

Toxicological and pharmacological evaluation of *Discaria americana* Gillies & Hook (Rhamnaceae) in mice

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Medicinal plants (e.g. *Discaria americana*) have been used by populations for centuries. However, popular use is not enough to validate these plants as safe and effective medicinal products. The present study sought to evaluate the acute and subacute toxicity as well as the anxiolytic and antinociceptive effects of *D. americana* root bark and aerial parts extracts in mice. In acute toxicity studies, mice were treated with single intraperitoneal doses of the aforementioned extracts. Subacute toxicity studies were performed by oral administration of the extracts over 14 days. Anxiolytic studies consisted of the elevated plus maze method, and antinociceptive studies were based on the hot plate test. The LD₅₀ value for *D. americana* aerial parts extract was established at >500 mg/kg, and for the root bark extract, 400 mg/kg. *D. americana* aerial parts extract produced anxiolytic (250 mg/kg) and antinociceptive effects (125, 200 and 250 mg/kg). Conversely, *D. americana* root bark extract showed neither anxiolytic nor antinociceptive effects in mice.

Descriptors: *Discaria americana*/pharmacognosy. Medicinal plants. *Discaria americana*/toxicological analysis. *Discaria americana*/acute toxicity. *Discaria americana*/subacute toxicity. *Discaria americana*/anxiolytic effect. *Discaria americana*/antinociceptive effect.

As plantas medicinais (*i. e. Discaria americana*) têm sido utilizadas pela população por séculos, entretanto, o conhecimento popular não é suficiente para validá-las como medicamentos seguros e/ou efetivos. Assim, o presente estudo teve por objetivo avaliar a toxicidade aguda e subaguda, bem como o efeito ansiolítico e antinociceptivo dos extratos da casca da raiz e das partes aéreas da *D. americana* em camundongos. A toxicidade aguda foi avaliada pela administração dos extratos, via intraperitoneal. Para o estudo da toxicidade subaguda os animais foram tratados oralmente com os extratos por 14 dias. O efeito ansiolítico dos extratos foi determinado através do modelo do labirinto em cruz elevado e o efeito antinociceptivo, mediante o teste da placa quente. O valor da DL₅₀ para o extrato das partes aéreas da *D. americana* foi definido como > 500 mg/kg, enquanto que para o extrato da casca da raiz foi estabelecido em 400 mg/kg. O extrato das partes aéreas da *D. americana* apresentou atividade ansiolítica (250 mg/kg) e antinociceptiva (125, 200 e 250 mg/kg). O extrato da casca da raiz da *D. americana* não apresentou efeito ansiolítico nem antinociceptivo.

Unitermos: *Discaria americana*/farmacognosia. Plantas medicinais. *Discaria americana*/análise toxicológica. *Discaria americana*/toxicidade aguda. *Discaria americana*/toxicidade subaguda. *Discaria americana*/efeito ansiolítico. *Discaria americana*/efeito antinociceptivo.

INTRODUCTION

Medicinal plants have been used by populations for centuries. Even now, many plants play a key role in world health (Calixto, 2000). However, popular use is not enough to validate these plants as safe and effective medicines

(Agra *et al.*, 2007). Toxicity studies are required to evaluate the toxicity levels and adverse effects associated with medicinal plants. In addition to toxicity studies, study of the pharmacological activity of these plants is also important.

Discaria americana Gillies & Hook is a shrub from the Rhamnaceae family. This plant is known by the common names “Quina-do-Brasil” and “Quina-do-Rio-Grande” (Brazil) or “Coronillo del campo” and “Quina del campo” (Uruguay) (Correa, 1984). *D. Americana* root bark is used by local populations against stomach and

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skin diseases, diabetes, fever (antipyretic), and as a tonic (Záchia, Moraes, 1999). However, almost no studies have assessed the toxicological and pharmacological properties of this plant; only one report, describing strong antioxidant activity of the essential oil extracted from the aerial parts of *D. americana* (Rodríguez, Murray, 2008), was located by a review of the literature.

The main chemical constituents of *D. americana* are pentacyclic triterpenes and steroids (Giacomelli, 2005). An important point is the presence of the triterpene ursolic acid, which has showed antioxidant (Ramachandram, Prasad, 2008), antinociceptive, anti-inflammatory (Tapondjou *et al.*, 2003) and anti-cancer activities (Shao *et al.*, 2011). The steroid β -sitosterol showed antioxidant (Yokota *et al.*, 2006), antinociceptive (Cechinel Filho, Yunes, 1998), anti-cancer (Jourdain *et al.*, 2006) and anti-inflammatory effects (Valeiro, Awad, 2011). Furthermore, betulinic acid, a triterpene, has known anti-cancer, antinociceptive, anti-inflammatory, anti-HIV and antimicrobial activities (Yogeeswari, Sriram, 2005), as well as anxiolytic effects (Durst *et al.*, 2002).

Cyclopeptide alkaloids and cyclopeptides (Giacomelli *et al.*, 2004; Giacomelli, 2005) have also been isolated from *D. americana*. However, almost nothing has been reported on the toxic and pharmacological effects of these compounds. Among the alkaloids, frangulanine has a sedative effect, whereas discarine A and discarine B reportedly show antibacterial activity (Maldaner, 2005).

Some Rhamnaceae plants have been reported to show high levels of toxicity and adverse effects. Liver and lung damage and death have been observed in mice given *Karwinskia humboldtiana* (Bermudez *et al.*, 1986). This plant also caused a decrease in ATP levels in the kidney and blood (Jaramillo-Juárez *et al.*, 2005) and central nervous system damage in rats (Becerra-Verdin *et al.*, 2009). *Rhamnus cathartica*, another plant from the same family, is known to be hepatotoxic in mice (Lichtensteiger *et al.*, 1997). Therefore, according to the toxicity shown by other Rhamnaceae, evaluation of the toxicity of *D. americana* is paramount.

Thus, the present study sought to evaluate the acute and subacute toxicity of *D. americana* aerial parts and root bark extracts, as well as the potential anxiolytic and antinociceptive effects of these extracts, in mice.

MATERIAL AND METHODS

Plant material

D. americana root bark and aerial parts were collected in Jaguari, RS, Brazil (29° 49' 70" S, 54° 69' 00" W) and authenticated by Dr. Renato Záchia, Department of

Botany, Universidade Federal de Santa Maria, RS, Brazil, where a specimen sample (SMDB 2688) is retained.

Chemicals

Dimethyl sulfoxide (DMSO) and methanol (MeOH) were obtained from VETEC Química Fina LTDA® (Rio de Janeiro, RJ, Brazil). Kits for clinical chemistry analysis of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were obtained from Labtest Diagnóstica S/A® (Lagoa Santa, MG, Brazil). Paracetamol was obtained from Labsynth® (Diadema, SP, Brazil). Diazepam (DZP) was obtained from União Química Farmacêutica Nacional S/A® (Jabaquara, SP, Brazil).

Preparation and identification of *D. americana* extracts

Dried ground root bark (1080 g) and aerial parts (1200 g) of *D. americana* were extracted with methanol (MeOH) (6 L) in a Soxhlet apparatus for 12 h. The resulting MeOH extracts were filtered and concentrated under vacuum, yielding the crude residues of the plant root bark and aerial parts (2.79 and 0.69% yield, respectively). The extracts were dissolved in dimethyl sulfoxide (DMSO) for administration in mice.

Four cyclopeptides were isolated from the root bark of *D. americana* (discarene C, discarene D, discarine M and discarine N), as well as nine cyclopeptide alkaloids (adoutine Y, adoutine Y', franganine, frangulanine, discarine A, discarine B, discarine C, discarine D and myrianthine A), betulinic acid, and three steroids (β -sitosterol, β -sitosterol-3-O-6- β -D-glucopyranoside and β -sitosterol-3-O-6-stearoyl- β -D-glucopyranoside). The steroids β -sitosterol and β -sitosterol-3-O-6- β -D-glucopyranoside, three pentacyclic triterpenes (betulinic acid, ursolic acid and ceanotic acid) and an ester derived from ferulic acid were isolated from the plant aerial parts. The isolated compounds were identified by direct comparisons with authentic samples by thin layer chromatography (TLC) and on the basis of NMR spectral data. TLC was performed on precoated silica gel 60 F₂₅₄ plates (Merck®) (mobile phase: mixture of CHCl₃ and MeOH) and detection was achieved by UV light (254 nm), by spraying with Dragendorff's reagent, and by spraying with 10 % H₂SO₄, followed by heating (Giacomelli *et al.*, 2004; Giacomelli, 2005).

Animals

Healthy mice (*Mus musculus*) of either sex (1:1), weighing 35-45 g, obtained from our own breeding fa-

cilities, were kept in an air-conditioned room (22 ± 2 °C), under a 12-hour light/dark cycle (lights on at 8:00 a.m.), with water and food (Nuvital® chow, PR, Brazil) *ad libitum*. A total sample of 270 mice was used for toxicity and pharmacology studies. For each test, animals were divided into groups of 10 animals. The experiments were carried out with the approval of the Universidade Regional Integrada do Alto Uruguai e das Missões Research Ethics Committee (protocol no. 023/07).

Acute toxicity studies

Groups of 10 mice were injected with single doses of *D. americana* root bark extract (50, 100, 300, 400 and 500 mg/kg), plant aerial parts extract (500 mg/kg) and DMSO (control). The dose of 500 mg/kg was established as the highest dose of the plant extract for determination of LD₅₀. Mice were injected intraperitoneally, as this route of administration is associated with rapid absorption, thus enabling evaluation of exposure during a short period of time.

Signs of general toxic effects, such as weight loss, seizures, or death, were observed continuously for 2 h and then monitored over a 48-hour period. After 48 h, animals were euthanized and necropsied for gross observation of the heart, kidneys and liver, as well as blood sample collection. The study was carried out as by Maciel *et al.* (2000), with the exception of exposure time (48 hours).

Subacute toxicity studies

D. americana root bark extract (50 and 100 mg/kg) and aerial parts extract (300 and 400 mg/kg) were administered to groups of 10 mice by once-daily gavage for 14 days. Control group animals received the vehicle (DMSO) under the same conditions. Toxic manifestations, such as seizures, weight loss, and death, were monitored daily. At the end of the 14-day period, animals were euthanized and necropsied for macroscopic observation of the heart, kidneys and liver, as well as blood sample collection. Again, the study was carried out as by Maciel *et al.* (2000), except for the route of administration.

Blood analysis

Blood was collected in heparinized tubes and used immediately. Tubes were centrifuged at 3000 rpm for 10 min to obtain serum, which was then analyzed. Transaminase (AST and ALT) activity was determined enzymatically as described by Bergmeyer (1978), using commercial kits from Labtest Diagnóstica S. A® in a semi-automatic LABQUEST® biochemical analyzer.

Evaluation of anxiolytic effect

The elevated plus maze (EPM) method was used to assess the anxiety level of mice. The maze consists of two opposed open arms and two opposed enclosed arms, bounded by 30-cm high walls. Each arm measures 50 x 10 cm, with a shared central area of 10 x 10 cm. The maze is kept 50 cm above the floor.

Single doses of *D. americana* root bark extract (50, 100 and 200 mg/kg), aerial parts extract (125 and 250 mg/kg), DMSO (placebo control) and diazepam 1.5 mg/kg (positive control) were administered intraperitoneally to groups of 10 mice.

One hour after treatment, mice were placed on the central area of the maze. The number of entries into the enclosed and open arms was recorded, as was the time spent in each arm. Time spent in the open arms was considered as the measure of anxiolytic effect (Pellow *et al.*, 1985).

Evaluation of antinociceptive effect

The experiment started 60 min (time zero) after intraperitoneal administration of *D. americana* root bark extract (50, 100 and 200 mg/kg) and aerial parts extract (125, 200 and 250 mg/kg) to groups of 10 mice. Animals were placed individually on a hot plate kept at a constant temperature of 55 °C. The time to first paw lick (henceforth “latency”) was recorded. Measurements were performed at 0, 15, 30 and 60 min after the first thermal stimulus. To avoid damage to the animals’ paws, the maximum time spent on the plate was 30 s. Therefore, a latency period of 30 s was defined as complete antinociception (Ankier, 1974). For comparison, the same experiment was conducted with paracetamol 400 mg/kg (positive control) and DMSO (control).

RESULTS AND DISCUSSION

Acute toxicity

To assess acute toxicity of *D. americana*, mice were injected with aerial parts and root bark extracts and with DMSO (control). During the experiment, the occurrence of adverse effects and the mortality of mice were observed. The maximum dose of 500 mg/kg of *D. americana* aerial parts extract did not cause death in mice; hence, the LD₅₀ value was established as >500 mg/kg (Table I).

According to Saraswat *et al.* (1996), ursolic acid, a triterpene constituent of *D. americana*, has hepatoprotective activity. However, acute administration of the plant aerial parts extract at the dose of 500 mg/kg caused

TABLE I - Effect of acute and subacute administration of *D. americana* extracts on mortality in mice. *N*=10

Treatments	Dose (mg/kg)	Mortality (%)
<i>Acute toxicity</i>		
Control	-	0.0
Root bark extract	50	0.0
	100	0.0
	300	40.0
	400	50.0
	500	60.0
Aerial parts extract	500	0.0
<i>Subacute toxicity</i>		
Control	-	0.0
Root bark extract	50	10.0
	100	20.0
Aerial parts extract	300	0.0
	400	20.0

a significant increase in transaminase (ALT and AST) levels (Table II). High serum levels of these enzymes are a marker of injury, usually liver damage. Furthermore, autopsy of these animals revealed a variable number (1 to 4) of opalescent oval lesions, approximately 0.5 cm in diameter, on the liver. These hepatic lesions were observed in 70% of animals.

TABLE II - Effect of acute administration of *D. americana* extracts on serum transaminase levels (U/L) in mice

Treatments	Dose (mg/kg)	ALT (SGPT)	AST (SGOT)
Control	-	52.4 ± 6.8	363.6 ± 28.6
Root bark extract	50	104.4 ± 33.6*	359.2 ± 57.9
	100	145.7 ± 6.9*	364.0 ± 40.9
	300	186.0 ± 38.5*	348.7 ± 41.8
	400	-	516.0 ± 80.4*
Aerial parts extract	500	101.4 ± 34.9**	425.8 ± 60.4**

Values are expressed as mean ± standard deviation. *N*= 4-10. (*) Statistically different from controls, *p*<0.05 (ANOVA/Tukey's test). (**) Student's *t* test.

Acute administration of *D. americana* root bark extract caused mortality proportional to the administered doses. At the highest dose level (500 mg/kg), the mortality rate was 60%. The LD₅₀ was established as 400 mg/kg. At this dose, there was a significant increase in AST levels.

At 300 mg/kg, 40% of mice died, and half of those that survived showed evidence of liver injury and significantly increased ALT levels. At the dose of 50 and 100 mg/kg, there was no mortality, but ALT levels were increased as compared to mice in the control group.

The weight of mice that survived acute exposure was evaluated 48 h after administration of the extracts. There was no significant weight loss in mice treated with *D. americana* extracts as compared to the control group. Furthermore, there was no significant difference in liver weight/body weight of mice treated with *D. americana* in relation to the control group (Table III).

TABLE III - Effect of acute and subacute *D. americana* exposure on weight and liver weight/body weight of mice

Treatments	Dose (mg/kg)	Initial weight (%)	Liver weight/body weight (%)
<i>Acute toxicity</i>			
Control	-	105.2 ± 7.3	5.01 ± 0.90
Root bark extract	50	95.0 ± 3.0	4.90 ± 0.23
	100	93.0 ± 6.0	5.64 ± 1.40
	300	90.6 ± 9.6	5.85 ± 0.87
	400	92.9 ± 2.5	6.37 ± 0.37
	500	88.0 ± 8.0	5.61 ± 0.13
Aerial parts extract	500	93.0 ± 8.1	5.27 ± 0.79
<i>Subacute toxicity</i>			
Control	-	104.0 ± 5.2	5.10 ± 0.49
Root bark extract	50	97.8 ± 4.4	4.86 ± 0.41
	100	91.5 ± 5.7*	5.30 ± 0.80
Aerial parts extract	300	105.2 ± 7.3	4.84 ± 0.52
	400	91.6 ± 7.2*	5.00 ± 0.61

Values are expressed as mean ± standard deviation. *N*= 4-10.

(*) Statistically different from controls, *p*<0.05 (ANOVA/Tukey's test).

No macroscopic changes were observed in the kidneys and hearts of mice treated with *D. americana* extracts. Furthermore, there was no significant difference in kidney weight or heart weight/body weight of mice treated with *D. americana* in relation to the control group. No seizure activity was observed in acute toxicity studies. There were no deaths or adverse effects in the control group.

Subacute toxicity

For assessment of subacute toxicity, *D. americana* extracts and DMSO were orally administered to mice for

14 days. General toxic effects and mortality were observed during the treatment period. At a dose of 400 mg/kg, the aerial parts extract caused 20% mortality (Table I), moderate weight loss (Table III), and a significant increase in ALT and AST levels (Table IV). At 300 mg/kg, there were no deaths or significant weight loss, although ALT and AST levels did increase.

TABLE IV - Effect of subacute administration of *D. americana* extracts on serum transaminase levels (U/L) in mice

Treatments	Dose (mg/kg)	ALT (SGPT)	AST (SGOT)
Control	-	88.7 ± 18.2	360.2 ± 65.7
Root bark extract	50	119.0 ± 25.7	482.1 ± 46.3*
	100	169.3 ± 40.2*	491.5 ± 31.0*
Aerial parts extract	300	128.8 ± 33.7*	493.8 ± 72.8*
	400	191.4 ± 43.4*	516.0 ± 33.2*

Values are expressed as mean ± standard deviation. *N* = 8-10. (*) Statistically different from controls, *p* < 0.05 (ANOVA/Tukey's test).

At a dose of 100 mg/kg, *D. americana* root bark extract led to significant weight loss and increased ALT and AST levels, as well as a 20% mortality rate. A dose of 50 mg/kg was associated with 10% mortality and increased serum AST levels, but no significant weight loss.

The greater toxicity of *D. americana* root bark extract as compared to the aerial parts extract can be attributed to the alkaloids present in the root bark. A review of the literature showed that some of these alkaloids have known toxic effects in mice (Tabosa *et al.*, 2006). Furthermore, some alkaloids are toxic – mainly hepatotoxic – in humans (Bolzan *et al.*, 2007).

No macroscopic changes were observed in the liver, kidneys and heart of mice treated with *D. americana* extracts. There were no significant differences in the ratio of weight of these organs to body weight in mice treated with *D. americana* as compared to the control group. No seizure activity was observed in subacute toxicity studies. There were no deaths and no adverse effects in the control group.

Although important parameters were evaluated in these acute and subacute toxicity studies, further assessments are needed before intake of *D. americana* can be considered safe in humans.

Pharmacology

This study evaluated the anxiolytic effect of *D. americana* in mice using the elevated plus maze (EPM)

method. The potential antinociceptive effect of the plant was evaluated with the hot plate test. No toxic doses or doses that showed lower toxicity effects were used in the pharmacology portion of this study. These doses were determined according to acute toxicity studies. For *D. americana* root bark extract, 50 mg/kg (1/8 LD₅₀), 100 mg/kg (1/4 LD₅₀) and 200 mg/kg (1/2 LD₅₀) doses were used. The aerial parts extract of *D. americana* showed a LD₅₀ of >500 mg/kg. At this dose, toxic manifestations were observed; therefore, half of this dose (250 mg/kg) was used for pharmacology studies. The 125 and 200 mg/kg doses were also used in pharmacological studies in order to evaluate the possibility of a dose-dependent effect.

In the anxiolytic studies, the control group spent most of the 10 min in the enclosed arms of the EPM, indicating anxiety. Data showed an increase in time spent in the open arms among mice treated with *D. americana* aerial parts extract at the dose of 250 mg/kg. This reduction in anxiety could be associated with ursolic acid, an important component of the aerial parts of *D. americana* that has known anxiolytic properties (Durst *et al.*, 2002). A similar effect occurred in the positive control group (DZP 1.5 mg/kg). *D. americana* root bark extract had no anxiolytic effect (Table V).

TABLE V - Time spent in open arms of the elevated plus maze after exposure to *D. americana* extracts

Treatments	Dose (mg/kg)	Time spent in open arms (%)
Control	-	23.0 ± 7.4
	50	42.2 ± 12.8
Root bark extract	100	37.8 ± 14.5
	200	42.6 ± 13.7
Aerial parts extract	125	39.1 ± 13.8
	250	72.0 ± 18.1*
Diazepam	1.5	81.8 ± 22.8*

Values are expressed as mean ± standard deviation. *N* = 10. (*) Statistically different from controls, *p* < 0.05 (ANOVA/Tukey's test).

Antinociceptive studies revealed that mice treated with *D. americana* aerial parts extract at a dose of 125 mg/kg had a significant reduction in nociceptive response at 0 and 15 s. The 200 mg/kg dose caused a significant increase in latency at all periods analyzed. At a 250 mg/kg dose, the nociceptive response was > 30 s. A trend toward dose-dependent increase in the analgesic effect of *D. americana* aerial parts extract was observed. This striking antinociceptive potential of *D. americana*

TABLE VI - Effect of *D. americana* extracts on hot plate test latency in mice

Treatments	Dose (mg/kg)	Latency (s)			
		0	15	30	60
Control	-	12.7 ± 5.6	13.5 ± 5.2	18.2 ± 7.6	22.0 ± 3.3
Root bark extract	50	18.7 ± 5.1	17.0 ± 3.7	29.0 ± 2.0	28.5 ± 2.3
	100	25.7 ± 4.9	23.0 ± 5.5	26.2 ± 7.5	27.5 ± 5.0
	200	24.2 ± 11.5	18.2 ± 9.8	27.0 ± 3.8	29.5 ± 1.0
Aerial parts extract	125	28.7 ± 2.5*	29.7 ± 0.5*	21.0 ± 2.0	26.0 ± 5.2
	200	29.5 ± 0.5*	28.7 ± 1.8*	29.5 ± 0.5*	30.0 ± 0.0*
	250	30.0 ± 0.0	30.0 ± 0.0	30.0 ± 0.0	30.0 ± 0.0
Paracetamol	400	30.0 ± 0.0	30.0 ± 0.0	30.0 ± 0.0	30.0 ± 0.0

Values are expressed as mean ± standard deviation. *N*=10.

(*) Statistically different from controls, *p*<0.05 (ANOVA/Tukey's test).

can be ascribed to three components that are present in the plant: the triterpenes ursolic acid (Tapondjou *et al.*, 2003) and betulinic acid (Yogeeswari, Sriram, 2005) and the steroid β -sitosterol (Cechinel Filho, Yunes, 1998), which are known antinociceptive agents. Pretreatment of animals with paracetamol (400 mg/kg), the positive control used in this study, also caused a significant antinociceptive effect (nociceptive response >30 s). Conversely, *D. americana* root bark extract did not reduce the nociceptive response in mice (Table VI).

In conclusion, the present study showed that *D. americana* root bark extract produces toxic effects at low doses and was not successful as an anxiolytic or antinociceptive agent. Conversely, an aerial parts extract of the same plant showed low toxicity even when administered at the highest doses, and successfully reduced anxiety and nociception in mice. However, to protect human health, further toxicity studies are necessary if this plant is to be used as a herbal remedy. On the basis of the findings reported herein, *D. americana* can be considered a promising source for development of new anxiolytic and analgesic agents.

ACKNOWLEDGMENTS

The authors would like to thank the National Council for Scientific and Technological Development (CNPq), the Rio Grande do Sul State Department of Science and Technology (SCT), and Fundação Regional Integrada do Alto Uruguai e das Missões (FURI) for their financial support.

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Received for publication on 17th August 2011

Accepted for publication on 13th March 2012