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Chemical Compounds and Some Acute Toxicological Parameters of *Bauhinia platypetala*

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ABSTRACT

Bauhinia platypetala Burch Leguminosae is widely used in Brazil as a medicinal agent for treating diabetes, parasitic infestations, and also acts as a diuretic. This study investigated the safety of ethanol extract (EE) and the ethereal (EF), aqueous (AF), and ethyl acetate (EA) fractions from *B. platypetala*, determining their toxicological effects in mice. For each experiment, mice were randomly divided into five groups: the control group was treated with vehicle; four groups were treated 30 min before the behavioral tests and acute toxicity with only one dose of EE, EF, AF and EA (0.05 g/kg), and a reference group treated with DZP 0.001 g/kg. In EF, EA, and AF fractions the main components were phytol, stearic acid and myo-inositol, respectively. Our experiments could not determine the ED_{50}, since no death was detected in acute toxicological tests. Ethanol extract and fractions did not cause alteration in motor activity, behavioral and respiratory rates. In open field test, only EE decreased the number of square crossed. Acute administration of the *B. platypetala* produces no change of hematological and biochemistry parameters. Collectively, the results indicate that the extract and fractions from *B. platypetala* may be atoxic, showing that this can be used as a folk medicine.

Keywords: *Acute toxicity, Bauhinia platypetala Burch, Behavior, Biochemical parameters, Ethanol extract, Hematological parameters*

INTRODUCTION

Herbal medicine, a natural remedy has become universally popular in primary healthcare, particularly in developing countries including Brazil [1-5]. Herbal sources from medicinal plants are presumed to be safe without any compromising health effect, thus widely used as self-medication [6]. However, since there is a lack of scientifically proven studies on the toxicity and adverse effect of these remedies, further investigations are vitally needed and the same applies to *Bauhinia platypetala* Burch Leguminosae.

Brazil possesses a large potential for biodiversity and has a wealth of traditional knowledge accumulated by local people who have direct access to nature and natural products [7,8]. Traditional knowledge related to medicinal plants is the basis in Brazil folk medicine, which is derived from a mixture of Brazilian indigenous with the European and African cultures influences from the colonization period [9].

Among the plants used in popular medicine, there is *B. platypetala*, a species presented as small trees or shrubs of 4-10m

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high from 1.5 to 3m high, leaves obcordiforme, flowers have thin tube calyx and broad-linear petals or oblong fruit is a pods flat, 4-7 inches in length and number of seeds [10].

Studies conducted in Brazil found that *B. platypetala* is popularly used in the treatment of diabetes. However, the mechanism of antihyperglycemic has not been demonstrated yet. Other Bauhinia species such as *B. forficata* Link subsp. forficate have been used as a potent antidiabetic, diuretic, hypo-cholesteremic and used against bladder infections and intestinal parasites [11].

Despite the evidence of *B. platypetala* consumption to treat diabetes, the present study investigated its safety to human health, since it is used in folk medicine with no details regarding toxic effects of the ethanol extract and fractions of *B. platypetala*. Thus, we evaluated the effects of ethanol extract and fractions of *B. platypetala* on motor activity in mice as well as toxicological aspects after acute administration to prove scientifically that it is safe to use in Northeastern Brazil´s folk medicine.

Material and Methods

Plant materials

The species also known as cow's foot or "capa-bode", was collected in March 2008 by Francisco José Borges dos Santos in areas around the Federal University of Piauí (UFPI), Teresina, Piauí (Brazil), and stored under medium temperature, dried before use. The plant was identified by Dra. Ângela Maria Studart da Fonseca Vaz at the Botanic Garden Research Institute of Rio de Janeiro, in the state of Rio de Janeiro, Brazil. A voucher specimen (ICN TEPB 24875) was deposited in Graziella Barroso Herbarium of the Federal University of Piauí, Brazil.

Phytochemical screening and gas chromatography-mass spectrometry (GC-MS)

The leaves collected were dried under medium temperature and grounded into a course powder. Dry powder (1300g) was macerated exhaustively in 95% ethanol (w/w), for sixteen days. The resulting ethanol extract (5.9%) was filtered and concentrated under pressure. The concentrated part (800 g) was partitioned into diethyl ether and ethyl acetate, respectively, resulting in the aqueous (13.79%), ethereal (17.24%) and ethyl acetate (3.45%) fractions. The ethanol extract and fractions (ethereal, aqueous and ethyl acetate) were lyophilized. The dried extract and fractions were kept at 4 °C in airtight bottles until use.

In order to determine the chemical constituents, qualitative phytochemical screening of the ethanol extract of *B. platypetala* was carried out following standard procedures routinely used in the laboratory [12,13]. Pretreatment of the fractions in BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane) was performed as previously described [14]. Analysis of the fractions was performed on Shimadzu GC-17A/MS QP5050A (GC-MS system): DB-5HT capillary column (30 m x 0.251 mm, 0.1 μm film thickness); carrier gas: helium 1.7 mL/min; column inlet pressure 107.8 kPa; column flow = 1.7 mL/min; linear velocity = 47.3 cm/s; total flow 24 mL/min; carrier flow 24 mL/min; injector temperature 280 °C; detector temperature 300 °C; column temperature 100 (1 min) – 310 °C at 10 °C/min (15 min). Mass spectrometer operating conditions were 70 eV of ionization energy. Mass spectra were recorded from 40-450 m/z. The percent of area was obtained electronically from the GC-MS response without the use of an internal standard or correction factors.

Animal procedures

Adult *Swiss* albino mice (*Mus musculus*, 20-30 g) were obtained from the animal facilities of Federal University of Piauí, Brazil. They were kept in well-ventilated cages (ALESCO, São Paulo) under standard conditions of light (12 hours with alternative day/night cycles) and temperature (26 ± 1 °C) and were housed with access to commercial rodent stock diet (Purina, São Paulo, Brazil) and water ad libitum. All behavioral tests were conducted in quiet rooms at the same controlled conditions referred above and isolated from external noise. For each experiment, mice were randomized into five groups (10 mice per group): one control group treated with the vehicle (Tween-80 0.05% dissolved in 0.9% saline), four groups treated with ethanol extract (EE), ethereal (EF), aqueous (AF) and ethyl acetate (EA) fraction at 0.05 g/kg. Treatment with EE, EF, AF, EA or vehicle was performed by intraperitoneal (i.p.) route (1 mL/kg) and all animals were observed during 24 hours [15-18]. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals [19] and are in accordance with COBEA (Colégio Brasileiro de Experimentação Animal) and International Standard on the care and use of experimental animals (EEC Directive of 1986, 86/609/EEC). The project was previously approved by the Animal's Ethics Committee of the Federal University of Piauí (Protocol number # 044/09).

Experimental protocol and behavioral screening

After the treatment, each animal was submitted to a series of tests. Firstly, the animal was observed in a closed room, at constant temperature (26 ± 1°C). Then, the animal was placed in the open field area for 5 min. Finally, its temperature was taken with a digital thermometer. All tests were performed between 08:00 and 10:00 a.m. Behavioral screening was performed following established parameters [20] and animals were observed 24 hours after i.p. administration with EE, EF, AF and EA from *B. platypetala* (0.05 g/kg). After 24 hours, they were observed about occurrence of the following general signs of toxicity: piloerection, prostration, writhing, evacuation, grooming, dyspnea, sedation, analgesia and palpebral ptosis.

Open field test

The open field area was made of acrylic (transparent walls and black floor, 30 x 30 x 15 cm) divided into nine squares of equal area. The open field was used to evaluate the exploratory activity of the animal [21]. The observed parameter was the number of squares crossed (with the four paws).

Toxicological evaluation of ethanol extract and fractions from *B. platypetala*

Acute toxicity studies in mice

The ethanol extract and fractions from *B. platypetala* were

administered by intraperitoneal route (n = 10 animals/group) at a dose of 0.05 g/kg body weight, while the control group received the vehicle (Tween-80 0.05% dissolved in 0.9% saline). The general behavior of mice and signs of toxicity were observed continuously for 1, 4 and 24 hours after the intraperitoneal treatment [17]. Mice were further observed once a day up to 24 hours for following treatment for behavioral changes and signs of toxicity and/or death and the latency of death. The LD_{50} value was determined by a method previously described by Litchfield and Wilcoxon [22].

Measurement of biochemical and hematological parameters in blood

The hematological and the biochemical analyses were performed at a large nationally accredited commercial laboratory. Blood cell parameters (hematocrit, hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV)) were determined by a counter (Advia 120/ Hematology, Siemens Diagnostics, Tarrytown, NY). Blood smears stained with May-Grünwald-Giemsa were used to stain and differential count of white blood cells (neutrophils, erytrocytes, eosinophils, lymphocytes, monocytes) and platelets. Serum glucose, uric acid, urea, creatinine, alkaline phosphatase, triglycerides, total protein, total bilirubin, direct bilirubin, total cholesterol, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined enzymatically by standard methods using specific Labtest® kits and measurement of optical density at the corresponding wavelength with a Labmax 240.

Results

The GCMS analysis indicated the presence of various constituents in the *B. platypetala* in the ethereal (Figure 1), ethyl acetate (Figure 2) and aqueous (Figure 3) and fractions.

In non-polar fraction (ethereal) the following components were detected as main components: phytol (30%), hexadecanoic acid (22.1%), neophytadiene (8.2%) and p-cymene (6.1%). To fraction ethyl acetate the main components were octa-decanoic acid or stearic acid (36.04%), hexa-decanoic acid or palmitic acid, (20.87%) and myo-inositol (6.44%). In the aqueous fraction, myo-inositol (60.90%), maltose (22.80%) and 6,7-di-hydroxycoumarin-β-d-glucopyranoside or esculin (4.60%) (Table 1), were detected as main components.

Ethanol extract, ethereal fraction, aqueous fraction and ethyl acetate fractions did not cause alteration in motor activity, behavioral and respiratory rate at the dose of 0.05 g/kg. Lower doses (0.025 mg/kg) did not produce any noticeably motor or behavioral changes and death within 24 hours after administration. In this open field test, only ethanol extract (0.05 g/kg) significantly ($p<0.001$) decreased the number of square crossed (Figure 4).

However, ethanol extract induced a reduction only in the number of square crossed (Figure 4, $p<0.05$). Spontaneous motor activity of each animal was measured in open field test. The animals were submitted to a first trial in the chambers 60 min after vehicle, DZP, ethanol extract and fractions from *B. platypetala* treatments. Twenty-four hours later, the animals were tested in the same activity chamber used in the previous day without drug treatment for 5 min, and the number of squares crossed was recorded again. In acute experiments, the *B. platypetala* ethanol extract and fractions were administered 30 min before the second trial. The motor activity in the first trial was used as an index of sedative/excitatory effect during the activity. Mice treated with DZP or ethanol extract showed a decrease in the motor activity (number of squares crossed), which indicated sedative effect [23].

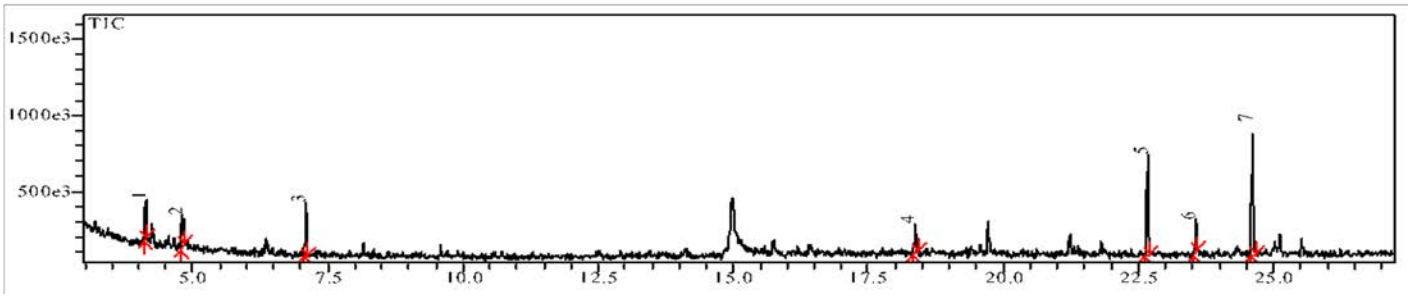

Figure 1. *Chromatogram of the ethereal fraction from Bauhinia platypetala. (1) - p-cymene; (5) – Hexadecanoic acid; (6) – neophytadiene; (7) - phytol.*

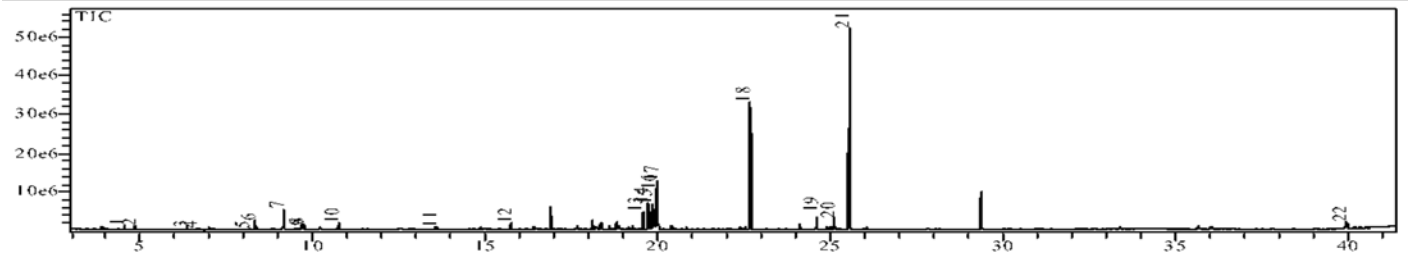

Figure 2. Chromatogram of the ethyl acetate fraction from *Bauhinia platypetala.* **(17)** – Lactone mannonic acid; **(18)** – Hexadecanoic acid; **(20)** – α–Linolenic acid; **(21)** – Octadecanoic acid**.**

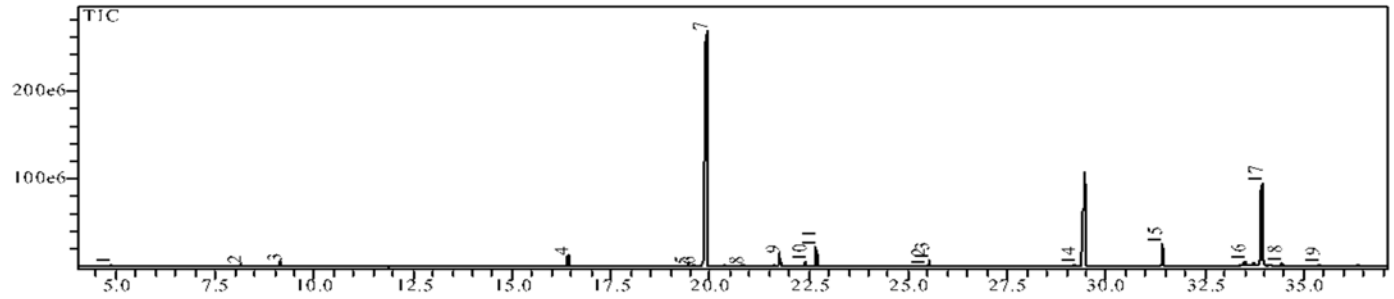

Figure 3. Chromatogram of the aqueous fraction from *Bauhinia platypetala.* **(4)** – Lactone Arabinonic acid; **(7)** – Myo-inositol; **(11)** - Hexadecanoic acid or palmitic acid; **(13)** - Octadecanoic acid or stearic acid; **(15)** – Esculin; **(17)** – Maltose.

The absence of effects of acute administration from *B. platypetala* on hematological parameters is presented in Table 2. Only MCHC was reduced ($p<0.05$) in the group treated with ethanol extract. Biochemical parameter profiles of the treated and vehicle groups are shown in Table 3. After 24 hours of the intraperitoneal administration of ethanol extract and fractions of the *B. platypetala* no changes in the biochemistry serum profile were noticed.

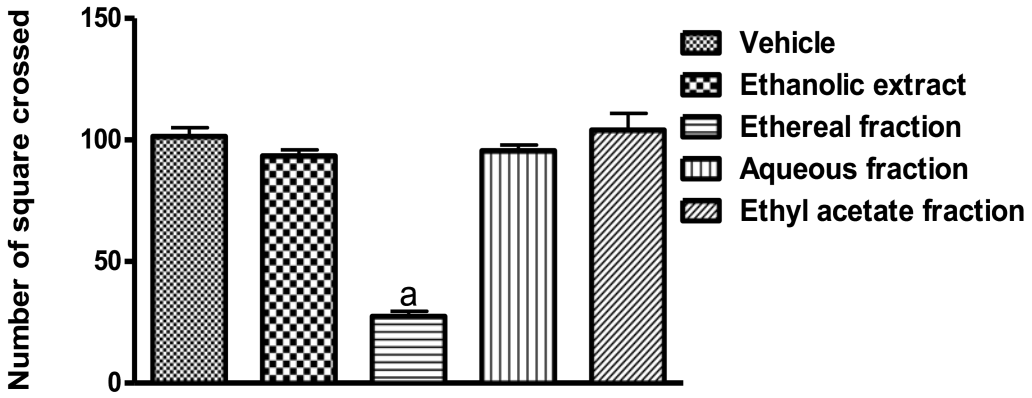

Figure 4: Open-field test of groups of mice which received vehicle, ethanolic extract and fractions (0.05 g/kg, i.p.) from *Bauhinia platypetala*. The results are presented as mean ± S.E.M. of the number of squares crossed. *n* = 10 animals per group; Vehicle = Negative control, Ethanolic extract = EE, Ethereal fraction = EF, Aqueous fraction = AF, Ethyl Acetate fraction = EAF. Significant difference compared with vehicle [a]$p<0.001$ (ANOVA and Student-Newman-Keuls's as the *post hoc* test).

Table 1: Main Chemical compounds (%) of the fractions obtained of the ethanol extract of leaves from *B. platypetala*.

Fractions	Class	Compounds	Relative Area (%)	Identification
Aqueous	Alcohol	Myo-Inositol	60.90	MS
	Lactone	Arabinonic acid lactone	2.40	
	Fatt acid	Hexadecanoic acid or Palmitic acid	3.30	
		Octadecanoic acid or stearic acid	1.18	
	Coumarin	6,7-dihydroxycoumarin-β-d-	4.60	
	Carbohidrat	glucopyranoside or esculin	22.80	
		Maltose		
Ethyl acetate	Fatt acid	Octadecanoic acid or stearic acid	36.04	MS
		Hexadecanoic acid or Palmitic acid	20.87	
		α linolenic Acid	1.98	
	Lactone	Mannonic acid	7.56	
	Alcohol	Scyllo-Inositol	6.44	
	Steroid	Stigmasterol	1.70	
Ethereal	Fatty acid	Hexadecanoic acid or Palmitic acid	22.10	MS
	Terpene	Phytol	30.00	
		Neophytadiene	8.20	
		p-Cymene	6.10	

GC-MS, tentatively identified based on computer matching of the mass spectra of peaks with WILEY 229 and compared to those of the literature.

Table 2: Hematological values of mice in acute toxicity of the ethanolic extract and fractions (0.05 g/kg, i.p.) of the leaves from *Bauhinia platypetala*.

Hematological parameter	Vehicle	Ethanolic extract	Ethereal fraction	Aqueous fraction	Ethyl acetate fraction
Hemoglobin (g/dl)	14.43 ± 0.20	14.00 ± 0.84	13.20 ± 0.20	13.20 ± 0.73	14.60 ± 0.68
MCH (pg)	16.57 ± 0.53	17.00 ± 1.58	15.80 ± 0.84	17.00 ± 0.71	16.60 ± 1.52
MCHC (g/dl)	36.43± 0.79	32.60 ± 1.14[a]	34.60 ± 1.52	33.80 ± 1.92	35.60 ± 2.97
Hematocrit (%)	44.29 ± 1.11	43.40 ± 1.82	43.00 ± 0.71	43.60 ± 2.07	42.80 ± 1.92
MCV (fl)	49.71 ± 0.49	47.60 ± 1.14	49.40 ± 2.07	47.80 ± 1.64	49.20 ± 1.48
Platelets (10^3/µl)	290.7 ± 4.79	295.6 ± 2.41	296.8 ± 10.18	295.2 ± 1.92	298.2 ± 12.60
Neutrophils (%)	18.71 ± 0.49	18.00 ± 1.00	18.20 ± 0.45	18.40 ± 0.89	18.20 ± 2.17
Eosinophils (%)	0.40 ± 0.07	0.30 ± 0.07	0.32 ± 0.04	0.36 ± 0.05	0.44 ± 0.17
Lymphocytes (%)	78.29 ± 1.11	77.80 ± 1.30	78.40 ± 1.52	77.40 ± 0.55	81.20 ± 4.49
Monocytes (%)	2.29 ± 0.49	2.40 ± 0.55	2.40 ± 0.55	2.00 ± 0.0	3.00 ± 1.23
Erythrocytes (mm^3)	8.57± 0.53	8.00± 0.71	8.00± 0.0	8.20± 0.84	8.20± 0.84

Mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean corpuscular volume (MCV). The results are presented as mean ± S.E.M. Significant difference compared with vehicle (Tween 80 0.05% dissolved in 0.9% saline; egative control); *n* = 10 per group. [a]p<0.001 (ANOVA and Student-Newman-Keuls's as the *post hoc* test).

Discussion

Natural remedies originated from plants have become universally popular in primary healthcare, particularly in developing countries, including Brazil. Herbal sources from medicinal flora are presumed to be safe without any compromising health effect, thus widely used as self-medication [8,24]. However, since there is a lack of scientifically proven studies on the toxicity and adverse effect of these remedies [17], further investigations are vital to confirm the safety relevance of *B. platypetala* substances.

An estimate of safety of drugs and plant products is currently performed in animals. A good correlation has been reported between toxicological insults in rats and humans and there is a weak correlation between humans and mice [25]. Therefore, numerous studies investigate the acute effects of high doses in mice and the chronic effects of lower doses in rats including the doses potentially usable in humans [17,26-28].

Acute toxicity tests often involve three animal species, being adequate the evaluation with a non-rodent species and in two routes of administration that are able to represent the therapeutic use and to ensure absorption. In order to assess preliminary toxic effects, we initially investigate the acute toxicity of the ethanol extract and fractions of *B. platypetala* only one rodent species with a single dose [29]. Based on these results, we are going to carry out, in future chronic and sub-chronic studies, a more detailed investigation of the toxicity of this plant.

In acute toxicity studies, mice which were administered doses up to 50 mg/kg did not exhibit signs of adverse effects. Higher doses induced mortality and symptoms of pronounced adverse

Table 3: Biochemistry data of mice after single dosage of negative control, ethanolic extract and fractions (0.05 g/kg, i.p.) of the leaves from *Bauhinia platypetala*.

Biochemistry parameter	Vehicle	Ethanolic extract	Ethereal fraction	Aqueous fraction	Ethyl acetate fraction
Glicose (g/dl)	88.43± 0.79	88.00 ± 0.71	87.80 ± 0.45	88.40 ± 0.55	88.20 ± 0.84
Urea (mg/dl)	55.29 ± 1.50	56.20 ± 1.92	56.40± 1.14	55.80± 2.59	54.20± 0.84
Triglycerides (mg/dl)	106.90 ± 0.38	107.00 ± 3.67	106.60 ± 2.07	107.80 ± 2.39	106.20 ± 1.92
Creatinine (mg/dl)	0.357± 0.05	0.38 ± 0.15	0.38 ± 0.08	0.38± 0.13	0.36 ± 0.11
Uric acid (mg/dl)	2.59 ± 0.13	2.50 ± 0.16	2.70 ± 0.16	2.58 ± 0.26	2.46 ± 0.15
Total cholesterol (mg/dl)	86.00 ± 2.24	85.00 ± 2.92	84.60 ± 1.52	83.80 ± 1.92	84.60 ± 2.88
Total protein (mg/dl)	6.63 ± 0.11	6.52 ± 0.31	6.56 ± 0.34	6.56 ± 0.21	6.52 ± 0.28
AST (U/ml)	90.71± 6.55	88.00 ± 9.49	88.80 ± 9.47	89.40 ± 10.19	97.80± 4.09
ALT (U/ml)	57.71± 2.98	57.80 ± 0.84	58.40 ± 0.55	58.00 ± 1.00	58.40 ± 0.55
Alkaline phosphatase(U/l)	157.3± 3.68	155.6± 5.90	156.2± 4.60	157.4± 2.07	150.8± 8.26
Total bilirubin (mg/dl)	0.17± 0.05	0.26 ± 0.11	0.20 ± 0.07	0.28 ± 0.13	0.22 ± 0.13
Direct bilirubin (mg/dl)	0.17± 0.07	0.24 ± 0.11	0.18 ± 0.08	0.22 ± 0.08	0.18 ± 0.08

The ethanolic extract and fractions of the plant was given acute by the intraperitoneal route to groups of Swiss mice (*n* = 10 per group) at the following doses of vehicle (Tween 80 0.05% dissolved in 0.9% saline; control negative) for 24 h.

effects. The LD_{50} for the intraperitoneal administration of the *B. platypetala* ethanol extract was not estimated in mice since a dose can be much higher than that popularly used dosage. In consequence, ethanol extract and fractions of the *B. platypetala* leaves could be considered practically atoxic or at worst slightly toxic according to the toxicity categories established for humans by Hodge and Sterner [30]. In the acute analyses, we evaluated the effects of ethanol extract and fractions from *B. platypetala* and a negative control group was treated with the vehicle used to dissolve the ethanol extract and fractions to ensure that the toxicological effects observed are associated with substances extracted from *B. platypetala*.

Gender differences in acute mortality of ethanol extract and fractions of *B. platypetala* were not observed. Sexual characteristics effects have been observed for other plant extracts, being the difference in susceptibility, as here for *B. platypetala*, in a narrow range of doses. For example, the female were more sensitive to plant extracts of other medicinal plants [26,31].

Although leaves of *B. platypetala* are used as a drug in popular medicine, experimental studies referring to the risks of the *B. platypetala* oral use and its pharmacological studies are deficient. The present investigation was carried out to estimate safety limits of intraperitoneal administration of the ethanol extract and fractions of the leaves of *B. platypetala* through toxicological assessment in rodents.

Initially, we consider that the estimated doses are safe since no death was observed among animals treated with ethanol extract and fractions of *B. platypetala*. From these data, further studies were conducted with the fraction that induced changes in biochemical and hematological parameters in larger doses, LD_{50} to determine and evaluate the sub-chronic and chronic toxicity in rodents in order to strengthen primary data on the safety of this species for human health.

Multiple constituents are usually responsible for the therapeutic effects of medicinal plants, based on the synergic response of their constituents. Therefore, it is desirable to standardize the processing conditions in order to achieve uniformity and enhance the quality of the herbal products [32].

Several researches have revealed the beneficial effects of several flavonol glycosides in relation to diabetes mellitus, through acting at multiple sites of glucose regulatory pathways, e.g. glucose tolerance, lipid profile, glycogen biosynthesis, glucose up take and insulin release [33,35]. Flavonoids can reduce serum glucose levels after acute treatments being able to manage glucose utilization through different pathways [34]. Phytochemical screening and GC-MS analysis of the ethanol extract indicated the presence of flavonoids, coumarin, triterpenoids/steroids, and tannins. Although further investigation in the future is essential, since in the present study we evaluated only the motor activity and toxicity profile by hematological and biochemical parameters.

The GC-MS analysis indicated the presence of various constituents in the *B. platypetala* in the aqueous (myo-inositol, a acid lactone, hexadecanoic acid or palmitic acid, octadecanoic acid or stearic acid, 6,7-dihydroxycoumarin-β-d-glucopyranoside or esculin and maltose), ethyl acetate (octadecanoic acid or stearic acid, hexadecanoic acid or palmitic acid, α-linolenic acid, mannonic acid, scyllo-inositol and stigmasterol) and ethereal (hexadecanoic acid or palmitic acid, phytol, neophytadiene and *p*-cymene) fractions. Our results suggest that the compounds identified by GC-MS analysis can provide a profile of low toxicity to humans, since it does not determine toxic effects on hematologic and biochemical parameters of mice.

Analysis of blood parameters is relevant to the assessment of alterations in the hematological system [17,25]. The absence of changes on hematological and biochemical parameters of mice treated with *B. platypetala* can be attributed to the plant extract. The nonexistence of modifications in blood glucose level ethanol extract and fractions-treated groups shows absence of hypoglycemic effect of the specie studied after 24 hours of treatment. However, other species of the genus Bauhinia such as *Bauhinia forficate* Link, *Bauhinia monandra* and *Bauhinia purpurea* [32,36,37] exhibit hypoglycemic activity [38]. Thus, further studies should be carried out to investigate the effects of *B. platypetala* on blood glucose level.

Conclusion

In this study, it was observed the absence of alterations in motor activity in animals treated with ethanol extract and their fractions from *B. platypetala* leaves, which may be atoxic in high doses. In general, this study provides a valuable preliminary data on the toxicity profile that should be useful for planning of future pre-clinical studies with this medicinal plant. Further studies with the isolated compounds are in progress to ensure its safe use in humans.

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Author's Contributions

ABC (PhD student) contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, analysis of the data and drafted the paper. DEF contributed to biological studies. GH contributed in plant identification and herbarium confection. IJ contributed to chromatographic analysis. SE contributed to critical reading of the manuscript. KLM contributed to plant collection. NO and PQR designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the

authors have read the final manuscript and approved the submission.

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