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Research Paper

Evaluation of the Subchronic Toxicity of Two Extracts of Tusca Leaves (*Acacia aroma)* on Rats' Kidneys, Liver, and Stomach

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ABSTRACT

Background: *Acacia aroma* (*A. aroma*), or Tusca, is a South American plant frequently used in medicine. We recently reported the antiulcer effect of two Tusca leaf extracts in rats: a 5% infusion and a 10% hydroalcoholic. This study aimed to investigate the *in vitro* cytotoxicity and the *in vivo* oral subchronic toxicity of these two extracts in rats.

Methods: Cytotoxicity was evaluated through cell viability assays using the 50% lethal concentration (LC_{50}) on lung cells, which was calculated for each extract. Oral subchronic toxicity was studied in Wistar rats, and the clinical parameters were determined during a 90-day treatment period. Following the experimental period, biochemical parameters were analyzed, and histopathological analyses of the stomach, liver, and kidneys were performed.

Results: The *in vitro* cytotoxicity was low in both extracts (LC_{50}=966.78±48.34 µg/ml for the 5% infusion and 562.28±28.11 µg/ml for the 10% hydroalcoholic extract). The *in vivo* clinical, behavioral, and biochemical parameters, body weight, and food intake had no significant differences ($P \geq 0.05$) compared to the control group. There were also no histopathological alterations in the analyzed organs.

Conclusion: The present study contributes to expanding the knowledge on the safety of the two extracts derived from *A. aroma* (5% infusion and 10% hydroalcoholic). Therefore, the extracts can possibly be used in the treatment or prevention of gastric diseases in future studies.

Keywords: *Acacia aroma*, Cytotoxic activity, Preclinical study, Subchronic toxicity, Tusca leaf extracts

Introduction

Acacia is the second largest genus within the *Fabaceae* family of plants, with more than 1450 species. Within *Acacia* species, *Acacia aroma* (*A. aroma*) Gillies ex Hook & Arn. (syn. *Vachellia aroma*; Seigler & Ebinger, 2006) is a small tree that commonly grows from northern Argentina to southern Ecuador [1, 2]. The vernacular name of *A. aroma* is Tusca, and it is used for numerous properties. The aerial parts, especially the bark, have traditionally been used for their antimycotic, abortifacient, diuretic, wound healing, antitussive, anti-inflammatory, and antibacterial properties [3, 4]. In a recent study, we reported the antioxidant activity of Tusca polar extracts, as well as their gastroprotective effects, using an ethanol-induced gastric ulcer model in rats [5].

Several studies have described the phytochemical profile of Tusca. Among the main constituents of *A. aroma* leaf are polysaccharides, polyphenols, hydrolyzable and condensed tannins, and flavonoids, such as apigenin and rhamnetin [3, 5, 6]. Linamarin has also been found in dried Tusca leaves, which is a cyanogenic glucoside that causes neurotoxic effects and damages the kidneys and liver tissues in animal models [7]. Traditional and scientific knowledge about the use of Tusca extracts and their phytoconstituents is currently available. It is known that two polar extracts from Tusca leaf lack cytotoxicity and genotoxicity [8]. In a previous study, we reported that the 10% hydroalcoholic and the 5% infusion of Tusca leaf do not cause acute toxicity in normal Wistar rats [5]. However, a comprehensive study of the potential long-term toxicity of Tusca leaf extracts is still lacking. The importance of performing such a study lies in the fact that long exposure to some *Acacia* metabolites or phytoconstituents accumulated in human tissues causes adverse effects that are not necessarily evident upon shorter exposure periods [9].

Aim of the Study: This study aimed to evaluate the *in vitro* cytotoxicity and the *in vivo* oral subchronic toxicity of two extracts of *A. aroma* leaves: 5% oral infusion and 10% hydroalcoholic.

Materials and Methods

Plant Material and Extraction Process: The *A.*

aroma (Gill ex Hook & Arn.) leaves were collected in September 2019 from wild specimens in Silípica, Santiago del Estero, Argentina. Voucher specimens (No: 615923) were deposited in the herbarium of Fundación Miguel Lillo, Tucumán, Argentina. First, the leaves were dried in an oven with an air stream at 40°C, and then they were powdered. The powder was used to prepare 5% infusion and 10% hydroalcoholic extracts, according to Farmacopea Argentina 7° Ed. [10]. The extracts were passed through Whatman filter paper No. 4 and then concentrated under vacuum at 40°C. The infusion and hydroalcoholic extracts were both lyophilized and stored at -20°C until further use for the experiments.

Quantitative Analyses

Proanthocyanidins: These compounds were quantified according to the method developed by Porter *et al*. [11]. The proanthocyanidin content was expressed as mg of tannic acid equivalents (TAE) per g of dried extract.

Hydroxycinnamic Acid: The hydroxycinnamic acid (AHCT) content was quantified according to the method described by Martino *et al*. [12]. The results were expressed as mg of chlorogenic acid equivalents (CAE) per g of dried extract.

Ortho-dihydroxyphenols: The ortho-dihydroxyphenol (ODF) content was quantified according to the method described by Johnson and Schall [13]. The results were expressed as mg catechol equivalent (CE) per g of dried extract.

Cytotoxic Activity: The cytotoxic activities of the two Tusca leaf extracts (5% infusion and 10% hydroalcoholic) were tested using the 50% lethal concentration (LC_{50}) on a human lung fibroblast cell line. The cells were grown in Roswell Park Memorial Institute culture media with 10% fetal calf serum, 1% streptomycin or penicillin, and 10% L-glutamine added at 37°C under humidified atmospheric conditions of 5% CO_2 and 95% air. The cells were incubated in 96-well microplates containing 100 µl medium per well. After 24 h, the media were removed, and the cells were exposed to various concentrations of each extract (200, 400, and 800 µg/ml). The pure culture medium was used as the negative control.

The cell viability was determined by the MTT assay method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [14]. The viability was also determined as percentages, based on the following equation: $Viability\% = (A_P - A_b / A_c - A_b) \times 100$ where

A_P=Absorbance of treated wells, A_c=Absorbance of control wells, and A_b=Absorbance of the blank wells. The LC_{50} was determined by linear regression analysis. All of the *in vitro* assays were performed in triplicate.

Animals: Adult male and female Wistar rats (n=42) aged 8-12 weeks and weighing 180±20 g were selected for the experiments. They were colony-bred animals acquired from Instituto Superior de Investigaciones Biológicas (INSIBIO, CONICET-UNT), Tucumán, Argentina. The animals were acclimated for seven days before the experiments were performed. During this period, the animals were individually examined to ensure they were in good physical health and had appropriate body weights (b.w.), which were two prerequisites for inclusion in the study. The animals were placed in cages with air circulation, maintained at a room temperature of 22±2°C and 60-70% relative humidity. They were kept under a 12-hour light/dark cycle from 07:00 a.m. to 7:00 p.m. Powdered, certified rodent food obtained from a commercial source (Standard Food, Asociación de Cooperativas Argentinas - S.E.N.A.S.A. No. 2706) and potable water were available to the rats *ad libitum*. There was no food or water contamination that could interfere with the study. To avoid bias, the daily treatment order was randomized.

The animals were handled, and the procedures were performed in compliance with the current research standards of Argentina (Ethical Framework of Reference for Biomedical Research in Laboratory Animals, Resol. D N° 1047 annexe II, 2005) and ARRIVE guidelines. The study protocol was approved by the Institutional Committee on Animal Care and Use of the Facultad de Bioquímica, Universidad Nacional de Tucumán, Argentina (N°0015-2017). The number of animals used in this study was statistically adequate and provided reliable data for later analyses. We also made every effort to reduce the animal suffering during the experiments.

Subchronic Toxicity Study: The male and female Wistar rats were divided into seven groups (6 rats/group, 3 of each sex). Animals were randomly assigned to either control or experimental groups with a computer-generated randomization schedule. Group I received 0.5 ml of water at each treatment cycle and was considered the control group. Groups II, III, and IV received the 5% infusion extract at 75, 150, and 300 mg dry extract/kg b.w., respectively. Groups V, VI, and VII received the 10% hydroalcoholic extract at 75, 150, and 300 mg/kg b.w., respectively. The minimum effective dose within the tested range (150 mg/kg b.w.) was included [5]. The extracts at each dose were dissolved in 0.5 ml of distilled water and administered orally to the rats using intragastric tubing for 90 days before eating their food at 6 p.m. every day. All animals received standard rat food and water *ad libitum*.

During the experimental period, the rats were weighed weekly, and their food intakes were recorded daily. Clinical examinations, behavioral profile (stereotypes, irritability, and sedation), neurological status (posture, exploratory movements, and presence of clonic and/or tonic movements), and autonomic activity (salivation, piloerection and lacrimation, pupil size, breathing pattern, abdominal contortion, emesis, and diarrhea) were checked daily. Once a month, blood samples were obtained from each rat from the tip of the tail under

fasting conditions and used for hematological and biochemical analyses.

At the end of the experimental period, the animals were fasted overnight but allowed access to water *ad libitum* and were euthanized by an intraperitoneal (i.p.) overdose of ketamine and xylazine (150:5 mg/kg b.w.). Immediately after, the last blood sample was collected by cardiac puncture, and finally, the animals were dissected. A macroscopic examination of the organs was carried out *in situ*, and the liver, kidneys, and stomach were excised, weighed, and fixed in 10% formaldehyde for histopathological examinations [15].

Hematological and Clinical Analyses: For hematological analyses, blood samples were collected during the experiments and kept in tubes containing ethylenediamine tetraacetic acid. Standard hematological parameters were measured in each sample using an automated blood analyzer (CELL-DYN 3700, Abbott).

For clinical determinations, blood samples were kept in glass tubes without anticoagulants, and after retraction of the clot, the samples were centrifuged at 5000 rpm for 10 min to obtain the sera [16]. Diagnostic kits (Wiener Lab Group, Rosario, Argentina) were used to measure the concentration of glucose (mg/dl), creatinine (mg/l), total proteins (g/dl), urea (g/l), aspartate aminotransferase (AST, U/l), and alanine aminotransferase (ALT, U/l) activities.

Histopathological Examinations: After fixation, the liver, kidneys, and stomach tissue samples were dehydrated in a decreasing concentration of alcohol series, cleared in xylene, and embedded in paraffin wax at 56°C. Using a microtome, the paraffin blocks were cut into 6 µm slices, mounted on glass slides pretreated with Histo-Grip (Zimed Lab, Inc., USA), stained with hematoxylin and eosin (H&E), and examined under a light photomicroscope (Nikon, Fluophot, Japan). Photomicrographs were taken using an 8 MP digital camera attached to the microscope (FX-35A, Nikon, Japan). To eliminate bias, a skilled and experienced observer, unaware of the identification of the specimens, performed the histopathological examinations [5].

Statistical Analyses: The data from all of the assays were expressed as mean±standard deviation (SD) and evaluated by one-way analysis of variance (ANOVA) followed by an unpaired Student's t-test. We used Statistical Package for the Social Sciences (version 12.0; SPSS Inc., Chicago, IL, USA) for these analyses. For all statistical analyses, $P<0.05$ was considered statistically significant.

Results

Quantitative Phytochemical Analyses: The yields of Tusca leaf extract were 8.40 g dry residue/100 g leaves (8.40%) for the 5% infusion and 14.90 g dry residue/100 g leaves (14.90%) for the 10% hydroalcoholic extract. Proanthocyanidins were not detectable in the 5% infusion extract, while the 10% hydroalcoholic extract resulted in 1.52±0.08mg TAE/g of dry extract. Regarding the total hydroxycinnamic acids, the 5% infusion contained 70.93±0.85 mg CAE/g extract, and the 10% hydroalcoholic contained 79.88±0.71 mg CAE/g extract. Finally, the content of o-dihydroxyphenols detected in the 5% infusion was 87.17±0.83 mg CE/g extract, while in the 10% hydroalcoholic extract, it was 35.25±0.91 mg CE/g extract.

Cytotoxic Activity: Both Tusca leaf extracts demonstrated low cytotoxic activity based on the LC_{50} of the lung fibroblasts. The 5% infusion showed an LC_{50} of 966.78±48.34 µg/ml, whereas the LC_{50} for the 10% hydroalcoholic extract was 562.28±28.11 µg/ml. The cell viability was 95-100% for the controls.

Subchronic Toxicity Study: No clinical or behavioral abnormalities were detected during daily examinations of the rats at any of the tested doses with either Tusca leaf extracts (5% infusion or 10% hydroalcoholic extract). In addition, no deaths occurred in any group of the animals. The consumption of food and water in the control and treatment groups remained normal throughout the experiments. The body weight gain of animals treated with any of the extract doses (75, 150, and 300 mg/kg) of either Tusca leaf extract had no significant differences ($P>0.05$) compared to that of the respective control group, either in female or male rats. Moreover, no significant changes ($P>0.05$) in the relative weights of the kidneys and liver were detected between the control and treatment groups of either female or male rats (Table 1).

Hematological Analyses: The analyzed hematological parameters did not significantly vary between the groups treated with either Tusca leaf extract and the control group in either female or male rats ($P>0.05$) at the end of the experimental period (Table 2).

Clinical Chemistry Studies: After analyzing the rats' metabolism and function of certain organs, such as the liver and kidneys, no significant differences ($P>0.05$) were detected in the hepatic enzyme levels (ALT and AST) and renal function markers (urea and creatinine serum concentrations). In addition, no differences were noted in the serum glucose and total protein concentrations in the two Tusca leaf extracts (5% infusion and 10% hydroalcoholic extract) between the treatment and control groups (Table 3).

Histopathological Findings: Our findings revealed no significant macroscopic alterations in the rats' vital organs, such as the liver, kidneys, gastrointestinal (GI) tract, brain, lungs, heart, spleen, testes, prostate gland, uterus, or ovaries. Likewise, the rats treated with either of the Tusca leaf extracts presented organs in normal shape, color, size, and texture similar to those in the control animals. The microscopic examinations also revealed no other tissue abnormalities, as represented in Figure 1.

Table 1. Effect of subchronic oral administration of the 5% infusion and the 10% hydroalcoholic extract of *Acacia aroma* leaf on body weight gain and relative weights of liver, kidney, and stomach in rats after the 90-day treatment period

Experimental Group	Body weight gain (BWG)(g)	Relative liver weight (RLW)(g)	Relative kidney weight (RKW)(g)	Relative stomach weight (RSW)(g)
Male Rats				
Control	212.00±22.72	2.70±0.24	0.55±0.07	0.53±0.05
5% I 75 mg/kg	205.00±10.50	2.60±0.21	0.47±0.05	0.48±0.07
5% I 150 mg/kg	188.00±11.79	3.00±0.15	0.47±0.04	0.56±0.05
5% I 300 mg/kg	186.00±14.70	2.60±0.10	0.49±0.05	0.50±0.06
10% HE 75 mg/kg	202.00±12.12	3.10±0.18	0.48±0.06	0.53±0.05
10% HE 150 mg/kg	202.00±10.54	2.80±0.17	0.41±0.04	0.45±0.08
10% HE 300 mg/kg	203.00±9.90	2.80±0.14	0.43±0.05	0.49±0.07
Female Rats				
Control	170.00±20.40	3.10±0.22	0.59±0.07	0.58±0.06
5% I 75 mg/kg	174.00±15.66	3.00±0.12	0.62±0.09	0.55±0.05
5% I 150 mg/kg	176.00±8.80	3.10±0.16	0.56±0.06	0.58±0.07
5% I 300 mg/kg	173.00±12.11	3.10±0.18	0.54±0.08	0.57±0.06
10% HE 75 mg/kg	199.00±15.92	3.00±0.15	0.59±0.03	0.56±0.05
10% HE 150 mg/kg	198.00±11.88	3.30±0.23	0.53±0.05	0.57±0.08
10% HE 300 mg/kg	201.00±14.07	2.60±0.17	0.51±0.06	0.49±0.04

Data are represented as means±SD. n=6 animals (3 males, 3 female). *P≤0.05 was considered statistically significant compared to the control group. BWG=final body weight-initial body weight; RLW=final liver weight/final body weight; RKW=final kidney weight/final body weight; RSW=final stomach weight/final body weight. 5% I: 5% infusion of *Acacia aroma* leaf, 10% HE: 10% hydroalcoholic extract of *Acacia aroma* leaf

Table 2. Effect of the subchronic oral administration of the 5% infusion and the 10% hydroalcoholic extract of *Acacia aroma* leaf on hematological parameters in rats after the 90-day treatment period

Experimental Groups	RBC (10^6/µl)	Hto (%)	Hb (g/dL)	MCV (fl)	MCH (pg)	MCHC (g/dl)	WBC (10^3/µl)	Neutrophils (10^3/µl)	Eosinophils (10^3/µl)	Basophils (10^3/µl)	Lymphocytes (10^3/µl)	Monocytes (10^3/µl)	Plateletcount (10^3/µl)
Male Rats													
Control	7.34±0.37	38±2	13.50±0.68	51.4±2.6	18.5±0.9	35.9±1.8	9.1±1.2	1.09±0.15	0.27±0.07	0.00±0.10	7.37±1.11	0.36±0.05	742.00±146.14
5% I 75 mg/kg	7.57±0.38	38±2	13.80±0.69	50.2±2.5	18.3±0.9	36.5±1.8	8.0±0.5	1.12±0.16	0.16±0.04	0.16±0.10	6.24±0.61	0.32±0.05	844.00±67.52
5% I 150 mg/kg	7.71±0.39	39±2	14.40±0.72	50.3±2.5	18.7±0.9	37.1±1.9	9.1±0.4	1.00±0.15	0.27±0.04	0.18±0.10	7.28±0.46	0.36±0.05	1050.00±199.50
5% I 300 mg/kg	8.02±0.40	40±2	14.60±0.73	50.4±2.5	18.2±0.9	36.0±1.8	7.5±1.0	0.85±0.14	0.23±0.03	0.00±0.10	5.33±1.01	0.33±0.05	659.00±46.13
10% HE 75 mg/kg	7.16±0.36	37±2	13.50±0.68	51.0±2.6	18.9±0.9	37.1±1.9	10.8±0.9	1.08±0.15	0.32±0.05	0.10±0.10	8.75±0.44	0.43±0.06	730.00±58.40
10% HE 150 mg/kg	7.41±0.37	38±2	13.60±0.68	50.9±2.5	18.3±0.9	36.0±1.8	9.1±0.5	1.00±0.15	0.27±0.04	0.18±0.10	7.28±0.36	0.37±0.06	1074.00±193.32
10% HE 300 mg/kg	7.80±0.39	39±2	14.30±0.72	50.4±2.5	18.3±0.9	36.2±1.8	9.3±0.5	1.12±0.16	0.19±0.03	0.00±0.10	7.44±0.37	0.46±0.07	837.00±41.85
Female Rats													
Control	7.14±0.57	38±3	13.40±0.67	53.2±2.7	18.8±0.9	35.3±1.8	4.6±0.9	0.55±0.14	0.19±0.07	0.12±0.10	3.73±0.78	0.23±0.06	912.00±109.44
5% I 75 mg/kg	6.36±0.38	34±2	12.60±0.65	53.3±2.7	19.9±1.0	37.3±1.9	6.9±1.4	0.76±0.11	0.23±0.06	0.14±0.10	5.38±0.91	0.35±0.07	801.00±64.08
5% I 150 mg/kg	6.43±0.32	35±2	12.70±0.64	54.0±2.7	19.7±1.0	36.5±1.8	4.3±0.6	0.43±0.12	0.16±0.02	0.09±0.10	3.53±0.53	0.18±0.03	1072.00±117.92
5% I 300 mg/kg	6.48±0.32	35±2	12.80±0.64	54.0±2.7	19.7±1.0	36.6±1.8	5.8±0.5	0.64±0.19	0.11±0.03	0.12±0.10	4.76±0.71	0.17±0.03	853.00±59.71
10% HE 75 mg/kg	6.83±0.34	37±2	13.60±0.68	54.0±2.7	19.9±1.0	36.8±1.8	6.9±1.4	0.69±0.16	0.20±0.03	0.21±0.10	5.45±1.04	0.34±0.06	708.00±113.28
10% HE 150 mg/kg	6.37±0.32	35±2	12.80±0.64	54.7±2.7	20.1±1.0	36.7±1.8	5.3±0.8	0.74±0.16	0.11±0.03	0.11±0.10	3.92±0.20	0.26±0.04	866.00±43.40
10% HE 300 mg/kg	6.70±0.34	36±2	13.40±0.67	54.1±2.7	19.9±1.0	36.9±1,8	6.9±1.4	0.69±0.13	0.21±0.04	0.21±0.10	5.52±1.15	0.26±0.04	1020.00±91.80

Data are represented as mean±SD. n=6 animals (3 males and 3 female). *P≤0.05 was considered statistically significant compared to the control group. RBC: Red Blood Cells, Hto.: Hematocrit, Hb: hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, WBC: White blood cells. 5% I: 5% infusion of *Acacia aroma* leaf, 10% HE: 10% hydroalcoholic extract of *Acacia aroma* leaf

Table 3. Effect of subchronic oral administration of the 5% infusion and the 10% hydroalcoholic extract of *Acacia aroma* leaf on biochemical parameters in rats after the 90-day treatment period

Experimental Group	Glucose (mg/dl)	Total protein (g/dl)	Urea (g/l)	Creatinine (mg/l)	ALT (U/l)	AST (U/l)
Male Rats						
Control	87.58±5.26	6.55±0.46	0.38±0.08	3.18±0.64	50.46±7.06	194.88±23.39
5% I 75 mg/kg	90.84±5.45	6.66±0.47	0.34±0.07	2.37±0.47	45.24±6.33	201.84±24.22
5% I 150 mg/kg	91.59±5.50	6.70±0.47	0.38±0.08	2.18±0.54	52.20±7.31	168.78±20.25
5% I 300 mg/kg	97.86±5.87	6.27±0.44	0.34±0.07	2.87±0.57	55.68±7.80	184.44±22.13
10% HE 75 mg/kg	87.08±5.23	6.53±0.46	0.34±0.06	2.32±0.46	41.76±5.85	167.04±20.04
10% HE 150 mg/kg	95.35±5.72	6.49±0.45	0.31±0.05	3.52±0.90	43.50±6.09	186.18±22.34
10% HE 300 mg/kg	95.60±5.74	6.71±0.47	0.30±0.06	2.92±0.58	55.68±7.80	174.00±20.88
Female Rats						
Control	88.59±5.32	6.24±0,44	0.29±0.06	2.83±0,57	48,72±6.82	179.22±21.51
5% I 75 mg/kg	89.71±5.38	6.23±0.44	0.31±0.07	2.15±0.49	46.98±6.58	186.18±22.34
5% I 150 mg/kg	91.09± 4.29	6.27±0.43	0.30±0.06	2.16±0.52	53.94±7.95	177.48±21.30
5% I 300 mg/kg	96.11±7.89	6.49±0.45	0.29±0.05	2.75±0.55	50.49±7.07	172.26±20.67
10% HE 75 mg/kg	86.71±6.63	6.41±0.46	0.38±0.08	3.18±0.69	48.72±6.82	167.04±20.04
10% HE 150 mg/kg	93.85±6.69	6.25±0.44	0.36±0.07	2.56±0.51	40.02±6.60	179.22±21.51
10% HE 300 mg/kg	92.97±5.99	6.31±0.43	0.38±0.08	2.68±0.54	48.72±6.82	184.44±22.13

Data are represented as mean±SD. n=6 animals (3 males and 3 female). *P≤0.05 was considered statistically significant compared to the control group. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase. 5% I: 5% infusion of *Acacia aroma* leaf, 10% HE: 10% hydroalcoholic extract of *Acacia aroma* leaf

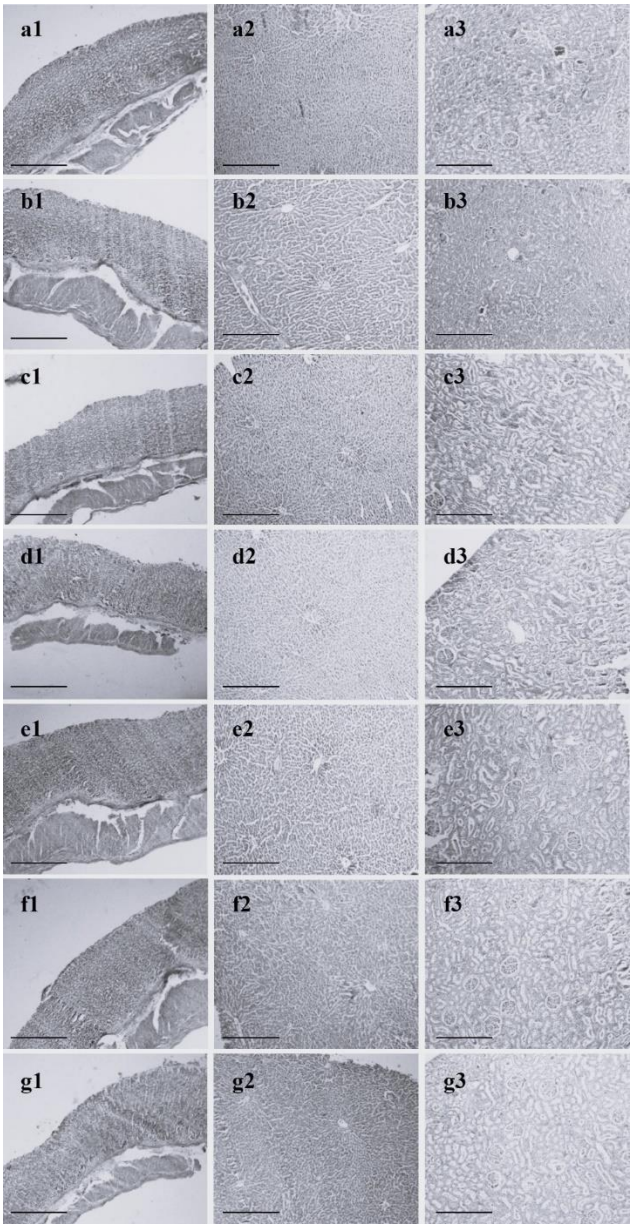

Figure 1. Histopathological analysis of rats' stomach, liver, and kidney from control and treated groups stained with hematoxilin and eosin
Images with number 1 are stomachs, 2 livers, and 3 kidneys. Experimental groups are: (a) Control group, (b) 5% I 75 mg/kg b.w., (c) 5% I 150 mg/kg b.w., (d) 5% I 300 mg/kg b.w., (e) 10% HE 75 mg/kg b.w., (f) 10% HE 150 mg/kg b.w., and (g) 10% HE 300 mg/kg b.w. Scale bar: 100µm.
5% I: Tusca leaf 5% infusion, 10% HE: Tusca leaf 10% hydroalcoholic extract
Keys: I: Infusion; HE: Hydroalcoholic extract

Discussion

In the present study, we found that most of the phenolic compounds in the 5% infusion and 10% hydroalcoholic extract were hydroxycinnamic acids and o-dihydroxyphenols. However, the 10% extract also contained proanthocyanidins. It is known that these secondary metabolites provide beneficial health effects, such as free radical scavenging activities. However, some kinds of hydroxycinnamic acids produce toxic effects at moderate concentrations [17]. In addition, compounds bearing the ortho-dihydroxyl groups (such as chlorogenic and caffeic acids) can cause direct DNA damage regardless of their antioxidant activities [18]. Further, it has been demonstrated that high doses of proanthocyanidins can produce toxic effects due to their ability to precipitate proteins [19]. Despite the presence of these potentially toxic compounds in the 5% infusion and 10% hydroalcoholic extract, we did not find any associated toxic effects in the cell culture *in vitro* or subchronic assays *in vivo* at any of the tested concentrations. These findings suggest that the amounts of phytoconstituents in both extracts were not sufficient to cause deleterious consequences.

Regarding the cytotoxicity of Tusca leaf extracts, as evaluated on the LC_{50} human lung fibroblast cell line, the LC_{50} of the 10% hydroalcoholic and the 5% infusion extracts was similar to that reported in *in vitro* cell cytotoxicity experiments [20]. According to the American National Cancer Institute, *in vitro*

cytotoxicity is categorized into four levels based on LC_{50} values. These include high cytotoxicity (LC_{50} lower than 20 µg/ml), moderate cytotoxicity (LC_{50} between 20 and 200 µg/ml), weak cytotoxicity (LC_{50} between 201 and 500 µg/ml), and no cytotoxicity (LC_{50} higher than 500 µg/ml) [21]. Therefore, based on our findings and following this categorization, we established that both Tusca leaf extracts were not toxic to human cells in culture.

The subchronic oral toxicity of the Tusca leaf extracts in rats was evaluated following the OECD guidelines. No nutritional effects were detected due to the Tusca leaf extract intake at various doses, including the minimum effective gastroprotective dose. The animals maintained an expected body weight gain consistent with their age and gender and demonstrated normal water and food intakes. In contrast, Smith *et al*. have shown that another *Acacia* species (*A. angustissima)* has produced a remarkable antinutritional effect, reducing food intake and causing severe weight loss in rats during the experimental period [22]. One possible explanation may be that there are qualitative and quantitative differences between the chemical compositions of *A. aroma* and *A. angustissima* extracts.

The subchronic exposure of rats to toxic substances usually leads to damaging effects in major organs, such as the liver, kidneys, and GI tract. The liver is a key organ in the detoxification and metabolism of plant-derived compounds and drugs. Alterations in hepatocytes, such as necrosis or apoptosis, are signs of hepatic cell dysfunction, and such changes can cause an increase in ALT and AST enzymes and other hepatic serum markers [23]. However, we found no elevation in serum transaminases or alterations in the relative weight of the liver. Likewise, we did not find histological alterations compatible with any pathology or liver damage in any of the experimental groups.

Kidney function is essential for removing toxic waste products from the blood. The inherent toxicity of natural products can be manifested in this organ, causing damage through their chronic accumulations [24]. In the current study, we did not find any sign of nephrotoxicity from Tusca leaf extracts since all experimental groups showed normal concentrations of serum urea and creatinine. Similarly, the rats' kidneys had normal relative weights, and no pattern of renal injury was found upon the microscopic histopathological examinations. Unlike some species within the *Acacia* genus, such as *A. nilotica*, which cause renal and hepatotoxic injuries [25, 26], the rats' subchronic exposure to *A. aroma* extracts did not produce damage to the two key organs, the kidneys and liver.

The blood-formed elements and their precursors in the hematopoietic organs are a frequent target for many toxic substances, including natural products. Some studies have also described the *in vitro* hemolytic activity of the aqueous extracts of selected *Acacia* species [27, 28]. Therefore, the evaluation of such parameters allowed us to determine the safety of *A. aroma* extracts. The subchronic administration of both Tusca leaf extracts (5% infusion and 10% hydroalcoholic extract) to rats did not cause any hematotoxic effects, nor did they alter such a sensitive process as hematopoiesis.

Finally, based on our experimental findings, the two Tusca leaf extracts did not induce any toxicity or adverse effects on the rats' GI tract, especially in the stomach lining. This finding was especially important because we have previously demonstrated the gastroprotective properties of these extracts [5]. Moreover, we applied a more accurate method that consisted of the administration of the extracts before feeding instead of incorporating them into the animals' diet. In this context, the methodologies used in the current study allowed for controlling the concentration of the extracts in the stomach and the associated absorption ratio [29].

Conclusions

The present study contributes to expanding the knowledge about the safety of two extracts derived from *A. aroma* (5% infusion and 10% hydroalcoholic extract). They lacked cellular toxicity *in vitro* and did not produce any alteration in clinical and biochemical parameters *in vivo* during a subchronic period in doses up to 300 mg/kg. Therefore, histopathological alterations were not observed in the rats' liver, kidneys, or stomach. This study provides the possibility for the use of these two extracts in the treatment or prevention of gastric diseases in future studies.

Conflict of Interests

The authors declare that they had no conflict of interest with any internal or external entities in conducting this study.

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Compliance with Ethical Guidelines

All animal handling and procedures in this study complied with the current research standards in Argentina, based on the Ethical Framework of Reference for Biomedical Research in Laboratory Animals, Resol. D N° 1047 annexe II, 2005, and ARRIVE guidelines. The current study protocol was approved by the Institutional Committee of Animal Care and Use of the Facultad de Bioquímica, Universidad

Nacional de Tucumán (N°0015-2017). We used the minimum number of animals required to ensure statistically reliable results and made every effort to reduce animal suffering.

Authors' Contributions

FFT: Methodology, investigations, formal analyses, writing, reviewing, and editing. NCH: Conceptualization, methodology, project administration, writing, reviewing, and editing. SBG: Conceptualization, supervision, writing the original draft, and funding acquisition.

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