong et al. 2004).

The EE*As* (5 and 100 mg/kg p.o.) did not alter any of the hematological parameters evaluated (WBC, RBC, Hb, Ht, platelets, absolute lymphocytes and relative lymphocytes) and did not cause any toxic effects on the bone marrow and homeostasis of the to circulatory system.

The autopsy revealed no changes in the stomach, intestines, liver, spleen, heart, lungs and kidneys and no alterations in the relative organ weight after exposure to EE*As* (5 and 100 mg/kg, p.o.). These findings are in accordance with the results observed in the

Table 3: Effect of subchronic oral administration of ethanolic extract from *Aspidosperma subincanum* Mart. stem bark (EE*As*) for 30 days on the hematological and biochemical parameters of Wistar rats

Parameter	Control group	EE*As* (mg/kg)	
		5	100
Hematological parameters			
White Blood Cells -WBC (10^3/UL)	7.66 ± 0.92	8.35 ± 1.03	$7.43 \pm 0{,}83$
Red Blood Cells - RBC (10^6/UL)	7.03 ± 0.20	$6.91 \pm 0{,}20$	7.17 ± 0.62
Hemoglobin (g/dL)	14.46 ± 0.29	14.56 ± 0.25	14.46 ± 0.47
Hematocrit (%)	41.31 ± 1.204	40.63 ± 1.40	38.80 ± 1.17
Platelets (10^3/UL)	605.17 ± 23.98	534.17 ± 61.41	564.03 ± 28.56
Absoluts Lymphocytes (mm^3)	6.16 ± 0.73	6.66 ± 0.81	6.03 ± 0.53
Relative Lymphocytes (%)	80.25 ± 1.50	80.75 ± 2.82	82.43 ± 2.57
Biochemical parameters			
Glucose (mg/dL)	80.0 ± 5.34	84.50 ± 7.27	78.83 ± 3.60
Urea (mg/dL)	37.00 ± 4.43	41.17 ± 5.02	39.00 ± 4.73
Uric acid (mg/dL)	1.70 ± 0.34	1.08 ± 0.12	2.00 ± 0.61
AST (U/L)	179.50 ± 12.36	181.33 ± 11.25	187.83 ± 16.63
ALT (U/L)	50.16 ± 2.24	61.83 ± 12.21	56.50 ± 7.97
Total Cholesterol (mg/dL)	70.50 ± 10.67	78.16 ± 9.62	71.66 ± 2.33
Triglyceride (mg/dL)	81.33 ± 6.02	77.00 ± 10.2	75.83 ± 10.16
Albumin (mg/dL)	4.28 ± 0.11	4.31 ± 0.07	4.28 ± 0.13

One way ANOVA

hematological and biochemical parameters, and in functional tests of vital organs when administered for 30 days.

To evaluate the cytotoxicity of EE*As* we performed the brine shrimp assay. LC_{50} of the EE*As* calculated was 1340 ± 428 μg/mL. Taking into account the possible relationship between active brine shrimp assay and possible human acute toxicity to the plant, we can suggest the innocuousness of EE*As*. Extrapolation of doses from animals to human is based on multiple assumptions about the compound's behavior across species. A commonly used approach is based on the no observable adverse effect level – NOAEL(Reigner and Blesch 2002). The safe dose, in our study, could be estimated using the formula $1/10 \times$ NOAELx 70 kg x/1/10, where NOAEL corresponds to 300 mg/kg. The estimated safe dose in our study was 210 mg/70 kg/day. When comparing the safe one with that dose usually taken as domestic preparations by people (25 mg/70 kg/day) we can deduce that the people are taking doses just eight times less and thus its use is relatively free from health risk.

Phytochemical studies carried out on the stem bark of *A. subincanum* demonstrated the presence of indole alkaloids, steroids, terpenoids, saponins and organic acids and the isolation and identification of two main compounds, oleic acid and guatambuine.

In general, oleic acid is encountered in several higher plants and reputed for their antioxidant property and low toxicity (Waterman and Lockwood 2007).

Indole alkaloids are largely present in various species of the genus *Aspidosperma* that exhibit a range of biological activities, including antimicrobial, trypanocidal, leishmanicidal, anticancer, anti-hypertensive and stimulant for the central nervous system (Kutney 1981; Obitz et al. 1997; Oliveira et al. 2009). Cytotoxicity of the different indole alkaloids from *Aspidosperma* was shown on a, NIH 3T3 (fibroblasts) cultured human cell line (Mitaine-Offer et al. 2002). Despite the wealth of indole alkaloids in the genus *Aspidosperma*, there is no report in the literature for *in vivo* toxicological activity of the alkaloids present in *Aspidosperma subincanum* (Morais et al. 2005).

The toxic effects, especially stimulation of the central nervous system and death, observed only with high doses of EE*As* in the acute toxicity test may be due to the presence of indole alkaloids.

In summary, with the doses used in the toxicological preclinical studies in rodents, the EE*As* did not show any remarkable toxic effects in acute doses until 300 mg/kg p.o. Doses until 100 mg/kg administered for 30 days, did not cause alterations in rat's behavior, nor changes in hematological and biochemical parameters, nor in relative vital organs weight. The EE*As* showed low cytotoxicity tested with *Artemia salina* Leach. The extrapolation of results obtained in the toxicological preclinical study to humans, allows to say that domestic preparations used by the population have relative security.

4. Experimental

4.1. Animals

Male albino Wistar rats (180–200 g) and male Swiss mice (25–30 g) were used. The animals were maintained in propylene cages at $25 \pm 2\,^{\circ}C$ in a 12 h light-dark cycle and had free access to standard pellet chow and water. Groups of three to ten animals were used for experimentation. Nauplii of the brine shrimp (*Artemia salina* Leach) were used in the bioassay of cytotoxicity. The protocol for these experiments was approved by the Animal Ethics Committee of the Federal University of Mato Grosso State, Brazil.

4.2. Plant material

The stem bark of *A. subincanum* was collected (5.35 kg) in Cuiabá municipality area of Mato Grosso State, Brazil, in a reserve of native Cerrado. The botanical identity was confirmed by Ms. Harri Lorenzi, of the Plantarum Institute, in São Paulo, Brazil. A voucher specimen (n° 30487) was deposited in the Herbarium of Federal University of Mato Grosso.

4.3. Preparation of extract

The stem bark was cleaned and dried at 40 °C for 3 days to constant weight (4.7 kg) and later triturated. The dried powdered stem bark (2 kg) was macerated with ethanol/water (3:1 w/v) at room temperature for 7 days. After this period, the macerate was filtered through a filter paper and the filtrate was evaporated using a rotary evaporator ($45 \pm 1\,^{\circ}C$) under reduced pressure (625 mmHg) to yield a residue of 208.2 g (10.41%).

4.4. Phytochemical screening

In order to verify the presence of different chemical classes, the ethanolic extract was subjected to a standard screening test (Matos 1988). A conventional protocol for detecting the presence of alkaloids, flavonoids, saponins, tannins, xanthones, steroids, antraquinones, triterpenes, etc., was used.

4.5. Fractionation and isolation of constituents

Dried methanolic extract (63 g) was successively partitioned with solvents of increasing polarity to give the following fractions and yields: n-hexane (1.4 g; 0.07%), dichloromethane (1.0 g; 0.05%) and ethyl acetate (0.8 g; 0.04%). n-Hexane and dichloromethane fractions were combined because they exhibited a similar chromatographic profile by TLC and were chromatographed using a silica gel column eluted with n-hexane:acetone gradient, yielding pure compounds (oleic acid, 5 mg; guatambuine, 11 mg) identified on basis of their spectral data (IR, NMR-1H and 13C).

4.6. Hippocratic Screening Test

Male mice (3/group) were used for this test. Signs and symptoms were observed after oral - p.o. (100 – 2500 mg/kg) or intraperitoneal - i.p. (100 – 1000 mg/kg) administration of EE*As*. Following treatment, the animals were observed at 5, 30, 60, 120 and 240 min and clinical signs and mortality, if any, were verified in a period of one week (Malone 1977).

4.7. Lethal Dose 50 % (LD_{50})

Increasing doses of EE*As* were administered in groups of 10 mice (5 males and 5 females) each, weighing 25 – 30 g, 100 – 2500 mg/kg p.o. or 100 – 475 mg/kg i.p. Signs of toxicity and mortality within 24 h were noted. The LD_{50} was calculated from graph of percent mortality against probit log dose of extract (Miller and Tainter 1944).

4.8. Subchronic toxicity

The subchronic toxicity study was performed through daily and single oral exposition of Wistar rats (150 – 250 g), to the vehicle (1 mL/100 g) or EE*As* (5 and 100 mg/kg), for a period of 30 days (Bautista et al. 2004). For the remainder of 30 days study period, animals were monitored daily for mortality and any changes in food and water consumption, and any additional behavioral or clinic signs of toxicity. At the end of the period, EDTA blood samples were collected from the inferior vena cava. The vital organs were removed, autopsied and weighed for the determination of the relative weight [(weight of the organ/body weight) $\times 100$].

4.9. Brine Shrimp Lethality Bioassay

The eggs of *A. salina* were hatched with 3% marine salt and then they were incubated at room temperature for 48 h. After 24 h, ten nauplii (hatched larvae) were placed in each test tube containing the test sample. The EE*As* were tested at the concentration levels (0.1–1000 μg/mL). Survivors were counted after 24 h and the lethality fifties (LC_{50}) for each assay were calculated by taking average of three experiments using probits analysis. Quinidine sulfate was used as positive control (Meyer et al. 1982).

4.10. Statistical analysis

Data are presented as mean ± S.E.M. Significance differences between control and experimental groups were assessed by one way ANOVA. *P*-values less than 0.05 were considered to be significant.

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