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Acute toxicity study of the isomeric mixture of alpha and beta amyrin from *Protium heptaphyllum* (Aubl.) Marchand

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ABSTRACT. The isomeric mixture of alpha and beta amyrin (AMY), present in the resin of *Protium heptaphyllum*, is popularly used as anti-inflammatory and anti-ulcer. The literature has been demonstrating pharmacological activities of these triterpenes in the central and peripheral nervous systems, and in the gastrointestinal and immunological systems. This study traces a toxicological profile of amyrin, aiming to provide information that may clarify its safety. Nine female Wistar rats (170 to 200 g) were divided into three groups of three animals each (control, amyrin 300 and amyrin 2000 mg kg^{-1}, p.o.), which were evaluated by protocols preconized by the Organization for Economic Co-operation and Development (OECD). Open field Test and Malone Hippocratic Screening Scale were performed. AMY, mostly at 2000 mg kg^{-1}, reduced the number of crossings by 57% *vs*. saline (22.67 ± 2.40) and the number of rearing by 53% vs. saline (42.67 ± 2.96), but increased the number of grooming by 26% *vs*. saline (1.66 ± 0.33). AMY (2000 mg kg^{-1}) increased the serum glucose by 77% *vs*. saline (126.70 ± 4.33 mg dL^{-1}), triglycerides by 50% *vs.* saline (78.67 ± 2.18 mg dL^{-1}) and uric acid by 65% *vs.* saline (0.73 ± 0.03 mg dL^{-1}). AMY induced vascular congestion and hemorrhage in the liver, spleen and cerebral cortex. Renal changes (cellular damage, inflammatory infiltrate, tubular protein deposition and glomeruli atrophy) were also seen. In conclusion, AMY decreased rat locomotor activity, caused minor biochemical changes, and altered the morphology of the kidney. The present study may contribute to deepen the knowledge about the safety of AMY, aiming the development of a novel pharmacological product.

Keywords: toxicological evaluation; Burseraceae; triterpenes; morphological analysis; behavioral response.

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Introduction

Medicinal plants are used worldwide to treat many diseases, and the research for new drugs of plant origin continue to be developed. Its preparations can be formulated into many forms, including liquids, that have been used for many years (Gupta, Bleakley, & Gupta, 2008). The misuse of phytotherapies or the use of medicinal plants without scientific basis that adequately support conclusions of safety and efficacy are potentially dangerous to humans (Mir, Sexena, & Malla, 2013). As the chemical composition of medicinal plants is complex, some moderate to severe side effects may arise from its use. Therefore, it is important to establish the medicinal plant safety using well-controlled and scientifically validated toxicity protocols, since these studies provide information on toxic doses and therapeutic index of drugs and xenobiotics (Rahman et al., 2014). In addition, prior to any novel medicine administration to humans, its safety must be investigated in animals (Robinson et al., 2009).

Protium heptaphyllum Aubl. (Burseraceae), known as 'almécega', 'breu branco' or 'almíscar', is a plant originated in South America, easily found in the Amazon forest and known to produce an amorphous resin composed by α- and β-amyrin (AMY), taraxastan-3-oxo-20-ol and sitostenonein. The triterpenes α- and β-AMY from *P. heptaphyllum* exhibit pharmacological activities in several systems (Nogueira, Oliveira, Adjafre, Moraes, & Aragão, 2019). These activities include gastroprotective effects (Oliveira et al., 2004), antinociceptive properties (Oliveira et al., 2005a, Holanda, Pinto, Guedes, & Cunha, 2008), hepatoprotective effects (Oliveira et al., 2005b), anxiolytic and antidepressant effects (Aragão et al., 2006), anti-inflammatory (Aragão, Pinheiro, Bandeira, Lemos, & Viana, 2007, Melo et al., 2011), antihyperglycemic and hypolipidemic effects (Santos et al., 2012) and anticonvulsant activity (Aragão et al., 2015).

Despite of the wide pharmacological effects demonstrated for these triterpenes, no study has been performed on its oral toxicity, which would provide proper safety information. Thus, the aim of the current study was to evaluate for 14 days the safety of the *per oral (p.o.)* treatment with a single dose in rats with the isomeric mixture of α- and β-AMY.

Materials and methods

Collection of botanical material

The resin (20 g) was collected from the wild tree *P. heptaphyllum* in Crato - CE, Brazil by incisions of the stem of the plant submitted to cutting. The plant was identified by the Prof. Dr. Afrânio G. Fernandes and a voucher specimen (Nº 28509) deposited in the Herbarium Prisco Bezerra of the *Universidade Federal do Ceará* (UFC). *Protium heptaphyllum* was firstly described by Vidensk, Meddel, Naturhist, Foren, & Kjøbenhavn, 1873.

Isolation and characterization of α- and β-amyrin

The secreted material was stored in a dark flask at room temperature, and then it was washed in water to remove fragments and fractionated by chromatography (silica gel column) with hexane, chloroform, ethyl acetate and methanol. The fractions obtained with chloroform (5.2 g) were repeatedly chromatographed on silica gel and eluted with increasing amounts of hexane-ethyl acetate (1:1). Fractions were analyzed by thin-layer chromatography (TLC), giving 450 mg of α- and β-amyrin (Figure 1). The fraction rich in α- (67%) and β-amyrin (33%) was submitted to further purification. The identification of these isomers was performed by infrared spectrophotometry (KBr) Vmax cm^{-1} (3300, 1480 and 1050), ^{1}H-NMR (500 MHz, $CDCl_3$), ^{13}C (125 MHz, $CDCl_3$), melting point (179-181°C) (Mahato & Kundu, 1994). The final product, a mixture of α-AMY and β-AMY, was a white amorphous powder with slight odor. It had low solubility in water, but was soluble in organic solvents. The product was used immediately after dilution.

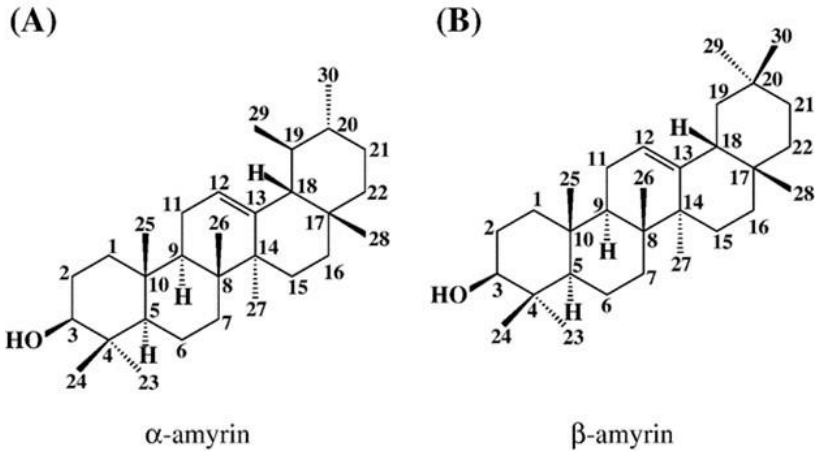

Figure 1. Chemical structure of α-amyrin (A) and β-amyrin (B).

Chemicals and reagents

Ketamine and xylazine were obtained from Sigma (St. Louis, MO, USA) and clinical diagnostic kits from Labtest Diagnóstica S.A., Lagoa Santa, MG, Brazil. All other chemicals and reagents used were of analytical grade.

Animals

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies (Tveden-Nyborg, Bergmann & Lykkesfeldt, 2018). The ethical standards followed the Directive 86/609/EEC, 'European convention for the protection of vertebrate animals used for experimental and other scientific purposes', 1986, and the 'Guiding principles in the use of animals in toxicology' and 'Guide for the care and use of laboratory animals'. For the acute oral toxicity test, 9 female Wistar rats, divided into 3 groups of 3 animals each (170-200 g), 5-6 weeks of age, were maintained with free access to water and food at 22-26°C, 12/12 hours light/dark cycle. The experimental protocols were approved by the Animal Care and Use Committee of the *Universidade Estadual do Ceará* (nº 2591767/2017).

Experimental design

The acute toxicity test was performed following the guidelines of the Organization for Economic Co-operation and Development (OECD-423) for testing of chemicals (Organization for Economic Co-operation and Development [OECD], 2002). Nine female rats were randomized into three groups (n=3 per group) and treated *per oral* with AMY (300 or 2000 mg kg^{-1}) diluted in 0.5% Tween 80 (25 µL kg^{-1}) or vehicle (0.9% NaCl + 0.5% Tween 80, 0.05 mL 10 g^{-1} body mass). These doses were chosen since most of the studies of amyrin pharmacological activities used the rage of 3 and 200 mg (Nogueira et al., 2019).

The animals were observed for mortality and clinical signs of toxicity (general behavior, respiratory pattern, cardiovascular signs, motor activities, muscle tone, reflexes and changes in skin and fur texture) at 30 min., 1, 2 and 4 hours after drug administration, and daily along 14 days. On day 15, after 4 hours of fasting (water allowed), the animals were anesthetized, and blood samples were collected by retro-orbital puncture in heparinized and non-heparinized tubes for hematological and biochemical analysis, respectively.

After blood collection, the animals were euthanized using CO_2 followed by cervical dislocation. The brain, liver, kidney and spleen were weighed and then submitted to pathological examination.

Behavioral tests (open field, Malone hippocratic screening)

For the Open Field test, rats were individually placed in the open-field apparatus, consisting of an acrylic box (100 x 50 x 90 cm) with the floor divided into 9 squares. The number of squares crossed with all paws (crossing), rearing and grooming was counted during 6 min (Archer, 1973). Animals were treated with vehicle or AMY 60 min before evaluation and submitted to the same test at the 7 and 14^{th} days.

For the Hippocratic screening test, rats treated with AMY were observed for mortality, behavioral changes and visible signs of toxicity in an open field apparatus during 2 min. after 5, 15, 30, 60, 120 and 240 min. of administration. The clinical signs of toxicity (general behavior, respiratory pattern, cardiovascular signs, motor activities, muscle tone, reflexes, changes in skin and fur texture, and mortality were also evaluated once a day, for a period of 14 days. The intensity of the events was classified as absent (-), rare (+), discrete (++), moderate (+++) and intense (++++) (Malone & Robichaud, 1962, Malone, 1977).

The body weight of animals was recorded immediately before AMY administration and again, at the end of each week. The percentage of body weight change was calculated according to the following Equation 1:

$$\frac{\text{Body weight at the end of each week} - \text{initial body weight x } 100}{\text{Initial body weight}} \quad (1)$$

Biochemical analysis

Plasma samples were centrifuged (3500 rpm, 10 min.) for analysis of the following parameters: glucose, total cholesterol, triglycerides, total protein, albumin, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphate, bilirubin, creatinine, amylase, urea and uric acid (Labtest, diagnostic kits, Brazil).

Hematological analysis

Hemoglobin, red blood cells, hematocrit, platelets, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBC), neutrophils, lymphocytes, monocytes, and eosinophils were quantified by automated hematology analyzer (SDH-3, Labtest).

Histopathological analysis

Brain, liver, kidney and spleen were removed from the euthanized rats, fixed in 10% buffered formaldehyde for 24 hours and transferred to 70% ethanol. After tissue processing, 5-µm median-sagittal paraffin sections were stained with hematoxylin-eosin (H&E). The slides were observed under light microscopy (Nikon Microscope Eclipse Nis, Software Nis 4.0) and the magnified images of tissues structures were captured. The relative organ's weight (ROW) was calculated and recorded in proportion to the body weight according to the following Equation 2:

$$\frac{\text{ROW: Absolut organ weight X } 100}{\text{Body weight at sacrifice}} \quad (2)$$

Statistical analysis

Results are represented as mean ± S.E.M and analyzed by ANOVA and Tukey posttest (Prism 7.0 GraphPad Software Inc., California, USA). Values of $p<0.05$ were considered significant.

Results

Effects of amyrin in the open field test

After 60 min. of treatment, AMY reduced the number of crossings by 33% at 300 mg kg^{-1} (35.33 ± 3.84 *vs.* saline: 52.67 ± 6.22) and by 57% at 2000 mg kg^{-1} (22.67 ± 2.40) (Figure 2A). AMY also reduced the number of rearing by 43% at 300 mg kg^{-1} (24.33 ± 2.33) and by 53% at 2000 mg kg^{-1} (20.00 ± 2.51), when compared to saline (42.67 ± 2.96) (Figure 2B). However, the number of grooming was increased by AMY at 2000 mg kg^{-1} (6.33 ± 0.33 *vs*. saline: 1.66 ± 0.33) (Figure 2C). The analysis of days 7 and 14 post treatment showed no alterations on these parameters. AMY (mg kg^{-1})

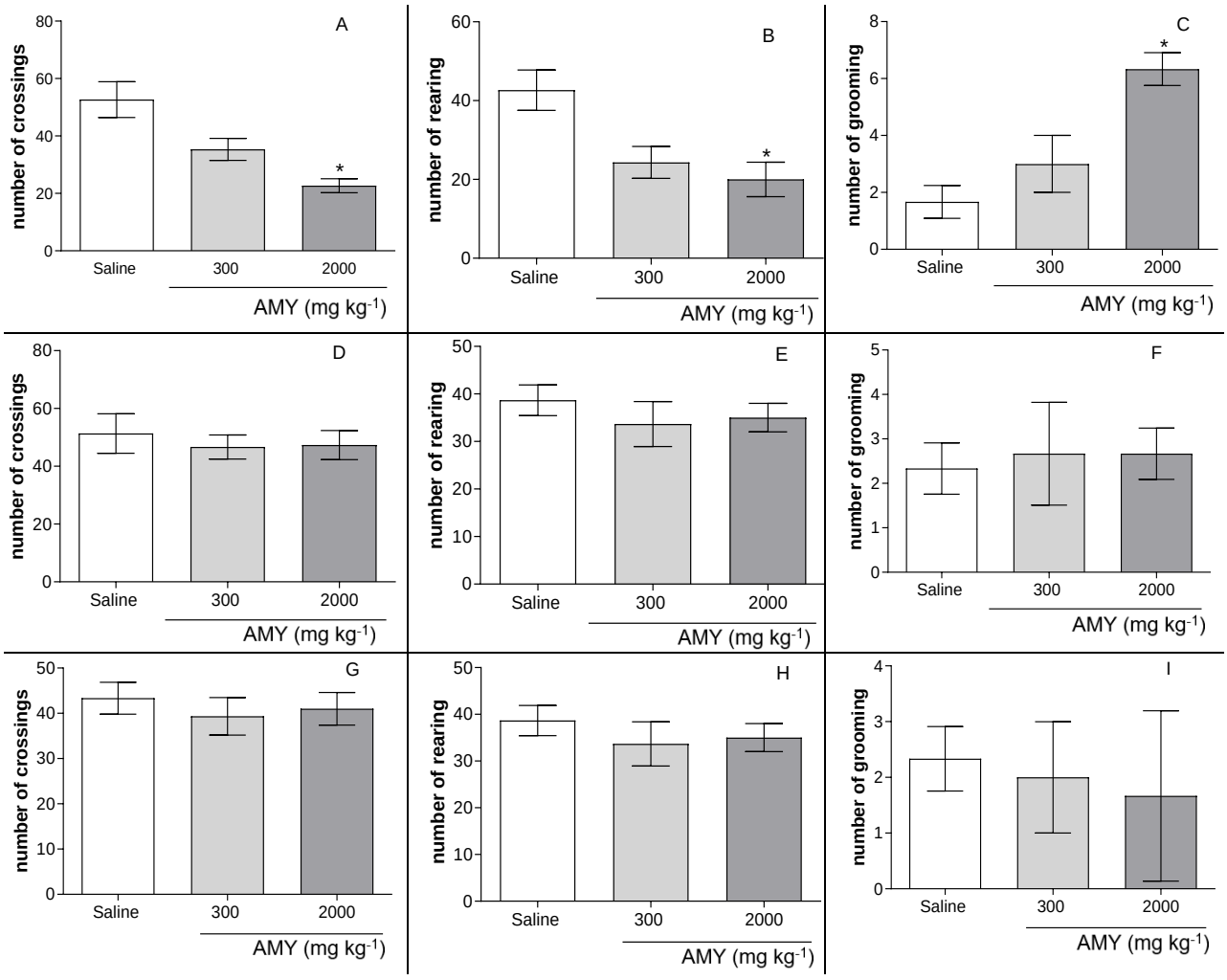

Figure 2. Effect of the isomeric mixture of α- and β-amyrin (AMY) in the Open Field Test. Rats received *per oral* vehicle (0.9% NaCl + 0.5% Tween 80, 0.05 mL 10 g^{-1} body mass), AMY (300 mg kg^{-1}) or AMY (2000 mg kg^{-1}) for evaluation of the number of crossing (A), rearing (B) or grooming (C) 60 min. after AMY (300 mg kg^{-1} or 2000 mg kg^{-1}, *p.o.*) and after 7 (D, E and F) and 14 (G,H and I) days of administration. Mean ± S.E.M (n= 3) *p<0.05 *vs.* Saline (ANOVA and Tukey test).

Effect of amyrin in the Malone hippocratic scale

AMY caused no significant behavioral alterations on the parameters evaluated in the Malone scale 30 minutes after treatment. In the central nervous system, the number of grooming was increased within the first hours after AMY, especially at 2000 mg kg^{-1}, but not within days. In the autonomic nervous system, only the exploratory activity was reduced in the first hours (Table 1).

Table 1. Effect of the isomeric mixture of α- and β-amyrin (AMY) in rat behavioral parameters: Malone Hippocratic scale.

Parameters	Groups (n=3)	Time (min.) 30	60	120	180	240	Time (Days) 2	3	4	5	6	7	8	9	10	11	12	13	14
Autonomic Nervous System																			
Breathing	R1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R2	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-
	R3	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fecal cake	R1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R2	++	+	+	-	-	++	+	+	+	+	+	-	-	-	-	-	-	-
	R3	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+
Diuresis	R1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R2	+	+	-	-	-	+	+	+	+	-	-	+	-	-	+	+	+	++
	R3	+	++	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-
Sialorrhea	R1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Exploratory	R1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	R2	+	+	-	-	-	++	++	++	++	++	++	++	+++	++	+++	+++	++	++
	R3	+	-	-	-	-	++	+++	++	++	+++	++	++	++	++	+++	++	++	++
Prostration	R1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Exophthalmia	R1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sedation	R1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Analgesia	R1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Central Nervous System																			
Grooming	R1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R2	++	++	++	++	++	+	+	+	+	+	+	+	+	++	+	+	+	+
	R3	++++	++++	+++	++++	++++	++	+	+	+	+	+	+	+	+	++	++	++	-
Catatonia	R1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tremors	R1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Seizures	R1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: R1 = Saline, R2 = AMY (300 mg kg^{-1}), R3 = AMY (2000 mg kg^{-1}). Scores: - = Absent, + = Rare, ++ = Discrete, +++ = Moderate and ++++ = Intense.

Effect of amyrin on animal's body mass and organs weight

Rats treated with AMY (300 and 2000 mg kg^{-1}, *p.o.*) during 14 days survived until the end of the experiment (data not shown). Besides, AMY did not alter the animal water and ration consumption, or the weight of most organs, except for the kidney, which was increased by 43% by AMY at 2000 mg kg-1 (Table 2).

During the follow-up period of AMY treatment, the animals body weight was not significantly changed at 300 or 2000 mg kg^{-1}, respectively, when compared to the initial weight (Figure 3). However, when compared to saline, the treated animals showed lower weight. Clinical abnormalities that would suggest toxicity were not observed, such as external and eye lesions, or changes in hair color and distribution (data not shown).

Table 2. Relative organs weight of rats 14 days after *per oral* treatment with and - amyrin (AMY).

Groups	Brain	Liver	Kidney	Spleen
Saline	0.93 ± 0.03[a]	4.24 ± 0.02	0.70 ± 0.05	0.37 ± 0.01
AMY (300 mg kg^{-1})	0.98 ± 0.02	3.89 ± 0.21	0.87 ± 0.02	0.34 ± 0.01
AMY (2000 mg kg^{-1})	1.01 ± 0.05	3.72 ± 0.13	1.23 ± 0.15*	0.37 ± 0.04

[a]Mean ± SEM (n=3) *p < 0.05 *vs.* Saline (ANOVA and Tukey test).

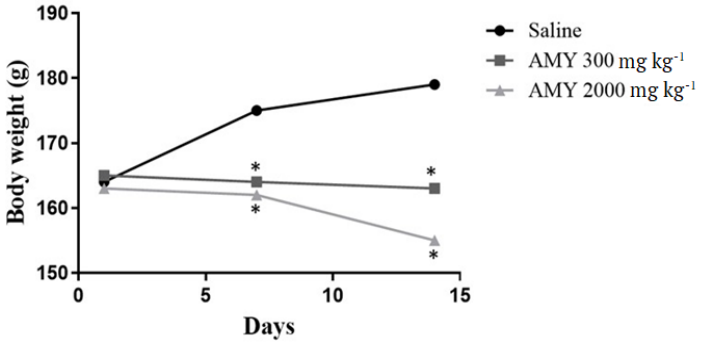

Figure 3. Effect of the isomeric mixture of α- and β-amyrin (AMY) on the weight gain. Rats received *per oral* vehicle (0.9% NaCl + 0.5% Tween 80, 0.05 mL 10 g^{-1} body mass), AMY (300 mg kg^{-1}) or AMY (2000 mg kg^{-1}) and were weighed (g) daily until day 14. Mean ± SEM (n=3) *p < 0.05 vs. Saline (ANOVA and Tukey test).

Effect of amyrin on hematological and biochemical parameters

The hematological parameters were unaltered by AMY at 300 or 2000 mg kg^{-1} (Table 3), being the erythrocytes normocytic and normochromic.

Table 3. Hematological parameters of rats 14 days after *per oral* treatment with α- and β-amyrin (AMY).

Blood Count	Saline	AMY (300 mg kg^{-1})	AMY (2000 mg kg^{-1})
RBC (10^6 mm^{-3})	7.79 ± 0.26[a]	7.42 ± 0.29	8.23 ± 0.08
HGB (g dL^{-1})	14.37 ± 0.44	13.00 ± 0.45	14.63 ± 0.18
HCT (%)	46.18 ± 0.64	42.62 ± 1.21	46.35 ± 0.61
MCV (μ)	59.33 ± 1.20	59.67 ± 0.33	56.33 ± 1.20
MCH (pg)	18.43 ± 0.17	17.33 ± 0.38	17.80 ± 0.36
MCHC (g dL^{-1})	31.13 ± 0.63	30.07 ± 0.49	31.60 ± 0.32
RDWc (%)	14.40 ± 0.10	13.33 ± 0.65	13.43 ± 0.16
Leukogram	Saline	300 mg kg^{-1}	2000 mg kg^{-1}
WBC	9.93 ± 2.85	7.40 ± 2.10	13.28 ± 3.41
LYM	7.23 ± 1.73	7.02 ± 2.75	9.66 ± 2.13
MID	0.68 ± 0.43	0.45 ± 0.26	0.97 ± 0.47
GRA	2.01 ± 0.72	0.88 ± 0.08	2.68 ± 1.08
LYM (%)	74.83 ± 3.91	66.75 ± 5.85	73.83 ± 3.38
MID (%)	5.76 ± 2.16	5.50 ± 1.50	6.40 ± 2.98
GRA (%)	19.40 ± 3.00	18.00 ± 3.29	19.73 ± 4.87
Platelets	Saline	300 mg kg^{-1}	2000 mg kg^{-1}
PLT	811.0± 93.82	651.3 ± 265.9	748.0 ± 137.8
PCT (%)	0.56 ± 0.06	0.43 ± 0.18	0.51 ± 0.10
MPV (μ)	6.90 ± 0.36	6.63 ± 0.23	6.76 ± 0.24
PDWc (%)	34.50 ± 1.06	32.27 ± 1.14	33.23 ± 1.38

[a]Mean ± SEM (n=3) *p < 0.05 *vs.* Saline (ANOVA and Tukey test). Red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), white blood cell (WBC), mean corpuscular hemoglobin concentration (MCHC), distribution of red blood cells (RDWc), white blood cell (WBC), lymphocytes (LYM), MID cells (MID), neutrophils, monocytes, eosinophils, and basophils (GRA), platelet (PLT), (PCT), mean platelet volume (MPV), and platelet distribution width (PDWc).

AMY did not induce alterations in the function biomarkers of pancreas (α-amylase), kidney (urea, creatinine), and liver (ALT: alanine aminotransferase, AST: aspartate aminotransferase, AP: alkaline phosphatase, TB: total bilirubin, DB: direct bilirubin, cholesterol). However, albumin was increased by AMY only at 300 mg kg^{-1}, while serum glucose, triglycerides and uric acid were increased at 2000 mg kg^{-1} (Table 4).

Effect of amyrin on the organs histological pattern

AMY induced alterations in the liver, such as vascular congestion, areas of lobular or portal hemorrhage. However, the morphometric evaluation of this organ showed no significant differences regarding the total hepatocellular degeneration area (Figure 4A-C). Vascular congestion and hemorrhage in spleen and cerebral cortex were also observed (Figure 4D-I). In addition, renal changes, such as cellular damage, presence of inflammatory infiltrate, tubular protein deposition and glomeruli atrophy were detected in the animals treated with AMY at 300 and 2000 mg kg^{-1} (Figure 4 J-R).

Table 4. Serum biochemical parameters of rats 14 days after *per oral* treatment with α- and β- amyrin (AMY).

Parameters	Saline	AMY (300 mg kg^{-1})	AMY (2000 mg kg^{-1})
ALT (U L^{-1})	42.67 ± 3.18[a]	47.00 ± 9.29	52.67 ± 6,48
AST (U L^{-1})	45.47 ± 11.46	50.95 ± 16,02	41.09 ± 20.21
Direct Bilirubin (mg dL^{-1})	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.00
Total Bilirubin (mg dL^{-1})	0.17 ± 0.02	0.19 ± 0.10	0.54 ± 0.04
Creatinine (mg dL^{-1})	0.37 ± 0.03	0.42 ± 0.02	0.37 ± 0.02
Alkaline Phosphatase (U L^{-1})	365.30 ± 32.05	364.00 ± 36.66	381.3 ± 13.8
Cholesterol (mg dL^{-1})	49.33 ± 1.02	56.67 ± 5.81	63.67 ± 9.28
Triglycerides (mg dL^{-1})	78.67 ± 2.18	78.67 ± 6.93[#]	156.7 ± 9.28*
Glucose (mg dL^{-1})	126.70 ± 4.33	144 ± 9.45	164.30 ± 6.88*
Amylase (U L^{-1})	427.70 ± 10.48	466.00 ± 7.00	464.30 ± 18.41
Albumin (g dL^{-1})	3.67 ± 0.06	4.33 ± 0.4*	4.0 1± 0.13
Total Protein	7.48 ± 0.12	8.57 ± 0.29*	9.38 ± 0.46*
Uric Acid (mg dL^{-1})	0.73 ± 0.03	0.96 ±0.06	1.11 ± 0.06*
Urea (mg dL^{-1})	32.00 ± 4.58	34.00 ± 2.64	34.33 ± 3.38

[a]Mean ± SEM (n=3). ALT: Alanine aminotransferase. AST: Aspartate aminotransferase, *p<0.05 *vs.* saline, [#]p <0.05 *vs.* 2000 mg kg^{-1} (ANOVA and Tukey test)

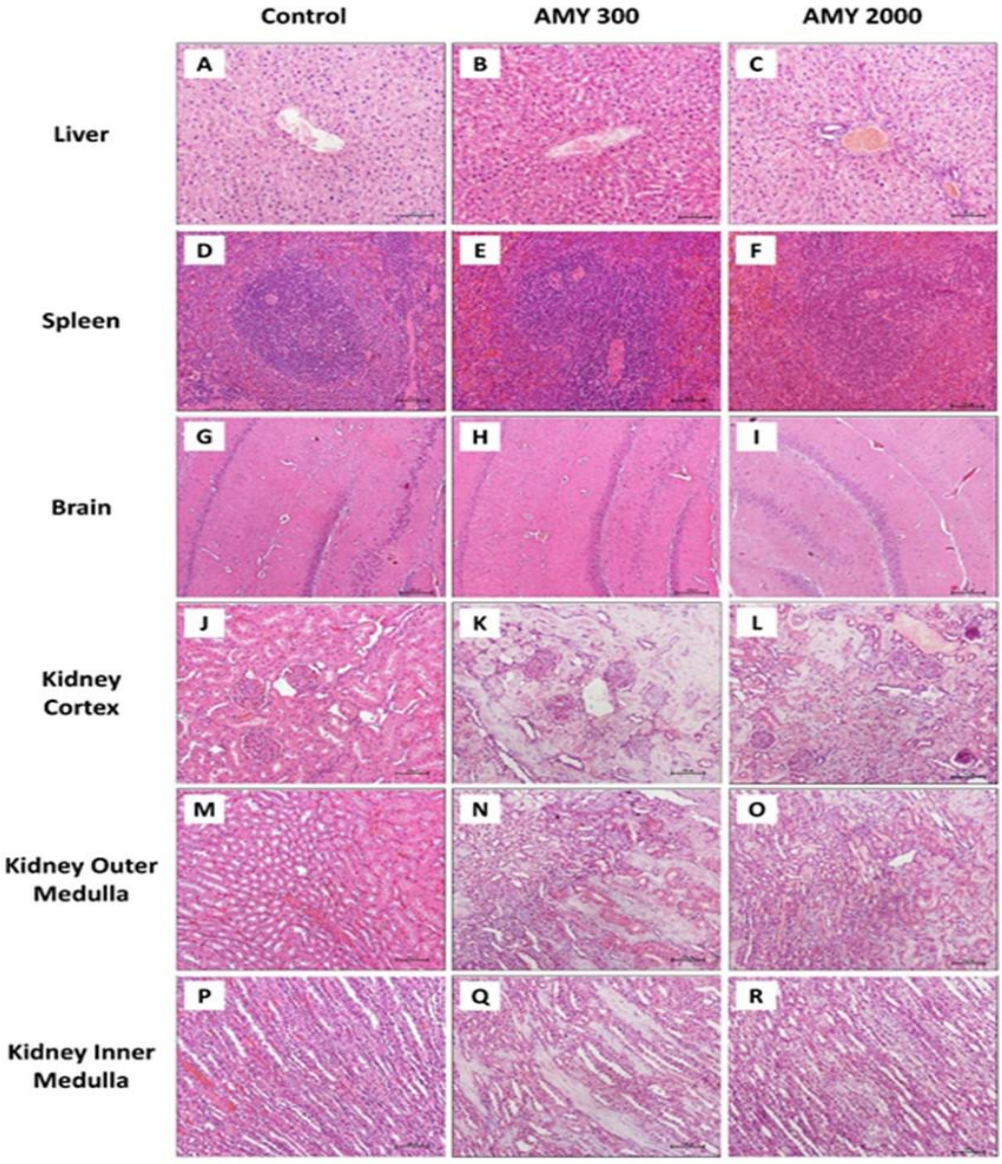

Figure 4. Photomicrographs of organs 14 days after *per oral* treatment with amyrin (AMY). Rats received *per oral* vehicle (0.9% NaCl + 0.5% Tween 80, 0.05 mL 10 g^{-1} body mass), AMY (300 mg kg^{-1}) or AMY (2000 mg kg^{-1}) and were euthanized at day 15 for histopathological analysis of Liver (4A, 4B, 4C), Spleen (4D, 4E, 4F), Brain (4G, 4H, 4I), Kidney Cortex (4J, 4K, 4L), Kidney Outer Medulla (4M, 4N, 4O), Kidney Inner Medulla (4P, 4Q, 4R). Hepatocellular degeneration (4A-C). Vascular congestion and hemorrhage in spleen and cerebral cortex (4D-I). Renal changes (cellular damage, inflammatory infiltrate, tubular protein deposition and glomeruli atrophy (4J-L). H&E, 200 x, Nikon Microscope Eclipse Nis, Software Nis 4.0.

Discussion

This study evaluated for the first time the acute toxicity of the triterpene mixture α- and β- AMY in rats. AMY has been extensively studied in animal models, being demonstrated several pharmacological effects (Nogueira et al., 2019).

The present results demonstrated the inhibitory effect of AMY on rat locomotor and exploratory activities (crossing and rearing) in the open field test, which suggest an inhibitory effect on the central nervous system. In fact, it had been previously demonstrated the GABAergic effect of AMY in the inhibition of convulsions induced by pentylenetetrazole, along with the increase of GABA levels within the brain (Aragão et al., 2015). These data led us to hypothesize that the GABAergic action of AMY would also be accounted for its anxiolytic effect in a mice model of anxiety (Aragão et al., 2006). Corroborating these findings, it was showed that the use of the antagonist of GABAA receptors reverses its inhibitory effect on the central nervous system (Jeon et al., 2015). Curiously, it was observed that the number of grooming was increased after AMY administration. Besides, the self-grooming is in part pharmacologically regulated by glutamate and the administration of anti-glutamatergic agents induces grooming in rodents (Audet, Goulet & Doré, 2006). It was also demonstrated that AMY protects against glutamate toxicity *in vitro* (Brimson, Brimson, Brimson, Rakkhitawatthana, & Tencomnao, 2012), and it has been hypothesized that the anti-glutamatergic action of the substance may play a significant role in the induction of the stimulatory grooming effect. Moreover, evidences suggest that the serotonergic system contributes to the regulation of grooming in rodents, since the use of serotonergic drugs reduce this behavior. Accordingly, the serotonin levels are reduced in the brain of mice injected with AMY (Aragão et al., 2015), which led to the speculation that this may influence the increase in the number of grooming observed in this study.

The Malone Hippocratic screening provides an estimate of the general pharmacological/toxicological nature of active principles found in a crude drug, along with their safety ratio (Malone & Robichaud, 1962). AMY evoked no alteration on the behavioral parameters evaluated in the Malone Hippocratic scale long-term. Only at the day one, AMY reduced the exploratory activity and increased the grooming pattern, without affecting any other parameters over the following days.

The reduction of animals ponderal gain caused by AMY in this study had been previously observed, along with the anti-obesity effect in murine models via modulation of enzymatic and hormonal response (Nasution, Mustanir, Marianne, & Marzuki, 2016). Additionally, the kidney lesion observed in the histopathological analysis might have caused protein loss and potentially impacted the weight gain. The kidney doubled its weight in response to the treatment with AMY at 2000 mg kg-1, which suggest renal toxicity (Sellers et al., 2007). However, it was not observed significant alterations in spleen, liver or brain. It is worth noting that α- and β-AMY presents *in vitro* cytotoxicity in tumoral lines (Barros et al., 2011), which could explain the renal cytotoxic effect observed by AMY at the highest dose. It is also probable that the vascular congestion and hemorrhage observed in liver, spleen and cerebral cortex are not related to the toxicity of the drug, but due to the animal euthanasia.

The blood is one of the major homeostatic systems of the body in animals. AMY, at any doses evaluated, did not alter the hematological parameters, suggesting no impact on the animal's hematopoiesis. Despite of some level of renal tissue impairment caused by AMY at 2000 mg kg-1, there was no change in the blood levels of urea, creatinine or albumin, except for the increase in uric acid concentration at this dose. However, a study using the triterpenes lupeol and its esters showed increased excretion of renal metabolites such as calcium, oxalate and uric acid (Vidya, Lenin, & Vralakshmi, 2002).

The present study may contribute to deepen the knowledge about the safety of AMY, aiming the development of a novel pharmacological product.

Conclusion

AMY administered *per oral* in female rats during 14 days decreased rat locomotor activity, did not cause lethality, nor severe hepatic or cerebral damages. However, vascular congestion and hemorrhage in spleen and cerebral cortex were observed. Furthermore, the highest dose of AMY showed more evident signs of renal cytotoxicity.

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