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BIOMEDICAL SCIENCES

Gait analysis with muscular fibrosis and treatment with *Alpinia zerumbet* essential oil in immobilized rats

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Abstract: The analysis of gait in animals is important for understanding movement disorders in various human pathologies, especially those that develop muscle fibrosis. In the search for treatment alternatives for this problem, essential oils have been studied. Among them, research involving the essential oil of *Alpinia zerumbet* (EOAz) has been shown to promote relaxation and improve muscle function. Therefore, this study aimed to evaluate the effect of EOAz on gait with muscle fibrosis in immobilized rats. 30 rats (Wistar) were divided into five groups of six animals each: control group (without fibrosis and without treatment), immobilization group (with fibrosis and without treatment), and EOAz treatment groups (with fibrosis and with treatment). The animals were immobilized for 15 days with an ankle plantar flexion orthosis. After this period, they were treated with the oil cutaneously for 30 days. The analysis of behavioral tests before treatment indicated a significant increase in the means of the immobilized groups about to with concerning the control. We conclude that EOAz was effective in improving gait after inducing muscle fibrosis in immobilized rats. Studies are needed to assess the oil's effectiveness in the treatment of muscle fibrosis in human pathologies.

Key words: Immobilization, fibrosis, essential oil, *Alpinia*, gait.

INTRODUCTION

Functional analysis is important before introducing experimental treatments in humans (Wong & Shah 2019). Motor function plays an essential role in quality of life and a functional analysis would detect disturbances in the locomotor system (Timotius et al. 2019). Another function that deserves to be analyzed is gait, as any changes in this parameter could be used to identify and quantify locomotor changes.

Gait analysis has been performed in rodents, and is becoming more popular in pre-clinical behavioral trials (Lakes & Allen 2018). However, rodent gait has specific biomechanical characteristics, when compared to that of humans, as it is a quadrupedal gait, and should be analyzed with some considerations related to the animal's body posture, such as: rotation of paws about the body axes, angular displacement of limbs, parameters of stride length and width, speed and acceleration (Wong & Shah 2019).

Kinematic measures can be considered an effective method for the natural recording of animal behavior, providing quantitative results for the study of pathologies that present changes in gait (Lakes & Allen 2018). Muscular dystrophies, cerebral palsy and Parkinson's are frequently studied, and some of the changes observed are related to the duration of muscle immobilization (Ruan et al. 2013, Lieber & Fridén 2019).

Some factors lead to the prescription of immobilization during rehabilitation, particularly following sprains, lower limb injuries, fractures and ligament ruptures (Santos-Júnior et al. 2010), and this immobilized period can lead to decreased movement of the joints, muscle hypotrophy, a decline in muscle elasticity related to disuse of the locomotor system, and the development of fibrosis resulting from increased infiltration with overexpression and the accumulation of collagen in the connective tissue (Carvalho et al. 2013), leading to decreased muscle function (Smith & Barton 2014) and muscle contracture due to the proliferation of connective tissue (Yoshimura et al. 2017).

Pre-clinical studies have been carried out involving the immobilization of animal paws to analyze the changes that occur in muscles and joints. The most commonly used materials for making these orthoses were plaster (Yoshimura et al. 2017, Maezawa et al. 2017, Honda et al. 2017), steel screens (Carvalho et al. 2013), adhesive tape (Santos-Júnior et al. 2010) and metal (Bertolini et al. 2009).

In this context, countless changes occur in the muscle due to the immobilization process. The existing treatments for these changes include conventional physiotherapy and surgical procedures (Silva & Santos 2015, Carvalho et al. 2011). However, to date, there is no drug treatment for muscle fibrosis.

Research into medicines involving essential oils has shown promise in recent years and may identify alternatives to conventional medicines and therapeutics (Ghosh & Rangan 2013, Manion & Widder 2017). Among the oils studied, *Alpinia zerumbet* (Pers.) B.L.Burtt & R.M.Sm oil has stood out, possibly due to its availability and popularity, as it comes from a plant belonging to the Zingiberaceae family, originating in the East Indies, and found in the tropical and subtropical regions of South America, being particularly abundant in northeastern Brazil (Barcelos et al. 2010).

Some pharmacological actions involving the essential oil of *Alpinia zerumbet* have already been reported. For example, it may act as an endothelium vasorelaxant (Pinto et al. 2009), a cardiodepressor by modulating the activity of the L-type calcium channels, a muscle relaxant in spastic muscles (Santos et al. 2011a, Cândido & Xavier-Filho 2012, Cerqueira et al. 2015, Cândido et al. 2017), an anti-inflammatory and tissue repairer (Santos-Júnior et al. 2017) and a collagen quality modulator (Cerqueira et al. 2015). Therefore, this study aimed to analyze the effect of *Alpinia zerumbet* essential oil on the functionality of immobilized rats.

MATERIALS AND METHODS

Animals and ethical statement

Thirty adult female Wistar rats (*Rattus norvegicus albinus*) with an average body weight of 200–300 grams and conventional microbiological status, from the Bioterium of Tiradentes University, were used. The animals were housed in a closed system in polypropylene cages with appropriate metal grids (49x34x16cm), in groups of three. The local temperature was maintained at 21°C +/- 2°C with a relative humidity of 30% to 70%. Artificial lighting was provided with a light and dark cycle (12/12 h), and all animals had free access to food (balanced feed *ad libitum*) and a drinking fountain with drinking water. The study was submitted to and approved by the Ethics Committee on the Use of Animals (CEUA) of Tiradentes University (UNIT) as protocol nº 010418 9.

Experimental design

After one week of acclimation, the rats were randomly divided into groups of six (n = 6/ subgroup), being distributed as described below.

- Treatment groups (n = 18): Treatment groups involved immobilization of the gastrocnemius muscle and application of the EOAz, subdivided into: treatment 1 (0.0115 μg/g), treatment 2 (0.009 μg/g) and treatment 3 (0.0065 μg/g) (Santos et al. 2011a, Cândido & Xavier-Filho 2012). Each applied dose was calculated according to the animal's body weight and the concentration for its group according to Santos et al. 2011a.

- Immobilization group (n = 6): Muscle immobilization without treatment.

- Control group (n = 6): Healthy muscle without muscle immobilization.

Immobilization of paws to induce muscle fibrosis

For the immobilization of the rats' ankles, the animals were anesthetized with 10% ketamine hydrochloride (95 mg/kg) and 2% xylazine hydrochloride (12 mg/kg) with the intraperitoneal application (Cerqueira et al. 2015). After sedation, the animal's left ankle was immobilized in a complete plantar flexion position, using an orthosis made as follows: a 2.5 cm wide micropore tape was used to protect the animal skin and prevent injury. To fix the ankle in plantar flexion, a 5 cm length of ½ inch hose with a 1 cm opening was placed over the paw. The hose was lined with cotton gauze and a 2.5 cm wide plaster (Figure 1a). A steel mesh-covered at the ends with high-strength adhesive tape was placed over the hose (Figure 1b). The tape was used to wrap the entire mesh to prevent the animal from having direct contact with the steel mesh (Figure 1c). The animals were monitored daily to check the circulation conditions, swelling in the paws, and possible loosening of the orthosis. The orthoses remained in place on the animals' paws for 15 days.

After completing the 15 days of immobilization, the orthoses were removed for observation. A manual test was passively performed by the researcher, performing the movements of plantar flexion and ankle dorsiflexion to verify the presence of muscle

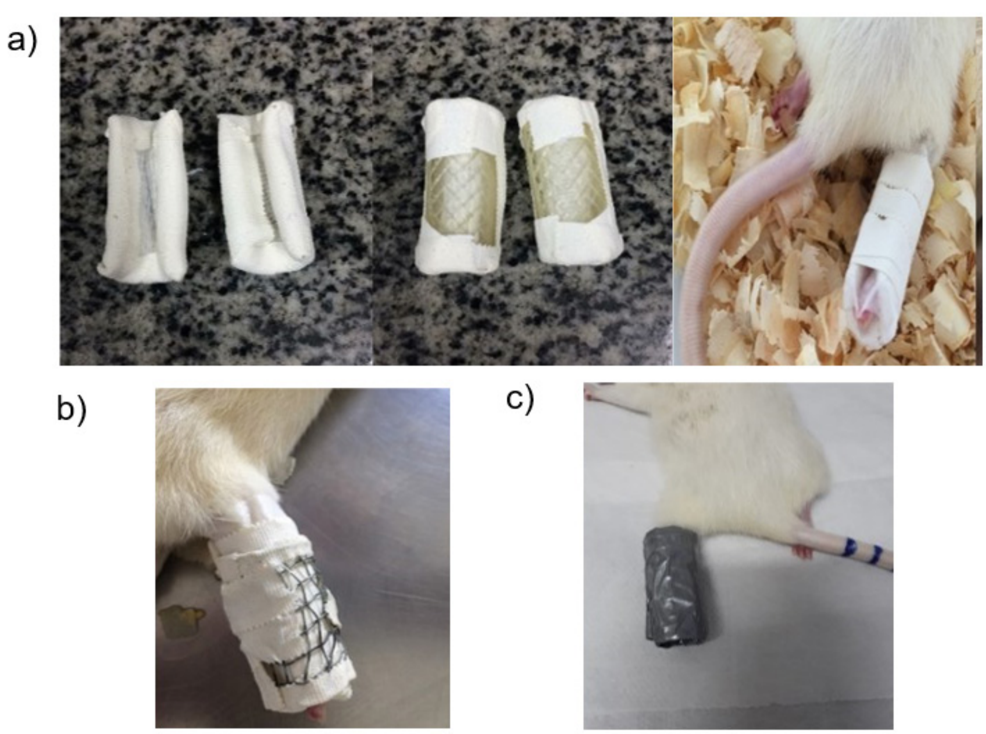


Figure 1. Sequence of the steps for fabrication of the orthosis model. a) part of the hose material lined with cotton gases and adhesive tape; b) part of the steel mesh reinforcing the hose; c) tape to protect the animal from the steel mesh.

resistance in the calf of the animal due to the possible development of fibrosis in the gastrocnemius muscle. The entire immobilization process was carried out by the same examiner. For the functional tests carried out before treatment, two other researchers participated. Two additional examiners participated in the reassessment. All thirty animals remained immobilized for 15 days, after which they were evaluated and reassessed with behavioral and functional tests including Paw Print, CVMob, and the Basso, Beattie, and Bresnahan (BBB) Locomotive Assessment. They were then euthanized as per the timeline shown in Figure 2.

Essential oil of *Alpinia zerumbet*

The essential oil of *Alpinia zerumbet* used in this study was obtained from Infan National Pharmaceutical Chemical Industry S/A, Brazil. The oil was extracted from the freshly harvested green leaves and stems of the *Alpinia zerumbet* (Pers.) B.L.Burtt & R.M.Sm. plant by steam distillation. In the chemical composition of this oil, the presence of three major components was observed: p-cymene (31%), 1,8-cineole (23.3%) and terpinen-4-ol (22.7%) (Batista et al. 2021).

Performance of behavioral tests

Functional gait assessment

Functional assessment of the rats' paws was performed using the Paw Print Test (Costa et al. 2008). For this, a transparent acrylic box measuring 1.11 m in length, 9 cm in width, and 10 cm in height was used. Sheets of white A4 paper folded in half lengthways were placed on the floor of the crate. The rats' hind legs were painted with non-toxic green paint and the animals were allowed to walk through the box. After the test was completed, the paper was scanned at 300 dpi. The first and last impressions of the paws created by the transfer of green paint were discarded. The index of rotation of the legs was obtained through two drawn lines, one from the heel to the midline of the paw and the other passing through the lateral edge of the paw. The meeting of these lines forms an angle that is measured in degrees. For this measurement, the Axio Vision LE64 Software was used, which

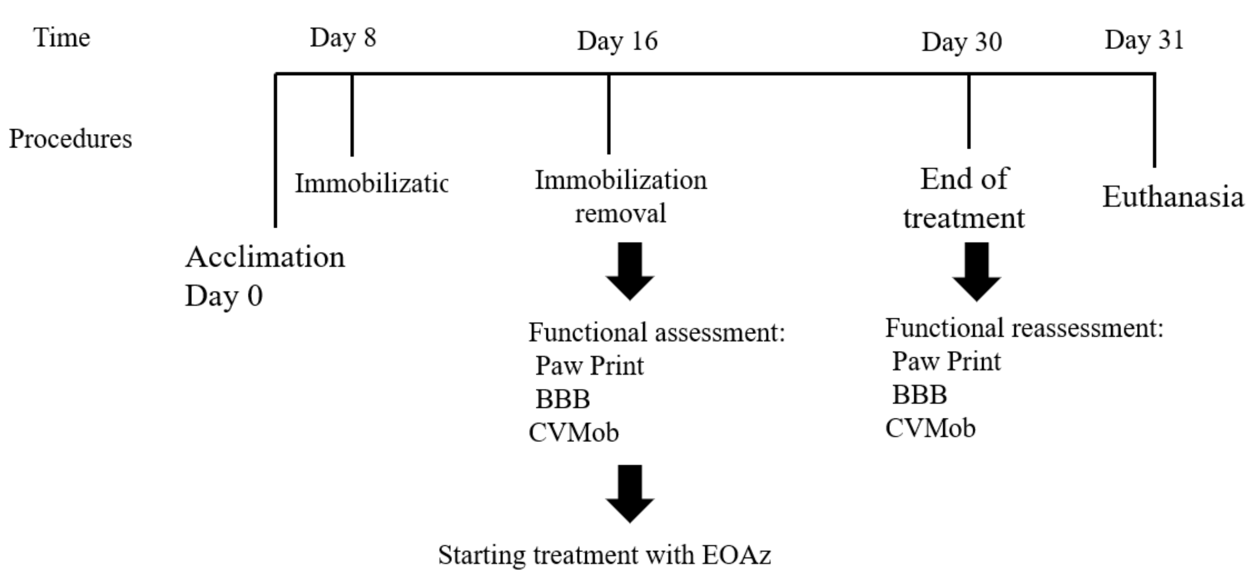


Figure 2. Scheme of the study timeline. The timeline displays the time in days and the procedures that took place.

allows measurement of the rotation angle of the legs.

Locomotive evaluation using Basso, Beattie and Bresnahan (BBB) scale

Two examiners participated in this evaluation, positioned opposite each other to observe both sides of the animal. This assessment was performed using the Locomotor Assessment scale of Basso, Beattie and Bresnahan (BBB) to measure the movements of the paws, coordination, firmness and behavior of the trunk, as well as movements of the posterior limbs, position of the trunk, abdomen, paw position, walking, predominant paw position, body instability and tail. The scores range from 0 (total absence of movement) to 21 (normal movement). To carry out the test, the animals were placed in an open field, surrounded by acrylic and with full visualization, each evaluation lasting 2 minutes (Costa et al. 2008). During the test, the animals were encouraged to move around and to avoid standing still for more than 20 seconds. All open field tests were recorded for 2 minutes for each animal with a Canon digital camera – Powershot Elph 180 20.0 Megapixel – Silver 1093C001.

CVMob

To analyze the speed and acceleration of the animals' gait, free software was used based on algorithms with computer vision acquired through the filming of the movement of the rats' paws. This software was developed by the Physics Institute of the Federal University of Bahia and is easily accessible and free of charge through the website https://sourceforge.net/projects/cvmob/. To obtain the data, videos of the animals' movements were made using the same acrylic box used in the Paw Print Test. To capture the image, a mark was made in the middle of the animal's ankle with a permanent black pen, and the animal was encouraged to walk through the crate throughout its length. The same video camera described above was used, placed 1 meter away from the animal.

Treatment and euthanasia

Before starting treatment with the OEAz, hair was shaved on the animal's left paw to expose the region to be treated. The treatment was performed daily for 30 days. Upon completing this period, the rats were reevaluated with the tests and then were sent for euthanasia in a carbon dioxide chamber at the Tiradentes University Bioterium.

Sample preparation and histomorphological analysis

After 30 days of treatment, the animals were euthanized and the biological material removed to make the slides. Muscle tissue was immersed in 10% buffered formaldehyde, the blocks were placed in the microtome, and 5-micrometer cuts were made before staining with Picrosirius stains. Histological sections were analyzed qualitatively using light microscopy to assess the pattern and intensity of tissue changes regarding the organization and properties of collagen (Cerqueira et al. 2015, Smith & Barton 2014).

Collagen visualization was performed using a Euromex iScope optical microscope (IS.1053-PLPOLi), and a 4X wide-field eyepiece to photograph the slides. The images were obtained by a Euromex photographic camera (CMEX5_WiFi) with 5X and/or 40X magnification and transmitted through the Image Focus Alpha\x64 software coupled to the microscope. The area was not specified since the entire muscle in the cross-section was compromised. The images were acquired with a resolution of 2592 x 1936 pixels and saved in TIF format with dimensions of 2584 x 1936.

For Picrosirius staining, the observational analysis of the collagen formation pattern was analyzed using the birefringence variable, where greenish or greenish-yellow colors identified collagen type III as immature and orange or reddish colors identified collagen type I mature.

Statistical analysis

The statistical program used was GraphPad Prism 6.01. All data are presented as mean ± standard deviation. Normality was tested by the Shapiro-Wilk test and a simple one-way ANOVA or KRUSKAL-WALLIS test was carried out to test differences between groups, followed by the Tukey post-test or Dunn's post-test. Results with a difference of $p < 0.05$ were considered significant.

RESULTS

Analysis of rotation of the rats' paws revealed that rats in the immobilization and treatment 1, 2, and 3 groups showed significantly higher rotation in relation to the control ($p<0.001$). However, a comparison of rotation before and after treatment revealed that rotation decreased in the three treatment groups ($p < 0.001$) after using the OEAz (Figure 3).

In the evaluation of the mean scores obtained on the BBB scale, it was observed that before treatment, the control group had higher scores in relation to the immobilization and treatment 1, 2, and 3 groups ($p < 0.001$). However, when comparing the values before and after treatment within groups, treatment group 1 showed a higher difference between before and after ($p < 0.001$) compared to treatment groups 2 ($p < 0.05$) and 3 ($p < 0.01$), as shown in Figure 4.

Analysis of the average gait acceleration of rats revealed that the control and treatment group 1 showed significantly higher rates of acceleration when compared to the immobilization group ($p < 0.05$), after the treatment. However, the highest average acceleration was observed in treatment group 3 ($p < 0.01$ when compared to the immobilization group), as can be seen in Figure 5a. Likewise, the average gait speed was significantly higher in the control and treatment group 1 in relation to the immobilization group ($p < 0.05$), as shown in Figure 5b.

In the analysis of the collagen histology of the perimysium of the rat gastrocnemius muscle, a greater orange or reddish color was observed in the immobilization group compared to the other groups. Among the treated groups, it was observed that treatment groups 1 and 3 showed

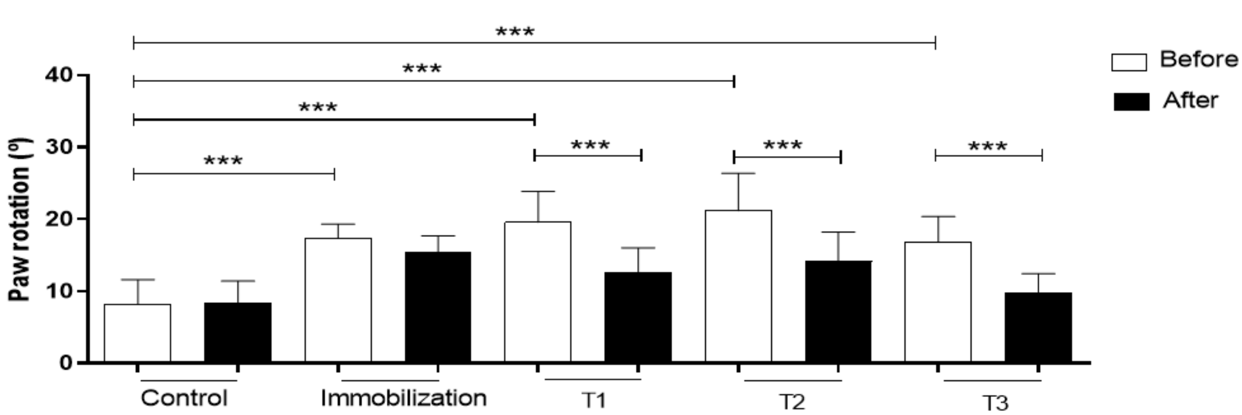


Figure 3. Comparative analysis of the rotation angle of the rats' paws between and within groups before and after the use of EOAz with the use of the Paw Print Test. Where T1 (treatment 1), T2 (treatment 2) and T3 (treatment 3). ANOVA One-Way test with Tukey post-test, with *** p <0.001.

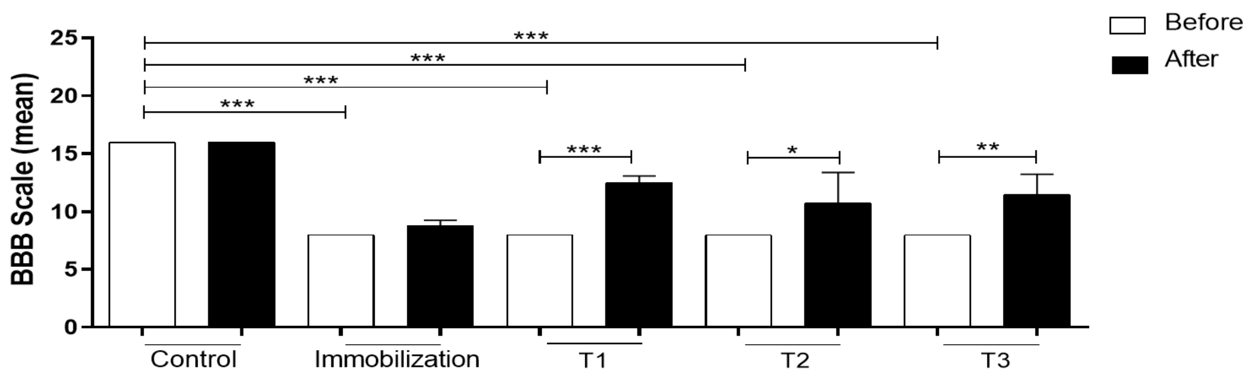
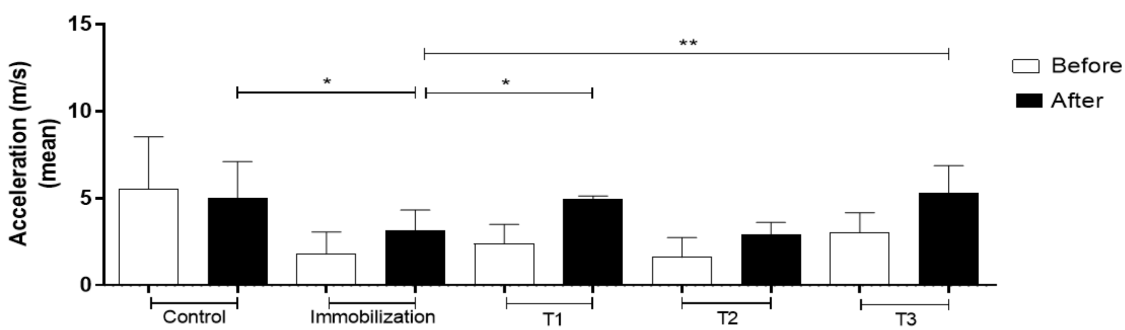


Figure 4. Basso Beattie and Bresnahan (BBB) score of experimental animals performed between and within groups in the period before and after 30 days of using EOAz. Where T1 (treatment 1), T2 (treatment 2) and T3 (treatment 3). The data were expressed as mean ± standard deviation. ANOVA One-Way test with Tukey post-test, with * p <0.05; ** p <0.01; *** p <0.001.

a)



b)

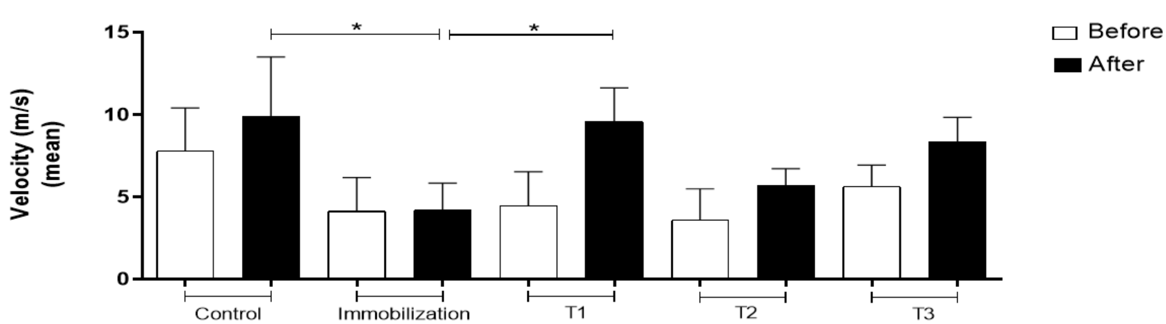


Figure 5. Analysis of data obtained with the CVMob of experimental animals carried out between and within groups in the period before and after 30 days of using EOAz. a) averages of gait acceleration of rats; b) average gait speed of rats. Where T1 (treatment 1), T2 (treatment 2) and T3 (treatment 3). ANOVA One-Way test with Tukey post-test, with * p <0.05; ** p <0.01.

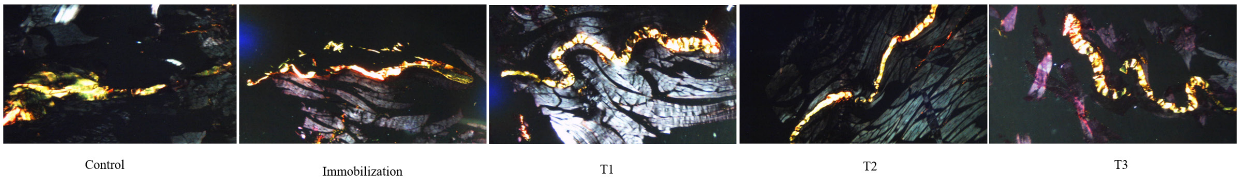


Figure 6. Histological analysis of collagen in the perimysium of the rat gastrocnemius muscle, showing variation in birificiality with green or greenish yellow (type III collagen) and orange or reddish (type I collagen) colors in the five studied groups (control, immobilized and treated).

a greenish or greenish-yellow color superior to that of treatment 2, as can be seen in figure 6.

DISCUSSION

The results of the present study showed a beneficial effect of the EOAz in the context of fibrotic muscle gait in immobilized rats, both in the rotation of the paws, speed, acceleration, and in the improvement of the movement pattern. Therefore, this study presents relevant data that suggest the possibility of using EOAz in fibrotic muscles submitted to immobilization for functional improvement of movement.

Most studies involving the use of orthoses to immobilize rodent paws used material such as plaster (Yoshimura et al. 2017, Maezawa et al. 2017, Honda et al. 2017). However, the use of this material may present a need for constant maintenance, as these animals can chew the plaster, thus damaging the orthosis, promoting loosening, and, thus, impairing its effectiveness. In order to prevent animals from loosening the orthosis, we used coated steel and this proved to be effective at immobilizing the rats' paws for the required period. Previous research comparing the effects of immobilization on the muscle over 7 and 14 days reported that changes in the muscle's morphology occur within 14 days of immobilization, this, therefore, being a suitable period when studying the effects of the immobilization of animal paws (Maezawa et al. 2017). In this study, the period of immobilization adopted was 15 days, in accordance with Bertolini et al. 2009, being long enough for the animals to present changes and locomotor difficulties caused by the orthosis.

Costa et al. 2008 and Franco et al. 2011 evaluated the gait of rats with sciatic nerve damage through paw prints on paper and observed greater rotation in the injured paws compared to those without injury. Ruan et al. 2013 analyzed gait in mice with osteoarthritis by transection of the cruciate ligament (TCL) and found a lower impression of the hind paw of the TCL group compared to the control group. The present study used the same type of test on immobilized muscles, showing similar results in terms of printing and rotation in immobilized paws without treatment. The rotation of the rats' paws is possibly related to the functional limitation of the ankle after immobilization. The use of EOAz in this analysis proved to be effective at promoting muscle relaxation and functional improvement in rats, thus decreasing the rotation angle. This functional improvement was confirmed by the histology analysis, which showed that the groups that received treatment presented staining for collagen type III more evident in relation to the immobilization group.

In the study by Santos et al. 2011b, the BBB locomotor scale was used in animal models of spinal cord injury and demonstrated functional improvement. In another study by Santos et al. 2017, this same scale was evaluated in a rat model of cerebral palsy and also demonstrated significant functional improvement. In a study by Cerqueira et al. 2015, BBB was used to assess gait after treatment with *Alpinia zerumbet* essential oil in rats with spinal cord injury and showed functional improvement in animals. In this study, the BBB scale was used for locomotor assessment of rats after muscle immobilization treated with the same type of oil used by Cerqueira et al. 2015 and a significant improvement in the mean of the functional scores was shown after treatment, demonstrating the scale to be reliable, objective and sensitive in its application. This functional improvement may be related to the modulating action of *Alpinia zerumbet* essential oil in the type L calcium channels, as suggested by Santos et al. 2011a, promoting relaxation in the muscles involved, and thus providing more mobility and functionality for animals.

Several studies have used video software to assess the gait of rodents. Timotius et al. 2019 and Ängeby et al. 2018 used the Cat Walk TM software to assess the functional motor gait of animals. Wong & Shah 2019 used a 3D kinematic evaluation for tracking gait movement in rats. However, Pena et al. 2013 opted for free software that analyzes movement (trajectory, speed, and acceleration) using techniques with computer vision, known as CVMob. In this study, CVMob software was used to analyze the acceleration and gait speed of rats, confirming the results of Pena et al. 2013, proving to be an effective instrument for the analysis of rodent gait.

In conclusion, the use of *Alpinia zerumbet* essential oil was shown to be beneficial in improving fibrotic muscle and gait functionality in a murine model. The three concentrations used were effective in all tests applied, both in decreasing the angle of rotation, increasing speed, and accelerating gait and in the highest scores for movement standardization. Although these results are very promising, further preclinical and clinical studies are still needed to evaluate the effectiveness of EOAz in the treatment of post-immobilization fibrotic muscle.

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Author contributions

TB: Conceptualization, Investigation, Methodology, Formal analysis Writing - original draft, Visualization; AO, LS, VN: Investigation; Methodology; Formal analysis. EC: Conceptualization; Methodology; Formal analysis; Resources; Visualization; Project administration; Funding acquisition; Supervision; Writing- Review and Editing. MB: Conceptualization; Formal analysis; Methodology; Resources; Visualization; Project administration; Supervision; Writing- Review and Editing; all authors wrote, read and approved the final version of the manuscript.