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Research Article

Safety Assessment of *Glyphaea brevis* Spreng. (Tiliaceae): Acute and Subacute Toxicity of the Leaf Aqueous Extract in Mice and Wistar Rats

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Abstract

Background: Despite the various medicinal applications of *Glyphaea brevis*, no toxicology data are available that could guarantee its safety or describe its possible toxic effects. We studied the acute and subacute toxicity of leaf aqueous extract of *G. brevis* (GbAE) in animal models.

Materials and methods: In acute toxicity study, mice were given a single oral administration of GbAE at doses of 1000, 2000 and 5000 mg/kg body weight (bw). The animals were monitored for behavioral changes and possible mortality over a 48-hour period, thereafter for 14 days. In the subacute toxicity study, rats were administered *G. brevis* extract at doses of 300, 600 and 1200 mg/kg bw daily, for 28 days. Tissue specimens of the liver and kidneys were subjected to histological examination using standard hematoxylin-eosin staining. An array of hematological (blood cells count and morphology) and biochemical assessments of blood (ALT, AST, urea, uric acid and creatinine) were performed.

Results: In acute toxicity study, GbAE at a dose of 5000 mg/kg caused some signs of toxicity and mortality was higher in males than in females. Subacute toxicity study revealed that GbAE at a dose of 1200 mg/kg caused significant increase of lymphocytes rate as well as serum rates of ALT and creatinin. Microscopically, there were slight hepatic and renal tissue inflammation that was reversible.

Conclusion: Some caution should be taken when *G. brevis* leaves are to be administered repetitively for long periods. Additional preclinical toxicological data should be acquired and ascertained over repeated long-term studies.

Keywords: *Glyphaea brevis*, toxicity, histopathology, liver, kidney

1. INTRODUCTION

Ancient manuscripts as well as oral tradition indicate very clearly that plants were used in the early moments of mankind to treat various health conditions. Plant-based traditional medicine is being used for thousands of years and has remarkably contributed in the development of modern pharmaceutical science. [1] Herbal products are viewed today as more 'natural' and 'soft' by a growing number of people and are still of interest in drug innovation. However, natural origin does not always guarantee their safety since there are no sufficient reports on their toxicity assessment in animals to support the nutritional or health claims made for many of these herbal products. As a matter of fact, several researchers have reported the safety and potential toxicity, as well as risks associated with the use of some vegetable species and herbal products. [2–4]

Glyphaea brevis Spreng. (Monach.) belongs to the family Tiliaceae and is widely distributed in Western and Central Africa where its leaves are consumed as a vegetable but are also valued in traditional medicine. Previous ethnobotanical surveys reported that the leaves of *G. brevis* are used in the traditional treatment of palpitations, hepatitis, poisoning, cough, heart and tooth diseases, convulsions, sexual impotency, sleepiness, bacterial infections, diabetes and some age-related brain disorders in West and Central African countries. [5–10]

Phytochemical investigations on *G. brevis* revealed the presence of alkaloids, polyterpens, sterols and phenolic compounds such as flavonoids in the leaves. [11–13] An array of studies highlighted many beneficial effects of *G. brevis* leaves such as anticonvulsant, antioxidant, α-amylase-inhibitor, antimicrobial, antimalarial, antihyperglycemic and antihyperlipidemic. [8,11,13–17] The findings from those previous studies strongly support the use *G. brevis* as an alternative medicine and as a source of compounds applicable to the treatment (or management) of various health conditions.

However, the only mention of the toxicological aspect of *G. brevis* is a study of the anticonvulsant properties of its leaf aqueous extract at the daily dose of 800 mg/kg body weight in mice. The authors noticed no mortality or behavioral change [8]. Scientific data regarding the safety of the use of *G. brevis* as an alternative medicine is however crucial in view of providing quality medicinal herbal therapy. Yet, there is no detailed report on the toxicity study of *G. brevis* leaves from the hematological, histological and biochemical points of view. Therefore, the present study was aimed at investigating both acute and subacute toxicity of *G. brevis* leaves in animals.

2. MATERIALS AND METHODS

2.1. Collection and identification of plant samples

Green adult leaves from the aerial part of several plants of *G. brevis* were collected between March and May in the City of Douala, Cameroon. Taxonomy was confirmed at Cameroon National Herbarium, Yaounde, Cameroon (voucher specimen no.10781/SRF/Cam).

2.2. Experimental animals

Swiss albino mice (*Mus musculus*) of both genders were used for the acute toxicity assessment. They were 12 week-old and weighing 25–30 g. Subacute toxicity study was performed with male and female Wistar albino rats (*Rattus norvegicus*), aged 10 weeks and weighing 150–180 g. All the animals were bred in our laboratory and maintained in cages under controlled conditions (25°C, 12/12-h dark/light cycle) with free access to food and water. Animals were randomly selected for use in the study and marked to provide individual identification. The procedures adopted in this study were in accordance with the internationally accepted principles for laboratory animal use and care as found in the United States guidelines (United States National Institutes for Health publication no. 85-23, revised in 1985). Ethical approval was obtained from the Institutional Ethics Committee for Research of the University of Douala (n° 1260 CEI-UDo/02/2018/T).

2.3. Aqueous extract preparation

The leaves of *Glyphaea brevis* were dried under shade at 40°C to constant weight and milled with a FFC-37 milling machine (Agro-Mac, Douala, Cameroon). Three hundred grams of the resulting powder was subjected to decoction with 4 liters of distilled water for 15 minutes followed by filtration and smooth evaporation at 50°C. The resulting powder (aqueous extract) was kept in a sealed flask at 2°C. Extraction yield was 19.2 % (w/w).

2.4. Study of acute toxicity

The study of acute toxicity effects was conducted according to the guidelines the Organization of Economic Co-operation and Development [18]. Forty-eight albino mice (24 males and 24 females) were randomly assigned into four groups with an equal number of males and females. After five-day acclimatization, mice were fasted for 12 hours with free access to water prior to the study. The first group served as control and received distilled water while the three other groups received aqueous extract at doses of 1000, 2000 and 5000 mg/kg body weight respectively. Water and extract were administered orally by gavage performed through a single gastric intubation. All mice were observed at the first, second, fourth and sixth hours thereafter once daily over 14 days for mortality and clinical signs of toxicity such as respiration, skin color, frequency and naturmovement, voluntary or involuntary contraction or seizures and loss of reflex etc. After the experimental period, all remaining mice were sacrificed under anesthesia and their internal organs including heart, lungs, livers, kidneys and spleen were weighted and taken for gross pathological examination. Blood samples collections were made using Eppendorf conic tubes for the group(s) in which toxic effects were observed. The samples collected were centrifuged at 2000 *g* for 10 min to obtain serum that was kept at −20 °C for biochemical analyses (transaminases and creatinine).

2.5. Study of subacute toxicity

This study was carried out according to the guidelines of the Organization of Economic Co-operation and Development (OECD) guidelines for testing of chemicals [18]. Since mortality was observed in acute toxicity at dose of 5000 $mg{\cdot}kg^{-1}$ body weight, aqueous extract was tested at three doses for subacute toxicity. Rats were randomly assigned into 5 groups of 10 animals (5 females and 5 males) each and marked to provide individual identification. The first group served as control and received distilled water. Three other groups received extract at doses of 300, 600 and 1200 $mg{\cdot}kg^{-1}$ body weight respectively by daily gastric intubation for four weeks. The last group served as satellite in order to observe reversibility, persistence or disappearance of potential toxic effects of extract. Rats from this group received aqueous extract at daily dose of 1200 $mg{\cdot}kg^{-1}$ body weight for the same period as the others and were allowed an additional period of two weeks without receiving extract. At the end of the study, rats were sacrificed under anesthesia after an overnight fast and blood was collected in EDTA tubes (for the measurement of hematological parameters) and in dry tubes (for biochemical analysis). Blood in dry tubes was centrifuged at 2000 *g* for 10 min to obtain serum that was kept at −20 °C for biochemical analyses. After blood collection, internal organs such as liver, kidney, heart, lung and spleen were removed, weighed and subjected to macroscopic observation. Liver and kidney, involved in body detoxication, were kept in 10% formalin for further histopathological examination.

2.6. Histopathological examination

Organs sections from rats of different groups were fixed in 10% formalin, dehydrated in graded alcohol and embedded in paraffin. Fine sections obtained were mounted on glass slides and stained with hematoxylin-eosin [19]. Stained specimens were observed microscopically with ×400-magnification, examined and photographed by a qualified histopathologist who had no knowledge of the treatment groups.

2.7. Hematological analyses

Hematological studies were bearing on total red blood cells (RBC) count, total white blood cells (WBC) count, hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) [20,21].

2.8. Biochemical analyses

Activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), as well as total protein and albumin concentrations were measured for the assessment of liver function. Serum urea, creatinine and total proteins concentrations were measured in order to assess the kidney function. All the measurements were done using commercially available test kits produced by Chronolab (Zug, Switzerland).

2.9. Statistical analysis

Results are expressed as means ± SD and were statistically analyzed with Student's *t*-test (STT) and one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The software SPSS for Windows (SPSS Inc., Chicago, IL, USA) version 10.1 was used for the analysis. *P*-values less than 0.05 were considered as significant.

3. RESULTS

3.1. Acute toxicity study

3.1.1. Mortality

GbAE given at doses of 1000 mg/kg and 2000 mg/kg body weight did not produce any mortality during the 14 days monitoring following extract administration (Table 1). However, at the higher dose of 5000 mg/kg, the mortality was 33.3% (4/12) in the first 48 h and was extended thereafter for male mice. At the end of the 14-day period, there were no male mice remaining and 33.3% (2/6) of females were killed.

3.1.2. Behavioral changes and adverse reactions

Clinical signs of acute toxicity of GbAE were observed only at the dose 5000 mg/kg and included immediate prostration upon extract administration (especially in male mice), and flabby skin as well as bristling hair after four hours (Table 1). Mice tended to isolate from each other as their mobility was decreasing and they were less sensitive to noise and touch. Stool became sticky during the hours following extract administration. Death occurred slowly with respiratory depression. Serum transaminases (ALT, AST) and creatinine concentrations were significantly higher ($p<0.001$) in mice treated with GbAE (5000 mg/kg) in comparison with control (Table 2). However, the necropsies performed did not show any macroscopic change in internal organs (liver, kidneys, heart, spleen and lungs).

3.2. Subacute toxicity

Oral administration of GbAE at daily doses of 300, 600 and 1200 mg/kg did not produce any mortality in tested animals. Apart from increased diuresis in GbAE-treated rats, no sign of behavioral trouble was detected during the experimental and recovery periods (satellite group).

3.2.1. Effects of GbAE on body weight

There was no significant difference in weight evolution of *G. brevis* treated rats in comparison with the control groups during the first 2 weeks of the study (Table 3). However, the total weight gain was significantly lower especially in groups treated with GbAE at doses of 600 and 1200 mg/kg body weight (Table 3).

3.2.3. Effects of GbAE on organ weights

There was no significant difference in organ weights of *G. brevis* treated rats as compared to the control rats at the end of the subacute toxicity study (Table 4).

3.2.4. Effects of GbAE on hematological parameters

Subacute administration of GbAE at dose of 1200 mg/kg triggered a significant increase in leucocyte rates in both female and male rats (Table 5). Such increase was not observed in the satellite group. The other hematological parameters were not affected by GbAE treatment.

3.2.5. Effects of GbAE on serum total proteins, albumin, urea and creatinine

There were no significant change in serum urea, total proteins and albumin concentrations in all GbAE-treated rats in comparison with those from the control group (Table 6). Serum creatinin concentrations were significantly increased in the GbAE 1200 mg/kg group, especially in males.

3.2.6. Effects of GbAE on serum marker enzymes

The enzyme activity of aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were not different from control group to tests groups (Table 7). However, the repeated administration of GbAE at dose of 1200 mg/kg daily significantly increased the activity of alanine aminotransferase (ALT) by 1.7 times ($p<0.01$) especially in male rats.

3.2.7. Effects of GbAE on liver histology

The liver cuts from the control group (Figure 1A) had a normal lobule with a uniform hepatic parenchyma, a central vein with normal diameter, and a normal portal space. Administration of the aqueous extract at doses of 300 and 600 mg/kg for 4 weeks did not result in significant impairment of liver histology in treated animals (Figure 1B and 1C). However, vascular congestion and mild periportal inflammation were noticed with the 1200 mg/kg dose in the liver (Figure 1D). Those conditions were absent in the satellite group (Figure 1E).

3.2.8. Effects of GbAE on kidney histology

The sections of the kidneys of rats in the control group had normal renal parenchyma with normal glomeruli, vessels and normal interstices, and visible tubules (Figure 2A). The administration of aqueous extract at doses of 300 and 600 mg/kg for 4 weeks did not result in significant impairment of kidney histology of treated animals (Figure 2B and 2C). However, administration of the aqueous extract at dose of 1200 mg/kg resulted in a glomerular inflammation in conjunction with tubular clarifications and interstitial edema (Figure 2D). In contrast, only the tubular clarifications have been noticed as abnormalities of renal histology in animals treated with the aqueous extract at a dose of 1200 mg/kg for 4 weeks and left under observation for 2 additional weeks (Figure 2E).

Table 1: Behavior of female and male mice in acute toxicity of *G. brevis* leaf aqueous extract (GbAE).

Treatment	*Sex*	*Time*	*Mobility*	*Aggressiveness*	*Sensitivity*		*Fecal consistency*	*Respiration*	*Mortality (%)*
					Auditory	Tactile			
Control	Female	2 h	N	N	N	N	N	N	-
		48 h	N	N	N	N	N	N	-
		7 days	N	N	N	N	N	N	-
		14 days	N	N	N	N	N	N	-
	Male	2 h	N	N	N	N	N	N	-
		48 h	N	N	N	N	N	N	-
		7 days	N	N	N	N	N	N	-
		14 days	N	N	N	N	N	N	-
GbAE 1000 mg/kg	Female	2 h	N	N	N	N	N	N	-
		48 h	N	N	N	N	N	N	-
		7 days	N	N	N	N	N	N	-
		14 days	N	N	N	N	N	N	-
	Male	2 h	N	N	N	N	N	N	-
		48 h	N	N	N	N	N	N	-
		7 days	N	N	N	N	N	N	-
		14 days	N	N	N	N	N	N	-
GbAE 2000 mg/kg	Female	2 h	R	N	N	N	N	N	-
		48 h	N	N	N	N	N	N	-
		7 days	N	N	N	N	N	N	-
		14 days	N	N	N	N	N	N	-
	Male	2 h	N	N	N	N	N	N	-
		48 h	N	N	N	N	N	N	-
		7 days	N	N	N	N	N	N	-
		14 days	N	N	N	N	N	N	-
GbAE 5000 mg/kg	Female	2 h	R	R	R	N	P	N	-
		48 h	R	R	R	R	P	N	16.67
		7 days	R	R	R	R	N	N	33.33
		14 days	N	N	N	N	N	-	33.33
	Male	2 h	R	R	R	N	P	S	-
		48 h	R	R	R	R	P	N	50
		7 days	-	-	-	-	-	-	100
		14 days	-	-	-	-	-	-	100

Behavioral components are expressed as follows: N= normal ; R= reduced ; S= slowed ; P= pasty. n=6 (females or males)

Table 2 : Biochemical markers of liver toxicity in mice during acute toxicity testing of *G. brevis* leaf aqueous extract (GbAE) at dose of 5000 mg/kg.

Parameter	Control	GbAE 1000 mg/kg	GbAE 2000 mg/kg	GbAE 5000 mg/kg
Females				
ALT (U/L)	32.48 ± 3.44	34.26 ± 3.01	37.11 ± 4.46	45.16 ± 6.92*
AST (U/L)	59.87 ± 2.27	62.35 ± 2.12	62.08 ± 2.18	69.12 ± 1.59*
Creatinin (mg/dL)	0.30 ± 0.02	0.34 ± 0.03	0.52 ± 0.07	0.83 ± 0.04*
Males				
ALT (U/L)	35.94 ± 2.71	40.32 ± 2.43	40.17 ± 2.10	-
AST (U/L)	61.58 ± 1.49	63.88 ± 2.18	67.01 ± 1.82	-
Creatinin (mg/dL)	0.34 ± 0.05	0.41 ± 0.08	0.56 ± 0.11*	-

Values are expressed as Mean ± SD, n=6 (females or males). *Significantly different from control (p<0.05 ; DMRT).

Table 3: Body weights (g) of female and male rats in subacute toxicity of *G. brevis* leaf aqueous extract (GbAE).

Treatment	Sex	Main study						Recovery period		
		Initial	Day 7	Day 14	Day 21	Day 28	Weight gained (%)	Day 35	Day 42	Weight gained (%)
Control	Female	160.65 ± 5.12^{a}	168.40 ± 4.86^{a}	175.43 ± 5.08^{a}	181.24 ± 6.02^{a}	188.56 ± 7.11^{a}	17.37	-	-	-
	Male	172.57 ± 5.93$^{\alpha}$	188.78 ± 6.47$^{\alpha}$	199.87 ± 5.66$^{\alpha}$	214.61 ± 6.48$^{\alpha}$	226.27 ± 6.07$^{\alpha}$	31.12	-	-	-
GbAE 300 mg/kg	Female	163.88 ± 5.96^{a}	170.55 ± 5.22^{a}	176.04 ± 4.17^{a}	180.31 ± 3.16^{a}	185.22 ± 4.29^{a}	13.01	-	-	-
	Male	176.97 ± 6.95$^{\alpha}$	191.28 ± 7.24$^{\alpha}$	204.68 ± 7.18$^{\alpha}$	219.22 ± 6.52$^{\alpha}$	232.62 ± 6.38$^{\alpha}$	31.44	-	-	-
GbAE 600 mg/kg	Female	162.47 ± 4.98^{a}	167.14 ± 6.28^{a}	172.84 ± 5.77$^{\alpha}$	177.11 ± 6.44^{ab}	181.17 ± 5.34^{ac}	11.51	-	-	-
	Male	173.91 ± 5.82$^{\alpha}$	185.45 ± 6.39$^{\alpha}$	198.06 ± 6.11$^{\alpha}$	209.44 ± 5.63$^{\alpha}$	223.18 ± 4.80$^{\alpha}$	28.33	-	-	-
GbAE 1200 mg/kg	Female	158.35 ± 5.81^{a}	162.26 ± 7.55^{a}	164.59 ± 5.86^{a}	167.98 ± 4.17^{b}	171.35 ± 3.88^{b}	8.21	-	-	-
	Male	175.40 ± 4.19$^{\alpha}$	186.37 ± 5.52$^{\alpha}$	196.12 ± 3.94$^{\alpha}$	207.89 ± 5.44$^{\alpha}$	213.52 ± 3.29$^{\beta}$	21.73	-	-	-
GbAE 1200 mg/kg+	Female	161.23 ± 6.11^{a}	165.06 ± 6.48^{a}	168.42 ± 5.99^{a}	170.55 ± 4.32^{b}	175.48 ± 2.16^{bc}	8.83	182.64 ± 5.33	190.51 ± 6.28	18.16
	Male	170.63 ± 3.13$^{\alpha}$	180.77 ± 4.56$^{\alpha}$	192.58 ± 4.16$^{\alpha}$	203.18 ± 5.08$^{\alpha}$	211.41 ± 4.22$^{\beta}$	23.90	220.38 ± 5.14	228.92 ± 5.79	34.16

Values are expressed as Mean ±SD, n=5 (females or males). In each column and for each sex considered, values not sharing the same superscript letter are significantly different (p<0.05 ; DMRT).

Table 4: Relative organ weights of rats in subacute toxicity of *G. brevis* leaf aqueous extract (GbAE)

Organ	Control	GbAE 300 mg/kg	GbAE 600 mg/kg	GbAE 1200 mg/kg	GbAE 1200 mg/kg+
Females					
Liver	2.88 ± 0.16	3.10 ± 0.34	2.73 ± 0.18	2.94 ± 0.12	3.06 ± 0.31
Kidney	0.48 ± 0.04	0.52 ± 0.08	0.55 ± 0.08	0.51 ± 0.10	0.54 ± 0.07
Heart	0.31 ± 0.05	0.32 ± 0.07	0.34 ± 0.05	0.37 ± 0.11	0.33 ± 0.08
Lung	0.68 ± 0.04	0.73 ± 0.03	0.72 ± 0.03	0.75 ± 0.09	0.71 ± 0.05
Spleen	0.27 ± 0.06	0.24 ± 0.07	0.25 ± 0.05	0.26 ± 0.05	0.24 ± 0.03
Males					
Liver	3.46 ± 0.34	3.19 ± 0.82	3.62 ± 0.73	3.52 ± 0.26	3.41 ± 0.34
Kidney	0.54 ± 0.06	0.59 ± 0.10	0.68 ± 0.09	0.62 ± 0.11	0.61 ± 0.05
Heart	0.30 ± 0.04	0.34 ± 0.12	0.38 ± 0.08	0.35 ± 0.13	0.37 ± 0.10
Lung	0.73 ± 0.06	0.76 ± 0.03	0.78 ± 0.04	0.74 ± 0.02	0.77 ± 0.06
Spleen	0.24 ± 0.07	0.23 ± 0.02	0.22 ± 0.05	0.24 ± 0.03	0.23 ± 0.02

Values are expressed as Mean ±SD, n=5 (females or males).

Table 5: Hematological values of rats in subacute toxicity of *G. brevis* leaf aqueous extract (GbAE)

Parameters	Sex	Control	GbAE 300 mg/kg	GbAE 600 mg/kg	GbAE 1200 mg/kg	GbAE 1200 mg/kg+
RBC ($\times 10^6/\mu l$)	Female	7.08 ± 0.28	7.02 ± 0.49	7.26 ± 0.35	7.33 ± 0.21	7.07 ± 0.26
	Male	8.12 ± 0.67	7.70 ± 0.39	7.96 ± 0.44	8.05 ± 0.52	7.82 ± 0.68
Hb (g/dL)	Female	14.48 ± 1.31	14.52 ± 1.54	14.43 ± 1.82	14.51 ± 0.99	14.64 ± 1.17
	Male	15.55 ± 1.13	14.42 ± 1.37	15.69 ± 1.42	15.38 ± 1.25	15.64 ± 1.69
HCT (%)	Female	41.00 ± 4.37	40.11 ± 3.88	40.68 ± 2.67	39.34 ± 3.83	41.66 ± 2.41
	Male	46.60 ± 5.22	43.33 ± 2.22	43.72 ± 2.73	45.62 ± 4.36	45.85 ± 3.54
MCV (μm^3)	Female	60.95 ± 5.45	59.83 ± 4.13	60.52 ± 3.55	59.47 ± 4.62	58.71 ± 4.76
	Male	57.28 ± 4.32	59.12 ± 1.78	58.84 ± 3.23	58.13 ± 3.77	59.79 ± 2.16
MCH (pg)	Female	21.74 ± 2.12	20.88 ± 2.27	21.49 ± 2.73	21.86 ± 3.01	22.63 ± 2.11
	Male	19.08 ± 2.03	19.72 ± 2.34	19.47 ± 2.11	19.78 ± 1.49	20.12 ± 2.57
MCHC (g/dl)	Female	35.68 ± 1.93	36.25 ± 2.57	35.52 ± 3.48	36.83 ± 2.59	37.06 ± 3.16
	Male	33.35 ± 3.18	31.62 ± 3.01	33.38 ± 3.32	34.47 ± 2.47	34.74 ± 2.99
WBC ($\times 10^3/\mu l$)	Female	6.20 ± 1.08	5.04 ± 0.93	5.23 ± 1.27	5.67 ± 0.78	5.18 ± 1.04
	Male	7.68 ± 0.75	7.39 ± 0.97	7.58 ± 0.85	7.24 ± 0.64	7.77 ± 1.19
LC (%)	Female	72.56 ± 3.16	71.28 ± 6.22	74.98 ± 7.74	80.33 ± 3.07*	70.67 ± 9.18
	Male	71.90 ± 4.04	69.74 ± 7.48	73.11 ± 5.26	80.19 ± 4.71*	77.12 ± 5.88
NP (%)	Female	19.44 ± 7.40	19.32 ± 5.18	17.89 ± 5.83	15.64 ± 7.50	17.47 ± 6.91
	Male	14.90 ± 6.08	15.29 ± 6.54	17.56 ± 4.38	13.78 ± 5.31	16.66 ± 6.29
MC (%)	Female	6.22 ± 1.96	6.07 ± 2.54	5.35 ± 1.86	5.81 ± 1.73	6.16 ± 2.28
	Male	5.10 ± 3.64	6.01 ± 2.26	5.28 ± 3.03	5.65 ± 2.58	5.98 ± 1.77
EP (%)	Female	0.78 ± 1.08	1.12 ± 0.77	0.98 ± 1.16	0.94 ± 1.25	1.06 ± 0.93
	Male	2.30 ± 2.04	2.23 ± 1.47	1.87 ± 1.11	1.16 ± 0.99	1.54 ± 1.21

RBC: red blood cell count; Hb: hemoglobin, HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; WBC: white blood cell count; LC: lymphocytes; NP: neutrophils; MC: monocytes; EP: eosinophils. Values are expressed as mean ± SD, n=5 (females or males). *Significantly different from control ($p<0.05$).

Table 6: Effects of subacute administration of *G. brevis* leaf aqueous extract (GbAE) on serum total proteins, creatinine, urea and albumin.

Parameters	Sex	Control	GbAE 300 mg/kg	GbAE 600 mg/kg	GbAE 1200 mg/kg	GbAE 1200 mg/kg+
Creatinine (mg/dL)	Female	0.41 ± 0.07	0.34 ± 0.08	0.39 ± 0.05	0.41 ± 0.12	0.34 ± 0.09
	Male	0.30 ± 0.08	0.22 ± 0.07	0.24 ± 0.09	0.57 ± 0.05*	0.55 ± 0.08
Urea (mg/dL)	Female	19.27 ± 1.94	20.02 ± 2.27	21.43 ± 1.15	22.08 ± 1.83	21.17 ± 2.24
	Male	22.26 ± 1.62	21.41 ± 3.24	22.07 ± 3.07	22.98 ± 2.29	23.97 ± 3.33
Total proteins (g/dL)	Female	4.95 ± 0.08	5.29 ± 0.17	5.19 ± 0.06	5.47 ± 0.13	5.11 ± 0.19
	Male	5.06 ± 0.04	5.13 ± 0.18	5.26 ± 0.15	5.42 ± 0.17	5.25 ± 0.14
Albumin (g/dL)	Female	3.57 ± 0.22	3.13 ± 0.28	3.22 ± 0.30	3.48 ± 0.19	3.59 ± 0.22
	Male	3.31 ± 0.29	3.72 ± 0.27	4.03 ± 0.32	3.41 ± 0.34	3.67 ± 0.25

Values are expressed as mean ± SD, n=5 (females or males). *Significantly different from control (p<0.05).

Table 7: Effects of subacute administration of *G. brevis* leaf aqueous extract (GbAE) on serum marker enzymes

Parameters	Sex	Control	GbAE 300 mg/kg	GbAE 600 mg/kg	GbAE 1200 mg/kg	GbAE 1200 mg/kg+
ALT (U/L)	Female	28.48 ± 6.23	32.29 ± 5.68	36.11 ± 4.23	33.48 ± 6.41	31.68 ± 7.35
	Male	24.62 ± 5.68	23.54 ± 7.18	27.86 ± 5.41	41.64 ± 4.27*	28.31 ± 3.99
AST (U/L)	Female	87.00 ± 8.34	75.47 ± 5.23	81.14 ± 7.08	84.29 ± 9.66	82.37 ± 8.71
	Male	82.14 ± 4.68	79.1 ± 10.02	78.43 ± 4.13	85.06 ± 5.21	80.27 ± 8.12
ALP (U/L)	Female	62.92 ± 10.44	59.33 ± 9.52	67.3 ± 11.47	64.1 ± 13.02	70.66 ± 9.68
	Male	68.2 ± 12.18	72.2 ± 11.11	77.1 ± 12.15	80.03 ± 9.64	70.2 ± 10.37

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase. Values are expressed as mean ± SD, n=5 (females or males). *Significantly different from control (p<0.05).

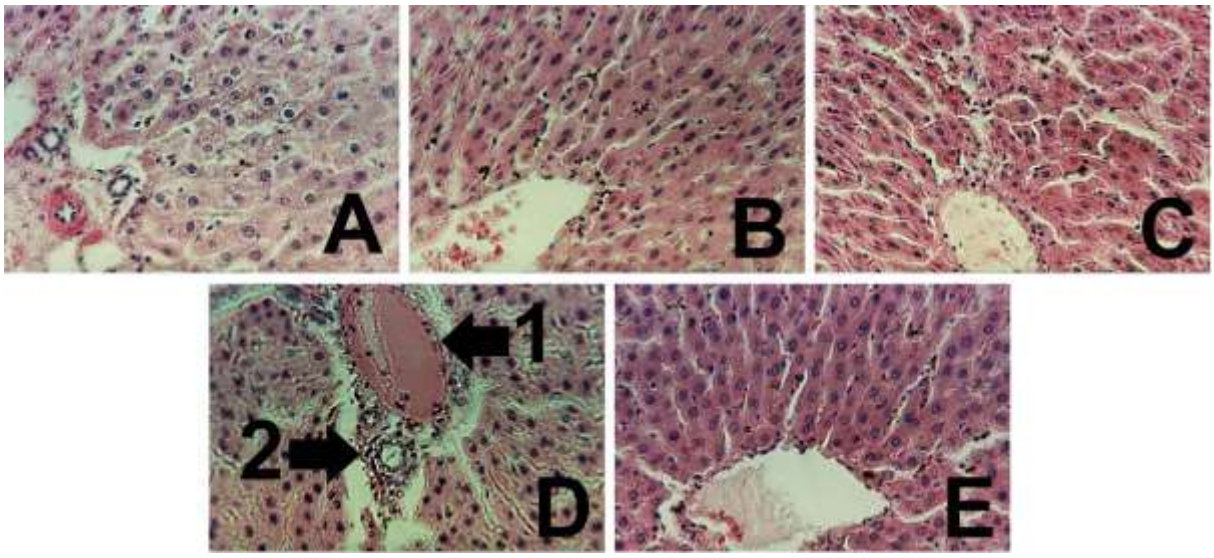

Figure 1: Liver histology of rats treated with *G. brevis* leaf aqueous extract (GbAE) at doses of: A) 0 (control), B) 300 mg/kg, C) 600 mg/kg, D) 1200 mg/kg and E) 1200 mg/kg+ (satellite) at the end of subacute toxicity study. 1: vascular congestion; 2: periportal inflammation.

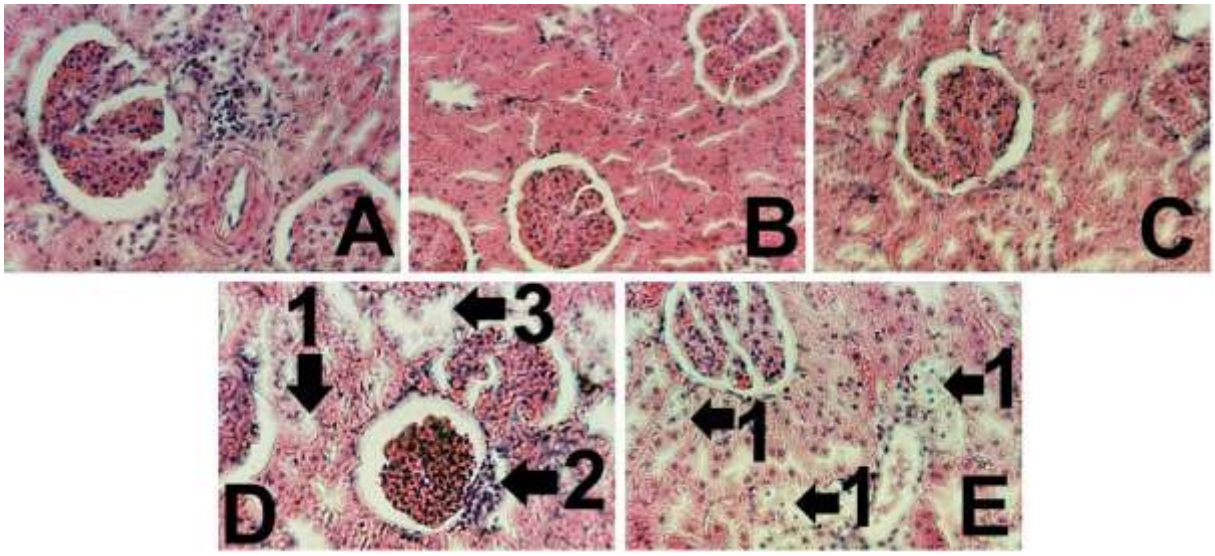

Figure 2: Kidney histology of rats treated with *G. brevis* leaf aqueous extract (GbAE) at doses of: A) 0 (control), B) 300 mg/kg, C) 600 mg/kg, D) 1200 mg/kg and E) 1200 mg/kg⁺ (satellite) at the end of subacute toxicity study. 1: tubular clarification; 2: glomerular inflammation; 3: interstitial edema.

4. DISCUSSION

The aim of this study was to investigate the safety of the aqueous extract of *Glyphaea brevis* (GbAE) by determining its toxicological effects after acute and subacute administration in mice and rats, respectively. It is crucial the therapeutic activity of any given substance being effective at doses that will not be harmful for the consumer. When a substance is toxic, some vital functions of the body are affected and the resulting disturbances may be evidenced by specific behavioral, histological, hematological and biochemical analyses.

In the acute toxicity study, a single dose treatment of 5000 mg/kg of GbAE in mice caused signs of toxicity and mortality (33.3%), suggesting that the LD_{50} of GbAE would be higher than 5000 mg/kg which corresponds to level 5 (practically non-toxic substances) in the Hodge and Sterner toxicity scale. [22] The mortality rate was found to be higher in male mice than in females. Such difference in mortality has been observed in other toxicity studies and is generally ascribed to hormonal differences between both genders [23]. Thus, the results of our study suggest a low toxicity of *G. brevis* extract.

In subacute toxicity, hematological study showed that the extract had no effect on red blood cells as well as white blood cells counts. However, the proportion of lymphocytes was slightly increased in the rats treated with GbAE at a dose of 1200 mg/kg but remained within the normal range, ie 65-84% [24], suggesting the absence of clinical significance at this level.

The damage to liver cells as a result of necrosis results in the release of intracellular components, including transaminases (ALT and AST) and alkaline phosphatase into the bloodstream [25,26]. Therefore, these components are ordinarily used as markers of hepatocellular injury. However, about 80% of the AST cellular pool is located in mitochondria while ALT is merely cytosolic. As a consequence, ALT is released faster than AST in the bloodstream and is viewed as a more reliable marker of liver damage. [27] Therefore, the increases in serum levels of transaminases observed in the groups treated with GbAE in the acute and subacute toxicity studies are indicators of liver damage as confirmed by liver histology (Tables 2 and 6, Figure 1). Although this increase is significant compared to the control group, all values are close to the reference value, i.e. 96-200 U/L for AST and 21-52 U/L for ALT [28]. This observation may explain the fact that no significant change in relative organ weight was observed among the groups. Alkaline phosphatase (ALP) is one of the most frequently measured enzymes in the screening of bile duct obstruction. An important activity of this enzyme is found in metabolically active cells such as liver and kidney, among others and is markedly increased in case of primary biliary cirrhosis, liver architecture destruction and disease characterized by inflammation and obstruction of the bile ducts. [29] Subacute administration of *G. brevis* aqueous extract resulted in no significant change in serum activity of ALP. This observation suggests that the extract would not favor the bile duct obstruction in subacute toxicity. Reference values of serum creatinin levels in rats are between 0.2 and 0.8 mg/dL. [24] Therefore, the values observed in the test groups suggest the absence of impairments to the renal function.

The relative organ weight of is an indication of whether an organ has been exposed to damage or otherwise. As a consequence, the assessment of the weight of various organs is an important step in toxicological studies. [30] Relative organ weight in the extract-treated animals was not changed significantly as compared to the organs (liver, kidney, heart, lung, spleen) of the rats from the control group. This observation suggests that the extract at the doses tested in this study does not bring about toxicity signs causing organ weight changes upon repeated doses of GbAE in subacute toxicity. However, the slight microscopic inflammatory lesions observed in tissues from detoxification organs indicate that GbAE may cause liver and kidney toxicity when administered orally for an extended period. However, the data we collected do not allow us to elucidate the mechanism by which this tissue damage occurs.

Body weight gain was significantly lower in the groups receiving the extracts as in males than in females. On the other hand, the food intake of rats receiving the extracts was not different from that of the rats from the control group (data not shown). Previous research has shown that GbAE had α-amylase inhibitory effects that impacted body weight gain in rats by decreasing the efficiency of dietary starch digestion and endogenous lipogenesis. [14]

5. CONCLUSION

In this study, we assessed the acute and subacute toxicity of *G. brevis* leaf aqueous extract (GbAE) administered orally to mice and rats respectively. The results suggest that acute single administration of GbAE at a dose of 5000 mg/kg and upwards are associated with signs of toxicity and mortality. Repeated administration GbAE at 1200 mg/kg and upwards is associated with mild liver and kidney inflammation evidenced by some histological, hematological and biochemical changes. As a consequence, some caution should be taken when *G. brevis* leaves are to be administered repetitively for long periods. Additional preclinical toxicological data should be acquired and ascertained over repeated long-term studies.

Conflicts of Interest: None.

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