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Advanced Glycation End Products Inhibitors from *Talisia esculenta*

Inibidores de Produto Final de Glicação Avançada de Talisia esculenta

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Advanced Glycation End Products (AGEs) are formed under hyperglycemic conditions but also during the natural aging process. AGEs are associated with skin aging, loss of skin elasticity, and some chronic diseases such as diabetes and Alzheimer's, being a research topic of great relevance. Several herbal supplements have beneficial effects on glycemic control and oxidative stress, with the consequent reduction in the volume of AGEs during aging. In this scenario, the search for natural products with AGEs inhibition activity has been gaining prominence due to their importance for innovation in pharmaceuticals and cosmetics. The present study describes the investigation of natural products from *Talisia esculenta* with antiglycation activity. Ten phenolic compounds were annotated by LC-MS, and the compounds present in the leaves extracts were isolated, characterized by NMR and submitted for further *in vitro* studies. Rutin and quercitrin were isolated and presented 44% and 86% antiglycation activity at 150 µg/mL. These results open new possibilities for the use of abundant natural compounds as AGEs inhibitors, and further studies should be performed to evaluate their use and efficacy in cosmetics formulations and even in the treatment of AGEs-associated diseases.

Keywords: Advanced glycation; natural products; *Talisia esculenta;* Sapindaceae; bioeconomy

1. Introduction

The great biological and chemical diversity in Brazil is one of the reasons for the interest in scientific research on biodiversity and natural products in the country. Science transforms biodiversity into a great provider of chemical knowledge, with the potential to generate products by identifying natural compounds beneficial to human well-being, such as medicines, food supplements, and cosmetics. [1,2,3] In the discovery and development of new drugs, natural products are an important source of active compounds.[4] Natural products are the most traditional source for developing new drugs, being an important player of therapeutic agents for centuries, providing frequent models for the chemical structures of drugs.[5,6]

The global cosmetics market was valued at US$380.2 billion in 2019, and is expected to reach a market cap of US$463.5 billion by 2027.[7] An important and emerging driver of this market is the aging population, with the world population over 60 years old expected to exceed 2 billion by 2050. The desire to maintain a youthful appearance has led to a demand for products to prevent skin wrinkles, dryness and blemishes. There are only a few innovative cosmetic products with anti-glycation activity launched by renowned companies like Chanel and Olay[8]. With the concept of bioeconomy that uses products of sustainable natural origin, new pharmaceutical bioproducts, food supplements, cosmetics, among others, drive scientific research in the area of natural products, in which the potential in Brazil is enormous, due to its extensive biodiversity.

Advanced Glycation End Products (AGEs) are associated with aging and loss of skin elasticity, in addition to some chronic diseases, such as diabetes and Alzheimer's. The glycation process is characterized by the formation of AGEs, which are formed under hyperglycemic conditions and during the natural aging process. The pathological effects of AGEs are related to changes in the chemical and functional properties of several biological structures. Through the generation of free radicals, the formation of cross-links with proteins, or interactions with cell receptors, AGEs can promote oxidative stress, morphofunctional changes, and increased expression of inflammatory mediators.[9]

Natural products, such as caffeic acid[10], curcumin[11], rutin[12], and emodin[13], have been reported with antiglycation activity. They are all phenolic compounds and therefore indicate that this class of compounds may be related to antiglycation activity. Phenolic derivatives are able to

neutralize free radicals, inhibiting oxidative stress, as they readily react with carbonyls. Some phenolic compounds can also break the cross-links formed during the glycation process, being a prospecting model for the search for natural antiglycation agents.[14]

Several plants from Brazilian biodiversity biosynthesize bioactive phenolic derivatives, such as flavonoids, anthraquinones, benzophenones, among others, which have antioxidant properties, In this context, it makes them a promising source for the search for compounds with potential antiglycation activity. *Talisia esculenta* is native to the Amazon region, although it is also cultivated in the North and Northeast regions of Brazil.[15] Its fruits are an important part of the extractive chain in the Amazon and Northeast and its seeds are responsible for the propagation of the species. The bioactivity-guided fractionation of the branches, tree bark, leaves, fruit peel and seeds of *T. esculenta*, a plant species understudied and known for producing natural compounds such as acids quinic, gallic and chlorogenic, catechin, epicatechin, rutin and quercetin[16,17] and considered promising to find compounds with the desired activity.

Therefore, the main goal of this study was to perform a bioactivity-guidedstudy of *Talisia esculenta* (Sapindaceae) extracts, popularly known as 'pitombeira'. Several compounds were annotated by LC-MS, and a deeper investigation was performed in order to identify the natural products capable of inhibiting the glycation process.

2. Experimental

2.1. Plant material and extraction protocol

The collection of branches, tree bark, and leaves of *T. esculenta* was carried out in Fortaleza, Ceará, Brazil, by Dr. Guilherme Julião Zocolo in october, 2018, SISGEN AA60DD3. In the extraction process, approximately 10 g of sample and 5 g of diatomaceous earth were added to a beaker for each extraction performed. Then, the homogenized mixture was transferred to the 66 mL extraction cell of the ASE (Accelerated Solvent Extraction), and the extraction procedure began. The operational conditions established were: 3 cycles of 5 min each, temperature of rinse of 200 s at 80%, and purge time of 200s. The solvent used was 99.9% pure ethanol. Extracts from leaves, branches and tree bark were obtained in 9.0%, 10.1%, and 6.9% yields, respectively. The fruit peel and seeds of the *T. esculenta* were purchased in João Pessoa, Paraíba, Brazil, in February 2017. Approximately 250 fruits (known as 'pitomba') were submerged in water for about an hour for cleaning. Then, fruit peel and seed were dried in a fume hood for two days and subsequently grounded in an analytical mill (Ika Labortechnink model A11 Basic). The crushed material, 450.01 g of fruit peel and 334.90 g of seed, was used for extraction by maceration for 24 h and stirring with solvents of increasing polarity: hexane (used to remove apolar compounds), ethyl acetate and methanol solvents. The supernatants were filtered and dried in a rotary evaporator. Extracts from fruit peel and seeds using ethyl acetate as solvent were obtained in 0.8 % and 0.2% yields, respectively. The extracts from methanol solvent were obtained in 1.0% and 3.2% yields for fruit peel and seed extracts, respectively.

2.2. Evaluation of antiglycation activity *in vitro*

The antiglycation activity was determined by the ability of the samples to inhibit AGEs formation. For this, an adaptation of the method described by literature[18,19] was used, in which the glycation reaction is promoted by the addition of bovine albumin protein (BSA) and methylglyoxal (MGO). The BSA solution was prepared with a concentration of 1 mg/mL in phosphate buffer and NaCl (150 mmol/L), with a concentration equal to 10 mg/mL and pH 7.4. A 1:1 ratio of DMSO (Dimethylsulfoxide) and H_2O was used to solubilize the extracts at concentrations of 150 µg/mL. The extracts and MGO (5 mmol L^{-1}) were added to the BSA solution to subsequent incubation for 72 h under agitation of 150 rpm and temperature of 37 °C. The intrinsic fluorescence of each sample was considered, as they were incubated in the presence and absence of MGO. After 72 h, the samples were introduced into a plate spectrometer at 370 nm (λexcitation) and at 440 nm (λemission) for fluorescence reading. Equation 1 was used to calculate the percentage of inhibition of AGEs, where FLCN and FLbCN are the fluorescence intensities of the negative control mixture (containing MGO and protein only) and its blank (without MGO), respectively, and FLS and FLbS are the fluorescence intensities of the extract and its blank, respectively. Aminoguanidine was used as a positive control. Equations 2 and 3 were used to determine the antiglycation activity in the tests performed.

$$\%\ \text{AGE formation inhibition} = \frac{(\text{FLCN} - \text{FLbCN}) - (\text{FLS} - \text{FLbS}) \times 100}{(\text{FLCN} - \text{FLbCN})} \quad (1)$$

$$\text{MGO (sugar)} + \text{BSA (protein)} = \text{Glycation} \quad (2)$$

$$\text{MGO (sugar)} + \text{BSA (protein)} + \text{extract (inhibitor)} = \text{Glycation with inhibition} \quad (3)$$

2.3. HPLC-MS/MS analyses

The extracts were analyzed in an HPLC Prominence (Shimadzu®, Japan) coupled to an ion trap mass amaZon SL Bruker Daltonics® (Bruker, Billerica, MA, USA), equipped with electrospray ionization (ESI) for dereplication purposes. The HPLC comprises an LC-20AD pump, DGU20-A3R online degassing unit, CTO-20A column oven, SIL-20AHT automatic injector and CBM-20 communication module. A Phenomenex® C18-Luna (250 mm × 4.6 mm, 5.0 µm) column was employed as stationary phase and the solvent system consisted of water (A) and acetonitrile (ACN, B),

both acidified with 0.1% (v/v) formic acid. The flow rate, column temperature, and injection volume were set to 1 mL/min, 30 °C, and 3 µL, respectively. The dried extracts were solubilized in ACN–H_2O (1:1, v/v) to a concentration of 1 mg/mL and further centrifuged for 5 min at 4500 g, and the supernatants were analyzed. A linear gradient of 5 to 64% B in 40 min was employed. The mass spectra were obtained in the negative ionization mode considering a mass range of 50–1200 Da, using the Auto MS(n) mode (n = 3). The mass spectrometer source parameters were set as follows: capillary voltage up to 4.5 kV, nitrogen used as the nebulizing and drying gas (50 psi, 10 L/min, 300 °C). The data were processed through Bruker Compass Data Analysis 4.3® software.

2.4. Isolation of substances by HPLC in preparative scale: extract of leaves of *T. esculenta*

The leaves extract (300 mg) was solubilized in 30 mL of MeOH:H_2O 1:1 (v/v) and submitted to filtration in an SPE C18 (*clean-up*). A Phenomenex C18-Luna column (150 x 21.2 mm, 5 µm) was used, with a mobile phase of H_2O (A) and ACN (B), both acidified with 0.1% formic acid, using a flow rate of 10 mL/min. The gradient method from 5 to 42% B in 25 minutes was employed. The major compounds were manually collected at elution times of 19.5 min (compound **6**, 7.6 mg) and 22.5 min (compound **8**, 3.2 mg) and subsequently the solvent was evaporated in a Speed vac. The chromatogram was monitored at 254 and 280 nm.

2.5. NMR analyses

The extracts and isolated substances were analyzed by Nuclear Magnetic Ressonance (NMR), with the Avance III HD 600 spectrometer (Bruker). The samples (3 mg) were solubilized in 200 µL of deuterated methanol and the tube used for analyses was 3 mm.

3. Results and Discussion

3.1. Antiglycation assay of *T. esculenta* extracts

The preparation of extracts from branches, tree bark, and leaves was performed using the ASE technique, in which the liquid solvent acts with a combination of high temperatures and pressures.[20] This methodology is highly in accordance with the green chemistry principles since, in addition to using a green solvent (ethanol), the volume of solvent used is reasonably reduced, and the extraction time is shorter compared to traditional methods, such as maceration.[21] As for the fruit peel and seed extracts, it was observed that, despite the yields being considered low, the amount of extract obtained is within the expected, considering the technique used.

An initial antiglycation assay screening was performed with extracts from leaves, branches, tree bark, fruit peel, and seed of the fruit of *T. esculenta*. Antiglycation activity was determined using fluorimetry as described in the literature,[18] due to the fluorescence displayed by AGEs. The leaves, branches, and tree bark extracts showed similar potency for glycation inhibition and considerably higher than the extracts of the peel and seed of the fruit, but not comparable to the positive control aminoguanidine (95.7%). The extract with the highest percentage of inhibition and, therefore, the most potent was obtained from *T. esculenta* branches (Figure 1).

Rutin was previously reported to decrease the production level of AGEs by 91% at 440 uM.[12] and emodin had an IC50 of 57.8 uM for the inhibitory effect of AGE formation.[13]

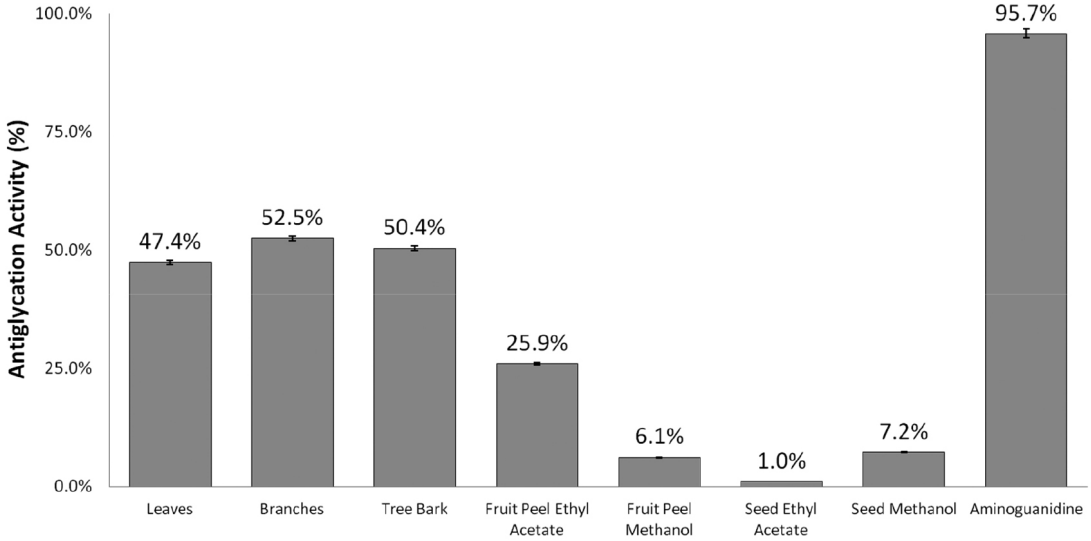

Figure 1. Results of antiglycation activity for extracts from leaves, branches, tree bark, fruit peel, and seeds of *T. esculenta* obtained in the screening assay at 150 µg/mL. Aminoguanidine was used as positive control. All experiments were performed in triplicate

3.2. HPLC-MS/MS analysis of *T. esculenta* extracts and annotation of known compounds

In order to annotate the known compounds present in *T. esculenta* extracts, they were analyzed by HPLC-MS/MS. The Base Peak Chromatograms are depicted in Figure 2, while Table 1 describes the annotated compounds. A total of ten compounds comprising the major peaks were annotated, all belonging to the flavonoid class. It is possible to observe that the chemical profiles differ for all the extracts obtained, in which the fruit peel extracts presented less polar compounds. Additionally, the methanolic seeds extract did not present intense peaks throughout the chromatogram, presenting only an intense peak near the dead volume (~3 min) relative to sugar portions. The putative annotation of the major secondary metabolites present in the extracts obtained was performed based on each compound's fragmentation patterns and comparison with previous reports.

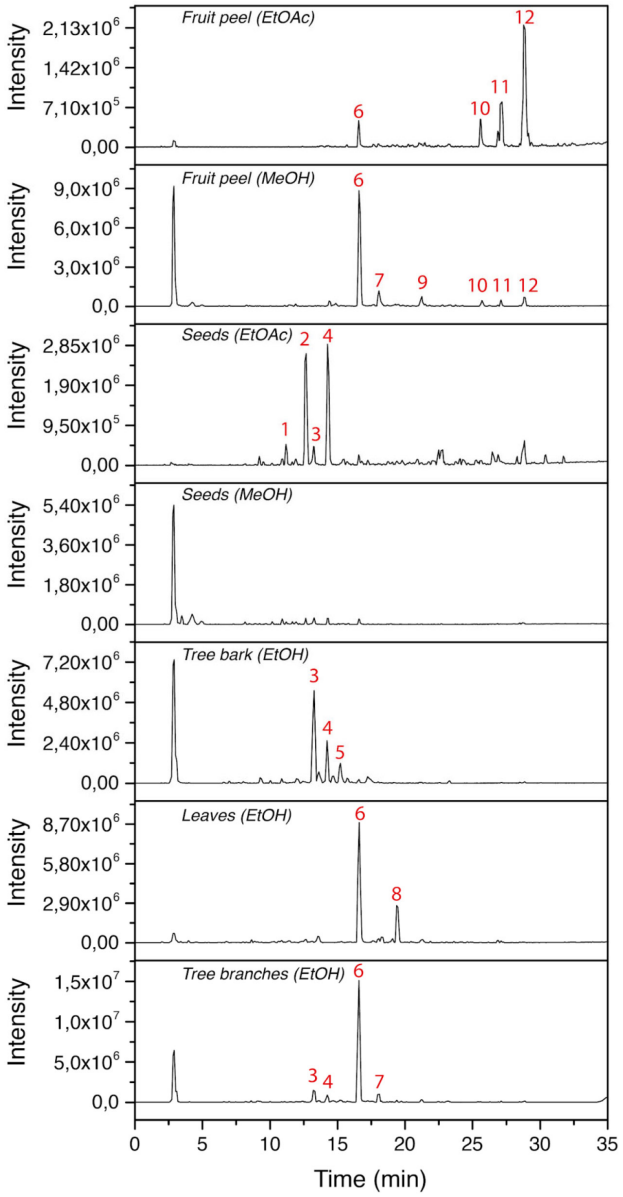

Figure 2. Base Peak Chromatograms (BPCs) obtained from the *T. esculenta* crude extracts

Compounds **2** and **4** (*m/z* 289 $[M–H]^-$) were annotated as catechin and epicatechin, which presented fragments relative to the loss of CO_2 (*m/z* 245) and the cleavage of the A-ring of flavan-3-ol (*m/z* 203and *m/z* 205).[22] These compounds were detected in higher amounts in the EtOAc extract seeds, in which compound **2** was exclusively detected in this sample, while compound **4** was also detected in the tree bark and branches ethanolic extracts. Similarly, compound **1** (*m/z* 305 $[M–H]^-$) was annotated as gallocatechin, also presenting the loss of CO_2 at *m/z* 261 and A-ring cleavage at *m/z* 219, being detected only in the ethyl acetate extract of *T. esculenta* seeds. Compounds **3** and **5** (*m/z* 577 $[M – H]^-$) were annotated as proanthocyanidin dimers. The fragment ion resulting from a Retro Diels-Alder (RDA) reaction was observed at *m/z* 425, and the cleavage of the interflavanic bond to obtain a (epi)catechin monomer resulted in the fragment ion at *m/z* 289 and its decarboxylated product ion at *m/z* 245.[18] These compounds were annotated in the seeds EtOAc extract, and in the tree bark and branches ethanolic extracts.

Several *O*-glycosylated flavonoids were also annotated, in which the neutral loss of 162 Da and 146 Da were relative to the loss of a hexose and a deoxyhexose portion, respectively.[18,24] Compound **6** (*m/z* 609 $[M – H]^-$) was putatively annotated as quercetin-*O*-hexoside-deoxyhexoside (rutin), presenting the main fragment ion at *m/z* 301 representing the loss of its entire glycosidic portion (146 Da + 162 Da). The MS^3 fragments also showed the fragments relative to the RDA reaction (*m/z* 151).[18] This compound was detected in the leaves and branches of ethanolic extracts and in the fruit peel extracts (EtOAc and MeOH). Compound **8** (*m/z* 447 $[M – H]^-$) also presented a fragment ion relative at *m/z* 301 (neutral loss of 146 Da) consistent with the aglycone quercetin, and the MS^3 fragments also showed the characteristic RDA fragments.[18] Therefore, compound **8** was annotated as quercetin-*O*-deoxyhexoside, being exclusively observed in the leaves extract.

Luteolin-*O*-hexoside-deoxyhexoside was annotated as compound **7** (*m/z* 593 $[M – H]^-$) and presented the aglycone fragment ion at *m/z* 285, consistent with luteolin. This compound was detected in the tree branches and in the fruit peel methanolic extract. Another compound exclusively detected in the fruit peel MeOH extract, **9** (*m/z* 623 $[M – H]^-$), presented an intense fragment ion at *m/z* 315 (total loss of 308 Da), which is consistent with a methoxylated quercetin.[25] Therefore, this compound was putatively annotated as methoxy-quercetin-O-hexoside-deoxyhexoside. Finally, compound **10** was putatively annotated as the aglycone quercetin[23], with the expected fragment ions for this compound, only detected in the fruit peel extracts (MeOH and EtOAc). Compounds **11** and **12** were not annotated.

Table 1. Compounds putatively annotated from the *T. esculenta* extracts in HPLC-MS/MS analyses: peak number, retention time (Rt), $[M–H]^-$ and MS^n fragments

#	Rt (min)	$[M–H]^-$	MS/MS fragments (% abundance)	Metabolite	Reference
1	11.2	305	MS^2 (305): 305 (100), 261 (7), 219 (15), 179 (5) MS^3 (305→219): 219 (100), 177 (7)	Gallocatechin	22
2	12.6	289	MS^2 (289): 289 (100), 245 (7) MS^3 (289→245): 205 (5), 203 (10)	(Epi)catechin	22
3	13.2	577	MS^2 (577): 451 (21), 425 (100), 407 (70), 289 (24), 245 (5) MS^3 (577→425): 407 (100), 381 (5), 339 (5), 273 (10)	Proanthocyanidin dimer	18
4	14.2	289	MS^2 (289): 289 (100), 245 (3)	(Epi)catechin	22
5	15.2	577	MS^2 (577): 491 (40), 451 (13) , 425 (7), 407 (5), 289 (100), 245 (16) MS^3 (577→289): 245 (100), 205 (41), 179 (15), 137 (6)	Proanthocyanidin dimer	18
6	16.6	609	MS^2 (609): 301 (100) MS^3 (609→301): 271 (78), 255 (35), 179 (100), 151 (42)	Quercetin-*O*-hexoside-deoxyhexoside	18
7	18.1	593	MS^2 (593): 285 (100) MS^2 (593→285): 267 (33), 257 (100), 241 (24), 239 (13), 229 (29), 213 (21), 163 (12) , 151 (10)	Luteolin-*O*-hexoside-deoxyhexoside	
8	19.4	447	MS^2 (447): 301 (100) MS^3 (609→301): 271 (48), 255 (37), 179 (100), 151 (76)	Quercetin-*O*-deoxyhexoside	18
9	21.3	623	MS^2 (623): 315 (100) MS^3 (623→315): 300 (100), 287 (13), 271 (10), 256 (8), 207 (7), 193 (40), 165 (60)	Methoxy-quercetin-*O*-hexoside-deoxyhexoside	
10	25.9	301	MS^2 (447): 301 (100), 215 (6), 179 (3), 151 (8)	Quercetin	23
11	27.0	327	MS^2 (327):327 (100), 229 (5), 211 (4), 171 (2)	Not identified	–
12	28.8	329	MS^2 (329): 329 (100), 229 (8)	Not identified	–

In general, the extracts obtained from *T. esculenta* tree (bark, branches, and leaves) seemed to have simpler chemical profiles compared to the fruit peel and seeds, but since the extraction methods were different this cannot be a direct comparison. Particularly for the leaves extract, there was a selective extraction of compounds **6** and **8**, and very few compounds eluting near the dead volume. Therefore, this extract was chosen to perform the isolation of its major compounds to confirm their structure and bioactivities.

3.3. Isolation of substances by HPLC on a preparative scale: extracts from leaves of *T. esculenta*

Preliminary HPLC-UV analyses of the extracts were performed, showing that similarly to what was observed in the BPCs from the LC-MS analyses leaves and branches presented the chromatograms with fewer compounds. These extracts presented similar activities, and shared the same major peak. In addition, the leaves extract was the only one in which the presence of tannins was not observed (Supplementary Information, Figure S1-S2). Therefore, substances **6** and **8** present in the leaves extracts were isolated, being obtained both as yellow amorphous powder.

The isolated compounds were subjected to NMR analysis for structure characterization by ^1H and TOCSY 1D experiments (Supporting Information, Figures S3-S6). Compound **6** was characterized as rutin, while substance **8** was elucidated as quercitrin.[26] Both rutin and quercitrin are flavonoids derived from quercetin, containing one and two sugars in its structure, respectively. Rutin is a well-known flavonoid and is commonly used as positive controls in antioxidant assays.

3.4. Antiglycation assay of the isolated substances

The isolated compounds were subjected to the antiglycation assay to determine their bioactivity. It was possible to observe a relevant activity when the pure compounds were evaluated, especially for substance **8**, indicating that these compounds are responsible for the bioactivity observed in the leaves extract. Substance **8** (86.1%) showed greater antiglycation activity than substance **6** (44.4%). However, substance **8** was present in smaller amounts in the pitombeira leaves and, therefore, the feasibility of isolating it must be evaluated, since there must be an expansion of the isolation scale for cosmetic applications.

4. Conclusions

The performance of HPLC and LC-MS analysis of the extracts allowed an initial exploration, as well as the isolation of substances with the highest antiglycation activity present in the extract of *T. esculenta*. The antiglycation assays of the extracts and of the pure compounds were fundamental to guidethe isolation of the most active substances based on the bioactivity. The results obtained indicated that the

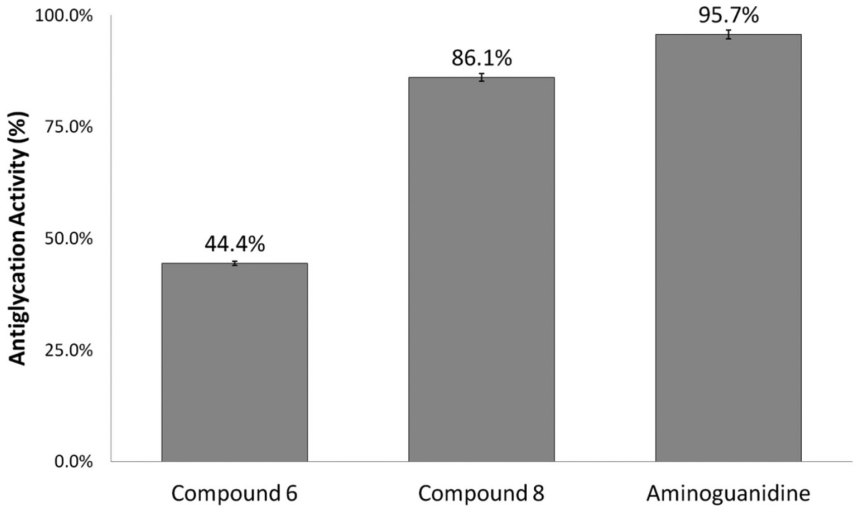

Figure 3. Results of antiglycation activity obtained for substances **6** (rutin) and **8** (quercitrin). Aminoguanidine was used as positive control. All experiments were performed in triplicate

antiglycation activity was more expressive in the extracts of the branches, leaves and bark of *T. esculenta* when compared to the activity of the fruits and seeds. In addition, the major composition of the leaves was determined as rutin, and quercitrin. The stem extract was also analyzed and the substances present in it were tentatively identified by LC-MS/MS, corresponding to mostly phenolic compounds. The present study opens up new possibilities for the use of fruit residues (peel and seed), since rutin is a commercial standard, adding value to these discarded products and contributing to the concept of bioeconomy.

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