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SYSTEMIC AND LOCAL TOXICITY ASSAY OF AN AQUEOUS EXTRACT OF *LARREA DIVARICATA* CAV.: ROLE OF NDGA

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ABSTRACT: *Larrea divaricata* Cav. (Zygophyllaceae) is an autochthonous plant of South America, widely distributed in Argentina. It is used in folk medicine as an anti-inflammatory drug. By other way, presents well documented antitumoral and immunomodulatory effects. Nordihydroguaiaretic acid (NDGA), an antioxidant compound, had been previously described in this plant. Nowadays a formulation for hair growth based on an extract of *L divaricata* is commercialized in Argentina. Taken these into account, the objective of this work was to determine the systemic and local toxicities of the aqueous extract of *L divaricata*, by giving it under oral and topical administration. Also, it was proposed to analyze the participation of NDGA in the systemic toxicity of the plant extract. The extract presented low acute toxicity, and the histopathological examination of the tissues did not show any signal of toxicity at the same time that did not affect biochemical parameters except eosinophils which were decreased by the treatment. Which is more; the extract did not exert dermatological or ocular irritation. It is important to note that in relation to its innocuousness and pharmacological properties other phytotherapy formulations could be prepared in a future.

INTRODUCTION: *Larrea divaricata* Cav. (Zygophyllaceae) is an autochthonous plant of South America widely distributed in Argentina. It is used in folk medicine as an anti-inflammatory drug. The aqueous extract possesses well documented activities such as antiproliferative, antimicrobial [1, 2] and antioxidant activity [3, 4]. The antiproliferative and immunomodulatory activities of the aqueous extract (AE) of the leaves had been well documented "*in vitro*" [5, 6].

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Also, "in vivo" antitumoral activity of the aqueous extract, in a dose from 25 to 250 mg/kg, was shown in female rats with mammary carcinomas chemically induced with N-nitroso-N methylurea [5, 7]. By other way, the "in vivo" immunomodulatory activity in mice was demonstrated at a dose of 15 mg/kg [4, 8].

By other way, nordihydroguaiaretic acid (NDGA), which is known to be an antioxidant compound, previously described in this plant [9] was prohibited by FDA due to its hepatic and renal toxicities. Previously an acute toxicity study was performed with the extract but NDGA was not studied in comparison with this [7]. Nowadays a formulation, based on an extract of *L divaricata*, used as a topical hair growth increaser, is commercialized in Argentina. It is worth try to study the toxicity of the

plant extract in regards to its pharmacological properties and the fact that other phytotherapy formulations could be prepared in a future.

Taken into account, the objective of this work was to determine the systemic and local toxicities of the aqueous extract from *L divaricata* by giving the extract under oral and topical administration. Also, it was proposed to analyze the participation of NDGA in the systemic toxicity of the plant extract. This study revealed that the extract was not toxic.

MATERIALS AND METHODS:

Plant material and preparation of the extract:

Leaves of *Larrea divaricata* Cav. were collected in the province of Córdoba, Argentina and identified using morphological, anatomical and histochemical analyses. A voucher specimen (BAFC N° 38) was deposited in the Museum of Pharmacobotany, School of Pharmacy and Biochemistry, University of Buenos Aires.

An aqueous extract (AE) of the leaves was prepared at 7.5 %. For this the air-dried leaves were extracted for 10 min with boiling distilled water, then, the extract was filtered and lyophilized. The final yield was 26.6 g % of plant material. AE was aliquoted and stored at -20°C until used [5].

Phytochemical studies:

HPLC analysis:

The HPLC analysis was performed in a Varian Pro Star instrument equipped with a Rheodyne injection valve (20 µl) and Photodiode array detector set at 280 nm A reversed-phase column Phenomenex – C18 (2) Luna (250 mm x 4.6 mm and 5 µ dp) was used. As mobile phases, water and acetic acid (98:2, mobile phase A) and methanol and acetic acid (98:2, mobile phase B) were employed, The gradient was from 15 % B to 40 % B in 30 min; 40 % B to 75 % B in 10 min; 75 % B to 85 % B in 5 min and 100 % B in 5 min. Mobile phase B was kept at 100 % for 10 min before restoring the initial conditions. Mobile phases were delivered with a flow rate of 1.2 ml/min. The chromatographic procedure was performed at room temperature (18-25ºC). Pure standard of NDGA (Sigma (USA)) was used for identification and quantification by comparing retention times and by plotting peak areas respectively [9].

Determination of total polyphenols:

The lyophilized extract was weighted and dissolved in distilled water. Total polyphenols was determined by spectrophotometry according to the Folin-Ciocalteu method [10] using gallic acid as standard. Briefly, 1.0 ml of this sample extract was transferred to separate tubes containing 7.0 ml distilled water, 0.5 ml of Folin–Ciocalteu´s reagent, and 1.5 ml of a 20% anhydrous sodium carbonate solution. The mixture was then allowed to stand at room temperature for 60 min and then the absorbance at 765 nm was measured by employing a spectrophotometer. The concentration of polyphenols in samples was derived from a standard curve of gallic acid ranging from 10 to 50 µg/ml (Pearson´s correlation coefficient: r^2 = 0.9996).

Animals:

Seven week old female and male C3H/He mice were provided by Dr. Norberto San Juan (Department of Microbiology Faculty of Medicine, University of Buenos Aires and maintained on a standard laboratory diet and water “ad libitum”. Animals were housed at the Animal Resource Facilities, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, in accordance with institutional guidelines. The experimental designs were approved by the institutional animal research committee from Faculty of Pharmacy and Biochemistry, University of Buenos Aires, (Number: 220612-1) and experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care (Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Systemic toxicity studies:

Acute toxicity:

A modified method of Litchfield and Wilcoxon was used to assess the acute toxicity of *L. divaricata* and NDGA. Female mice were fasted for 16 h and divided into three groups of five animals per dose. The treatments were administered orally by using an intubation’s cannula. *L. divaricata* extract was administered from 20 to 50000mg/kg, and NDGA from 2.5 to 1000mg/kg. The control group received 0.2 ml of physiological saline orally. Both, the test and control animals were then allowed to access to food

and water "ad libitum" and were observed over a period of 15 days for signs of acute toxicity. The number of deaths in each group of animals within the period of observation was recorded. On day 15, mice were euthanized, gross observations were recorded and necropsies were performed.

The median lethal dose (LD_{50}) of the plant extract was calculated from data extrapolated from concentration-response curves, using a mathematical method based upon the principles of a right-angled triangle: LD_{50}: D- [(A-50 % max response). X]/ Y; A: immediately superior response of 50 % max response; D: log concentration corresponding to A response; X: D-C; Y: A-B; B: immediately inferior response of 50 % max response; C: log concentration corresponding to B response.

Sub-chronic oral toxicity:

The sub-chronic oral toxicity was performed in accordance to the instructions laid down by OECD test guideline number 407[11]. Fifty mice were divided into 5 groups of 10 animals (5 females and 5 males). The extract, re-suspended in distilled water was administered to different groups of mice at the dose levels of 25, 50, 250mg/kg respectively, 1mL of different concentrations of the extracts were administered by using an intubation's cannula, one group that receive physiological saline was considered as control. The doses were selected in accordance to the "in vivo" previous studies [5, 7, 8, 4]. Other group was treated with a dose of NDGA 1.45mg/kg (this dose represents the approximate amount of NDGA found in 250mg/kg of AE, quantified by HPLC).

At the end of the study, blood was collected, from cardiac puncture of 5 females and five males per group, into EDTA coated and uncoated containers. A complete blood test using an automated analyzer Coulter LH 750 (Beckman, Pasadena, California, USA) was performed. Red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), white blood cells (WBC), neutrophil, eosinophil, basophil, lymphocyte, monocyte and platelets were determined. Alanine aminotransferase (ALT) activity (IU/L) was determined in serum of extract/NDGA-treated or untreated mice using a commercial kit (Wiener Lab. Rosario. Argentina), cholesterol (mg %) and triglycerides (mg %) were evaluated by colorimetric methods. Also creatinine (mg/dl) was determined in serum of mice using a kinetic-colorimetric method.

Immediately after blood extraction, treated and untreated animals were sacrificed and autopsied. Briefly, organs were weighted and 3–5 mm thick of several tissue pieces were fixed in 10% formal-saline (0.9% NaCl in 10% formaldehyde) for 24 h and washed in running water for another 24 h. Samples were dehydrated by passing through 50, 70, 90, and 100% alcohol over a 2-day period, and then cleared in benzene to remove alcohol until the tissues became transparent. Then the samples were stained with haematoxylin–eosin and examined by using light microscope.

CNS toxicity:

Tail suspension test:

To evaluate the toxicity in central nervous system, such as the depressant or stimulant action, 30 animals were separated into three groups of ten animals (5 females and 5 males), one group was treated with AE (250mg/kg), other with NDGA (1.45mg/kg) and a third group with saline (control group) by oral administration as explained before. After a treatment of 28 days, animals were suspended by the tail with adhesive tape placed at 1 cm from the tip of the tail. Immobility time during a period of time of 6 min was recorded with an automated electromechanical strain gauge device and stored on a computer equipped with the relevant software (Med Associates Inc., Georgia, USA)[12]

Forced swimming test:

For this test, 30 animals were separated into three groups of ten animals (5 females and 5 males), one group was treated with AE (250 mg/kg), and other with NDGA (1.45 mg/kg) and a third group with saline (control group) by oral administration as explained before. The animals were individually placed in a beaker (16 cm in diameter) filled with 20cm water maintained at 23.5–24.5 °C. A mouse was judged to be immobile when it floated in the

water, in an upright position, and made only small movements to keep its head above water. The duration of immobility was recorded during the last 4 min of the 6-min test. A decrease in the duration of immobility is indicative of an antidepressant-like effect. The test period was automatically recorded by a camera mounted above the cylinders and stored on a computer equipped with the relevant software (Viewpoint, Viewpoint Life Sciences, France).

Local toxicity

Primary acute-irritation dermal test:

To test the irritant and toxic properties of *L. divaricata* extract on the skin, the Draize test was used. Briefly, 0.5 ml of *L. divaricata* extract (1000 mg/ml, concentration used for local uses) was applied to the intact and abraded skin of six male New Zealand White (NZW) rabbits. The treated zones were covered and observed 24 and 72 h after treatment. The erythema and edema scores for each site were monitored. The results were expressed considering the following reference values of dermic irritation: 0-1: No irritant; 1-2: Minimally irritating, 2-6: Moderately irritating; > 6: Extremely irritating

Ocular irritation test:

The acute ocular irritation study was performed to assess the potential irritancy of *L. divaricata* extract in the rabbit eyes. Twelve NZW rabbits, weighing 2.13–2.58 kg and approximately 12–20 weeks of age, were acclimatized for at least 5 days prior to the study. A volume of 0.1 ml of the test material (1000 mg/ml) was instilled into right eyes. The left eye remained untreated and was used for control purposes. Assessment of ocular damage/irritation was made at approximately 1, 24, 48 and 72h following treatment, according to the numerical evaluation given in Appendix I of Draize. Any other ocular effects were also noted. Examination of the eyes was facilitated by using a standard ophthalmoscope. An additional observation was made on day 7 to assess the reversibility of the ocular effects[13].

Statistical analysis:

Student´s *t* test was used to compare mean values between two groups and for multiple comparisons, ANOVA plus Dunnett´s test was used. A $p \leq 0.05$ was considered statistically significant.

RESULTS:

Firstly, the total polyphenol content was determined to standardize the extract. The extract presented a concentration of polyphenols of 11.395 ± 0.57 mg GAE/ g extract. The chromatographic profile of AE showed a peak corresponding to NDGA (0.30 g % w/w) with a retention time at 43.2 min (**Fig.1**).

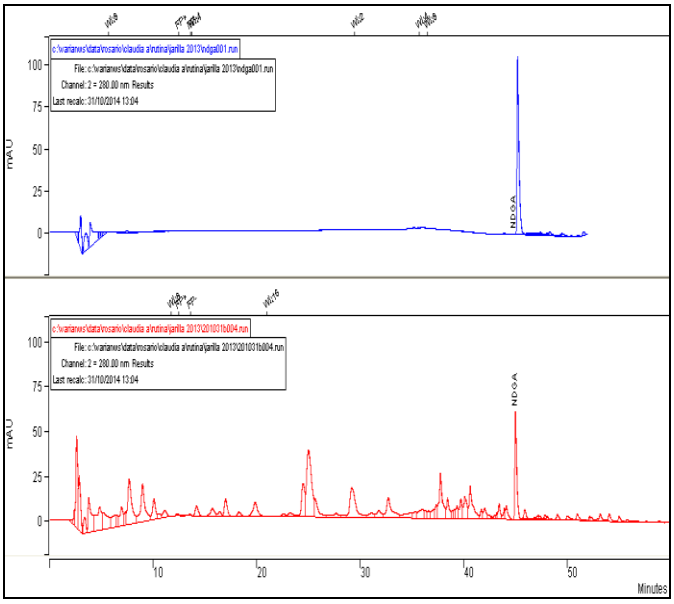

FIG. 1: CHROMATOGRAPHIC PROFILE OF AE FROM *L. DIVARICATA* CAV. AND NDGA STANDARD BY HPLC.

Secondly, the acute toxicity was determined but only in female mice. The LD_{50} of AE was 4466 mg/kg meanwhile NDGA presented a value of 77 mg/kg (**Fig. 2**).

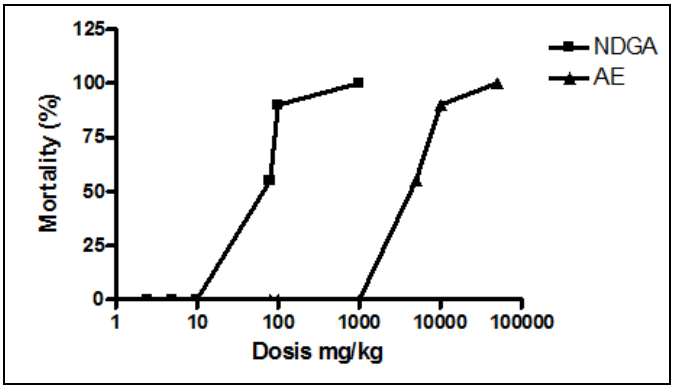

FIG. 2: MORTALITY CURVES OF *L. DIVARICATA* AND NDGA. *L. DIVARICATA* OR NDGA WAS ADMINISTERED IN FEMALE MICE AT DIFFERENT DOSES. THE NUMBER OF DEATHS IN EACH GROUP OF ANIMALS WITHIN THE PERIOD OF OBSERVATION WAS RECORDED. DOSE/RESPONSE (% DEATH) CURVE WAS THEN USED TO CALCULATE THE MEDIAN LETHAL DOSE LD50

No deaths were registered with control animals. None of the treated animals showed any clinical sign of abnormalities during their gross examination of skin, fur, eyes, mucous membrane, respiratory, circulatory, autonomic, central nervous systems and behavioral pattern. Even no signs of tremors, convulsions, salivation, diarrhea and coma were recorded after the administration of the extract. Patho-physiological observations of the organs revealed no visible lesions in any animals.

Then, the sub-acute toxicity was determined at selected doses, chosen from the animal's "in vivo" studies. Treated animals did not show any sign of mortality during the repeated dose 28-day oral toxicity study. No abnormality was observed in the ophthalmic, autonomic, central nervous system, somato-motor activity and behavior pattern that were considered to be biologically significant either with AE or NDGA (**Table 1**).

TABLE 1: PRELIMINARY OBSERVATION AND SCREENING EFFECT OF AE UP TO 48 HS AFTER ADMINISTRATION

Clinical Signal	Time (Hs)					
	0.5	1	2	6	24	48
			CNS stimulation			
Hyperactivity	-	-	-	-	-	-
Piloerection	-	-	-	-	-	-
Twitchting	-	-	-	-	-	-
Rigidity	-	-	-	-	-	-
Irritability	-	-	-	-	-	-
Jumping clonic	-	-	-	-	-	-
Convulsions	-	-	-	-	-	-
Tonic convulsions	-	-	-	-	-	-
Ptosis	-	-	-	-	-	-
			CNS depression			
Sedation	-	-	-	-	-	-
Loss of pinna	-	-	-	-	-	-
Reflux catatonia	-	-	-	-	-	-
Loss of muscle tone	-	-	-	-	-	-

Remarks: - No effect

No significant change of terminal bodyweight was recorded between the normal control and the treatment groups of both male and female animals with both treatments (data no shown). The hematology and biochemistry blood parameters did not appear to be affected by AE in any dose assayed or by NDGA as no significantly differences were noticed between treatments and controls. The only parameter affected with the treatments was eosinophils number which was decreased by AE and NDGA in all doses (**Table 2** presented results obtained with the maximum dose assayed).

TABLE 2: BLOOD SIMPLE ANALYSIS FROM TREATED AND UNTREATED MICE WITH AQUEOUS EXTRACT OF *L. DIVARICATA* 250 MG/KG AND NDGA 1.45 MG/KG.

A

	RBC	WBC	HGB	HCT	MCVC	MCH	MCHC	RDW	N	E	B	L	M	P
C F	7.2 ± 0.6	5.8 ± 0.3	12.2 ± 1.0	36.0 ± 3.0	49.0± 5.0	16.8 ± 1.0	34.3± 3.0	12.8 ± 1.0	37.5 ± 9.0	5.0 ± 0.6	0 ±0	56.3 ± 8.5	9.0 ± 0.8	727.0 ± 60
C M	8.2 ± 0.9	6.0 ± 0.6	13.2 ± 1.0	38.0 ± 3.0	51.0± 5.0	18.8 ± 1.0	36.3± 3.0	13.8 ± 1.0	38.5 ± 9.0	6.0 ± 0.6	0 ± 0	58.3 ± 8.5	9.8 ± 0.8	737.0 ± 70
NDGA F	7.3 ± 0.5	4.8 ± 0.5	11.9 ± 0.9	33.0 ± 3.0	48.0 ± 4.0	17.5 ± 2.0	35.5± 4.0	13.0 ± 0.9	30.5 ± 5.0	1.8 ± 0.6**	0 ± 0	62.5 ± 6.0	7.5 ± 7.0	715.0 ± 70
NDGA M	8.3 ± 0.5	6.8 ± 0.5	14.9 ± 0.9	34.0 ± 3.0	50.0 ± 6.0	19.5 ± 2.0	37.5± 5.0	13.6 ± 0.9	36.5 ± 5.0	1.9 ± 0.5**	0 ±0	59.5 ± 6.0	8.5 ± 7.0	730.0 ± 70
AE F	7.0 ± 0.6	4.9 ± 0.4	12.3 ± 1.0	34.0 ± 2.0	49.7 ± 5.0	18.1 ± 1.0	36.3± 4.0	12.7 ± 1.0	32.0 ± 5.0	2.0 ± 0.2 **	0 ±0	64.8 ± 7.5	6.8 ± 8.0	788.0 ± 70
AE M	8.0 ± 0.6	6.9 ± 0.4	14.3 ± 1.0	38.0 ± 2.0	55.7 ± 5.0	19.1 ± 1.0	38.6± 4.0	14.7 ± 1.5	35.0 ± 5.0	2.8 ± 0.2 **	0 ±0	62.8 ± 7.5	7.5 ± 8.0	786.0 ± 80

B

	Cholesterol (mg%)	Triglycerides (mg%)	Creatinine (mg/dl)	Alanine transaminase (IU/L)
C F	91.0 ± 4.2	146± 5.6	0.32 ± 0.02	28.0 ± 6.0
C M	92.0 ± 5.0	150± 6.6	0.37 ± 0.04	29.0 ± 5.0
NDGA F	92.5 ± 4.0	152 ± 9.0	0.34± 0.02	29.0± 6.8
NDGA M	94.0 ± 8.0	158 ± 10	0.36± 0.03	30.0± 5.0
AE F	95.5 ± 3.5	154 ± 5.6	0.28 ± 0.01	20.0 ± 5.0
AE M	96.0 ± 9.5	155 ± 10	0.32 ± 0.04	25.0 ± 5.0

Results represented mean ± S.E. of five animals. F: female mice; M: male mice. ** $p \leq 0.01$ respect to control value. C: control; NDGA: nordihydroguaiaretic acid AE: aqueous extract, RBC: Red blood cell, HGB: hemoglobin, HCT: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red blood cell distribution width, WBC: white blood cells, N: neutrophil, E: eosinophil, B: basophil, L: lymphocyte, M: monocyte and P: platelets

Moreover, no histological changes were observed in the organs analyzed with AE in different doses or NDGA (**Table 3** presented results with the maximum dose assayed).

TABLE 3: HISTOPATHOLOGICAL INVESTIGATION OF SEVERAL ORGANS FROM TREATED AND UNTREATED MICE WITH AQUEOUS EXTRACT OF *L. DIVARICATA 250* MG/KG AND NDGA 1.45 MG/KG.

	Liver		Lung		Glandular and un-glandular stomach		Kidney		Adrenal Bladder		Spleen		Pancreas		Small and large Intestine		Mouth organs		Genital organs	
	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
C1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
C2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
C3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
C4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
C5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
NDGA1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
NDGA2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
NDGA3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
NDGA4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
NDGA5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
AE1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
AE2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
AE3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
AE4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		
AE5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-			-	-			-	-					-	-		

- No alterations. C: control, NDGA: nordihydroguayaretic acid, AE: aqueous extract. F: female mice; M: male mice.

By other way, even though no signals of CNS depression or stimulation effects were observed at the naked eye, AE and NDGA treated animals were studied deeper by two methods commonly used to determine effects on CNS: the forced swimming and tail suspension tests. The time of immobility did not change with both methods in tests (female and male animals) in comparison with controls. The values obtained in female mice are represented in **Fig. 3** as example.

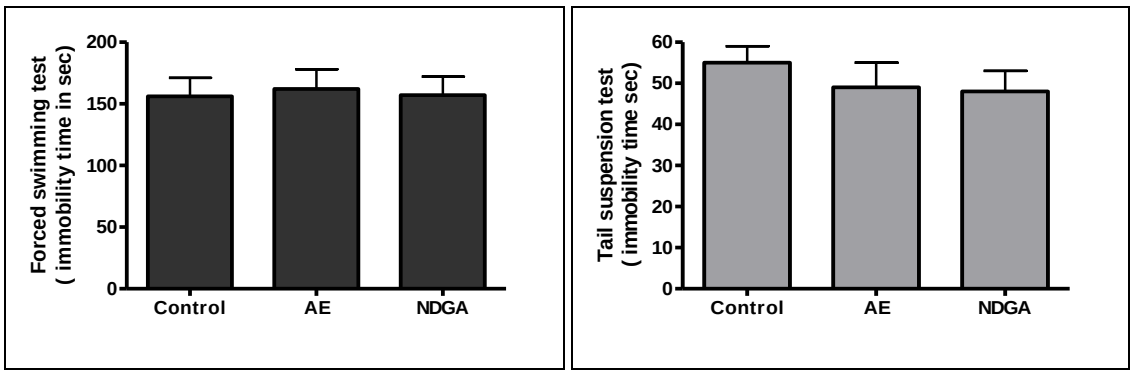

FIG.3: FORCED SWIMMING TEST (A) AND TAIL SUSPENSION TEST (B) ASSAYED ON GROUPS OF FIVE FEMALE MICE TREATED WITH (AE), NDGA AND UNTREATED (CONTROL). RESULTS WERE EXPRESSED AS MEAN ± SEM.

Finally, AE did not show dermal or ocular irritation (Table 4).

TABLE 4. "*IN VIVO*" IRRITATION TESTS IN RABBITS WITH AQUEOUS EXTRACT OF *L. DIVARICATA*.

A

Dermic irritation test		
Reference scores for dermal irritation		**AE score**
0-1	No -irritant	0.75
1-2	Minimum irritation	
2-6	Moderate irritation	
> 6	Hightly irritation	

B

Scores	Ocular irritation test				
	Time after treatment				
Signals	**24 h**	**48 h**	**72 h**	**4 days**	**7 days**
* corneal opacity	-	-	-	-	-
** Iritis	-	-	-	-	-
*** Conjuntival inflammation	-	-	-	-	-
**** all signals	-	-	-	-	-

Remarks: - No effects

DISCUSSION: In this study it was demonstrated that AE did not present systemic or local toxicity. By HPLC it was demonstrated that NDGA was the majority polyphenol compound found. NDGA is known to have antioxidant properties [14], because of this it had been employed in the conservation of fats and butter. Then, it was prohibited by FDA as consequence of its hepatotoxic effects observed at high concentrations. Despite the fact that NDGA was the majority compound, it is soluble in ethanol, slightly soluble in hot water and insoluble in cold water [26], this last explained why the amount of this compound extracted in the aqueous extracts was very low in comparison with alcoholic extracts [27].

The concentrations of NDGA related to hepatotoxicity in tissues are in the order of 50- 100 µM (15- 30 µg/ml) or more[28]. In contrast, low concentrations between 1- 10 µM (0.3- 6µg/ml) are related to beneficial properties, which are of potential use for humans, such as: enzyme inhibitor, antimicrobial, protective of neuro and bladder toxicity[29], anticancer and antimutagenic [30]. Furthermore, it was shown that it possesses antimitogenic effect in mammalian hepatocytes "in vitro" and "in vivo" [31].

The extract presented low acute toxicity because its LD_{50} was high (4.4 g/kg), previously, the acute toxicity of *L. divaricata* extract was determined in females and male mice but it was not compared with NDGA [10]. Accordingly to the previously study, the LD_{50} in male mice (LD_{50}: 10000 (8196 to 12200 mg/kg)) was higher than in female mice (LD_{50}: 4000 (3030 to 5276 mg/kg)). As female mice were more sensible than male mice in the present work females mice were selected to perform acute toxicity study with NDGA.

According to Kennedy et al[32], substances that present LD_{50} in the order of 5.0 g/kg given by oral route may be considered practically non-toxic. In regards with NDGA, as the amount of this compound present in the LD_{50} of AE, was in the order of 13.5 mg, quantity that did not produce any death or toxic signals, it could be said that NDGA did not contribute to LD_{50} of the extract.

When the sub-chronic toxicity was performed, with the exception of eosinophils number, hematological and biochemical parameters remained under the reference range for control animals as no statistical significant changes were observed between groups. Analysis of blood parameters is relevant for risk evaluation, as any change in the hematological and biochemical systems have a higher predictive value for human toxicity, when data are translated from animal studies [33].

The eosinophils are located in the connective tissues under the epithelial layer of the skin, bronchi, gastrointestinal tract and the wall of the uterus [34]. In healthy individuals, only a small number of eosinophils, circulate in the blood,

where they remain for a short period (3 to 8 h) before entering the tissues. Blood and tissue eosinophilia are markedly augmented in individuals with allergic diseases or with helminthes infections [35]. Eosinophils secrete a variety of mediators which might affect the development of the inflammation and are involved in allergic reactions. The most important of these mediators are the 5-lipoxygenase metabolites of arachidonic acid: 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene C4 (LTC4) [36] which are involved in the recruitment of other eosinophils. Some drugs such as corticosteroids induce eosinophenia in part associated with the inhibition of the synthesis of leukotrienes [37]. Since the extract presented NDGA, which is known to inhibit 5-lipoogygenase, this compound could be involved in the eosinophenia induced by the extract.

Because the extract did not modify the level of transaminases (ALT), cholesterol and creatinine, which are good indicators of liver and kidney functions respectively [38], and did not induce any histological damage in these organs, it could be hypothesize that the extract was not toxic for these organs.

By other way, it did not exert CNS toxicity in relation to depressant or stimulant activity. The methods used in this work are widely used to detect potential antidepressant effects of drugs. For example, the forced swimming test is based on the observation that when rats are exposed to water, after initial intense escape-directed behavior, such as swimming and climbing, they stop struggling and show passive immobile behavior. A wide range of antidepressant treatments have been shown consistently to reduce the amount of immobility time in the test by increasing active escape behaviors. In addition the extract would be safe for topic administration as it did not exert irritation signals by the methods studied.

CONCLUSION: Taken together these data, it could be suggested that, the aqueous extract of the plant did not present systemic or local toxicities and that NDGA content was very low to exert any toxicity at the dose assayed. These results could stand the safely medicinal use of this plant in folk medicine and in phytomedicines.

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