Antioxidant activity and hypoglycemic effect assessment of the leaves from Syzygium cumini (L.) Skeels in Wistar rats

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ABSTRACT. Syzygium cumini (L.) Skeels was adapted to the climatic conditions and soil types in Brazil. Its fruits, leaves and inner bark are used in folk medicine due to their high antioxidant, anti-inflammatory, anticarcinogenic and antidiabetic activities mainly associated with the presence of phenolic compounds. It is estimated that at least 300 million people worldwide develop diabetes and approximately 11 million people are carriers of the disease in Brazil. The objective of this work was to evaluate the in vitro antioxidant activity, as well as the hypoglycemic action of hydroethanolic extract (HEE), the ethyl acetate (EAF) and hydromethanical (HMF) fractions from leaves of S. cumini (L.) Skeels in rats. All assays were carried out in three replications. Data were expressed as mean ± SD and significance was evaluated by ANOVA and Bonferroni test (p < 0.05). The results indicate a significant (p < 0.05) total phenol content (207 ± 2.3 GAE mg g⁻¹) and antioxidant activity (EC₅₀ = 9.05 ± 0.170 µg mL⁻¹) for EAF. HEE and its fractions showed no significant (p > 0.05) action to modulate glucose by the OGTT assay in nondiabetic mice compared to control. Thus the use of the plant against diabetes in individuals is not proven.

Keywords: Syzygium cumini; oxidative stress; natural product.

Introduction

Medicinal plants that are part of biodiversity, are widely used to treat and cure several human diseases since early civilizations. For many communities and ethnic groups, the popular knowledge about plants symbolizes therapy in many cases. Currently, about 80% of the population treats several diseases with folk medicine, using techniques and forms of use that have been passed down for generations. In this context, Brazil is a country of invaluable biodiversity, which, at the present time, is a source for the isolation of natural molecules used for this purpose (Firmo et al., 2011; Nunes, Castro, & Ruiz, 2014; Dias & Carvalho, 2017).

The extensive use and study of plant species is associated with the fact that they are an abundant supply of natural compounds having diverse chemical and biological properties, with a spectrum of anti-inflammatory, antioxidant, antiallergic, antidiabetic and antimicrobial activities. They are an alternative for treating several diseases due to the presence of secondary metabolites such as terpenoids, alkaloids, phenolics, tannins and flavonoids, among others (Souza et al., 2009; Chhikara et al., 2018; França et al., 2019).

The antioxidant capacity of a plant is related to its power to neutralize free radicals and prevent oxidative stress. Damage to health caused by chemical instability, and lipids and DNA oxidation can be caused by superoxide anion (●O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (●OH), which are known as reactive oxygen species, as well as reactive nitrogen species (Cheng, Wang, Wang, & Hou, 2017; Sobeh et al., 2018; Chagas et al., 2019).

Prevention and/or treatment of various diseases are associated with the action of medication in the body, with emphasis on diabetes mellitus (DM) and metabolic syndrome. Studies indicated that secondary metabolites produced by plants are associated with different actions related to symptoms and consequences of DM. Among these compounds, polysaccharides, proteins, steroids, terpenoids, alkaloids, flavonoids, glycosides, oils, vitamins, saponins, peptides and amino acids can be indicated either for direct use in
treatment or for use as raw material to discover new therapeutic agents (Dornas et al., 2009; Ayyanar, Subash-Babu, & Ignacimuthu, 2013; Hasenclever, Paranhos, Costa, Cunha, & Vieira, 2017). Substances such as saponins (calendasaponins 1A, 1B, 1C, 1D e 1F), sesquiterpenes (officinosides C and D), alkaloids (cryptolepine analogues), flavonoids (isoorientin) and phenolic compounds (4-hydroxybenzoic acid) were previously reported for their hypoglycemic effect (Ayyanar et al., 2013; Baldissera et al., 2016; Jacob & Narendhirakannan, 2019). Diabetes treatment involves the use of oral hypoglycemic drugs with or without insulin, and even with the use of medicinal plants and herbs, which are rich in active compounds that act by raising insulin release to change glucose metabolism, inhibiting hyperglycemic elements, inhibiting or stimulating the synthesis of enzymes and reducing diabetic complications (Ayyanar et al., 2013; Chagas et al., 2019; Jacob & Narendhirakannan, 2019).

Several species of plants have shown antioxidant and hypoglycemic properties. Among those are the genus Syzygium, which possess about 500 species of trees and shrubs. Approximately 400 of them are found in Brazil. Its seeds, fruits, flowers, leaves and bark can be used (Ayyanar et al., 2013; Atale et al., 2017; Chhikara et al., 2018).

Given the above, the objective of this study was to evaluate the in vitro antioxidant activity and hypoglycemic action of the hydroethanolic extract and fractions from the leaves of S. cumini (L.) Skeels in normoglycemic rats. The hypothesis of the present study is that due to the phenolic compounds present in the plant, there is an antioxidant and hypoglycemic action.

**Material and methods**

**Collection and identification of plant material**

Leaves of S. cumini (L.) Skeels were collected in September 2011 in the municipality of Estancia, state of Sergipe. After botanical identification, a sample of the specimen was recorded in the herbarium of the Federal University of Sergipe under registration 22744 (ASE).

**Preparation of hydroethanolic extract and its fractions**

The collected material was dried in an oven (Marconi, model MA-037) with air renewal and circulation at 40°C for 48h and grounded in a knife mill to obtain a powder, which was submitted to extraction with cold ethanol 90% by maceration for five days. Then, the filtrate was evaporated under reduced pressure (rota evaporator LS Logen) at 50°C to obtain the hydroethanolic extract (HEE). Part of this crude extract (109.66 g) was dissolved in 40% methanol (v/v in water) and subjected to liquid–liquid extraction to obtain fractions of ethyl acetate (EAF) and hydromethanol (HMF).

**Determination of total phenol content**

The total phenolic content (TPC) was determined according to the methodology described by Ainsworth and Gillespie (2007), with modifications. An aliquot of 500 μL of HEE, EAF and HMF solutions (1 mg mL⁻¹ in methanol) was mixed with 500 μL of the Folin–Ciocalteu reagent and 6 mL of distilled water for 1 min. Then, 2 mL of 15% Na₂CO₃ were added to the mixture, agitated for 50 sec with an additional 5 mL of distilled water, and added to the mixture after agitation. The absorbance of the samples was measured after 2 hours at 750 nm, using as blank methanol and all reagents except the extract/fraction. TPC was determined against a calibration curve (Y = 0.1993X - 0.2158; where Y is the concentration of gallic acid, X is the absorbance at 750 nm, R² = 0.9031) constructed with standard gallic acid (10 to 200 μg mL⁻¹) and expressed as GAE (gallic acid equivalents) mg per g of extract.

**Evaluation of antioxidant activity by DPPH•**

The antioxidant activity was determined by the reaction of stable radical 2,2-diphenyl-1-picrilidrazina (DPPH•) in methanol solution with control, extracts and fractions, by monitoring the decrease in consumption of the radical absorbance (Brand-Williams, Cuvelier, & Berset, 1995). Thus, 50 mL of stock solution of DPPH• in methanol at a concentration of 40 mg mL⁻¹ was prepared. HEE, EAF and HMF from S. cumini (L.) Skeels were solubilized in methanol to give a stock solution of 0.5 mg mL⁻¹. Aliquot part of these solutions were taken to obtain final concentrations of 5, 10, 15, 20, 25 and 50 mg mL⁻¹ in 5 mL of the DPPH• solutions. Absorbance of all solutions was determined at 515 nm after 1, 5, 10, 20, 30, 40, 50 and 60 min. of reaction, using a blank composed of methanol and extract/fractions and gallic acid as positive control.
control. The percentage of remaining DPPH+ was calculated using the absorbance values and data of a calibration curve at 60 min., according to the equation Brand-Williams et al. (1995):

$$\%\text{DPPH}_{REM} = [\text{DPPH}]_T / [\text{DPPH}]_0 \times 100$$

where [DPPH]T corresponds to the concentration of the DPPH radical in the medium after the reaction with the extract, and [DPPH]0 is the initial concentration of the radical DPPH.

The amount of antioxidant required at 30 mg mL⁻¹ to decrease the initial concentration of DPPH in 50% (EC50) was calculated by DPPHREM% at 60 min, in contrast with the concentrations of samples (Sousa et al., 2007). Results were expressed in g mL⁻¹ ± standard deviation. In addition, results were also expressed as inhibition percentage (IP) and antioxidant activity index (AAI) calculated according to Scherer and Godoy (2009) by the equation:

$$\text{IAA} = \text{DPPH stock (mg mL}^{-1})/\text{EC50 (mg mL}^{-1})$$

**Animals and experimental design**

This study used 32 male Wistar rats, weighing between 200 and 300 g, obtained from the Federal University of Sergipe. Animals were treated and kept in their cages at 25 ± 1°C, with controlled light-dark cycle of 12 h and free access to food and water.

The analysis of HEE and EAF antidiabetic potential was performed by the oral glucose tolerance test (OGTT) in normoglycemic rats, according to the protocol described by Villaseñor and Lamadrid (2006) and Sousa et al. (2009), with modifications. Besides HEE, EAF was chosen for this test because of its antioxidant activity. Rats were deprived of food and water for 12h before the experiments and randomly divided into 4 groups consisting of 8 animals each, as described below:

Group 1 (negative control) - Dimethyl sulfoxide (DMSO) and distilled H₂O (0.5 mL 100 g⁻¹ of body weight); Group 2 (positive control) - Glibenclamide (0.28 mg 250 g⁻¹ of animal); Group 3 (hydroethanolic extract) - 200 mg kg⁻¹ HEE; Group 4 (ethyl acetate fraction) - 200 mg kg⁻¹ EAF;

HEE and EAF (200 mg) were solubilized with 10% DMSO (400 μL) and the final volume of 4 mL was reached by adding 3600 μL of distilled water to the mixture. These solutions were prepared immediately before use and administered by gavage (p.o.) with a circular end needle, avoiding animal trauma or pain. The same dosage was used to allow a comparison of their effects. Initially, glucose content was determined in fasting blood before treatment and defined as time 0 (zero). After that, animals received their respective treatments and glucose was then administered at a dosage of 50 mL 100 g⁻¹ animal. Afterwards, blood glucose was measured at 15, 30, 60, 90 and 120 min after administration of glucose using reagent strips (ACCU-CHECK Active, Roche) coupled to a digital portable glucometer from blood samples collected from the tail vein. After the experiment, animals were sacrificed by the intraperitoneal administration of a lethal dosage of sodium thiopental (120 mg kg⁻¹). All procedures were approved by the Ethics Committee for Animal Use at the same institution (19/2015) and were in accordance with the Guidelines of the Brazilian College of Animal Experiments (COBEA).

**Statistical analyses**

Data were expressed as mean ± standard deviation (SD) and the differences were considered significant when p < 0.05 after one-way ANOVA and Bonferroni’s or Tukey’s test analysis using GRAPHPAD PRISMTM software version 5.0. Standard gallic acid quantification was performed by linear regression. All experiments were performed in triplicate with three repetitions.

**Results and discussion**

**Determination of total phenol content**

Phenolic compounds possess antioxidant properties due to the presence of hydroxyl groups linked to a single aromatic ring or coupled aromatic rings. Thus, the structure of these compounds allows resonance of single electrons in the aromatic ring as their intermediates are formed due to their antioxidant action (Naczk & Shahidi, 2004; Cheng et al., 2017). These compounds may be synthetic or natural. When present in plants, they may be found in their free forms or complexed with sugars and proteins (Jacob &

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Narendhirakannan, 2019). In plants, they are classified as secondary metabolites such as simple phenols, lignans, lignins, flavonoids, stilbenes, hydrolysable and condensed tannins, coumarins, and phenolic acids (derivatives of cinnamic and benzoic acids). They can reduce lipid peroxidation and inhibit lipoxygenase in vitro by neutralizing or seizing free radicals and chelating transition metal, which may act in the beginning and the spread of the oxidative process (Naczk & Shahidi, 2004; Chhikara et al., 2018; França et al., 2019).

In the present study, TPC for EAF (207 ± 2.3 GAE mg g⁻¹) was significantly (p < 0.05) higher than HEE and HMF, being 172.50% and 249.4% higher than these two, respectively (Figure 1). In the study by Faria, Marques and Mercadante (2011), TPC determined in fruits of S. cumini (148.3 ± 32.4 GAE mg 100 g⁻¹) was 140 times lower than the one observed for EAF. In the study published by Luzia and Jorge (2009), in which S. cumini seeds were analyzed, TPC was found to be 150.56 GAE mg 100 g⁻¹, while Rufino et al. (2010) found 185 mg ± 3.8 GAE mg 100 g⁻¹ in fresh fruits of S. cumini. However, Ruan, Zhang, and Lin (2008) showed high TPC in the methanol extract of S. cumini leaves, which was considered greater than the results found in HEE and the EAF and HMF.

Figure 1. Total phenol content for HEE, EAF and HMF obtained from the leaves of S. cumini (L.) Skeels. Values with different letters indicate means with significant differences (p < 0.05) compared by one-way ANOVA followed by the Tukey’s post-hoc test.

Antioxidant activity by DPPH•

Stable at room temperature, DPPH• is a free radical producing a violet solution in ethanol and in the presence of antioxidants is reduced, producing discoloration of those solutions (Mensor et al., 2001; Donatini, Ishikawa, Barros, & Bacchi, 2009). When coupled to substances that are antioxidant or radical species (R•), DPPH is reduced to form a yellow compound called diphenyl-picryl-hydrazine, which can be monitored by the decrease in absorbance at 515 nm (Sousa et al., 2007). Therefore, according to the data presented in Table 1, HEE and EAF showed the best antioxidant activity, with IP values higher than 90%, which were similar to positive control gallic acid. In addition, the lowest effective concentration to reduce 50% of DPPH was observed for EAF, while the highest AAI was also observed in this sample. According to Scherer and Godoy (2009), AAI expresses how strong an antioxidant agent is. The antioxidant activity is considered poor when the value of AAI is less than 0.5, moderate when the AAI is between 0.5 and 1.0, strong when the AAI is between 1.0 and 2.0, and very strong when the value of AAI is higher than 2.0. Therefore, HEE and EAF can be considered as very strong antioxidants, while HMF is a strong antioxidant.

A direct correlation can also be observed between TPC and EC50 and TPC and AAI, with both showing the order EAF > HEE > HMF. Regarding IP values, which were similar for HEE and EAF when these samples showed differences in EC50 and AAI, Ainsworth and Gillespie (2007) reported that interactions between metabolites in an extract/fraction as well as their competition for free radicals to be neutralized can increase or decrease their synergism and antagonism regarding one biological activity. Therefore, even if EAF does not contain the complete pool of substances present in HEE, its substances may be acting in a synergistic way to give a value of IP similar to HEE. The present findings are aligned to other studies regarding the antioxidant capacity of S. cumini (Ruan, Zhang, & Lin, 2008; Ayyanar & Subash-Babu, 2012; Ayyanar et al., 2015; Chagas et al., 2019). Luzia and Jorge (2009) found an IP value of 94.98% for the extract of S. cumini (L.)
Skeels seed, which is similar to HEE and EAF, while the EC50 of 118.66 mg mL⁻¹ indicated it was at least 4.4 times higher than the lowest EC50 observed in the present study (HMF). However, Donatini et al. (2009) found an EC50 value (5.68 mg mL⁻¹) for the hydroethanolic extract of S. cumini leaves that was 0.6 times the one observed for EAF.

Table 1. Antioxidant activity of HEE, EAF and HMF from leaves of S. cumini (L.) Skeels at 60 min. (30 µg mL⁻¹) determined by free radical DPPH⁺ reduction.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>EC50 (µg mL⁻¹ ± SD)</th>
<th>AAI</th>
<th>IP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEE</td>
<td>19.17 ± 0.594</td>
<td>2.09</td>
<td>90.25</td>
</tr>
<tr>
<td>EAF</td>
<td>9.05 ± 0.170</td>
<td>4.42</td>
<td>94.33</td>
</tr>
<tr>
<td>HMF</td>
<td>27.15 ± 5.132</td>
<td>1.47</td>
<td>55.04</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>1.14 ± 0.8</td>
<td>27.56</td>
<td>92.44</td>
</tr>
</tbody>
</table>

Note: The percent inhibition (PI) of the samples (30 mg mL⁻¹) and 50% effective concentration (EC50) were calculated at 60 min. The antioxidant activity index (AAI) was calculated by dividing the final concentration of the sample by the EC50 at 60 min. Statistical differences were determined by one-way ANOVA followed by Tukey's post-test (p < 0.05) and values with different letters indicate significant differences with media.

In addition to the parameters already discussed above, Figure 2 shows the kinetic behavior through the dosage-response curve relative to the decrease in DPPH remaining percentage (%DPPHREM) versus time (min), which indicates the speed of DPPH decay in presence of extract/fraction over time. Thus, EAF provides a rapid kinetics reaching almost the maximum consumption of DPPH at the first minute, with a percentage of remaining DPPH less than 50% when compared to the gallic acid, whereas the HEE and HMF showed a slower kinetics.

![Figure 2](image-url) Kinetic behavior of HEE, EAF and HMF from leaves of S. cumini (L.) Skeels (30 mg mL⁻¹) and positive control of gallic acid (50 mg mL⁻¹) using the DPPH free radical (40 mg mL⁻¹).

**Evaluation of the antidiabetic potential**

Mainly based on changes in fasting plasma glucose or after an oral glucose load, diabetes diagnosis allows the adoption of therapeutic measures to prevent the disease in patients with impaired tolerance to retard the onset of chronic complications in diagnosed individuals (Iacob & Narendhirakannan, 2019). One of the parameters for the diagnosis of DM is the oral glucose tolerance test (OGTT), which is measured in fasting plasma glucose (FPG) and 2 hours after oral dosage (75 g) of glucose (Cavagnolli, Gross, & Camargo, 2010). Thus, according to the data presented in Figure 3, it was possible to infer that there is no significant (p > 0.05) effect on the modulation of rat blood glucose levels of HEE and EAF, as the negative control (DMSO) was able to reduce glucose levels at the same level of the extract/fraction, which suggests the reduction is being caused by the organism instead of by these samples.

The present findings did not prove the folk medicine use of S. cumini bark to treat diabetes because HEE and EAF did not modulate blood glucose levels. Similar results were previously seen for extract and fractions of S. cumini leaves (Ayyanar et al., 2015). However, De Bona et al. (2011) showed that the aqueous extract of S. cumini leaves reduced the activity of enzymes acetylcholinesterase and adenosine deaminase and parameters of inflammation and oxidative stress in type 2 diabetes patients. Schoenfelder et al. (2010), using an ethanol extract of S. cumini leaves and similar methodology in an acute study with normoglycemic rats, found there was no effect to reduce blood glucose. In another study, Baldissera et al. (2016) showed...
that the administration of E. jambolana Lam to rats with streptozotocin-induced diabetes prevents urinary albumin excretion, increased urinary volume and renal hypertrophy, and did not alter serum creatinine.

Figure 3. Hypoglycemic effect of HEE and EAF obtained from leaves of S. cumini (L.) Skeels at 200 mg kg⁻¹ (p.o.) during the oral glucose tolerance test after 8 h fasting. Data represent mean ± SE and were analyzed by one-way ANOVA followed by the Bonferroni’s post-hoc test.

Conclusion

EAF from S. cumini (L.) Skeels has higher total phenolic content and better antioxidant activity against DPPH•. However, HEE and its fractions did not have any significant action on the modulation of blood glucose in nondiabetic mice. The hypothesis of the present study is that due to the phenolic compounds present in the plant, there is an antioxidant and hypoglycemic action. Further long-term studies to verify the behavior of the EAF associated with the symptoms and complications of diabetes are required.

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References


