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Anti-tryptic activity in seed and food product of chia (*Salvia hispanica L.*)

Atividade antitríptica em semente e produto alimentício de chia (*Salvia hispanica L.*)

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Abstract

Objective: This study has aimed to detect anti-tryptic activity in commercial chia seeds and flour and to isolate a trypsin inhibitor present in the seeds. *Methodology*: Protein extraction, ammonium sulfate fractionation, affinity chromatography, trypsin inhibition assay, protein quantification and sodium dodecyl sulfate polyacrylamide gel electrophoresis were performed. *Results and discussion*: From the retained protein peak in the affinity column, 51% and 64% of anti-tryptic activity for chia flour and seeds were respectively found. Large amounts of soluble proteins were not observed in the retained protein peaks of both chia seeds and flour when compared to the crude extract and protein fractions. The trypsin inhibitor in the seeds was isolated, with an estimated molecular mass of approximately 14.4 kDa. *Conclusions*: In view of the unprecedented results regarding the detection of anti-tryptic activity in commercial chia seeds and flour and isolation of the inhibitor in the seeds, further research is needed on this new molecule, regarding its bioactive properties and safe consumption.

Keywords: Flour. Protein. Enzyme Inhibitors. Trypsin.

Resumo

Objetivo: Este estudo objetiva detectar a atividade antitríptica em semente e farinha comerciais de chia e isolar um inibidor de tripsina presente na semente. *Metodologia:* Foram realizados extração de proteínas, fracionamento com sulfato de amônio, cromatografia de afinidade, ensaio de inibição sobre tripsina,

quantificação de proteínas e eletroforese em gel de poliacrilamida desnaturante. *Resultados e discussão:* A partir desses experimentos, detectou-se a atividade antitríptica no pico proteico retido na coluna de afinidade (RT), da farinha e semente, apresentando 51% e 64%, respectivamente. Porém, não foram verificadas grandes quantidades de proteínas solúveis no RT, tanto da semente quanto da farinha, quando comparadas ao extrato bruto e frações proteicas. O inibidor de tripsina de semente foi isolado, indicando massa molecular estimada em, aproximadamente, 14,4 kDa. *Conclusões:* Diante dos resultados relativos à detecção da atividade antitrípica em semente e farinha comercial de chia, e isolamento do inibidor na semente, faz-se necessário o aprofundamento nas pesquisas acerca dessa molécula quanto as suas propriedades bioativas e segurança do consumo.

Palavras-chaves: Farinha. Sementes. Inibidores de Proteases. Tripsina.

Introduction

In the search for functional foods that produce health benefits, the use of natural foods recognized as medicinal plants for disease prevention has been emphasized. Among these foods, *Salvia hispanica* L. seeds or, as they are more commonly known, chia, stand out.[1] The *Salvia hispanica* L. species belongs to the *Lamiaceae* family, *Nepetoideae*[2] subfamily. This family includes several medicinal plants used in popular medicine as raw material in manipulation of pharmaceuticals, herbal products and others, in solid, liquid and semi-solid forms, such as: powders, essential oils and creams, respectively.[3]

Cultivation of chia, therefore, has been of great interest for Brazil[4] and is gaining prominence in the Brazilian population as a functional food, with several attributes, such as hypocholesterolemic, satietyogenic and antioxidant effects, [5-7] besides the presence of omega-3 fatty acids, proteins of high biological value and of dietary fibers, mainly insoluble ones.[7,8] Chia also stands out as a source of important phenolic compounds.[9] Thus, it is widely consumed for its various health benefits, such as in prevention and control of cardiovascular problems, diabetes and obesity.[10,11]

Chia is considered as some food that is exempt from regulation in most countries due to the absence of health risks and historical consumption that it has. However, in Brazil, according to Brazilian government National Health Surveillance Agency (ANVISA, in the Portuguese abbreviation), because of therapeutic allegations, chia has been considered a basic functional food, although it does not have regulation yet.[12]

Currently, herbal and health food industries market chia as a dietary supplement and incorporate it into various food preparations.[1,13] Its seeds can be consumed in their fresh or ground forms, such as in flours, in addition to other applications, thus showing its economic and commercial importance.[14,15]

Due to their nutritional properties and health claims, such seeds stand out for the food industry, especially in formulation of healthy foods, with commercial flour being one of the promising products for use in fortified foods, according to a study by Pizarro et al.[16] Thus, food nutritional quality can be improved and good sensorial performance can be guaranteed.[17] By virtue of the benefits provided by chia, its consumption has been greatly increasing in the world, making these seeds a product in expansion.

However, among the factors interfering in an appropriate diet that can influence individuals' nutritional status are anti-nutritional ones, present in natural foods, mainly in the seeds, that can cause adverse physiological effects, such as toxicity and reduction of growth in experimental models, or decrease bioavailability of nutrients such as protease inhibitors, lectins, phenolic compounds, among others.[18] However, regarding enzymatic inhibitors, therapeutic potential has been observed in their heterologous use for treatment of some disease conditions.[19-24]

Although trypsin inhibitors are naturally present in the plants, in chia seeds there is no report in the literature about such presence. In addition, it is important to investigate whether these common seed inhibitors also present themselves and remain in its products such as flour. Even after industrial procedures to which some of these products are submitted, there is evidence that the process may not interfere with these molecules activity. Many inhibitors have proven thermostable properties, such as protease inhibitors.[19,25-28]

In this sense, it is important to evaluate the presence of anti-tryptic activity and isolate the trypsin inhibitor in chia commercial seeds and flour, parameters not yet studied, taking into account these inhibitors action and their importance for nutrition.

Methodology

Chia (*Salvia hispanica* L.) seeds and flour used for the experiment, both industrialized products, were randomly acquired in the local commerce of the Brazilian city of Natal, RN. Other reagents used were of analytical grade and obtained from Sigma, St Louis, USA and Brazilian company VETEC Química Fina Ltda., Rio de Janeiro, RJ, Brazil. Experiments were carried out in the Laboratory of Chemistry as a Function of Bioactive Proteins (LQFBP, in the Portuguese abbreviation) at the Department of Biochemistry at Brazilian university *Universidade Federal do Rio Grande do Norte* (UFRN, in the Portuguese abbreviation).

Protein extraction and fractionation with ammonium sulfate followed the protocols already established in laboratory routines. Initially, seeds were ground in a refrigerated grinder (6 ºC). For proteinaceous extraction of the commercial seeds and flour analyzed, buffer Tris-HCl 0.05 mol was separately added to each sample. L^{-1}, pH 7.5 in the ratio of 1:10 (m/v). Buffered samples were kept under constant stirring at room temperature for 3 hours, then centrifuged at 10,000 *x g*, 4 ºC, for 30 minutes, and filtered to obtain the crude extract (CE).

Crude extracts fractionation was carried out with addition of ammonium sulfate using three saturation bands, 0-30%, 30-60% and 60-90% under constant stirring at room temperature. At each salt addition step, samples were left overnight in a refrigerated chamber at 4 ºC and then centrifuged at 10,000 *x g* for 30 minutes at 4 ºC. Precipitates collected were dialyzed in membrane with pore 14 kDa for 48 h at 4º C against buffer Tris-HCl 0.05 mol. L^{-1}, pH 7.5. Thus, from each food product were initially obtained fractions 1 (F1) with saturation of 0-30% and the same procedures were performed to obtain fractions 2 (F2) and 3 (F3) with saturation of 30-60% and 60-90%, respectively. Finally, fractions were resuspended in buffer Tris-HCl 0.05 mol. L^{-1}, pH 7.5.

In the isolation of the commercial seeds and flour proteins analyzed, the affinity column with resin Sepharose CNBr 4B (Sigma, St Louis, United States) trypsin derivative was used, pre-equilibrated with buffer Tris-HCl 0.05 mol. L^{-1}, pH 7.5, and about 10 mg of the F2 proteins of the commercial seeds and flour analyzed were applied. The column was washed with buffer Tris-HCl 0.05 mol. L^{-1}, pH 7.5 for the removal of non-retained material. The material of interest retained in the column was eluted with HCl 0.05 mol.L^{-1} and 5-mL aliquots were collected at a volumetric flow rate of 1.0 mL min^{-1}.

All reagents used in the technique have met the specificities required by the resin manufacturer. Protein profile was determined by wavelength absorbance readings at 280 nm measured by a spectrophotometer *(Amersham Biosciences – Ultrospec 2100 pro)*. Then the aliquots which had the highest protein content were pooled and concentrated in a rotary evaporator under vacuum, resuspended in buffer Tris-HCl 0.05 mol. L^{-1} pH 7.5 in a minimum volume sufficient to solubilize the proteins, called trypsin-retained protein isolate (RT).

Proteins of CE, F1, F2, F3 and RT were quantified by the Bradford protein assay method[29] using bovine serum albumin (BSA) as a standard and absorbance readings were performed at wavelength at 595 nm by a spectrophotometer *(Amersham Biosciences – Ultrospec 2100 pro)*. Blank tests were performed and the tests were done in triplicate.

Anti-tryptic activity was determined by the enzyme trypsin at 0.3 mg.mL^{-1}, BApNA (benzoyl-DL-arginine-p-nitroanilide) at 1.25 mmol. L^{-1} dissolved in DMSO (dimethyl sulfoxide) (1%, v/v) as a substrate[30] and 100 L of CE, F1, F2, F3 and RT.

The result was measured by the formation of monitored p-nitroanilide by reading the absorbances at wavelength of 410 nm measured by a spectrophotometer *(Amersham Biosciences – Ultrospec 2100 pro)*. Blank tests were performed and the tests were done in triplicate. Following the same methodological steps, inhibitory activity for trypsin was repeated by pre-testing the RTs of seed and flour under denaturing conditions (100 °C for 10 minutes).

Results were expressed as percentage of inhibition and in IU (inhibitory unit) mg^{-1} of protein, with 100% of the enzymatic activity representing the trypsin enzyme action without the presence of the inhibitor, having, therefore, 0% of anti-tryptic activity. The inhibitory unit represents the difference between the enzymatic activity of the enzyme and the material tested, with 1 IU equal to 0.01 nm.

Evaluation of the degree of purity and determination of the molecular mass by electrophoresis in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were carried out according to Laemmli[31] in 4% concentration gel and 12% concentration separation gel. On the concentration gel were applied 15 µg of proteins of CE, F2 and RT. Gel was stained according to Weber & Osborne.[32] The dye used in this procedure was prepared with Coomassie blue R-250 at 1%, 40% methanol, 10% acetic acid in distilled water.

Subsequently, silver nitrate electrophoresis was developed based on the method described by Blum et al.,[33] using 50% methanol, 12% acetic acid and 37% formol in distilled water for fixation, 0.01% sodium thiosulfate, 30% ethanol and 50% in distilled water for washing and 0.2% silver nitrate and 0.075% formol in distilled water for coloring. Finally, development was carried out with 6% sodium carbonate, 0.05% formol and 0.01% sodium thiosulfate in distilled water.

The marker (Amersham Biosciences/GE Healthcare Life Sciences) used had the following known molecular weights: 97.0 kDa of phosphorylase B; 66.0 kDa of albumin (BSA); 45.0 kDa of ovalbumin; 30.0 kDa of carbonic anhydrase; 20.1 kDa of trypsin inhibitor and 14.4 kDa of α-lactalbumin.

The data represent at least three independent experiments and were expressed as mean and standard deviation, unless otherwise noted. Statistical data were processed in software GraphPad prism 6.0.

Results and Discussion

Chia is an extremely important seed and a number of health benefits have already been attributed to it. And it has also presented in its composition, in this research, trypsin inhibitors. In this study, crude extract (CE) obtained from chia commercial seeds and flour presented 60% (13 UIT/mg) and 82% (93 UIT/mg) of inhibitory activity for trypsin, respectively.

The three fractions F1, F2 and F3 obtained in the concentration ranges of ammonium sulfate (0%-30%, 30%-60% and 60%-90%) were also evaluated, presenting 64% (17 UIT/mg), 85% (44 UIT/mg) and 92% (44 UIT/mg) of inhibitory activity for seed, respectively. And the flour in the three fractions F1, F2 and F3 showed 78% (13 UIT/mg), 95% (310 UIT/mg) and 51% (100 UIT/mg) of inhibitory activity, respectively (Figure 1).

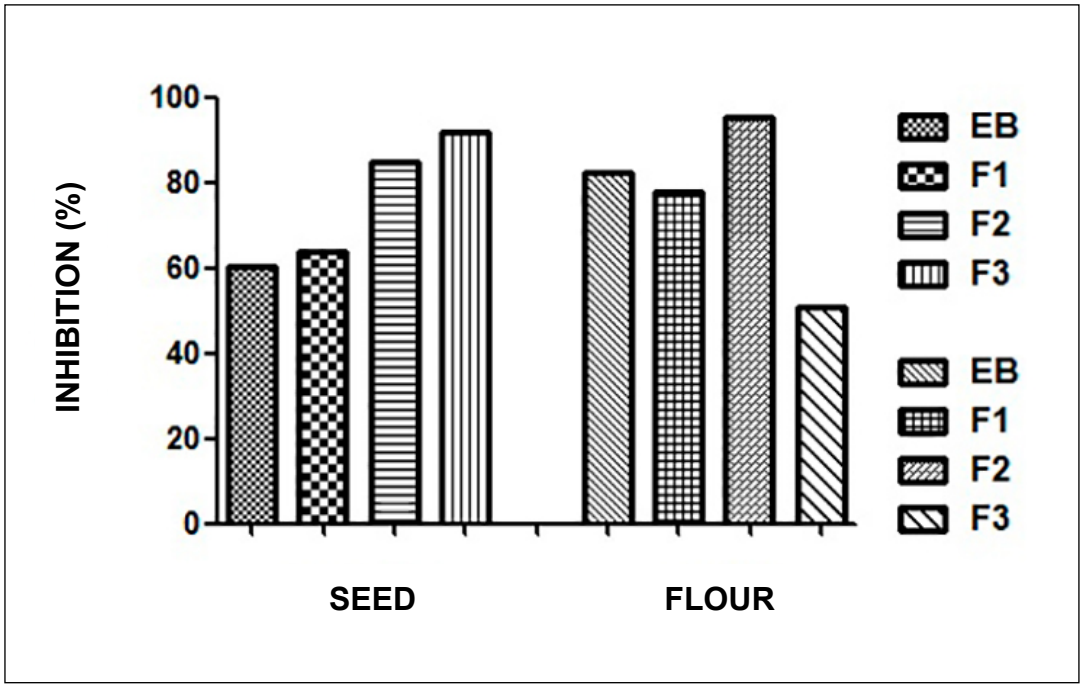

Figure 1. Inhibition percentage (%) of trypsin activity by crude extract and protein fractions F1, F2 and F3 (saturation with ammonium sulfate in 0-30%, 30-60%, 60-90%, respectively) of chia (*Salvia hispanica* L.) commercial seeds and flour. For the assay, 100 µl of the crude extract and protein fractions and BApNA 1.25 mmol were used. L^{-1} as a substrate.

Results regarding the amount of soluble proteins for the steps of inhibitor isolation in chia commercial seeds and flour revealed in the flour that CE (0.320 mg/mL), F1 (2.215 mg/mL) and F2 (0.112 mg/mL) indicated expressive amounts of soluble proteins. However, when compared with F3 (0.021 mg/mL), it can be seen that, in this fraction, no large amounts of proteins were detected, according to the method used. The seeds showed a number of proteins in CE (1.662 mg/

mL), F2 (0.701 mg/mL) and F3 (0.755 mg/mL) higher than flour and in F1 (1.390 mg/mL) lower than that perceived in flour.

For isolation of the chia commercial seeds and flour trypsin inhibitor, F2 (with 85% and 95% inhibition on trypsin, respectively) was chosen to apply to the affinity column because, in several studies, such as by Bezerra et al.[25] and Araújo et al.,[27] trypsin inhibitors were concentrated in a larger amount in this fraction. Ribeiro et al.;[21] Serguiz et al.;[22] Lima et al.;[23] Carvalho et al.[24] have also isolated trypsin inhibitors from F2.

In the isolation of this trypsin inhibitor in chia commercial seeds and flour, the protein profile was determined at 280 nm and the protein peak corresponding to the F2-retained proteins of the seed in the affinity column was found to achieve 64% inhibition for trypsin enzyme catalytic activity. On the other hand, the F2 retained protein peak of the flour had 51% inhibitory activity for trypsin. It can be seen in Figures 2A and C that in trypsin affinity chromatography RT showed a small protein peak, both in seeds and in flour, indicating a discrete amount of soluble proteins at this isolation step.

This data was confirmed by the Bradford protein assay method,[29] where RT, both of the seed and the flour, did not present expressive amount of proteins (0.001 mg/mL) when compared to the CE and the other protein fractions. Therefore, it was not possible to calculate the specific anti-tryptic activity for RT, since obtaining this result depends on quantification of proteins.

To estimate the molecular mass of the chia flour and seed trypsin inhibitor, SDS-PAGE was carried with CE, F1, F2, F3 and RT. Comparing with the molecular weights of the marker, it is possible to see in seeds that at in approximately 14.4 kDa there is a predominance of F2 proteins retained in trypsin affinity chromatography.

As for flour, it was not possible to visualize proteins in the material retained at the trypsin affinity chromatography as marked in Figures 2B and D, applying the same volume and quantity in the two gels of the seed and flour RT, differing from that observed in the RT of the chia seeds. However, even in flour, RT showed 51% inhibition for the catalytic activity of trypsin. This same anti-tryptic activity was not maintained when the chia flour RT was subjected to denaturing conditions, demonstrating a high selectivity of the trypsin affinity chromatography and, consequently, an excellent specificity of the proteins adsorbed to the column.

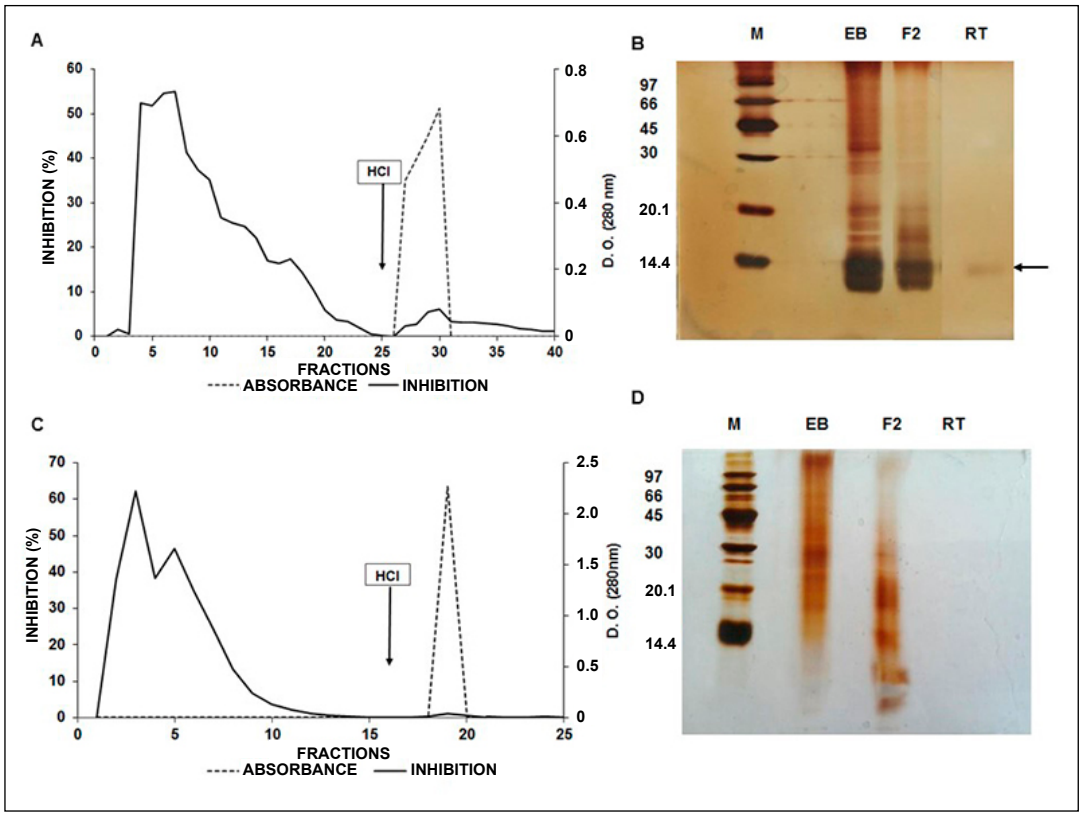

Figure 2. A and C) F2 Chromatographic profile of F2 of chia commercial seeds and flour (*Salvia hispanica* L.), respectively, in affinity chromatography trypsin-sepharose CNBr 4B. Anti-tryptic activity was verified using 100 μL of RT. B and D) SDS-PAGE at 12% stained with silver nitrate of isolation steps of the chia commercial seeds and flour trypsin inhibitor (*Salvia hispanica* L.), respectively. M: Marker; CE: Crude extract – 15 μg; F2: Saturation range with 30-60% of ammonium sulfate – 15 μg; RT: Protein isolate with anti-tryptic activity – 15 μg. The arrow in "B" refers to 14.4 kDa.

In view of the results, it was speculated that protein quantification using the Bradford reagent consisting of Coomassie blue R 250 might not have adequately estimated the amount of proteins in the isolation process, since there are substances that interfere with that method. Among these substances, it is known that polyphenols react with proteins, preventing the formation of their complex with the dye.[29,34]

However, the SDS-PAGE gel shows that this interference, specifically, could not have occurred in obtaining the flour RT, since gel staining was carried out with silver nitrate and it did not reveal proteins. However, in the crude extract and F2, also applied in the gel, the presence of protein bands is observed, thus demonstrating and confirming that the amount of these molecules in the material retained is negligible. In the RT of the seeds, proteins could be visualized, as well as in all steps of their isolation.

In amaranth seed, another pseudo-cereal, in the study by Valdes-Rodriguez et al.,[35] the trypsin inhibitor was purified and showed approximately 8 kDa. However, related to the specific activity of trypsin inhibitors in this same seed, in another study it was detected and ranged from 938 to 5,454 UIT/g of protein among different amaranth species and was considered low by the authors.[36]

These differences, among the most diverse molecular masses attributed to trypsin inhibitors, attest to what has been reported by Santos et al.[37] that protease inhibitors vary widely in their molecular mass and are classified according to their mass, there being a large variation among the families of the inhibitors and the molecular masses they present. Therefore, further studies related to its purification and characterization are necessary to better classify it among the families of inhibitors described in the literature.

It is known that the presence of enzymatic inhibitors in plant reserve organs is widespread, as in seeds, in which they can act as regulatory agents of endogenous proteases.[37] For nutrition, unlike the stigma relating them to anti-nutritional effects, these inhibitors have a heterologous and bioactive role in the protein digestion process, presenting effect in satiety control with consequent weight reduction in experimental model, considering that, from the decrease of the free trypsin activity and consequent reduction of the breakdown of proteins and peptides, there is an increase in the concentration of the satiety hormone CCK (cholecystokinin). [21,22,38-42]

Detection of anti-tryptic activity and the isolation of a trypsin inhibitor in chia seeds are unpublished findings, since previous studies that have analyzed the presence of these inhibitors were not found in the search of scientific literature in the databases. Chia proteins have been evaluated for possible biological activities[43,44] and the presence of peptides with inhibitory action on the angiotensin converting enzyme was even reported.[45] However, there is nothing about their anti-cryptic activity.

Due to the nutritional property of the seeds, chia commercial flour has been considered one of the promising products in the use in fortified foods.[16,17] It is important to emphasize that, with regard to chia commercial and industrial use, flour becomes a product of easy employment when compared to the seed, mainly for the possibility of its insertion in food products preparations.

Taking into account that chia is an important source of proteins and that the crude extract and the protein fractions presented inhibitory activity for trypsin, it is worth mentioning that its

consumption can bring benefits attributed to its nutritional composition and phenolic compounds. It is also worth mentioning that there are currently many studies on the action of enzyme inhibitors related to health effects, provided that the determination of quantitatively safe and healthy consumption is established.

Conclusion

Based on the present study, anti-tryptic activity was verified in chia commercial seeds and flour and it was possible to isolate a trypsin protein inhibitor from chia seeds and estimate its molecular mass. Since chia is of great importance due to its nutritional and functional composition and in view of the different activities reported for trypsin inhibitors, further research is needed to test safe amounts for human consumption and the influence of these inhibitors on body changes.

Contributors

De Souza APA and Nascimento LMAM have participated in collection, analysis and interpretation of data and elaboration of figures. De Lima VCO and de Carvalho FMC have participated in the statistical analysis and writing of the manuscript. Dos Santos EA has collaborated by providing the laboratory, financial resources and critical review of the manuscript. Morais AHA has participated in the study creation and design, obtaining financial resources, analysis and interpretation of data and writing the manuscript.

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