Reproductive toxicity of *Samanea tubulosa* on rats

Toxicidade reprodutiva de Samanea tubulosa em ratos

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Abstract

Samanea tubulosa is a plant used for medicinal and feeding purposes. However, ingestion of *S. tubulosa* pods has been associated with bovine abortion. Thus, the aim of this work was to investigate the effects of diet containing 5% of *S. tubulosa* pod meal on male and female Wistar rats. Diet was administered to male rats (n = 10) for 60 days before mating. Female rats (n = 10) received the treatment for 30 days, during cohabitation and from gestational day (GD) 0 to GD20. Treated animals were mated with untreated rats. In male rats, plant consumption caused decreased food consumption and 20% fertility index reduction. Litters from treated males presented lower body weight and crown–rump length. Female rats treated with the plant increased water and food intake and body weight. Decreases in fertility, fecundity and gestation indices and increase of placenta weight and mean number of corpora lutea were found. Thus, owing to the possible general and reproductive toxic effects, long-term consumption of *S. tubulosa* is not recommended for phytotherapic or food purposes.

Keywords: Cattle. Fertility. Phytotherapy. Plant. Reproductive toxicology.

Resumo

Samanea tubulosa é uma planta utilizada na fitoterapia e na alimentação animal. Entretanto, a ingestão de vagens de *S. tubulosa* tem sido associada à ocorrência de abortamentos em bovinos. Assim, o objetivo do trabalho foi investigar os efeitos da dieta contendo 5% de vagens de *S. tubulosa* em ratos machos e fêmeas Wistar. A dieta foi administrada para ratos machos (n = 10) por 60 dias antes do acasalamento. Ratos fêmeas (n = 10) receberam o tratamento por 30 dias, durante a coabitação e do dia gestacional (GD) 0 ao GD 20. As fêmeas tratadas foram acasaladas com ratos não tratados. Em machos, o consumo da planta causou diminuição no consumo de ração e redução de 20% no índice de fertilidade. A prole de machos tratados apresentou menor ganho de peso e comprimento cabeça cauda. Fêmeas tratadas com a planta apresentaram aumento do consumo de ração e água e do peso corporal. Ainda, foram observadas diminuição na fertilidade, fecundidade e no índice de gestação e aumento do peso da placenta e no número médio de corpos lúteos. Desse modo, em decorrência aos possíveis efeitos tóxicos sistêmicos e reprodutivos, o consumo prolongado de *S. tubulosa* não é recomendado para fins fitoterápicos ou alimentar.

Palavras-chave: Bovinos. Fertilidade. Fitoterápico. Planta. Toxicologia reprodutiva.

Introduction

Samanea tubulosa (Benth.) Barneby and Grimes is a multipurpose tree which belongs to the Fabaceae family. The plant is usually found in deciduous and semideciduous forests and savannahs (BARNEBY; GRIMES, 1996). *S. tubulosa* is commonly known in Brazil, Bolivia, Argentina and Paraguay as bordão-de-velho, chontaquiro, samán and maduvira, respectively (CARVALHO, 2007). *S. tubulosa* has been cultivated for ornamental and industrial purposes, especially for the furniture and paper industries (CARVALHO, 2007). *S. tubulosa* leaves are medicinally employed by Bolivian Indians to treat eye disorders (HAJDU;

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HOHMANN, 2012). The tree is also used on farms to provide shade to livestock and enhance soil fertility for crops, and its succulent pods are sweet and edible, making them useful for livestock grazing (BERG, 1986). In fact, the *S. tubulosa* pod meal has been used as forage in arid and semiarid regions of Brazil, especially during dry periods (ALVES et al., 2008). However, Brazilian farmers from the Amazonian and savannah ecotone have reported that ingestion of *S. tubulosa* pods at the end of the rainy season is associated with abortion outbreaks in cows (COSTA et al., 2011). The phytochemical screening of the ethanolic extract from *S. tubulosa* leaves revealed the presence of steroids, alkaloids, simple phenols, tannins, saponins, flavonoids, flavones and flavanones (SÁ; ARAÚJO; CHAVES, 2011). These chemicals may produce reproductive disorders. For instance, abortifacient, anti-implantation, antispermatogenic and antifertility effects have been ascribed to saponins (GUPTA et al., 2005; PADMASHALI et al., 2006); Indolizidine alkaloids may lead to abortion, infertility, fetal deformity and disturbances in placental circulation (JAMES, 1976); An flavonoid-rich fraction of *Vitex negundo* seeds has produced anti-androgenic activity in male dogs (BHARGAVA, 1989). Despite the possible presence of active substances in *S. tubulosa* that might be implicated in reproductive disruption and farmer reports of abortion outbreaks, there is little evidence to prove its abortifacient effect. Thus, the aim of this study was to investigate toxicological and reproductive effects after exposure to a diet containing 5% of *S. tubulosa* pods meal in male and female rats.

Material and Methods

Animals

Twenty-three-month-old male and 20 female Wistar rats, weighing 230-250 g, were obtained from the School of Veterinary Medicine and Zootechny, Federal University of Tocantins. Animals were randomized, assigned to treatment groups of 10 animals each and housed individually in plastic cages (40 x 50 x 20 cm) at controlled room temperature (20 ± 3°C) and humidity (45 - 65%) with a 12-h light–dark cycle (lights on 6:00 h) with free access to food and water. The experiment followed the protocols of the Guide for Care and Use of Laboratory Animal, National Research Council, USA (2011). For male and female reproductive evaluation, the Guidelines for Testing of Chemicals nº 421 described by the Organization for Economic Cooperation and Development were used with some modifications (OECD, 2015).

Plant material

S. tubulosa pods were collected in the region of Araguaína (Tocantins State, Brazil) and identified by Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA/Brasília. The voucher specimen was registered under the number HT9774 and kept in the University of Tocantins herbarium. The pods were oven-dried at 50°C, ground in a mill and placed in plastic bags to protect against humidity and luminosity.

Phytochemical analysis

The phytochemical analysis of *S. tubulosa* was performed according to methodologies described by Shriner et al. (1979), in order to verify the presence of secondary metabolism products: flavonoids, tannins, saponins and alkaloids.

Preparation of S. tubulosa pods

Every week, regular powdered chow (Purina®) was mixed with 5% of *S. tubulosa* pod meal, pelletized and stored in paper bags in a temperature-controlled room (22 - 25°C). The level of 5% *S. tubulosa* was established to avoid nutritional disturbances. Ten rats of each sex were given free access to either the commercial diet or a diet containing 5% of *S. tubulosa*. Female rats received the treatment for 30 days, during cohabitation and from gestational day (GD) 0 to GD 20. Male rats were treated during 60 days and cohabitation. Body weight, food and water consumption were monitored daily.

Estrous cycle phases evaluation

Control and experimental female rats had their estrous cycle monitored daily throughout the initial treatment period of 30 days. Vaginal secretion was collected every morning with a dropping pipette filled with 50 µL of saline (NaCl 0.9%) by inserting the tip into the rat vagina, expelling and drawing back the saline. Vaginal fluid was placed on glass slides and observed under a light microscope. The phases of the estrous cycle and the number of estruses during the period were recorded.

Female evaluation

From the 30th day of treatment, female rats in proestrus were cohabited overnight with untreated males. Males and females were separated the next morning and vaginal smears were collected. Presence of spermatozoa in the vaginal smear was considered as evidence of mating and the day of occurrence designated as GD 0. This procedure was repeated during two consecutive proestrous cycles. Females with no evidence of mating were maintained in treatment for 20 days. On GD 20, females were anesthetized with intraperitoneal injections of ketamine (90 mg/kg) and xylazine (10 mg/kg). Blood samples were collected and term Cesarean section was carried out and reproductive study was performed.

Male evaluation

Male fertility performance examination started after 60 days of continuous treatment. For this purpose, each male of both groups was caged overnight with one untreated female rat until mating or two weeks maximum. Female rats were kept in separate cages containing water and food *ad libitum* until GD 20 when reproductive studies were performed. After mating or at the end of the cohabitation period, male rats were anesthetized with i.p. injections of ketamine (90 mg/kg) and xylazine (10 mg/kg) for blood collection and necropsy.

Reproductive performance studies

On GD20, females were anesthetized and term Cesarean section was performed, ovaries were removed and number of corpora lutea was counted. Gravid and non-gravid uterine horns were weighed and the number of dead and live fetuses, number of implantations and early and late resorptions in the uterine horns were recorded. The number of male and female fetuses was counted and sex ratio (Nº of male pups/Nº of female pups) was calculated. Placenta and body weight of each fetus was registered and fetal crown-rump length was measured. In addition, male mating [(Nº of males with confirmed mating/ Nº of males cohabited) x 100], male fertility [(Nº of males impregnating a female/ Nº of males cohabited) x 100], female mating index [(Nº of females mating/ Nº of cohabited females) x 100], female fertility [(Nº of pregnant females/ Nº of females cohabited) x 100], fecundity [(Nº of pregnant females/ Nº of females with confirmed mating) x 100], and gestation [(Nº of litters with live pups/Nº of pregnancies) x 100] indexes were calculated. Implantation index [(Nº of implants/Nº of corpora lutea) x 100], preimplantation losses [(Nº of corpora lutea - Nº of implants)/Nº of corpora lutea] x 100 and postimplantation losses [(Nº of implants - Nº of viable fetuses)/Nº of implants] x100 were also evaluated [15].

Biochemical analysis

After anesthesia, blood samples were obtained from left common iliac vein and one aliquot of blood per animal was placed in a dry tube and centrifuged, serum was collected and stored at -20°C until assay. Glucose, total proteins (TP), albumin (ALB), cholesterol, triglycerides, urea, creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed using commercially available kits (Labtest®) and Microplate Reader, ASYS®, UVM 340.

Hormone determination

Testosterone, 17β- estradiol (E2) and progesterone (P4) concentration were measured using commercial

EIA kits (Enzo® Life Science, Farmingdale, NY, USA; EIA kits No.900-065, No.ADI-900-174, No. ADI-900-011). Samples were thawed and plated in antibody-coated 96 microplate wells in duplicate. The EIA kit protocol was then followed. Plates were read at 405 nm in Microplate Reader, ASYS®, UVM 340. The testosterone assay presented 5.0 pg/mL sensitivity, 10.8% intra-assay coefficient of variation (CV) and 9.3% interassay CV. The progesterone assay presented 6.5 pg/mL sensitivity, 4.4% intra-assay CV and 2.7% interassay CV. The estradiol assay presented 10.5 pg/mL sensitivity, 2.1% intra-assay CV and 8.3% interassay CV.

Gross and histopathological analysis

Necropsy was performed and samples of central nervous system (CNS), thymus, heart, lungs, liver, spleen, small and large intestine, kidneys, adrenals and mesenteric lymph nodes were collected from all animals for histological examination. Samples were fixed in 10% buffered formalin, routinely embedded in paraffin, cut into 3µm thick sections and stained with hematoxylin and eosin (HE) for light microscopic examination.

Statistical analysis

Kolmogorov and Smirnov normality test was applied to test for Gaussian distribution of the data. Accordingly, data are presented as mean values and standard errors of the mean (SEM) or as percentiles. Student's unpaired t test was applied for statistical comparison of the differences in data between the test groups ($P < 0.05$). The percentage of preimplantation and postimplantation losses, number of resorption sites and serum hormone level were analyzed by Mann-Whitney test ($P < 0.05$). Data were analyzed using GraphPad Instat 3.0 software.

Results

Phytochemical Analysis

The phytochemical analysis of *S. tubulosa* revealed the presence of tannins, flavonoids, saponins and alkaloids.

Effects of S. tubulosa in male rats

Treatment with 5% of *S. tubulosa* in male rats increased food consumption on the 19th (20.1 ± 0.481; 22.2 ± 0.573) day of treatment and decreased this parameter on days 39 (24.3 ± 1.096; 12.1 ± 0.997), 45 (22.7 ± 1.001; 17.8 ± 0.989), 47 (24.8 ± 0.711; 21.6 ± 0.791), 51(32.6 ± 1.204; 26.8 ± 0.827), 56 (22.5 ± 0.792; 18.8 ± 0.512) and 58 (20.3 ± 1.001; 17.6 ± 0.902) of treatment. Water consumption of experimental rats was reduced on days three (62.50 ± 0.845; 45.50 ± 0.609) and 32 (47.3 ± 0.430; 37.0 ± 0.291) and increased on day 38 (34.8 ± 0.585; 45.4 ± 0.819) of treatment. Serum concentrations of glucose, urea, creatinine and albumin were increased in experimental rats (Table 1). Serum testosterone concentrations (3.36 ± 0.0700; 3.37 ± 0.0660 $ng.mL^{-1}$) did not differ between experimental rats and control group. No significant differences were observed in other parameters evaluated.

Table 1 – Biochemical analysis of male rats treated with 5% of *S. tubulosa* in diet or just chow (control) during 60 days – Araguaína – 2010

Parameters	Control (n = 10)	*S. tubulosa* (n = 10)
Glucose (mg/dL)	90.3 ± 3.4	111.0 ± 2.5*
Urea (mg/dL)	52.5 ± 1.8	70.0 ± 2.8*
Creatinine (mg/dL)	0.54 ± 0.02	1.2 ± 0.18*
Total protein (g/dL)	6.2 ± 0.28	6.4 ± 0.47
Albumin (g/dL)	4.1 ± 0.41	7.0 ± 0.66*
ALT (U/L)	73.7 ± 3.1	76.9 ± 4.5
AST (U/L)	180.0 ± 1.0	183.8 ± 1.5
Cholesterol (mg/dL)	66.6 ± 4.0	62.0 ± 4.4
Triglycerides (mg/dL)	107.0 ± 1.6	111.0 ± 1.8

Data expressed as mean ± s.e.m. *$p < 0.05$, Student's unpaired t test

Effects of S. tubulosa in male reproductive performance

There were no significant differences between control and male treated rats for percentage of male mated and fecundity (Table 2). However, 20% pregnancy failure occurred and one dead fetus was detected in one female mated with an experimental male rat. Fetus body weight and crown-rump length of female and male offspring were decreased in the

group of dams mated with experimental rats (Table 2). No significant differences were observed in other reproductive parameters.

Table 2 – Reproductive performance of male rats treated with 5% of *S. tubulosa* in diet or just chow (control) during 60 days – Araguaína – 2010

Parameters	Control	Experimental
Male Reproductive Performance		
Male mating index (%) [a]	100	100
Male fertility index (%) [a]	100	80
Dams		
Nº of dams	10	10
Gestation index (%) [a]	100	100
Implantation index (%)[a]	100	100
Mean of maternal body weight [a]	345.8 ± 7.6	341.6 ± 7.4
Mean number of corpora lutea [a]	11.5 ± 0.93	12.0 ± 0.53
Mean number of implantations [a]	11.5 ± 0.93	12.0 ± 0.53
Mean of placental weight [a]	0.354 ± 0.027	0.357 ± 0.020
Preimplantation losses (%) [b]	0	0
Post-implantation losses(%) [b]	1.6	3.1
Nº of total dead fetuses	0	1
Nº of total live fetuses	113	93
Mean of reabsorption sites (%)[b]	0.20 ± 0.13	0.25 ± 0.16
Fetal parameters		
Mean of litter size [a]	11.3 ± 0.91	11.7 ± 0.55
Sex ratio [a]	1.23 ± 0.27	0.89 ± 0.39
Mean number of male per dam [a]	5.40 ± 0.74	4.25 ± 0.86
Mean number of female per dam [a]	5.80 ± 0.81	7.62 ± 0.94
Mean Body weight of fetuses(g) [a]	3.337 ± 0.09	2.726 ± 0.28*
Male [a]	3.439 ± 0.08	2.888 ± 0.29*
Female [a]	3.202 ± 0.13	2.636 ± 0.27*
Mean crown-rump length (cm) [a]	5.534 ± 0.04	5.113 ± 0.22*
Male [a]	5.562 ± 0.05	5.071 ± 0.25*
Female [a]	5.521 ± 0.03	5.083 ± 0.27*

Data shows mean ± S.E.M., where appropriate. [a] Student's unpaired t test. [b] Mann-Whitney test *$p < 0.05$ compared to control group

Effects of S. tubulosa in female rats

Significant increase of food (Figure 1A) and water consumption were observed (Figure 1B). No significant differences were observed in biochemical and anatomo-histological analysis. Serum estradiol concentrations (2.91 ± 0.473; 2.96 ± 0.644, ng.mL^{-1}) as well as progesterone (2.50 ± 0.076; 2.59 ± 0.055, ng.mL^{-1}) did not differ between experimental rats and control group. Other parameters were not affected by plant consumption.

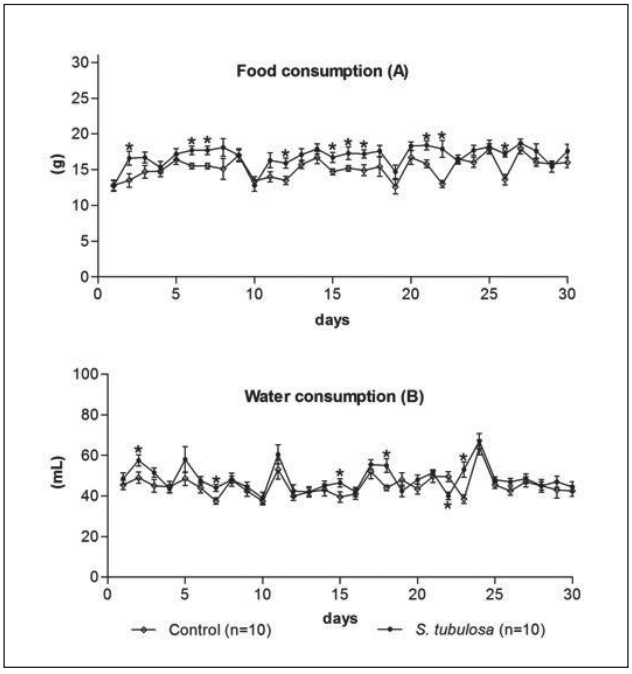

Figure 1 – Food consumption (A) and water consumption (B) of female rats that received 5% of *S. tubulosa* in diet or just chow (control) during 30 days. Data expressed as mean ± S.E.M.*$p < 0.05$, Student's unpaired t test
Source: (ARAÚJO et al., 2010)

Effects of S. tubulosa in female reproductive performance

Food consumption and body weight of the female treated rats were significantly higher than that observed in female control rats (Figure 2A and B). Estrous cycle phases were not affected by plant consumption. 20% of the females treated mated with untreated males did not become pregnant. Additionally, evaluation at day 20 of pregnancy revealed that all fetuses of one experimental rat were dead. The treatment produced increase in placenta weight and number of corpora lutea (Table 3). Other parameters were not affected.

Discussion

The incorporation of *S. tubulosa* in the diet at the level of 5% was chosen to produce a minimal degree of paternal and maternal toxicity once substances present in *S. tubulosa,* such as tannins, exerts biological properties and at high doses could lead to severe nutritional and absorption disorders

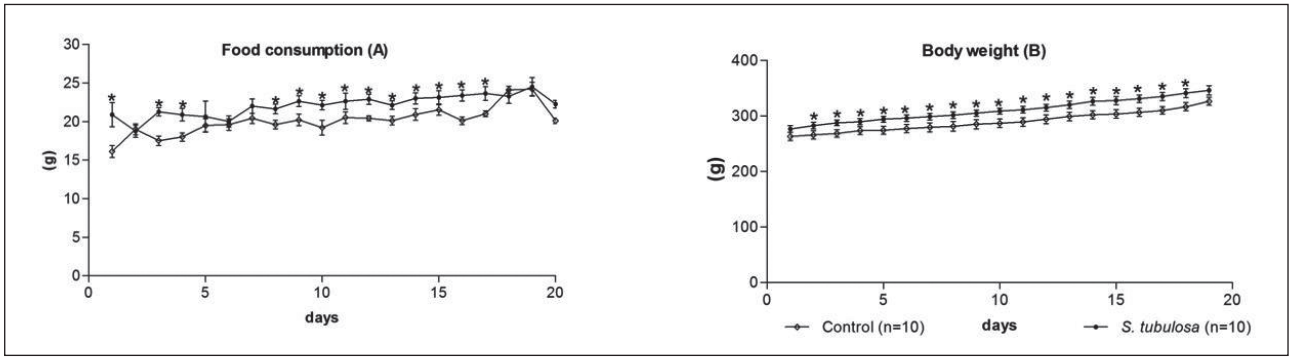

Figure 2 – Food consumption (A) and body weight (B) of female rats that received 5% of *S. tubulosa* in diet or just chow (control) during gestation. Data expressed as mean ± S.E.M. *$p < 0.05$, Student's unpaired t test
Source: (ARAÚJO et al., 2010)

Table 3 – Reproductive performance of the experimental dams that received chow mixed with 5% of *S. tubulosa* pod meal from GD0 to GD20 and control dams receiving commercial diet during the same period – Araguaína – 2010

Parameters	Control	Experimental
Female Reproductive Performance		
Nº of female examined	10	10
Female mating index (%)a	100	100
Female fertility index (%) a	100	80
Female fecundity index (%) a	100	80
Dams		
Gestation index (%) a	100	70
Implantation index (%) a	99.2	92.4
Mean of maternal body weight a	336.2 ± 8.5	354.0 ± 9.2
Mean number of corpora lutea a	12.5 ± 0.54	14.5 ± 0.42*
Mean number of implantations a	12.4 ± 0.54	13.5 ± 0.86
Mean of placental weight a	0.533 ± 0.026	0.649 ± 0.030*
Preimplantation losses (%) b	0.77	7.5
Post-implantation losses (%) b	5.7	21.5
Nº of total dead fetuses/litters affected	0	14
Nº of total live fetuses	117	85
Mean of reabsorption sites (%) b	0.70 ± 0.21	1.12 ± 0.29
Fetal parameters		
Mean of litter size a	11.7 ± 0.59	12.4 ± 1.10
Sex ratio	0.79 ± 0.16	0.93 ± 0.17
Mean number of male per dam a	5.2 ± 0.66	5.5 ± 0.84
Mean number of female per dam a	7.4 ± 0.58	7.0 ± 0.96
Mean Body weight of fetuses(g) a	3.421 ± 0.04	3.796 ± 0.27
Male a	3.529 ± 0.05	3.868 ± 0.26
Female a	3.403 ± 0.06	3.762 ± 0.25
Mean crown-rump length (cm) a	5.377 ± 0.04	5.452 ± 0.13
Male a	5.443 ± 0.04	5.551 ± 0.10
Female a	5.360 ± 0.06	5.446 ± 0.11

Data shows mean± S.E.M., where appropriate. a Student's unpaired t test. b Mann-Whitney test * $p < 0.05$ compared to control group

(MUELLER-HARVEY; MCALLAN, 1992; OECD, 2015). In fact, although fluctuations in water and food consumption were observed in male and female rats treated with *S. tubulosa*, these changes did not affect the weight gain. Paternal exposure to neuroendocrine disruptors, cytotoxic and mutagenic xenobiotics can disturb spermatogenesis and result in pre- and postimplantation losses, low birth weight, retarded growth, behavioral deficits, malformations and high neonatal mortality (COLBORN; VOM SAAL; SOTO, 1994; EPA, 1996; TRASLER; DOERKSEN, 1999). Correspondingly, female reproductive toxicity might be promoted by phytochemical compounds, including those with action in the hypothalamic-pituitary-gonadal axis and estrogen-dependent tissue, disrupting functional equilibrium causing ova expulsion from the uterus and producing interruption of communication among embryo, placenta and fetus, even at lower concentrations (PATTANAYAK; MAZUMDER, 2009; SOLOMON et al., 2010). It has been suggested that antiestrogens and progestin-like substances present in Fabaceae plant extracts such as tannins, flavonoids and alkaloids may act through antigonadotropic mechanisms by induction and competition with estradiol and progesterone receptors in vivo and in vitro (BENIE; THIEULANT, 2003; BENIE; THIEULANT, 2004). In fact, the presence of flavonoids, tannins, saponins and alkaloids in *S. tubulosa* pods reinforce this hypothesis. Toxicological

effects caused by subchronic administration of *S. tubulosa* might be related to direct action of plant toxic compounds on the reproductive system, producing abnormalities in spermatogenesis, suppressing follicular growth in the ovary and/or disrupting hormonal balance affecting implantation, pregnancy and gestation. Thus, *S. tubulosa* long-term consumption is not recommended for phytotherapy or food purposes.

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