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

Paola da Silva Balina, Flavia Carina Zanattaa, Bárbara Campos Jorgea, Maicon Leitaob, Roberto Mikio Kassuyab, Claudia Andrea Lima Cardosoc, Cândida Aparecida Leite Kassuyab, Arielle Cristina Areana

**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 LD50 of this extract was greater than 2000 mg/kg. In the subacute toxicity test, the tested doses produced no statistically 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 emulsifiers and in other applications (Freiman and Sabaa, 1999).

Cardoso et al. (2010) identified 35 compounds in the essential oil extracted from *B. balansae* fruits, with sesquiterpenes (94.6%)...
polyphenols in the human diet due to their diverse bene-
cficialities, including anti-oxidant, anti-diabetes and anti-
therapeutic or toxic ef-
is. Extract was
centrated under vacuum. The isol-
ation of the di-
agalcohol was made according to the methodology
previously (Traesel et al., 2014). The intakes of water and food and
body weight were measured daily (OECD, 2001). After euthanasia, the

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Acute toxicity</th>
<th>Subacute toxicity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control 2000 mg/kg</td>
<td>Control 25 mg/kg</td>
</tr>
<tr>
<td>Initial weight (g)</td>
<td>284.72 ± 6.11</td>
<td>278.54 ± 9.66</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>351.32 ± 12.21</td>
<td>339.48 ± 14.49</td>
</tr>
<tr>
<td>Body weight gain (%)</td>
<td>21.22 ± 1.78</td>
<td>20.10 ± 1.08</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>31.64 ± 1.78</td>
<td>26.67 ± 0.23</td>
</tr>
<tr>
<td>Water intake (mL/day)</td>
<td>43.78 ± 3.71</td>
<td>41.14 ± 0.72</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. For acute toxicity – Student’s test.

Table 1

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Acute toxicity</th>
<th>Subacute toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 2000 mg/kg</td>
<td>Control 25 mg/kg</td>
</tr>
<tr>
<td>Liver</td>
<td>4.11 ± 0.09</td>
<td>3.74 ± 0.11</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.40 ± 0.01</td>
<td>0.37 ± 0.00</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.26 ± 0.03</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>Heart</td>
<td>0.36 ± 0.01</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>0.66 ± 0.08</td>
<td>0.56 ± 0.04</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>Testis</td>
<td>0.44 ± 0.02</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>Epididymis</td>
<td>0.14 ± 0.01</td>
<td>0.13 ± 0.00</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>0.05 ± 0.00</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>Prostate</td>
<td>0.13 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
</tbody>
</table>

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.

2. Materials and methods

2.1. Plant material, preparation and isolation of ethanolic extract

Bromelia balansae fruits were collected (latitude 22012’37, 7th south and longitude 54055’03, 2nd 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 (UFGR).

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 Instituto 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
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.
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 formalin 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). Additionally, to calculate the sperm number per testis and the sperm transit time, 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 a 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 (Decoster 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 mice) 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-rhamnopyranoside, kaempferol-3-O-a-lrhamnopyranosyl-(1→6)-b-d-glucopyranoside, quercetin-3-O-a-lrhamnopyranosyl-(1→6)-b-d-glucopyranoside, 6-hydroxytolyein-7-O-a-rhamnose and kaempferol 3,7-di-O-a-lrhamnopyranoside).

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 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 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 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

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Table 3

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>25 mg/kg</th>
<th>100 mg/kg</th>
<th>400 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>153.23 ± 15.02</td>
<td>157.32 ± 6.45</td>
<td>181.07 ± 9.30</td>
<td>219.56 ± 19.95</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>39.10 ± 8.33</td>
<td>38.23 ± 6.01</td>
<td>56.12 ± 4.44</td>
<td>46.60 ± 10.14</td>
</tr>
<tr>
<td>Gamma glutamyltransferase (U/L)</td>
<td>0.63 ± 0.40</td>
<td>0.88 ± 0.49</td>
<td>0.50 ± 0.34</td>
<td>0.66 ± 0.45</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dL)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Indirect bilirubin (mg/dL)</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>6.53 ± 0.16</td>
<td>6.83 ± 0.15</td>
<td>6.77 ± 0.07</td>
<td>6.56 ± 0.07</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.08 ± 0.08</td>
<td>4.20 ± 0.11</td>
<td>4.18 ± 0.05</td>
<td>4.04 ± 0.07</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>2.45 ± 0.16</td>
<td>2.65 ± 0.11</td>
<td>2.58 ± 0.06</td>
<td>2.52 ± 0.06</td>
</tr>
<tr>
<td>Albumin/globulin ratio</td>
<td>1.68 ± 0.12</td>
<td>1.58 ± 0.07</td>
<td>1.63 ± 0.07</td>
<td>1.64 ± 0.06</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dL)</td>
<td>47.45 ± 1.49</td>
<td>46.87 ± 1.36</td>
<td>48.75 ± 1.62</td>
<td>45.38 ± 2.11</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.36 ± 0.00</td>
<td>0.37 ± 0.02</td>
<td>0.38 ± 0.02</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Sodium (mmol/dL)</td>
<td>139.45 ± 0.91</td>
<td>139.28 ± 1.54</td>
<td>140.98 ± 0.50</td>
<td>138.72 ± 1.46</td>
</tr>
<tr>
<td>Potassium (mmol/dL)</td>
<td>7.25 ± 0.22</td>
<td>7.58 ± 0.36</td>
<td>7.32 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>10.12 ± 0.15</td>
<td>10.13 ± 0.19</td>
<td>10.02 ± 0.19</td>
<td>9.98 ± 0.20</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>66.22 ± 3.75</td>
<td>60.03 ± 3.73</td>
<td>60.83 ± 4.33</td>
<td>64.94 ± 3.54</td>
</tr>
<tr>
<td>HDL Cholesterol (mg/dL)</td>
<td>24.77 ± 2.12</td>
<td>27.13 ± 2.82</td>
<td>25.47 ± 2.08</td>
<td>34.30 ± 3.74</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>133.22 ± 6.66</td>
<td>143.35 ± 6.22</td>
<td>137.85 ± 1.62</td>
<td>138.72 ± 1.46</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. ANOVA/Tukey's test. * p < 0.05 compared to control group; n = 8 animals/group.
The 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 addition, the histological analysis of the reproductive organs (testis and epididymis, sperm transit time, and sperm morphology (Table 4). In addition, the histological analysis of the reproductive organs (testis and epididymis, sperm transit time, and sperm morphology (Table 4).

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...

**Table 4**

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>25 mg/kg</th>
<th>100 mg/kg</th>
<th>400 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily sperm production (x10⁶/testis/day)</td>
<td>23.34 ± 2.21</td>
<td>25.65 ± 2.55</td>
<td>22.93 ± 2.73</td>
<td>22.52 ± 1.91</td>
</tr>
<tr>
<td>Sperm count in the testis (x10⁶/organ)</td>
<td>142.36 ± 13.45</td>
<td>156.48 ± 15.54</td>
<td>139.84 ± 16.66</td>
<td>137.38 ± 11.64</td>
</tr>
<tr>
<td>Sperm count in the testis (x10⁶/g/organ)</td>
<td>112.44 ± 14.64</td>
<td>101.80 ± 6.04</td>
<td>111.85 ± 2.81</td>
<td>104.36 ± 6.84</td>
</tr>
<tr>
<td>Sperm count in the caput/corpus epididymis (x10⁶/organ)</td>
<td>81.74 ± 8.40</td>
<td>97.33 ± 8.24</td>
<td>77.58 ± 20.39</td>
<td>75.16 ± 9.79</td>
</tr>
<tr>
<td>Sperm count in the caput/corpus epididymis (x10⁶/g/organ)</td>
<td>268.12 ± 29.09</td>
<td>310.21 ± 32.61</td>
<td>255.00 ± 53.96</td>
<td>265.00 ± 16.77</td>
</tr>
<tr>
<td>Sperm count in the cauda epididymis (x10⁶/organ)</td>
<td>97.84 ± 13.82</td>
<td>100.37 ± 13.38</td>
<td>88.52 ± 19.81</td>
<td>87.01 ± 17.45</td>
</tr>
<tr>
<td>Sperm count in the cauda epididymis (x10⁶/g/organ)</td>
<td>612.50 ± 89.32</td>
<td>613.06 ± 107.26</td>
<td>540.83 ± 100.82</td>
<td>552.00 ± 51.04</td>
</tr>
<tr>
<td>Sperm transit time in the caput/corpus epididymis (days)</td>
<td>3.68 ± 0.52</td>
<td>3.92 ± 0.39</td>
<td>3.10 ± 0.68</td>
<td>3.32 ± 0.28</td>
</tr>
<tr>
<td>Sperm transit time in the cauda epididymis (days)</td>
<td>4.24 ± 0.54</td>
<td>4.13 ± 0.55</td>
<td>3.67 ± 0.74</td>
<td>3.75 ± 0.50</td>
</tr>
<tr>
<td>(Normal sperm (%))</td>
<td>97.01 (90.0 - 99.5)</td>
<td>99.33 (98.5 - 100.0)</td>
<td>95.3 (95.0 - 100.0)</td>
<td>99.00 (96.5 - 100.0)</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SEM. ANOVA/Tukey’s test. p > 0.05; n = 8 animals/group.

**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.

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.

**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 (LD₅₀) higher than 2000 mg/kg) (OECD, 2001).
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 effects 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.), 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.

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. Each bar represents the mean ± SEM. ANOVA/Newman-Keuls test. * p < 0.05, ** p < 0.01 comparing to control group. n = 6 animals/group.

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 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 LTβ4 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 a vector 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 prostanoids 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-l-rhamnopyranosyl-(1→6)-b-d-glucopyranoside could also be found as nicotifolin (more currently used name), kaempferol-3-O-rutinoside, nicotifloroside, kaempferol-3-O-beta-rutinoside, 3-rutinosylkaempferol, kaempferol 3-O-rutinoside, kaempferol 3-O-beta-rutinoside. Nicotifolin 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), antilglycation (Lal Shyaula et al., 2012), protects against cerebral surgical action of rutin and other described that the main mechanism of inflammatory action is associated to NF-kB, 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-

References
Czekalski, S., 1969. Results of application of the preparation Lespenephryl Lyophilise in inflammatory models were 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.

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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-inflammation assays; CALK and ACA performed data analyses and wrote the manuscript. All authors read and approved the final manuscript.