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Original Article

Chemical characterization and effects of volatile oil of *Alpinia zerumbet* on the quality of collagen deposition and caveolin-1 expression in a muscular fibrosis murine model

Caracterização química e efeitos do óleo volátil de *Alpinia zerumbet* na qualidade da deposição do colágeno e expressão da caveolina-1 em modelo murino de fibrose muscular

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Abstract

This study evaluated the effect of the volatile oil of *Alpinia zerumbet* (VOAz) on caveolin-1 gene expression and muscular fibrosis. The rats were immobilized to induce fibrosis of the gastrocnemius muscle, and they were treated with VOAz. Collagen quality was assessed by histology and the expression of the caveolin-1 (CAV-1) gene was evaluated using qPCR. Histomorphological analysis indicated a significant reduction in the perimeter, width, and intensity of collagen in the treated groups, thus showing that the oil was effective in regulating the quality of collagen at the three concentrations. The results of expression levels suggested a decrease in the lesioned group and in two treatment groups (0.0115 µg/g and 0.009 µg/g). However, with the lowest concentration (0.0065 µg/g), no significant difference was observed, with levels similar to those found in healthy tissue. Therefore, the results showed that VOAz has the potential to be a non-invasive and low-cost alternative to aid in the treatment of muscular fibrosis.

Keywords: fibrosis, *Alpinia*, volatile oil, Caveolin-1, gene expression.

Resumo

Este estudo avaliou o efeito do óleo volátil de *Alpinia zerumbet* (OVAz) na expressão do gene da caveolina-1 e na fibrose muscular. Os ratos foram imobilizados para induzir a fibrose do músculo gastrocnêmio, e foram tratados com OVAz. A qualidade do colágeno foi avaliada com histologia e à expressão do gene caveolina-1 (CAV-1) foi avaliada usando qPCR. A análise histomorfológica indicou uma redução significativa no perímetro, largura e intensidade do colágeno nos grupos tratados. Os resultados dos níveis de expressão sugeriram diminuição nos grupos de lesão e em dois grupos de tratamento (0,0115 µg/g e 0,009 µg/g). No entanto, com a menor concentração (0,0065 µg/g), não foi observada diferença significativa, apresentando níveis semelhantes aos encontrados em tecido saudável. O uso do OVAz foi eficaz para reverter as alterações do colágeno causadas pela fibrose, e sua menor concentração apresentou uma possível tendência de aumento na expressão do CAV-1. Portanto, os resultados mostraram que o OVAz tem potencial para ser uma alternativa não invasiva e de baixo custo para auxiliar no tratamento da fibrose muscular.

Palavras-chaves: fibrose, *Alpinia*, óleo volátil, Caveolina-1, expressão gênica.

1. Introduction

Alpinia zerumbet (Pers.) B.L.Burtt & R.M.Sm. is an herbaceous, perennial and aromatic plant. Although found in abundance, *Alpinia zerumbet* is not endemic to Brazil, it originated in Asia. It is an exotic ornamental plant, which was introduced in the national territory, and does not form spontaneous populations. In Brazil, the use of *Alpinia zerumbet* is common because of its antipyretic, anti-inflammatory, analgesic and hypotensive properties (Barcelos et al., 2010; Cândido and Xavier-Filho, 2012; Cartaxo et al., 2010).

Preclinical studies have been carried out to determine the toxicological, pharmacological, and physiological actions of this plant. Some important medicinal effects and actions were discovered, including relaxing effect

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of basal tone, protective action against cardiovascular diseases, and anti-inflammatory properties (Barcelos et al., 2010; Cândido and Xavier-Filho, 2012; Tao et al., 2013; Santos-Junior et al., 2017; Paulino et al., 2019; Araújo et al., 2021). Maia et al. (2016) and Cândido et al. (2017), when assessing the feasibility of using the volatile oil of *Alpinia zerumbet* (VOAz) in spastic muscles, observed functional improvement in patients. An observed positive effect of VOAz was on the organization and orientation of collagen fibers (Cerqueira et al., 2015; Santos-Junior et al., 2017). Santos et.al. (2011) on the other hand, observed that the oil acted by modulating the L-type calcium channels when evaluating the effect of VOAz on cardiac function. These L-type calcium channels were also studied by Yang et. al. (2012), who observed that the calcium concentration in these channels was responsible for regulating the expression of caveolin-1 (Cav-1).

Cav-1 is a transmembrane protein of the caveola family, being the most abundant and necessary protein for its formation in non-muscle cells (Castello-Cros et al., 2011). Cav-1 is also involved in several cellular mechanisms, such as the regulation of intracellular Ca^{2+}, proliferation, and the deposition of extracellular matrix in the muscle (Kunert-Keil et al., 2011). There are countless studies investigating the role of Cav-1 in fibrotic diseases such as cardiac fibrosis (Miyasato et al., 2011), keloid scars (Zhang et al., 2011), systemic sclerosis (Galdo et al., 2008), pulmonary fibrosis (Tourkina et al., 2008; Kulshrestha et al., 2019; Kulshrestha et al., 2020), pathologies involving connective tissue (Castello-Cros et al., 2011) and neurodegenerative diseases (Kunert-Keil et al., 2011). In a review carried out by Shihata et al. (2017), the authors explored the antifibrotic influences of Caveolin-1 in cardiac, pulmonary, renal and liver fibrotic pathologies and its potential as a therapeutic target, observing that Cav-1 has a dynamic role in fibrosis, but its therapeutic effects still need to be better understood.

Cav-1 activity in the fibrosis process has been related to the regulation of transforming growth factor beta 1 (TGF-β1), which occurs through physical interaction with membrane receptors, leading to an effective reduction in TGF-β1 signaling (Gvaramia et al., 2013). A negative correlation between TGF-β1 and Cav-1 was observed in mice deficient in Cav-1, and in skin tissues of patients with systemic sclerosis (Galdo et al., 2008; Miyasato et al., 2011). In contrast, it was observed that the re-introduction of Cav-1 expression led to the suppression of TGF-β1 signaling, improving fibrosis (Miyasato et al., 2011; Zhang et al., 2011). It is known that in fibrosis there is excessive deposition of extracellular matrix, especially collagen (Smith and Barton, 2014). Cav-1 involvement in cell signaling events associated with tissue repair in fibrosis has been studied focusing on elucidating the negative correlation between TGF-β1 and collagen overexpression (Castello-Cros et al., 2011; Tourkina et al., 2008; Zhang et al., 2011).

Different studies have shown positive results with the use of VOAz to treat spastic muscles, likely due to the capability of VOAz to alter the conformation of collagen and modulate L-type calcium channels. These channels have already been shown to have a role in modulating the expression of the CAV-1 gene. However, the effects of the VOAz in muscular fibrosis and its action on CAV-1 gene expression are still unknown. Therefore, this study aimed to assess the effects of the VOAz on histomorphological changes in collagen and the expression of the CAV-1 gene in muscular fibrosis in a murine model.

2. Material and Methods

2.1 Collection of leaf samples and volatile oil preparation

The VOAz used in this study was obtained from Infan Industria Quimica Farmaceutica Nacional S/A. The oil was prepared from *Alpinia zerumbet* (Pers.) B.L.Burtt & R.M.Sm. (Magnoliopsida, Zingiberales, Zingiberaceae Martinov, *Alpinia* L.), an herbaceous and exotic plant grown in the northeastern hinterland, municipality of Itacuruba, Pernambuco, Brazil, latitude 8º49'6" and longitude 38º41'57". It was obtained in an industrial way from freshly harvested leaves and stems of *Alpinia zerumbet* plant at 6:00 A.M., and transported in an air-conditioned car. The oil was extracted by water vapor drag hydrodistillation at 105°C and filtration. After condensation of vapors, the volatile oil was separated from the aqueous phase, obtaining the hydrolate, according to Koketsu and Gonçalves (1991).

2.2 Analysis of the oil samples

A sample of this oil was subjected to chromatographic analysis in order to identify the composition of the product. Chemical characterization was performed using LTPRI (Linear Temperature Programmed Retention Indexes), which is used for chromatographic analyzes with a linear temperature program, and the aid of a solution with a homologous series of n-alkanes (C8-C30), using the Equation 1 (Van Den Dool and Kratz, 1963):

$$LTPRI = 100n + 100\frac{t_{R(i)} - t_{R(n)}}{t_{R(n+1)} - t_{R(n)}} \quad (1)$$

where: n = number of carbons in the n-alkane prior to analyte; $t_{R(i)}$ = analyte retention time; $t_{R(n)}$ = retention time of n-alkane prior to analyte; $t_{R(n+1)}$ = n-alkane retention time after analyte.

This identification was completed by injection of 1 µL of a sample solution in a Shimadzu model GCMS-QP 2010 gas chromatograph coupled to a mass spectrometer (GC-MS - Gas Chromatography-Mass Spectrometry), at a concentration of 1.000 ppm, and the solution of n-alkane standards (C8-C30). For the analysis, a ZB-5 column (53 m x 0.25 mm x 0.25 µm) and helium of 99.99% purity was used as a carrier gas. The programmed conditions were: temperature in the injector and interface set to 300ºC, temperature of the ion source set to 250ºC, gas flow in the column of 1.0 mL min^{-1}, split injection mode with a 1:20 ratio, and a scan interval of 40 to 450 m/z. The initial oven temperature was 100ºC for 1 min, following a heating ramp up of 3ºC min^{-1} until reaching a temperature of 300°C, requiring 67.67 minutes per chromatographic run.

The identification of the constituents of the sample was carried through the analysis of the retention indices (Cândido et al., 2021) and description of mass spectra

(Adams, 2007) and the mass spectra of the Wiley library (version 229).

2.3 Animals

The study was approved by the Ethics Committee on the Use of Animals of Tiradentes University under the number 010418. Thirty adult Wistar rats (*Rattus norvegicus albinus*) were used, each weighing between 200-300 grams, from the Animal Farm of the Tiradentes University. The animals were housed in appropriate polypropylene cages with metal grids in groups of three. The local temperature was maintained at 21°C, varying +/- 2°C, with relative humidity of 30% to 70%. The artificial lighting took place with a light and dark cycle (12/12 h), and all animals had free access to food (balanced feed *ad libitum*) and water fountain.

A total of 30 animals were used in the present study with 6 in each subgroup (n = 6 per subgroup). For the current experiment, the animals were randomly distributed as described below:

VOAz Group (n = 18): Induction of fibrosis in gastrocnemius muscle and the dermal application of VOAz using concentrations of 0.0115 µg/g, 0.009 µg/g, and 0.0065 µg/g.

Lesioned Group (n = 06): Induction of fibrosis in muscle without treatment.

Healthy Group (n = 06): healthy muscle without inducing fibrosis in muscle.

The doses were calculated based on a dilution series from the lowest concentration used by Santos et al. (2011), who demonstrated VOAz modulation in L-type calcium channels in heart muscles. Based on the differences of the muscles, the doses used in the present study were adjusted to be equivalent to 46, 36 and 26% of the doses used by Santos et al. (2011), and they ranged in logs from 25 x 10^{-4} to each other. The final volume used in each animal was calculated with a simple rule of three according to the animal's body weight and the concentration for its subgroup.

2.4 Induction of muscular fibrosis and VOAz treatment

For fibrosis induction, each rat was anesthetized with 10% ketamine hydrochloride (95 mg/kg) and 2% xylazine hydrochloride (12 mg/kg) with intraperitoneal application (Cerqueira et al., 2015). After sedation, the animal's left ankle was immobilized in a position of complete plantar flexion using a modified orthosis, based on Carvalho et al. (2013). The animals were monitored daily in order to avoid slack in the orthosis and excessive compression of the paw, causing poor blood circulation. The animals remained with the left paw immobilized in a shortened position of the gastrocnemius muscle for 15 days. After this period of immobilization, the orthoses were removed and a daily treatment with topical application was performed, where the pipette with the respective dose with the oil was placed directly on the animal's calf and spread with the researcher's index finger. This application was carried out for 30 days, according to Cândido and Xavier-Filho (2012).

2.5 Sample preparation and histomorphological analysis

After 30 days of treatment, the animals were euthanized, and biological material was removed for analysis. 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 and Masson's Trichrome 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 and Barton, 2014).

The analysis of the collagen neoformation pattern was performed using a Euromex iScope light microscope (IS.1053-PLPOLi), and a 4X wide field eyepiece was used to photograph the slides. The images were obtained by an 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 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 descriptive analysis of the pattern of collagen formation was analyzed by the birefringence variable, where the greenish or yellow-greenish colors identified type III collagen as being immature and the orange or reddish colors identify mature type I collagen. For Masson's Trichrome staining, the amount of fibrosis was assessed based on the amount of collagen deposition (represented by blue color throughout the perimysium) in the muscle. The Image J and Axion Vision LE64 software was used to analyze and quantify the collagen with the two stains.

2.6. Analysis of CAV-1 gene expression

The extraction of the total RNA from the muscle was performed using the ReliaPrep RNA Miniprep Systems kit (Promega), following the manufacturer's guidelines. The reverse transcription (RT) reaction was performed using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific), following the manufacturer's guidelines. For the RT reaction, the following conditions were used in a thermocycler: 25ºC for 10 minutes, 37ºC for 120 minutes and 85ºC for 5 minutes, repeated for 40 cycles.

After the RT reaction, CAV-1 gene expression was performed through quantitative reverse transcription polymerase chain reaction (RT-qPCR) using the Power SYBR Green Master Mix kit (ThermoFisher Scientific), following the manufacturer's guidelines. Caveolin-1 F (5'-GACCCCAAGCATCTCAACGA-3') and Caveolin-1 R (5'-GCCATAGGGATGCCGAAGA-3') primers were used (Xiong et al., 2016). β-MG gene expression provided a reference and was measured using F (5'-TGTCTCAGTTCCACCCACCT-3') and R (5'-GGGCTCCTTCAGAGTGACG-3') primers (Krasteva et al., 2006). For these reactions, 48-well plates were used. In each well, 10 µL of the mix, 1.5 µL of the primers (for the β-MG and caveolin-1 genes), 2 µL of the cDNA (of the group samples), and 5 µL of nuclease-free H2O were mixed.

The RT-qPCR conditions were as follows: 95°C for 10 min, then 95°C for 15 s, 60°C for 1 minute, repeated for 40 cycles. The melting curve was generated at the end of each reaction to verify the formation of a single peak and to

exclude the possibility of non-specific product formation. CAV-1 gene expression was performed in triplicate. For the calculation of the ratio, the average of the CT values for the target and reference genes of all groups were determined. The delta CT method (ΔCT) was used for normalization, using the difference between target and reference genes in all samples. For the calculation of the delta-delta CT (ΔΔCT), the mean of the healthy group was used for the calculation, using the difference between the samples of the groups and this average value. For the analysis of CAV-1 relative gene expression, the $2^{-\Delta\Delta CT}$ method was used (Livak and Schmittgen, 2001).

2.7. Statistical analysis

GraphPad Prism 6.01 was used for statistical analysis. All data were presented as mean ± standard deviation. Test of Shapiro-Wilk it was used to analyze the normality of the variables studied, analyses of multiple comparisons between groups were performed using the simple analysis of variance by ANOVA ONE-WAY followed by the Tukey post-test or Dunnett post-test. Differences with $p < 0.05$ and $p < 0.001$ were considered statistically significant and highly significant, respectively.

3. Results

3.1. Chemical composition of the volatile oil of Alpinia zerumbet

Through the chromatographic analysis of the VOAz, it was possible to identify 24 compounds (Table 1), totaling 96% of the total analytes detected in the sample. Of this percentage, 77% corresponds to three major components (Figure 1), being p-cymene (31%), 1,8-cineole (23.3%) and terpinen-4-ol (22.7%).

3.2. Effect of VOAz on muscle perimysium collagen

The effects of VOAz on the collagen of the gastrocnemius muscle perimysium were observed and compared between the groups. Histomorphological changes in the collagen were analyzed. When analyzing the collagen perimeter, it

Table 1. Compounds identified in the essential oil of *Alpinia zerumbet*.

Compounds[a]	RI[b]	LRI[c]	Retention time (min)	Area %
α-thujeno	929	924	10.26	2.62
α-pinene	937	932	10.57	1.66
Camphene	953	946	11.16	0.24
α-sabinene	977	969	13.05	2.01
β-pinene	982	974	12.25	1.72
Myrcene	991	988	12.60	0.36
α-phellandrene	1009	1002	12.30	0.15
α -terpinene	1020	1014	13.80	0.65
p-cymene	1028	1022	14.13	31.02
α-limonene	1032	1024	14.33	2.55
1,8-cineole	1035	1026	14.47	23.34
γ-terpinene	1062	1054	15.62	3.77
α-terpinolene	1092	1086	16.97	0.65
Linalool	1101	1095	17.33	0.56
Terpinen-4-ol	1183	1174	21.11	22.66
p-cimene-8-ol	1294	1289	21.36	0.29
α-terpineol	1195	1186	21.67	1.15
Mirtenol	1203	1194	22.01	0.08
Trans-piperitol	1212	1207	22.43	0.08
Cuminal	1246	1238	23.97	0.02
Thymol	1286	1289	25.80	0.03
Isopulegol acetate	1291	1283	26.03	0.15
p-cimene-7-ol	1294	1289	26.18	0.07
Carvacrol	1303	1298	26.55	0.20
Total				96.00

[a]Elution order on ZB-5 column; [b]RI: Retention indices on ZB-5 column; [c]LRI: Literature retention indices.

was observed that in the lesioned group (7.80 ± 1.92) it was significantly higher than the healthy group (5.08 ± 2.55; $p < 0.01$), and when compared to the treatment groups, 0.009 µg/g (3.83 ± 1.77), 0.0065 µg/g (1.44 ± 0.69), and 0.0115 µg/g (2.92 ± 1.12) it also showed a highly significant increase ($p < 0.001$) as shown in Figure 2a.

When analyzing collagen width, the lesioned group (0.40 ± 0.15) was significantly wider in relation to the healthy group (0.24 ± 0.08). When compared to the treatment groups, 0.0115 µg/g (0.19 ± 0.05), 0.009 µg/g (0.22 ± 0.06), and 0.0065 µg/g (0.15 ± 0.05) the width showed also changes highly significant ($p < 0.001$) as shown in Figure 2b.

The intensity of the total collagen was analyzed to determine the integrated optical density, and it was observed that the lesioned group (45.85 ± 64.00) presented a higher intensity relative to the healthy groups (8.51 ± 7.22) and the treated groups with 0.0115 µg/g (7.74 ± 5.20) and 0.009 µg/g (8.07 ± 3.89; $p < 0.05$). However, the treated group with 0.0065 µg/g (14.16 ± 9.98) did not show a significant difference. It is possible that the lack of significance observed with this concentration could be

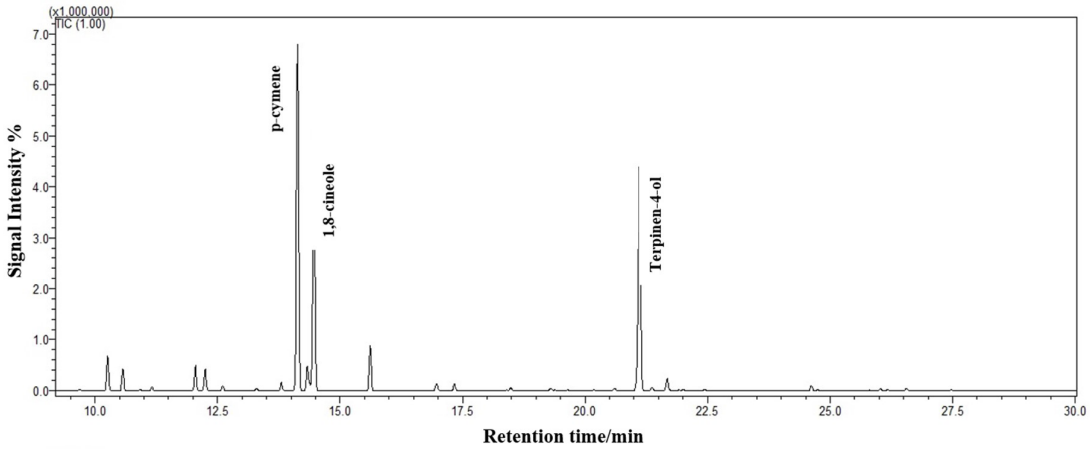

Figure 1. Chromatogram of the analysis of the volatile oil of *Alpinia zerumbet* using gas chromatograph coupled to a mass spectrometer (GC-MS - Gas Chromatography-Mass Spectrometry).

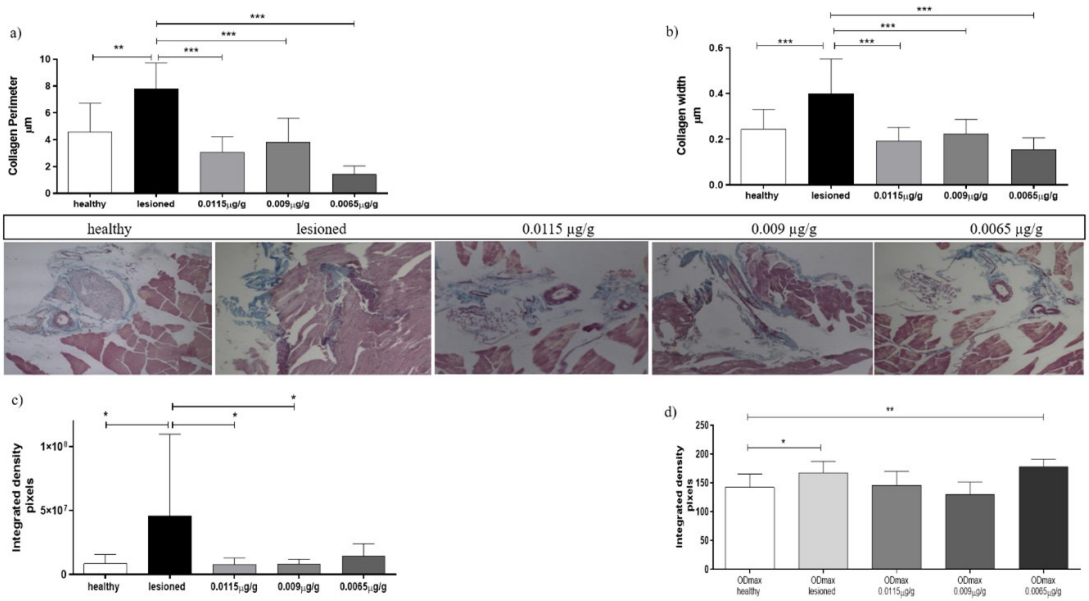

Figure 2. Characterization of collagen content in rat muscles after 30 days of treatment with the VOAz. Representative examples of Masson's Trichome staining inside the gastrocnemius muscles, where a) perimeter and b) width were analyzed using Axio Vision LE64, c) integrated optical density and d) maximum optical density using Image J. Statistical analysis was performed by the ANOVA One-Way Dunnett post-test, being *p<0.05; **p<0.01; ***p<0.001.

due to experimental variability as suggested by the error bar in the lesioned group, as can be seen in Figure 2c.

In the analysis of the maximum optical density (ODmax) in total collagen, it was observed that the lesioned group (167.8 ± 19.52) presented a higher density when compared to the healthy group (142.1 ± 22.98; $p < 0.05$). The treatment group with 0.0065 µg/g (178.1 ± 13.13) showed a higher density compared to the healthy group as well ($p < 0.01$), as shown in Figure 2d.

In the analysis of the predominance of collagen types I and III in the perimysium of the gastrocnemius muscles, it was shown that the lesioned group (89.06 ± 3.07) presented a higher percentage of collagen area, in pixels, for type I collagen relative to the other groups, healthy (12.46 ± 11.05), 0.0115 µg/g (10.15 ± 8.97), 0.009 µg/g (84.68 ± 41.05) and 0.0065 µg/g (18.25 ± 11.55; $p<0.001$). For type III collagen, it was observed that the lesioned group (10.59 ± 6.59) had a lower percentage of collagen compared to the healthy groups (58.21 ± 7.53), 0.0115 µg/g (77.08 ± 6.40), 0.009 µg/g (92.03 ± 2.65) and 0.0065 µg/g (90.00 ± 7.04) ($p <0.001$), as shown in Figure 3a.

To determine the integrated density of the two types of collagen, the intensity in each group was analyzed. The results showed that type III collagen was significantly more intense in the healthy group (1.180e+10 ± 7.393e+09) compared to the treated groups 0.0115 µg/g (1.386e+07 ± 5.845e+06), 0.009 µg/g (1.038e+10 ± 5.704e+09) and 0.0065 µg/g (8.094e+06 ± 5.164e+06; $p<0.001$). When comparing the healthy group to the lesioned group (3.505e+009 ± 1.924e+009), the healthy group showed a higher but not statistically significant intensity.

When analyzing type I collagen, the healthy group was more intense than the lesioned group and the 0.0115 µg/g group ($p < 0.05$ and $p < 0.001$, respectively). The 0.009 µg/g group was significantly more intense when compared to the other treatment groups 0.0115 µg/g and 0.0065 µg/g ($p < 0.001$), which can be seen in Figure 3b. The results showed that the VOAz provided a greater predominance of a more modeled collagen (type III) in the treated groups, and the lesioned group showed a greater predominance of a denser collagen (type I) in relation to the others, associated with the structural change in the fibrosis process in the muscle.

3.3. Analysis of CAV-1 relative gene expression

RT-qPCR analysis was performed to compare the relative levels of mRNA expression of the CAV-1 gene in the five groups after 30 days of treatment with VOAz. The results showed a significant decrease in the lesioned group (0.5223 ± 0.3976; $p < 0.01$) compared to the healthy group (1.155 ± 0.5586). It was also observed that the treated group with 0.0115 µg/g (0.5338 ± 0.3378) presented a similar result to that of the lesioned group. The treatment group with 0.009 µg/g (0.3829 ± 0.3366) had significantly lower expression than the healthy group ($p<0.001$). This data is shown in Figure 4.

However, in the treatment group with 0.0065 µg/g (0.7513 ± 0.4111), even though the lowest concentration of VOAz was applied, it was observed that its effect on CAV-1 gene expression levels was close to the relative gene expression found in the healthy group (Figure 4). The healthy group was composed of animals that did not develop muscular fibrosis and did not receive treatment with the oil, serving as a control group for measuring the effectiveness of the VOAz in the expression levels of the CAV-1 gene. Statistical analysis was also performed between the injured groups and the treated ones. However, no significant differences were observed between groups.

4. Discussion

The results of the present study showed a beneficial effect of the VOAz in the context of muscular fibrosis, both in the configuration and improvement in the quality of collagen. The treatment with the VOAz provided a more modeled collagen (type III), and the untreated group presented a denser collagen (type I), which can occur during

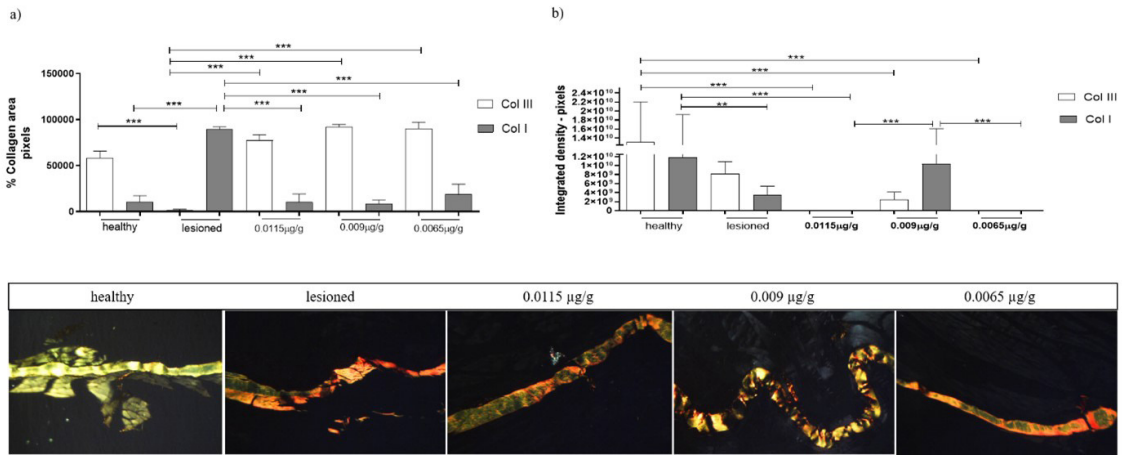

Figure 3. Characterization of collagen content in rat muscles after 30 days of treatment with the VOAz. Representative examples of Picrosirius staining inside the gastrocnemius muscles. a) percentage in collagen area in pixels, and b) integrated density in pixels were analyzed using Image J. The statistical analysis was performed by the ANOVA One-Way Tukey post-test, being **p<0.01; ***p<0.001.

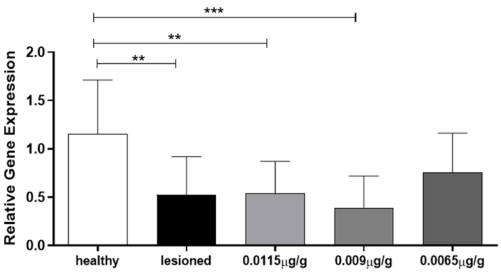

Figure 4. Relative expression of mRNA levels of the caveolin-1 gene in the Healthy, Injury and three treatment groups. Expression is reduced in groups in relation to the Healthy group. Expression levels were analyzed after 30 days of treatment with the VOAz and evaluated by RT-qPCR. Data are presented using the 2-ΔΔCT method in a column graph. The statistical analysis was performed by the ANOVA One-Way Tukey post-test, being **p<0.01; ***p<0.001.

the fibrosis process. In addition, the results showed that the lowest concentration of the VOAz may modulate the expression levels of CAV-1 gene by inhibiting the decrease of its expression, reaching values approximately similar to those of healthy tissues. Although further studies are needed, this study showed relevant data that suggests the possibility of using the VOAz in muscular fibrosis treatment.

The present study identified 1,8-cineole and terpene-4-ol as two of the major compounds of VOAz, as was observed by Barcelos et al. (2010), Cerqueira et al. (2015) and Santos et al. (2011). These compounds are the main monoterpenes found in *Alpinia zerumbet* (Barcelos et al., 2010; Cardoso et al., 2018). 1,8-cineole and terpene-4-ol are substances that have high dermal absorption (Sapra et al., 2008) and have been used to improve the transdermal absorption of drugs via interaction with lipids and keratin increasing oil solubility (Cal et al., 2006; Sapra et al., 2008).

Galdo et al. (2008), Smith and Barton (2014) and Honda et al. (2018), showed that collagen undergoes morphometric changes in fibrotic muscles. However, to the best of our knowledge, this is the first study that analyzed the effect of the VOAz on the collagen of fibrotic muscles, demonstrating the effectiveness of the oil in aiding the formation of collagen and in the regulation of its width, perimeter, and intensity. Furthermore, this study demonstrated that the VOAz treatment can improve collagen quality, making it more modulated.

Analyses involving the use of the VOAz in spastic skeletal muscles resulting from spinal cord injury in an animal model showed that type I collagen was found in a greater proportion in the group with the lesion and that type III collagen was more abundant in the lesioned groups that received treatment with the oil (Cerqueira et al., 2015). In the present study, the same type of oil was used in the fibrotic muscles, showing similar results regarding the predominance of collagen types I and III. In a previous study involving the use of the VOAz in the treatment of partial damage to the Achilles tendon in rats, it was demonstrated that the oil acted to aid tissue repair, stimulating the increase in the migration of fibroblasts after 14 days. After 30 days of treatment, the number of these cells decreased, providing the formation of a modeled tissue with better organization and orientation of the collagen fibers (Santos-Junior et al., 2017). The present study analyzed the effect of the VOAz in different tissues, observing similar results regarding the organization of its fibers. It is already known that the collagen content does not alter the passive mechanical properties of fibrotic muscle, however, during fibrosis, the collagen undergoes changes in terms of organization (Smith and Barton, 2014) which may contribute to the stiffness of the muscle tissue.

This study analyzed the expression of the CAV-1 gene in fibrotic muscles of rats submitted to treatment with the VOAz. An *in vitro* study observed that the use of the VOAz inhibited the extension of EndMT induced by TGF-β1 (Zhang et al., 2020). A negative involvement of the TGF-β1 has been reported in L-type calcium channels in cardiac muscles and in fibrotic cardiac pathology (Lei et al., 2011). Santos et al. (2011) observed that the VOAz was able to promote a cardio-depressor effect in the hearts of rats by modulating L-type calcium channels. It was also demonstrated that p-cymene had an antinociceptive effect by direct inhibition of calcium channels, including L-type, reinforcing the involvement of the VOAz in the modulation of these channels, as p-cymene is the main component in VOAz (Santos et al., 2019). These same L-type calcium channels were elucidated to increase the expression of the caveolin-1 gene (Yang et al., 2012; Yi-Chun and Anant, 2015). It is also known that during fibrosis there is an alteration of the collagen (Smith and Barton, 2014) and an increase in TGF-β1 (Zhang et al., 2011; Galdo et al., 2008), which in turn shows reduced caveolin-1 gene expression.

In this context, the results of this study showed that the lowest concentration of the VOAz presented a possible tendency to increase the expression levels of CAV-1 gene, approximately similar to the healthy group, which suggests the possibility of the VOAz to modulate the gene expression by inhibiting its decrease. Nevertheless, this possible inhibition was not observed in the 0.009 µg/g and 0.0115 µg/g treated groups. A possible explanation is that the histomorphological analysis showed a denser collagen formation (associated with a greater development of fibrosis) in the muscles of animals treated with 0.009 µg/g and 0.0115 µg/g concentrations of VOAz when comparing to the 0.0065 µg/g group. Therefore, the application of the VOAz in the time frame used, even at the higher concentrations, may not have been enough to inhibit the decrease in the expression levels.

The underlying molecular mechanisms have not yet been elucidated and further studies are needed to address the long-term effectiveness of administering the VOAz and regulating the caveolin-1 gene expression, as this study was conducted for only 30 days. Several mechanisms for the mode of action of caveolin-1 in fibrotic pathologies have already been proven, therefore, the intervention and regulation of caveolin-1 and its associated mechanisms may become a potential target for the treatment of muscular fibrosis.

The three concentrations of the VOAz treatment studied were effective in regulating the quality of collagen, and the lowest concentration suggested that the expression levels of caveolin-1 gene may be modulated by inhibiting the

decrease of its expression keeping the levels approximately similar to those in healthy tissues. Thus, although more studies are still needed, this study showed that the VOAz has the potential to be a non-invasive and low-cost alternative to aid in the treatment of muscular fibrosis.

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