Elevated Plus-Maze and the Hole Board test for use as a quality control assay of medicines based on *Passiflora incarnata* Linnaeus

Teste do Labirinto em Cruz Elevado e Hole Board para o uso como ensaios de controle de qualidade de medicamentos a base de *Passiflora incarnata* Linnaeus

Summary: In the search for the reduction of the side effects caused by allopathy, phytotherapeutic products are already on the veterinary market. This study aimed to evaluate Elevated Plus-Maze and Hole Board test as analytical methodologies for quality control of anxiolytic drugs based on *Passiflora incarnata*. The efficacy of three different brands of commercial *Passiflora incarnata* extracts was tested in the Elevated Plus-maze (for anxiolytic effect) and the Hole Board test (for locomotor and neophilia activity) in Wistar rats. Treatment with the *P. incarnata* brands revealed a significant 3-fold increase in the length of stay in the open arms in the LCE. In the ambulation, animals from bands 1 and 3 presented a significant increase, being brand 2 the only one that did not present this relevance. In the neophilia, *Passiflora* brand 1 presented a significant increase of 3 times and groups 2 and 3 an increase of approximately 2 times. The results showed that different brands of *Plassiflora* have a satisfactory quality standard related to biological activity and showed that the use of Elevated plus-maze/Hole board test can be an effective tool in the quality control of herbal medicines with anxiolytic activity associated with the phytochemical analysis.

Keywords: *Passiflora incarnata*; anxiety; anxiolytic; phytotherapic; Elevated Plus-maze; Hole Board test.

1. Introduction

Anxiety is characterized as a natural and essential behavior for self-defense, however, it can have a negative impact if it is excessive or prolonged, since pathological anxiety starts to be considered as a disease when it is exacerbated by the anxiogenic stimulus (SOUSA et al, 2018).

Several species of the genus *Passiflora* have evidence of their anxiolytic and sedative activity, as they have effects on the central nervous system (LEAL et al, 2016). *Passiflora incarnata*, has anxiolytic, sedative and muscle-relaxing properties in humans (GRUNDMANN et al, 2008), and these effects are also reproduced in dogs and cats (OZAKI & DUARTE, 2006, NOGUEIRA et al, 2010; SILVA, 2019). To reduce side effects caused by allopathy, herbal products are already on the veterinary market (OZAKI & DUARTE, 2006), and the use of phytotherapy in behavioral disorders in dogs has been associated with environmental and educational therapy (LANDSBERG et al, 2004). In

addition, the benefits gained from the use of medicinal plants are evident, their effectiveness associated with low toxicity and side effects demonstrate a remarkable cost / benefit ratio (OZAKI & DUARTE, 2006).

Herbal medicines have been growing progressively year after year at expressive rates, however, the sustainability of herbal medicine depends, among other factors, on maintaining a minimum quality of products. In this sense, adequate quality control of this type of product is essential to minimize any possible health risk for the consumer. Several studies have evaluated the quality of *Passiflora* spp., where the vast majority of them have focused on determining the phytochemical contents, as well as the presence of microbiological contaminants (REHWALD et al, 1994; ROMANI et al, 1999; SHUAYPROM et al, 2016; PASSARINHO et al, 2018**;** SEPÚLVEDA et al, 2018; SILVA et al, 2019**)**. Thus, it is important to evaluate complementary methodologies that can be associated with those already described to assess quality control through the pharmacological efficacy of products based on *Passiflora*.

Our study aims to evaluate the use of Elevated Plus-Maze and Hole Board test in rats as an analytical methodology for quality control of anxiolytic drugs based on *Passiflora incarnata*.

2. Materials and Methods

2.1 Animals

Male albino Wistar rats (3 and 5 weeks) were obtained from the Fundação Oswaldo Cruz breeding unit (Rio de Janeiro, Brazil). The animals were kept in plastic cages with free access to food and water ad libitum, light/dark cycle of 12 hours, humidity (55% ± 5) and ambient temperature 23ºC ± 3ºC. They were submitted to an acclimatization period of 7 days before the beginning of the behavioral tests. Groups of 10 rats were randomly assigned to different treatment groups and tested in varied order, all under the same experimental conditions. All experimental procedures were performed according to The Committee on Ethical Use of Laboratory Animals of the Fundação Oswaldo Cruz (FIOCRUZ, Brazil; license LW-30/14).

2.2 Drugs and treatment

Before administering the treatments, the animals were weighed and separated into 5 groups (Phosphate Saline Buffer-PBS, Diazepam, *Passiflora* brand 1, *Passiflora* brand 2, and *Passiflora* brand 3). Each animal received a solution according to its weight, with a standardized administration volume of 10 mL/kg of body weight. We designate Passiflora 1, Passiflora 2, and Passiflora 3, as they were drugs from three different manufacturers. After recording the weight gain, the animals received the specific substance for each group via gavage, half an hour before being introduced to the tests. The animals received 3 doses at different times, half an hour before the Elevated Plus-Maze test in the first week, half an hour before the Hole Board test in the second week, and 24 hours before euthanasia after the last weighing in the third week. The dosages used were: Diazepam at a dose of 1.5 mg/kg diluted in PBS for the positive control group. According to previous studies, we used the dosage of 400 mg/kg *Passiflora* extract diluted in PBS for all animals of the three groups *Passiflora* brands.

2.3 Elevated Plus-Maze test

The anxiolytic activity was measured using the Elevated Plus Maze test. The apparatus consists of two open arms (50 cm long x 10 cm wide) and two closed arms (50 cm long x 10 cm wide x 40 cm high for closed arms), perpendicularly arranged and interconnected by a center square (10 cm long x 10 cm wide). The maze was elevated 50 cm above the ground. 30 minutes after the dose administration, the animals were placed individually in the central square of the maze with the face facing the open arm, with their back to the evaluator, being able to explore the environment during a 5-minute test period. All sessions were recorded using a video system. The time it took the animal to move from the open arm to any of the closed arms was recorded by the evaluator. The videos were watched and the time spent in each arm was recorded with the aid of two STOP WATCH® stopwatches, counting the time in the open and closed arms. After each rat, the box was sanitized with 20% alcohol to prevent traces of the previous animal from interfering with the decisions of the next subsequent rat.

2.4 Hole Board test

The Hole Board test is frequently used since the 70s to investigate the effect of substances that may interfere with the natural exploratory tendency (neophilia). The apparatus consists of a box (metallic or acrylic) closed in the shape of an arena, measuring 80 x 60 x 50 cm. Inside, there are 20 evenly spaced holes (3 cm in diameter). Each animal submitted to the test was placed individually in the device, on the same edge of the artifact and with the snout facing the wall. Rats were given 5 minutes to freely explore the environment. Dipping behavior ("head dipping") was evaluated by the number of times an animal inserted its head into a hole at least at eye level. The number of quadrants displaced by the animal (virtual space between one hole and another) was also recorded. After each rat, the box was sanitized with 20% alcohol to prevent traces of the previous animal from interfering with the decisions of the next subsequent rat.

2.5 Acute toxicological analysis

The animals were weighed on a PRECISA® model 3000D semi-analytical scale with a sensitivity of 0.1g to adjust the substances according to body weight. The rats were weighed at three different times: on the day of the Elevated Plus Maze test, on the day of the Hole Board test, and finally 24 hours before being euthanized. The animals' blood was collected by puncture of the descending aorta artery after euthanasia with a solution containing the drugs Ketamine (140 mg/kg) and Xylazine (20 mg/kg) applied intraperitoneally. After collection, an aliquot of 500 µl of whole blood was placed in microtubes with anticoagulant EDTA K2 brand Vacuplast® for hematological analysis and another 500µl aliquot in microtubes containing separating gel and activator of clot brand Biocon® for biochemical analysis. Soon after coagulation in the tubes for biochemical analysis, they were centrifuged in a ZENTRIFUGEN® model MIKRO20 microcentrifuge at 13000 rotations per minute for 10 minutes to separate the serum. After centrifugation, serum was collected in 0.5 mL polypropylene tubes. The hematological parameters analyzed were: red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, average corpuscular hemoglobin concentration, leukocyte count, and

platelet count. For biochemical analysis, the following parameters were measured: glucose, urea, creatinine, creatine, albumin, Aspartate Aminotransferase (AST), Alanine aminotransferase (ATL), total proteins, uric acid, cholesterol, and alkaline phosphatase. Hematological analyzes were performed using the automatic counter brand SYSMEX® and KOBE®, and the biochemical parameters were measured using the device brand JOHNSON & JOHNSON®.

2.6 Statistical analysis

The anxiolytic and locomotor activity were analyzed using the ANOVA® statistical test and Tukey post-test. For all tests, a value of $p < 0.05$ was considered statistically significant. The statistical analyses were evaluated using Graph Pad Prism Program 3 version 5.0.

3. Results

3.1 Elevated Plus-Maze test

In order to evaluate the effectiveness of the different commercial brands of passiflora extract concerning anxiolytic activity, we carried out the elevated plus-maze (EPM) test. As expected, diazepam (dose of 1.5 mg/kg) reduced behavioral indices of anxiety in rats, characterized by a 3-fold increase in the time spent on open arms when compared to the negative control group. The animals treated with each of the 3 different brands of *Passiflora incarnata* extract (dose of 400 mg/kg) showed an anxiolytic effect similar to diazepam. In addition, we did not observe any significant difference in the anxiolytic effect between the 3 brands evaluated (**Figure 1**).

3.2 Effects of Passiflora incarnata on the locomotor activity of rats

To assess whether the effect observed in the EPM test is related to a possible alteration in locomotor activity induced by treatment with *Passiflora*, we analyzed this variable using the Hole Board behavioral test. As shown in **Figure 2**, animals treated with diazepam presented a significant increase in displacement behavior, as already shown in the literature. Animals treated with brands 1 and 3 of *Passiflora* also had an increase in displacement behavior, similar to animals treated with diazepam. However, animals treated with brand 2 were the only group that had no change in locomotor activity.

Based on these findings, we observed that none of the treatments with *Passiflora* brands, which had an anxiolytic effect, did negatively alter the locomotor activity of the treated animals.

3.3 Effects of Passiflora incarnata on the head-dipping behavior

Another evaluated variable in the Hole Board test was the head-dipping behavior. Less anxious animals tend to be more willing to explore the artifact freely and consequently to put their snouts in the hole. As presented in **Figure 3**, all treated groups performed significantly more head-dipping behavior compared to the control group (PBS). The diazepam and *Passiflora* brand 1 group had a 3-fold increase compared to the PBS group, while the *Passiflora* brands 2 and 3 had a 2-fold increase when compared to PBS. In addition, the displacement and exploratory behavior effects were more prominent in diazepam and *Passiflora* brand 1 over brands 2 and 3.

3.4 Hematology and Biochemistry

To assess whether commercial extracts of *Passiflora incarnata* generates changes in hematological and biochemical parameters, blood samples were collected for laboratory analysis (data are shown in **Table 1**). From the parameters observed in hematology, the concentration of mean corpuscular hemoglobin (CHCM) showed significant differences in the groups diazepam (33.35 ± 0,7 g/dL), *Passiflora* brand 1 (33.05 ± 0,4 g/dL), brand 2 (33.46 ± 0,4 g/dL) and brand 3 (33.04 ± 0,3 g/dL) compared to the PBS group (32.3 ± 0,4 g/dL). Albumin concentrations in the brand 1 (3.1 ± 0,14 g/dL), brand 2 (3.2 ± 0,13 g/dL) and brand 3 (3.4 ± 0,17 g/dL) groups were significantly higher than PBS group (2.9 ± 0,19 g/dL). The same data were found in the analysis of Total Protein, where brand 1 (5.7 ± 0,17 g/dL), 2 (5.7 ± 0,16 g/dL) and 3 (6.0 ± 0,18 g/dL) showed higher values than the PBS group (5.4 ± 0,26 g/dL).

4. Discussion

Passiflora species have several applications in popular medicine, but their most useful properties are anxiolytics and sedatives (GOSMANN et al, 2011). Experimental animal models are essential as tools for the recognition of anxiolytic compounds and issues involved in the basis of psychic problems (BARBOSA & LIMA, 2016). Rodents have several characteristics similar to human beings such as their anatomy, biochemical and cellular levels, their brain functions such as anxiety, fear, hunger and memory (MEER & RABER, 2016). In this way, rodents are valuable examples, since we can replicate symptoms and anxiety characteristics similar to that of humans (BALEOTTI, 2017).

Extracts of leaves, flowers and branches showed an anxiolytic effect, with the best results being achieved with leaf extract (DHAWAN et al, 2001). When evaluating the three different brands of *P. incarnata* to compare them concerning the anxiolytic activity of the rats in the elevated plus-maze test, we observed an increase in time spent in open arms compared to the PBS control group. In our results, all the three different brands behaved an anxiolytic activity in the same way. Moreover, the three different brands of *P. incarnata* at a dose of 400 mg / kg demonstrated an anxiolytic effect similar to diazepam 1.5 mg / kg, similar results were found by Grundmann and colleagues (2008) using *P. incarnata* at a dose of 375 mg / kg versus diazepam (at a dose of 1.5 mg / kg).

The results observed in the hole board test in the assessment of neophilia and displacement suggested that *P. incarnata* triggered behavioral alterations characteristic of a state of relaxation corresponding to the anxiolytic effect, once the number of times the animal placed the snout in the hole (head dipping) and moved around the environment was up to twice as large as the animals in the PBS group. When comparing the tested brands and diazepam with the PBS group, it was demonstrated that these alterations are within the expected parameters of increasing the anxiolytic effect by our positive control, thus corroborating that the tested brands are as efficient as the reference benzodiazepines. In the comparison between the *Passiflora* groups, the *Passiflora* brand 1 obtained the most prominent results, with animals being most curious and mobility. The *Passiflora* brand 2 was the one that presented the lowest number of head-dipping and ambulation, when compared to the PBS group, it is worth noting that its result was statistically different from the Passiflora brand 1. The anxiolytic effects from *P. incarnata* are demonstrated in the literature (LEAL et al, 2016) and the species is also used in folk medicine to treat this disorder (SANTOS et al, 2006). In veterinary medicine, Benzodiazepines are used for several diseases, but their side effects are quite serious in some species, such as in cats, which has recently been reported fulminant hepatotoxicity

in animals that received Diazepam orally. In addition, its mortality is high if the treatment is of prolonged use. In one of the case reports observed, eight out of eleven animals undergoing treatment died (ADAMS, 2016). In contrast, studies on the toxicity of *Passiflora incarnata* are still poor (TUROLLA & NASCIMENTO, 2006; OZAKI & DUARTE, 2006).

The evaluation of hematological parameters is of paramount importance, especially in the evaluation of toxicity, since hematopoiesis has a sensitivity to toxic agents, especially for those who have the capacity for mutation or cytotoxicity, resulting in alterations that can control the use of medications (MEDEIROS et al, 2009). Thus, the present study evaluated the hematological parameters in order to observe whether *P. incarnata* has a toxic effect on the animal organism. However, only the mean corpuscular hemoglobin concentration in the three brands showed a significant difference compared to the PBS group, but it was still within the reference values.

Among the biochemical parameters evaluated, Alanine aminotransferase (ALT) and Aspartate transaminase (AST) are good markers, in cases of liver disease they increase in 90% of cases (MEDEIROS et al, 2009). One of the parameters that showed alteration was albumin, although the rodents of the five groups had hypoalbuminemia, there was a significant increase in the *Passiflora* brands 1, 2 and 3 compared to the PBS and diazepam groups. Albumin can be related to nutritional status, but it can also be linked to liver synthesis and kidney disease (BROCK et al, 2016). Finally, total proteins are part of the blood plasma and have the function of oncotic regulation and responses to inflammatory processes (VANNUCCHI et al, 2016). In this parameter, we observed that there was a significant increase in the *Passiflora* groups in comparison to the PBS, but still the results remained within the normal range. Added that, there are some limitations in this study, such as the sample size and the lack of detail of the concentration of the composition of each brand, which makes it difficult to attribute the observed effect only to the active principle of *P. incarnata*. However, as the test was done with the finished product in its usual administration, it does not invalidate the observed data.

5. Conclusion

Based on the above, we observed that the three different brands of *Passiflora incarnata* showed anxiolytic activity in the elevated plus-maze. When comparing the anxiolytic effect of *P. incarnata* with that of diazepam, similarities were observed between the two drugs. The results showed that different brands of *Plassiflora* have a satisfactory quality standard related to biological activity and showed that the use of Elevated plus-maze/Hole board test can be an effective tool in the quality control of herbal medicines with anxiolytic activity associated with the phytochemical analysis.

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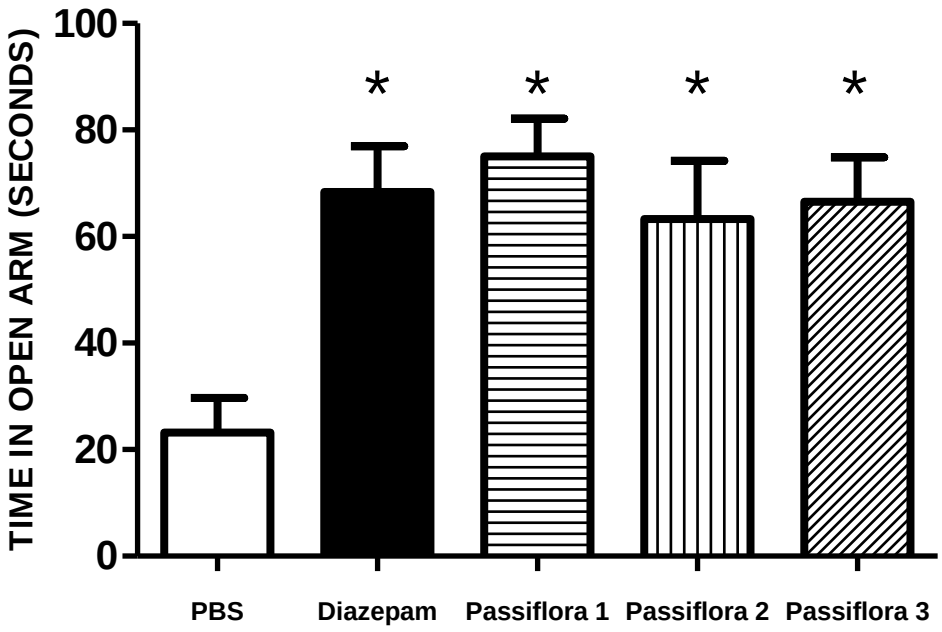
Legends

Figure 1: Anxiolytic effect of *Passiflora incarnata* extracts on the Elevated Plus-Maze in male Wistar rats. Time in seconds spent in open arms (n=10/group). PBS: Phosphate saline solution, Diazepam: 1.5 mg/kg, Passiflora 1.2 and 3: 400 mg/kg of three different brands. The statistical analysis used was the ANOVA test followed by the Tukey post-test. Differences were considered significant when $p< 0.05$ (*).

Figure 2: Anxiolytic effect of *Passiflora incarnata* extracts on the displacement of male Wistar rats in the Hole Board test. Total ambulation (X12cm) (n=10/group). PBS: Phosphate Saline Solution. Diazepam: 1.5mg/kg. Passiflora 1, 2, and 3: 400 mg/kg from three different brands. The statistical analysis used was the ANOVA test followed by the Tukey post-test. Differences were considered significant when $p< 0.05$ (* and +), $p< 0.01$ (**) and $p< 0.001$ (***).

Figure 3: Anxiolytic effect of *Passiflora incarnata* extracts on the displacement of male Wistar rats in the Hole Board test. *Head dipping* (n=10/grupo). PBS: Phosphate Saline Solution. Diazepam: 1.5mg/kg. Passiflora 1, 2, and 3: 400 mg/kg from three different brands. The statistical analysis used was the ANOVA test followed by the Tukey post-test. Differences were considered significant when $p< 0.05$ (* and +), $p< 0.01$ (**) and $p< 0.001$ (***).

Figure 1.


TIME IN OPEN ARM (SECONDS)
100
80
60
40
20
0
*
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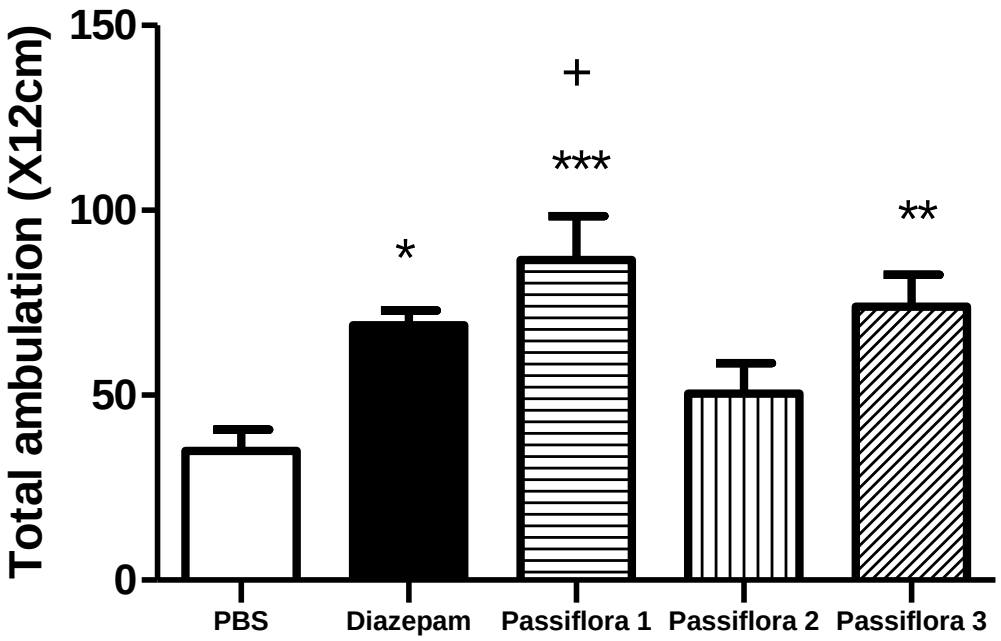
PBS
Diazepam
Passiflora 1
Passiflora 2
Passiflora 3

Figure 2.


Total ambulation (X12cm)
150
100
50
0
+
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*
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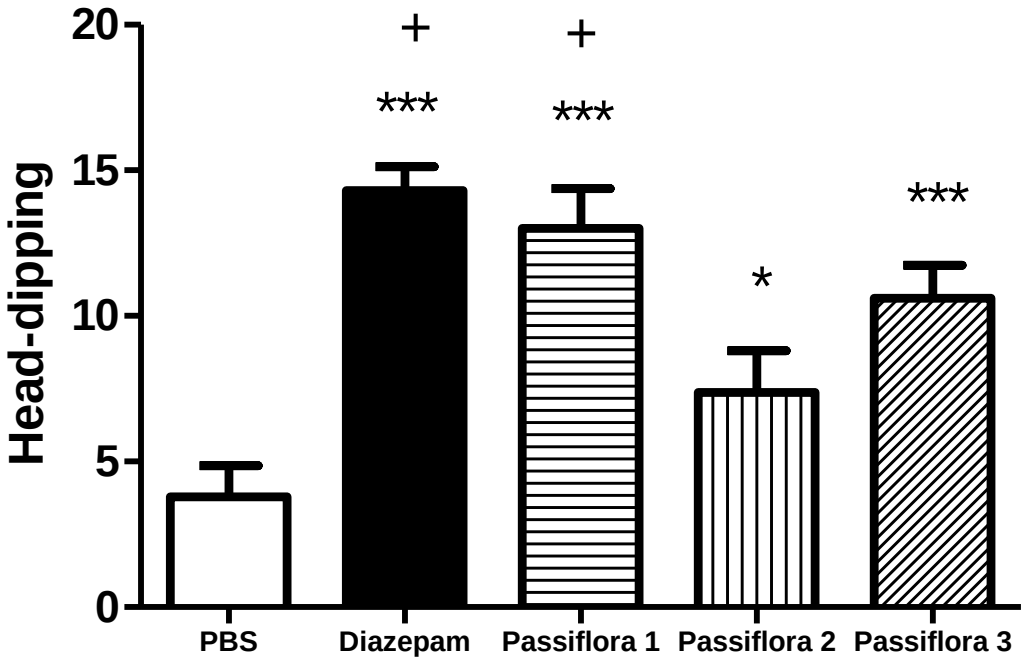
PBS
Diazepam
Passiflora 1
Passiflora 2
Passiflora 3

Figure 3.


Head-dipping
20
15
10
5
0
+
+
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PBS
Diazepam
Passiflora 1
Passiflora 2
Passiflora 3

Table 1.

Parameters	PBS	DIAZEPAM	PASSIFLORA 1	PASSIFLORA 2	PASSIFLORA 3
Red blood cells (millions/mm³)	7.7 ± 0.64	7.67 ± 0.2	7.64 ± 0.4	7.63 ± 0.2	7.35 ± 0.3
Hemoglobin - (g/dL)	15.5± 1.3	15.5±0.4	15.6±0.7	15.6±0.5	15±0.7
Hematocrit (%)	48.01±3.9	47.15±1.6	47.23±1.8	46.6±1.7	45.37± 2.0
Mean corpuscular volume (fm³)	62.31± 1.5	61.47± 1.4	61.82± 1.5	61.16± 2.5	61.17± 1.9
Mean Corpuscular Hemoglobin (pg)	20.13± 0.4	20.3± 0.3	20.41± 0.5	20.46± 0.6	20.42± 0.6
Mean corpuscular hemoglobin concentration (g/dL)	32.3± 0.4	33.35± 0.7*	33.05± 0.4 *	33.46± 0.4*	33.04± 0.3*
Leukocyte Count (thousand/mm³)	6.7± 1.3	7.1± 2.0	7.5± 1.6	7.9± 1.4	7.3± 1.0
Red blood cells count (thousand/mm³)	1099.17±126.9	1036.13± 94.7	1122.75± 97.7	1017.33± 127.4	1097.67± 131.0
Glucose (mg/dL)	256.6± 59.8	242.6± 49.1	297.7± 78.4	244± 53.5	242.7± 51.9
Uric acid (mg/dL)	3.2± 1.8	2.4± 0.57	3.5± 1.9	3.33± 1.6	3.9± 1.5
Albumin (g/dL)	2.9± 0.19	2.8± 0.03	3.1 ± 0.14*	3.2± 0.13*	3.4± 0.17*
Alanine aminotransferase (ALT) (U/L)	67.4± 3.5	62± 5.9	82.1± 4.3	70.40± 14.6	67.1± 6.2
Aspartate Transaminase (AST) (U/L)	178.8± 28.1	107.5± 7.5	174±13.6	104.1± 48.9	91.5± 10.3
Total proteins (g/dL)	5.4± 0.26	5.4± 0.08	5.7±0.17*	5.7± 0.16*	6.0± 0.18*
Creatinine (mg/dL)	0.2± 0.04	0.2± 0	0.2±0	0.2±0	0.2±0

Table 1: Hematological and biochemical parameters evaluated in male Wistar rats (n=10/group). The data were represented by Mean and ± Standard deviation. The statistical analysis used was the ANOVA test followed by the Newman Keuls post-test. Differences were considered significant when $p < 0.05$ (*).