Journal of Ethnopharmacology 222 (2018) 79–86

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Toxicological evaluation and anti-inflammatory potential of an ethanolic extract from *Bromelia balansae* (Bromeliaceae) fruit

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ARTICLE INFO

Keywords:
Bromelia balansae
Inflammation
Carrageenan
Toxicity

ABSTRACT

Ethnopharmacological relevance: *Bromelia balansae* is a relatively unexplored medicinal species that is used for nutritional purposes and in folk medicine to treat cough or wounds.
Aim of this study: This study assessed the anti-inflammatory activity of the ethanolic extract obtained from *Bromelia balansae* fruit (EEBB) as well as the toxicological potential of this extract after single and repeated exposure.
Materials and Methods: Male rats (Wistar) were gavaged with 2000 mg/kg of extract from the fruit of *B. balansae* for the acute toxicity test and with 25, 100, or 400 mg/kg of EEBB for the subacute toxicity test. The anti-inflammatory effect of EEBB was evaluated *in vivo* (30, 100, or 300 mg/kg) by carrageenan (Cg) induced-oedema and pleurisy in *Swiss* mice.
Results: A single oral dose of EEBB did not result in toxicity, demonstrating that the LD_{50} of this extract was greater than 2000 mg/kg. In the subacute toxicity test, the tested doses produced no significant changes in the haematological, biochemical or histopathological parameters of treated animals. Similarly, there were no statistically significant differences in the sperm parameters. A dose of 300 mg/kg of EEBB significantly reduced oedema formation, Cg-induced mechanical hypersensitivity and cold sensitivity, as well as leukocyte migration in the pleurisy model.
Conclusion: These results show that EEBB has an anti-inflammatory potential without causing acute or subacute toxicity. These data may contribute to the advancement of biopharmaceutical applications for this species.

1. Introduction

The acceptance and use of herbal medicines has increased in recent years, in part because of the belief that these products are “safe” because they originate from natural sources. However, many of these agents have not been adequately tested, so little is known about their mode of action, adverse effects, and contraindications, which compromises the safe use of these agents (Ekor, 2014).

Bromelia balansae Mez. (Bromeliaceae) (folk name caraguatá) is a herbaceous plant that is largely distributed through South America, including Argentina, Bolivia, Brazil, Colombia, and Paraguay (Pardo et al., 2001). The fruits of *B. balansae* can be used as food and medicine in the form of a syrup for the treatment of cough or wounds (Pott and Pott, 1994). In addition, species belonging to the Bromeliaceae family are considered to be of great importance to the pharmaceutical and food industries, as several proteolytic enzymes have been isolated and characterized. An important example is bromelain, an extract that is rich in cysteine endopeptidases, which possesses anthelmintic, anti-inflammatory and anti-cancer potentials (Maurer, 2001). In the food industry, these proteases are used in cheese and beer manufacturing, to tenderize meat, to produce of emulsifiers and in other applications (Freiman and Sabaa Srur, 1999).

Cardoso et al. (2010) identified 35 compounds in the essential oil extracted from *B. balansae* fruits, with sesquiterpenes (94.6%)

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https://doi.org/10.1016/j.jep.2018.04.049
Received 11 December 2017; Received in revised form 30 April 2018; Accepted 30 April 2018
Available online 03 May 2018
0378-8741/

Table 1
Body weight gain and food and water consumption of male rats treated orally with ethanolic extract of *B. balansae* fruits.

Parâmeters	Acute toxicity		Subacute toxicity			
	Control	2000 mg/kg	Control	25 mg/kg	100 mg/kg	400 mg/kg
Initial weight (g)	284.72 ± 6.11	278.54 ± 9.66	315.33 ± 11.70	319.53 ± 7.98	320.55 ± 13.58	329.82 ± 13.58
Final weight (g)	351.32 ± 12.21	339.48 ± 14.49	389.32 ± 9.89	378.00 ± 12.48	381.05 ± 14.38	367.58 ± 12.18
Body weight gain (%)	21.22 ± 1.78	20.10 ± 1.08	24.52 ± 2.54	19.92 ± 2.74	19.80 ± 1.88	15.39 ± 1.91
Food intake (g/day)	31.64 ± 1.78	26.67 ± 0.23*	52.74 ± 1.48	53.08 ± 1.18	52.36 ± 1.12	50.10 ± 0.83
Water intake (mL/day)	43.78 ± 3.71	41.14 ± 0,72	82.26 ± 1.38	83.19 ± 4.39	82.68 ± 4.40	79.58 ± 5.31

Values expressed as mean ± SEM. For acute toxicity – Student's test.
* $p < 0.05$ compared to control group; n = 5 animals/ group. For subacute toxicity - ANOVA/Tukey's test. $p > 0.05$; n = 8 animals/ group.

Table 2
Relative organ weight (g/100 g of body weight) of rats treated orally with ethanolic extract of *B. balansae* fruits.

Parameters	Acute toxicity		Subacute toxicity			
	Control	2000 mg/kg	Control	25 mg/kg	100 mg/kg	400 mg/kg
Liver	4.11 ± 0.09	3.74 ± 0.11*	3.35 ± 0.08	3.56 ± 0.04	3.46 ± 0.12	3.63 ± 0.05
Kidney	0.40 ± 0.01	0.37 ± 0.00**	0.36 ± 0.01	0.35 ± 0.01	0.34 ± 0.01	0.42 ± 0.05
Spleen	0.26 ± 0.03	0.01 ± 0.00	0.24 ± 0.04	0.24 ± 0.02	0.23 ± 0,01	0.21 ± 0.01
Heart	0.36 ± 0.01	0.34 ± 0.01	0.33 ± 0.01	0.35 ± 0.01	0.34 ± 0,01	0.34 ± 0.01
Lung	0.66 ± 0.08	0.56 ± 0.04	0.56 ± 0.01	0.53 ± 0.03	0.57 ± 0,02	0.50 ± 0.01
Adrenal gland	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0,00	0.01 ± 0.00
Testis	0.44 ± 0.02	0.42 ± 0.03	0.40 ± 0.02	0.45 ± 0.03	0.38 ± 0,04	0.41 ± 0.04
Epididymis	0.14 ± 0.01	0.13 ± 0.00	0.14 ± 0.01	0.15 ± 0.01	0.14 ± 0,01	0.15 ± 0.01
Vas deferens	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.03 ± 0,00	0.03 ± 0.00
Prostate	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.13 ± 0,01	0.11 ± 0.00

Values expressed as mean ± SEM. For acute toxicity – Student's test.
* $p < 0.05$ and.
** $p < 0.01$ compared to control group; n = 5 animals/ group. For subacute toxicity - ANOVA/Tukey's test. $p > 0.05$; n = 8 animals/ group.

(compounds with high therapeutic potential for the treatment of cardiovascular diseases and cancer) representing the most abundant class detected (Chadwick et al., 2013). Coelho et al. (2010) isolated four glycoside flavonols (kaempferol-3-O-α-l-rhamnopyranoside, kaempferol-3-O-α-lrhamnopyranosyl-(1→6)-β-d-glucopyranoside, quercetin-3-O-α-l-rhamnopyranosyl-(1→6)-β-d-glucopyranoside and kaempferol 3,7-di-O-a-l-rhamnopyranoside) in the methanolic extract of *B. balansae* fruits. These natural glycoside flavonols are among the most important polyphenols in the human diet due to their diverse beneficial properties, including anti-oxidant, anti-diabetes and anti-inflammatory activities (Xiao et al., 2014).

There is a lack of information available in the literature regarding the therapeutic or toxic effects of this species. According to Errasti et al. (2016), *B. balansae* fruits demonstrated anti-coagulant and procoagulant effects *in vitro*. Given the close relationship between haemostasis and inflammation, it is of interest to investigate the anti-inflammatory potential of this species. This study aimed to evaluate the toxicity and anti-inflammatory potential in an animal model of the ethanolic extract of *B. balansae*, a species with important bioactive compounds that is still unexplored.

2. Materials and methods

2.1. Plant material, preparation and isolation of ethanolic extract

Bromelia balansae fruits were collected (latitude 22012'37, 7" south and longitude 54055'03, 2" west) in March 2014. The plant material was identified by Vieira, MC and the voucher specimen was deposited (number 2467) in the DDMS herbarium of the Federal University of Grande Dourados (UFGD).

The fruits (9000 g) *in natura* of *B. balansae* were extracted successively with ethanol at room temperature. Extract was filtered and concentrated under vacuum. The isolation of the different fractions of the ethanolic extract was carried out according to the methodology proposed by Coelho et al. (2010).

2.2. Animals

Adult male Wistar rats (90 days old, 300 g, n = 42) were supplied by the Central Biotherium of São Paulo State University (UNESP). Adult male and female Swiss mice (60 days old, 20–30 g, n = 78) were used in inflammation study. The animals were maintained under a controlled temperature (23 °C), 12 h light-dark cycle and food and water *ad libitum*. The experimental procedures were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethics Committee for Animal Experimentation at the Institute of Biosciences of Botucatu/ UNESP (Protocol number: 852/2016).

2.3. Toxicity studies

All procedures were conducted according to internationally accepted guidelines (OECD, guidelines 423 and 407) (OECD, 2001, 2008). For these tests, we used the rat as this is the preferred rodent species for toxicity tests according to the OECD. The maximum volume of the extract administered did not exceed 1 mL/100 g per body weight in all tests.

2.3.1. Acute oral toxicity

The dose of 2000 mg/kg of ethanolic extract from *B. balansae* fruits (EEBB) was administered by gavage to one male following 8 h of fasting. After, at intervals of 48 h, the same dose was administered to four males, for a total of five treated animals. Negative control group was treated parallel with the vehicle (0.9% saline) to establish a comparative (OECD, 2001).

During the first 24 h after treatment the animals were observed periodically and daily for a total of 14 days. The Hippocratic screening parameters (Malone and Robichaud, 1962) were analysed as described previously (Traesel et al., 2014). The intakes of water and food and body weight were measured daily (OECD, 2001). After euthanasia, the

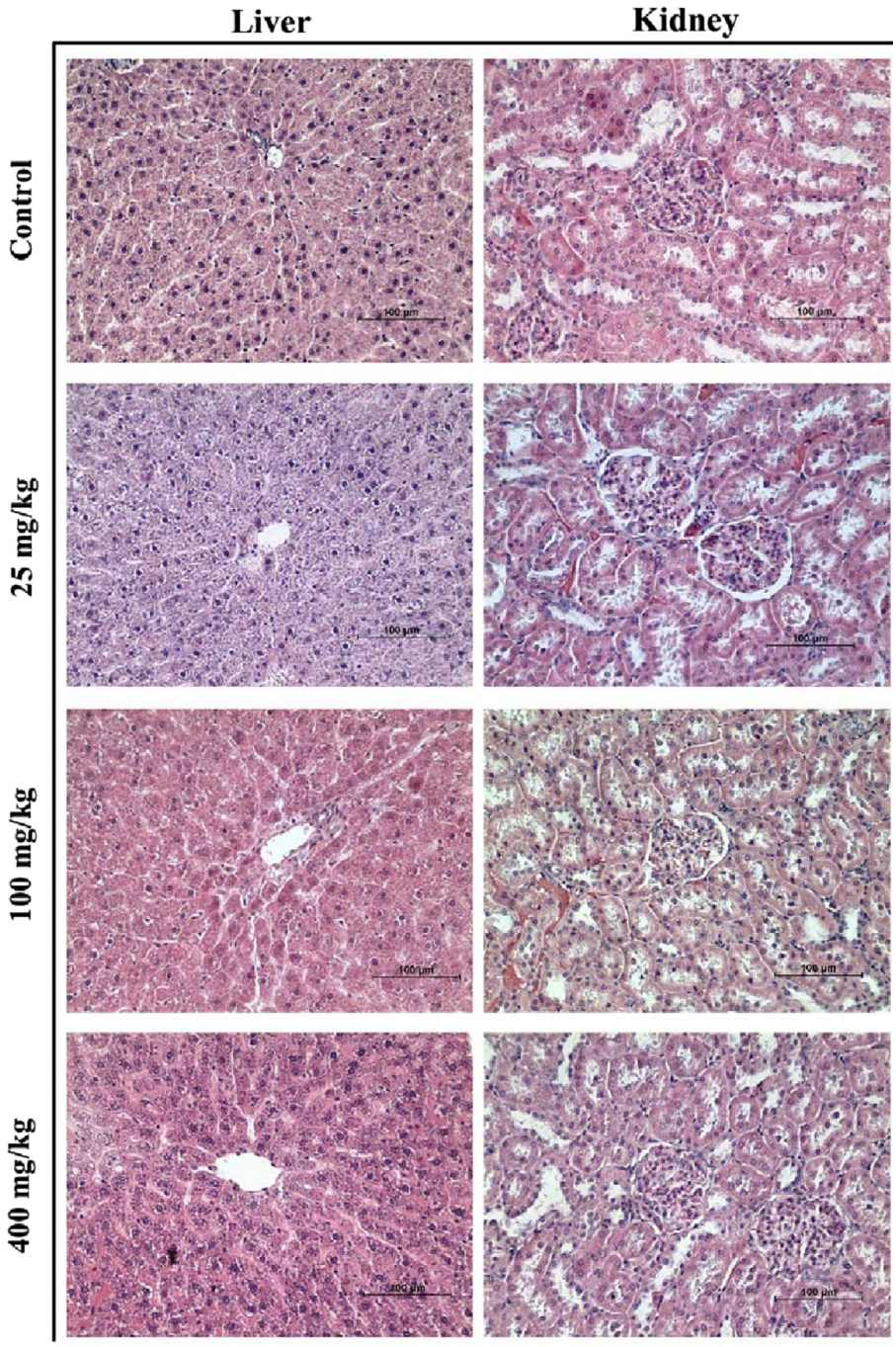

Fig. 1. Histopathological analysis of kidney and liver treated with ethanolic extract of *B. balansae* fruits in the subacute toxicity (H&E; X200).

organs (heart, lung, spleen, liver, kidney, adrenal, seminal gland, prostate, testis, epididymis, vas deferens) were removed, weighed and examined macroscopically.

2.3.2. Subacute oral toxicity

Male rats were divided into four experimental groups (n = 8 animals/group). Three groups were treated with different doses of EEBB (25, 100 or 400 mg/kg) by gavage, daily for 28 consecutive days. The control group was treated with vehicle (0.9% saline). In this study, it was determined that the highest dose would be 400 mg/kg/day, and based on that dose, the other two smaller doses were determined by applying a geometric ratio of ¼, for 100 and 25 mg/kg/day.

The body weight, food and water consumption, and clinical examination were performed daily during treatment, following the Hippocratic screening. At the end of the treatment, all animals were anaesthetized (xylazine at 10 mg/kg and ketamine at 25 mg/kg). Samples of blood were collected from large vessels after decapitation for subsequent biochemical analysis.

The biochemical parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (gamma-GT), bilirubins, albumin, total protein, blood urea nitrogen, creatinine, sodium, potassium, globulin, calcium, cholesterol, HDL cholesterol, and glucose, were analysed with the COBAS integra 400 plus (Roche Diagnostics).

Vital organs (heart, lung, kidney, adrenal, liver, and spleen) and reproductive organs (testis, epididymis, seminal vesicle and vas deferens) were weighed. The tissues (kidney, liver, testis and epididymis) were fixed in Bouin's solution. The pieces were embedded in paraffin

Table 3
Biochemical parameters of rats treated orally with ethanolic extract of *B. balansae* fruits in the subacute toxicity.

Parameters	Control	25 mg/kg	100 mg/kg	400 mg/kg
Aspartate aminotransferase (U/L)	153.23 ± 15.02	157.32 ± 6.45	181.07 ± 9.30	219.56 ± 19.95*
Alanine aminotransferase (U/L)	39.10 ± 8.33	38.23 ± 6.01	56.12 ± 4.44	46.60 ± 10.14
Gamma glutamyltransferase (U/L)	0.63 ± 0.40	0.88 ± 0.49	0.50 ± 0.34	0.66 ± 0.45
Direct bilirubin (mg/dL)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Indirect bilirubin (mg/dL)	0.02 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.04 ± 0.01
Total bilirubin (mg/dL)	0.02 ± 0.01	0.03 ± 0.00	0.02 ± 0.01	0.03 ± 0.01
Total protein (g/dL)	6.53 ± 0.16	6.83 ± 0.15	6.77 ± 0.07	6.56 ± 0.07
Albumin (g/dL)	4.08 ± 0.08	4.20 ± 0.11	4.18 ± 0.05	4.04 ± 0.07
Globulin (g/dL)	2.45 ± 0.16	2.65 ± 0.11	2.58 ± 0.06	2.52 ± 0.06
Albumin/globulin ratio	1.68 ± 0.12	1.58 ± 0.07	1.63 ± 0.07	1.64 ± 0.06
Blood urea nitrogen (mg/dL)	47.45 ± 1.49	46.87 ± 1.36	48.75 ± 1.62	45.38 ± 2.11
Creatinine (mg/dL)	0.36 ± 0.00	0.37 ± 0.02	0.38 ± 0.02	0.37 ± 0.02
Sodium (mmol/dL)	139.45 ± 0.91	139.28 ± 1.54	140.98 ± 0.50	138.72 ± 1.46
Potassium (mmol/L)	6.88 ± 0.13	7.25 ± 0.22	7.58 ± 0.36	7.32 ± 0.24
Calcium (mg/dL)	10.12 ± 0.15	10.13 ± 0.19	10.02 ± 0.19	9.98 ± 0.20
Cholesterol (mg/dL)	66.22 ± 3.75	60.03 ± 3.73	60.83 ± 4.33	64.94 ± 3.54
HDL Cholesterol (mg/dL)	24.77 ± 2.12	27.13 ± 2.82	25.47 ± 2.08	34.30 ± 3.74
Glucose (mg/dL)	133.22 ± 6.66	143.35 ± 6.22	137.88 ± 4.89	132.02 ± 4.75

Values expressed as mean ± SEM. ANOVA/Tukey's test.
* $p < 0.05$ compared to control group; n = 8 animals/ group.

and sectioned at 5 µm. The sections were stained with haematoxylin and eosin and observed by light microscopy to assess the general histopathological parameters (Cunha et al., 2009; Martey et al., 2010).

2.3.3. Sperm parameters

2.3.3.1. Sperm morphology. To evaluate the sperm morphology, sperm were recovered from the left vas deferens with a syringe and needle with 1.0 mL of saline formol solution. 200 spermatozoa per animal were analysed using a phase-contrast microscopy (X 200, total magnification) in wet preparations, according to Filler (1993).

2.3.3.2. Daily sperm production per testis, sperm number, and transit time in the epididymis. Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus and cauda epididymis were counted as described previously by Robb et al. (1978) with the adaptations adopted by Fernandes et al. (2007). To calculate the sperm transit time through the epididymis, the number of sperm in each portion was divided by the daily sperm production (DSP).

2.4. Anti-inflammatory activity

2.4.1. Paw oedema model of carrageenan induce inflammation

In this model 3 parameters of inflammation such as oedema (Winter et al., 1962), mechanical and cold hyperalgesia were analysed after carrageenan (Cg) intraplantar injection. The oral doses of EEBB chosen to treat male *Swiss* mice were 30, 100 and 300 mg/kg and were given 60 min before Cg. The Cg (300 µg) was administered by subcutaneous route in the right paw with an volume of 100 µL of a solution made with 0.9% saline in groups cited before and in control group (treated only with vehicle (0.9% saline)) and positive control group (treated with subcutaneous (s.c.) dexamethasone, 1 mg/kg). The left paw was injected with 100 µL of 0.9% saline. The measurement of paw volume changes was made with Digital Water Plethysmometer (from Panlab) at 1, 2, and 4 h after Cg while the Mechanical hyperalgesia (Vivancos et al., 2004) and cold sensitivity (Decosterd and Woolf, 2000) was assessed at 3 and 4 h after the carrageenan injection.

2.4.2. Pleurisy Induction

The same doses of EEBB (dissolved in 0.9% saline) described before were also used in different groups (3 groups with n = 6) of female *Swiss* mice by oral route (p.o.) while the control group received 0.9% saline (p.o.), the naïve group received 0.9% saline (p.o.) but not receive carrageenan by intrathoracic route (i.t) and the last group received dexamethasone (1 mg/kg positive control) by s.c. route (Vinegar et al., 1973). The i.t. injection of a solution of 1% of carrageenan (100 µL) and sterile saline (100 µL; only in naïve maice) was made. After 4 h the animals were killed and the thorax was opened to obtain and analyse (total leukocyte number (Sysmex KX-21N apparatus) and protein exudation).

2.5. Statistical analyses

The data are presented as the mean ± standard error of the mean (SEM). Student's *t*-test was used for comparisons between two experimental groups (acute toxicity). Differences among 3 or more groups were determined by analysis of variance (ANOVA) followed by Tukey or Newman-Keuls test or Kruskal-Wallis followed by Dunn's test. The significant differences were considered to be significant at $p < 0.05$. The graphs were produced using GraphPad Prism Software (San Diego, CA, U.S.A).

3. Results

Phytochemical investigation of EEBB has led to the isolation of six flavonoids (quercetin-3-O-rhamnopyranoside, kaempferol-3-O-a-l-rhamnopyranoside, kaempferol-3-O-a-lrhamnopyranosyl-(1→6)-*b*-d-glucopyranoside, quercetin-3-O-a-l-rhamnopyranosyl-(1→6)-*b*-d-glucopyranoside, 6-hydroxyluteolin-7-O-1-a-rhamnoside and kaempferol 3,7-di-O-a-l-rhamnopyranoside).

In the acute toxicity test, the dose of 2000 mg/kg (limit test - OECD, 2001) of *B. balansae* did not cause the death of any animal. The male rats exposed showed no behavioural or body weight gain changes during the treatment period; however, a reduction in food consumption, but not in water consumption, was observed in relation to the control group (Table 1). There was a statistically significant decrease in the relative weights of liver and kidney between the treated and the control groups (Table 2), while the relative weights of other organs analysed were similar. At necropsy, no macroscopic alteration was found in the organs.

Similarly, after prolonged exposure to the extract, the animals did not exhibit any signs of toxicity. In the same way, the consumption of water and food and the weight gain values (Table 1) did not differ among groups, and the relative weights of all organs examined were similar among the groups (Table 2). The macroscopic and histological analyses revealed no changes suggestive of toxic effects (Fig. 1). The

Table 4
Sperm parameters of rats treated orally with ethanolic extract of *B. balansae* fruits in the subacute toxicity.

Parameters	Control	25 mg/kg	100 mg/kg	400 mg/kg
Daily sperm production (x10^6/testis/day)	23.34 ± 2.21	25.65 ± 2.55	22.93 ± 2.73	22.52 ± 1.91
Sperm count in the testis (x10^6/organ)	142.36 ± 13.45	156.48 ± 15.54	139.84 ± 16.66	137.38 ± 11.64
Sperm count in the testis (x10^6/g/organ)	112.44 ± 14.64	101.80 ± 6.04	111.85 ± 2.81	104.36 ± 6.84
Sperm count in the caput/corpus epididymis (x10^6/organ)	81.74 ± 8.40	97.33 ± 8.24	77.58 ± 20.35	75.16 ± 9.79
Sperm count in the caput/corpus epididymis (x10^6/g/organ)	268.12 ± 29.09	310.21 ± 32.61	255.00 ± 53.96	265.00 ± 16.77
Sperm count in the cauda epididymis (x10^6/organ)	97.84 ± 13.82	100.37 ± 13.38	88.52 ± 19.81	87.01 ± 17.45
Sperm count in the cauda epididymis (x10^6/g/organ)	612.50 ± 89.32	613.06 ± 107.26	540.83 ± 100.82	552.00 ± 51.04
Sperm transit time in the caput/corpus epididymis (days)	3.68 ± 0.52	3.92 ± 0.39	3.10 ± 0.68	3.32 ± 0.28
Sperm transit time in the cauda epididymis (days)	4.24 ± 0.54	4.13 ± 0.55	3.67 ± 0.74	3.75 ± 0.50
‡Normal sperm (%)	97.01 (90.0 – 99.5)	99.33 (98.5 – 100.0)	95.5 (79.5 – 100.0)	99.00 (96.5 – 100.0)

Values expressed as mean ± SEM. ANOVA/Tukey's test. $p > 0.05$; n = 8 animals/ group.
‡Values expressed as median (Q1-Q3), Kruskal–Wallis/ Dunn. $p > 0.05$; n = 8 animals/group.

biochemical parameters analysed (ALT, gamma-GT, bilirubin levels, albumin, total protein, blood urea nitrogen, creatinine, sodium, potassium, globulin, calcium, cholesterol, HDL cholesterol, glucose) showed no statistically significant differences among the experimental groups. Only an increase in AST levels in the group receiving the higher dose (400 mg/kg) of extract was observed (Table 3).

The subacute exposure to EEBB did not alter any of the sperm parameters evaluated (DSP per testis, number of spermatozoa in the epididymis, sperm transit time, and sperm morphology (Table 4). In addition, the histological analysis of the reproductive organs (testis and epididymis) did not reveal any morphological changes related to the treatment (data not shown).

The animals treated with EEBB at a dose of 300 mg/kg showed a significant reduction in leukocyte migration induced by carrageenan in pleural exudate (Fig. 2A). Protein exudation was significantly decreased at doses of 100 and 300 mg/kg of EEBB (Fig. 2B), demonstrating a dose-dependent effect of the extract. The naive group, as expected, did not demonstrate inflammatory parameters in the analysis. As expect, the number of leukocyte and protein in pleural exudate were significantly reduced by treatment with dexamethasone.

In paw inflammatory model, a dose of 300 mg/kg of EEBB reduced the paw volume at 2 and 4 h after the carrageenan injection (Fig. 3A–C). EEBB (300 mg/kg) also significantly reduced mechanical hyperalgesia by 100% at 3 h after carrageenan injection (Fig. 4A-B). In the cold sensitivity test, EEBB decreased sensitivity to cold at doses of 100 and 300 mg/kg at 3 h and at a dose of 300 mg/kg at 4 h after carrageenan injection (Fig. 5A and B). As expect, all inflammatory parameters were significantly reduced in carrageenan induced inflammatory process by treatment with dexamethasone.

4. Discussion

Medicinal plants are widely used in folk medicine to treat inflammatory diseases. However, many of these plants are used without any scientific evidence to demonstrate their effectiveness and safety (Ekor, 2014). Thus, the present study may contribute to the safe use of *B. balansae*, an important species with a high therapeutic potential.

Toxicity tests are experimental screening methods that are used to confirm the safety of medicinal plants and drugs in assays with animal models. The acute toxicity test is usually the first analysis performed, and the information obtained from this test is used to identify any potential dangers and to manage the risks associated with the use of these products (Walum, 1998). In this study, although there was an alteration in food consumption and in the relative weights of the liver and kidney in animals receiving a single dose of EEBB, this alteration was not followed by changes in body weight gain in these animals or by mortality or behaviour changes, indicating that EEB is not acutely toxic. According to OECD Guideline 423, because all of the animals survived until the expected date of euthanasia, the extract can be considered to be of low toxicity and included in Category 5 (a substance with oral lethal dose (LD_{50}) higher than 2000 mg/kg) (OECD, 2001).

Toxicological evaluations after repeated exposures provide information on possible health risks after a minimum period of 28 days. This test is usually performed after the initial toxicity information is obtained in the acute toxicity test. After oral subacute exposure, all of the doses of EEBB used in this study (25, 100, or 400 mg/kg) did not cause death or changes during treatment that would indicate toxicity.

The main organ responsible for the biotransformation of xenobiotics in the body is the liver, and therefore, it is one of the main targets of the adverse effects caused by toxic substances. The serum enzymes AST and ALT are considered to be markers of hepatocellular toxicity, and their increased activity may indicate hepatic damage (Ramaiah, 2011). The balance between these enzymes is important for the homeostasis of the organism, and under physiological conditions, plasma AST is more active than ALT, whereas this condition is reversed in the case of a hepatic dysfunction. However, AST is also present in large amounts in muscular and cardiac tissues, and as such, it is not specific diagnosing

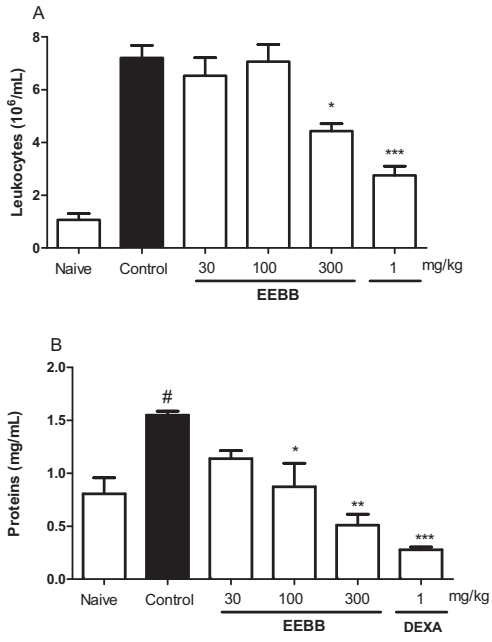

Fig. 2. Effects of oral administration of EEBB on leukocyte migration (A) and protein leakage (B) in the pleurisy test. The animals received EEBB (30, 100 or 300 mg/kg, v.o.), vehicle (control) or dexamethasone (DEX, 1 mg/kg, s.c.), and 1 h later, an intrathoracic injection of carrageenan was administered. The naïve group (# indicates a statistically significant difference from the vehicle group) received an intrapleural injection of sterile saline instead of carrageenan and was also treated with saline solution. Each bar represents the mean ± SEM. ANOVA/Newman-Keuls test. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, #$p < 0.001$ comparing to control group. n = 6 animals/group.

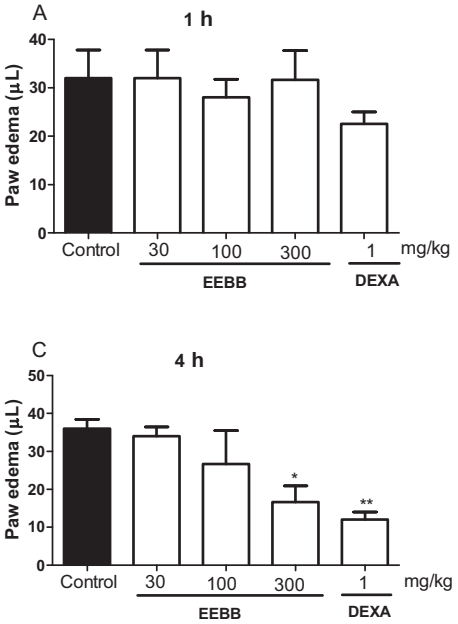

Fig. 3. Effect of oral administration of EEBB on the carrageenan-induced paw oedema in mice. The animals received EEBB (30, 100, and 300 mg/kg, v.o.), vehicle (control) or dexamethasone (DEX, 1 mg/kg, s.c.), and 1 h later, an intraplantar injection of carrageenan (300 µg/paw) was administered. Graphs (A), (B), and (C) represent the evaluation of the paw oedema at 1, 2, and 4 h, respectively, after carrageenan injection. Each bar represents the mean ± SEM. ANOVA/Newman-Keuls test. * $p < 0.05$, ** $p < 0.01$ comparing to control group. n = 6 animals/group.

hepatotoxicity; therefore, it is necessary to measure the amount of ALT activity (Burtis and Ashwood, 2001). In this study, it was observed that EEBB exposure caused an increase in AST activity only in the group treated with the highest dose (400 mg/kg), but did not alter the ALT activity. In addition, the histopathological examination did not show hepatic or renal damage that could be attributed to treatment with the extract.

Toxicity studies following repeated exposures can also provide important information on chemicals that affect the male and/or female reproductive system in animals and may give an indication of reproductive effects (OECD, 2008). Therefore, we evaluated the possible

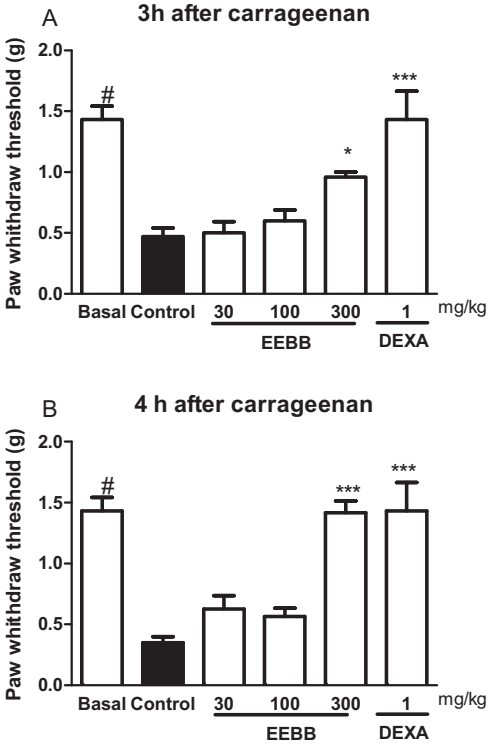

Fig. 4. Effect of oral administration of EEBB on the mechanical hyperalgesia in mice. The animals received EEBB (30, 100 and 300 mg/kg, p.o.), vehicle (control) or dexamethasone (DEX, 1 mg/kg, s.c.). The mechanical hyperalgesia was measured with a digital analgesymeter 3 and 4 h after carrageenan administration. Each bar represents the mean ± SEM, ANOVA/Newman-Keuls test. * $p < 0.05$, *** $p < 0.001$, #$p < 0.001$ comparing to control group. n = 6 animals/group.

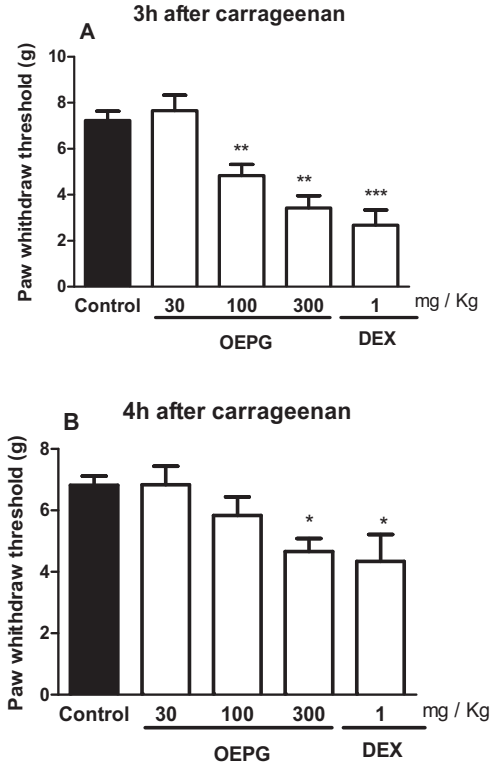

Fig. 5. Effect of oral administration of EEBB on the cold sensitivity induced by acetone in mice. The animals received EEBB (30, 100, 300 mg/kg, v.o.), vehicle (control) or dexamethasone (DEX, 1 mg/kg, s.c.). The cold sensitivity was measured 3 and 4 h after carrageenan administration. Each bar represents the mean ± SEM, ANOVA/Newman-Keuls test. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ comparing to control group. n = 6 animals/group.

effects of EEBB on the male reproductive parameters to better understand the risks to male fertility from using the extract. It was observed that the extract did not change the sperm parameters or the histology of the testis and the epididymis, suggesting an absence of male reproductive toxicity.

Several medicinal plants are used as popular remedies against signs or symptoms of inflammation. A cough and wounds also involve the inflammatory process, and *B. balansae* is popularly used to treat these symptoms. In the present study, 2 types of inflammatory models were used to validate the *B. balansae* anti-inflammatory effects. Both carrageenan-induced oedema and pleurisy are common models that are useful for the discovery of natural anti-inflammatory products. Carrageenan is a compound that elicits inflammation, as it increases several inflammatory mediators when injected into rodents. Intraplantar injection of carrageenan induces oedema (Winter et al., 1962), mechanical hyperalgesia (Draxler et al., 2014; Poole et al., 1995), and cold hypersensitivity (Draxler et al., 2014), as verified in Figs. 3–5, while intrapleural injection induces leukocyte migration and protein leakage, as shown in Fig. 2. All of the carrageenan-associated parameters were significantly inhibited by the *B. balansae* extract, but the potency of this extract was not high, as the extract was obtained from fruit. In pleurisy, neutrophils were the main leukocyte that migrated into the acute inflammation (first 6 h); then, the *B. balansae* extract inhibited the mediator factors responsible for neutrophil recruitment. Chemoattractant molecules, such as LTB4 and IL-8/KC, and adhesion molecules are important for early leukocyte migration (Ferreira et al., 2012) and could be modulated by natural or synthetic anti-inflammatory products. It is not possible to affirm the exact mechanism of action of the *B. balansae* extract, but this extract influenced neutrophil migration and protein leakage.

Inflammatory Cg pain is typically characterized by a fast onset of mechanical and cold hypersensitivity (Draxler et al., 2014). The C pain fibre responds to a multimodal stimulus and TRPM8, as well as to vesicular glutamate transporter 3-positive (VGluT3 +) primary afferents, which are important for the cold and mechanical responses (Draxler et al., 2014). In the paw inflammatory model induced by Cg, several inflammatory mediator, including prostaglandin are increased mainly at 3 and 4 h after Cg injection. The mechanism of action of *B. balansae* is inconclusive at the moment, but this extract inhibited mechanical and cold hyperalgesia. In the literature, the anti-inflammatory and anti-hyperalgesic actions of *B. balansae* is poorly described, but some studies described the mechanism of action of the flavonoids presents in the EEBB. Kaempferol-3-O-a-lrhamnopyranosyl-(1→6)-*b*-d-glucopyranoside could be also found as nicotiflorin (more currently used name), kaempferol-3-O-rutinoside, nicotifloroside, kaempferol-3-O-beta-rutinoside, 3-rutinosylkaempferol, kaempferol 3-O-rutinose, kaempferol 3-O-beta-rutinoside. Nicotiflorin has inhibition of IL-1β, TNF-α, IL-6 and IFN-γ and hepatoprotective effect induced by Concanavalin A-Induced and D-Galactosamine-Induced liver injury in mice (Zhao et al., 2017), antiglycation (Lal Shyaula et al., 2012), protects against cerebral ischemia/reperfusion injury-induced apoptosis *via* the JAK2/STAT3 pathway (Hu et al., 2017), protection against memory dysfunction, energy metabolism failure and oxidative stress (Huang et al., 2007), neuroprotection in permanent focal cerebral ischemia and in neuronal cultures acting in nitric oxide pathway (Li et al., 2006a, 2006b). Another compound found in EEBB is Lespedin or lespenephryl (kaempferol 3,7-di-O-a-l-rhamnopyranoside) that is widely investigated for renal problems (Ullrich, 1958; Zywiol, 1960; Kiczak and Wichert, 1964; Czekalski, 1969; Gau, 1969; Gessler, 1971; Graben and Bock, 1973) but no mechanism of action was described. The most studied compound found in EEBB is rutin or rutoside (quercetin-3-O-a-l-rhamnopyranosyl-(1→6)-*b*-d-glucopyranoside) and several studies indicated the biological action of rutin and others described that the main mechanism of inflammatory action is associated to NF-κB, iNOS, MAPKs or JNK or ERK1/2 pathway activation (Abdel-Aleem and Khaleel, 2017; Gul et al., 2018; Ma et al., 2018). Others compounds such as quercetin-3-O-rhamnopyranoside, kaempferol-3-O-a-l-rhamnopyranoside, 6-hydroxyluteolin-7-O-1-a-rhamnoside (Luteolin 6-hydroxy-7-O-beta-D-glucoside) are also found in EEBB but the mechanism of action is poorly described. The demonstration of the anti-oedematogenic, anti-migratory, and anti-hyperalgesic properties of the *B. balansae* extract could contribute to validation (at least in part) of the popular use of this plant against inflammatory diseases. The mechanism could be related to presence of rutin, nicotiflorin and other compounds that play an important role in anti-inflammatory action of EEBB.

In this study, fruits from *B. balansae* revealed the presence of six flavonoids. These results corroborate the results from Coelho et al. (2010), who also found flavonols in this species, reinforcing its therapeutic importance. Flavonoids are compounds that are very important for human health. Several studies have the attributed anti-oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic properties to flavonoids, as well as demonstrated their capacity to modulate key cellular enzyme functions (Panche et al., 2016; Xiao et al., 2011). Thus, these compounds may be related to the anti-inflammatory effects found in this study.

We demonstrated that the ethanolic extract obtained from *B. balansae* fruits did not display significant toxic effects when administered orally to male rats. Furthermore, the extract was rich in flavonoids and had potential anti-inflammatory activity, indicating that it may have biopharmaceutical applications. However, other studies should be conducted to evaluate the mechanisms of action and to identify the compound responsible for the anti-inflammatory activity, as well to investigate other aspects of toxicity.

Acknowledgements

The authors thank CAPES, CNPq and FUNDECT (59/300.596/2016) for the financial assistance.

Conflict of interest

The authors declare that there are no conflicts of interest.

Authors' contributions

All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript; PSB, FCZ and BCJ conducted the experiments; CALC were involved in the preparation and isolation of extract; ML and RMK performed the anti-inflammatory assays; CALK and ACA performed data analyses and wrote the manuscript. All authors read and approved the final manuscript.

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