



CARDIOPROTECTIVE PROFILING OF SYZYGIUM CUMINI: EVIDENCE IN EXPERIMENTAL MODEL OF ISCHEMIC INJURY

ORIGINAL ARTICLE

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RIBEIRO, Rachel Melo. *et al.* **Cardioprotective profiling of syzygium cumini: evidence in experimental model of ischemic injury.** Revista Científica Multidisciplinar Núcleo do Conhecimento. Year. 08, Ed. 07, Vol. 01, pp. 158-176. April 2023. ISSN: 2448-0959, Access link: <https://www.nucleodoconhecimento.com.br/health/cardioprotective-profiling>

ABSTRACT

Syzygium cumini is a medicinal plant traditionally used to treat cardiometabolic disorders. This study proposed to evaluate the cardiopreventive effect of the hydroalcoholic extract from the *S. cumini* leaves (HESc) on ischemic injury isoproterenol (ISO)-induced in rats. Male albino Wistar rats (*Rattus norvegicus*) were pre-treated with HESc or water for 15 days. They received a challenge with ISO (85 mg/kg) on the 14th and 15th days for induction of ischemic injury. For all animals were obtained electrocardiographic parameters predictive of the lesion in addition to evaluation of troponin I changes in animals. We also realized a phytochemical screening, and *in vitro* antioxidant activity including 1,1-Diphenyl-2-picrylhydrazyl radical. HESc was found to be rich in flavonoids, and high antioxidant potential. Additionally, increased the survival rate and reversed ventricular remodeling caused by isoproterenol-induced ischemic injury in a dose-dependent manner. Inhibition of ST segment elevation, impairment of QT interval prolongation, reduction of Troponin I levels also were observed in animals pretreated with HESc. Our results suggest that pretreatment with HESc reduces myocardial injury and simple phenols and flavonols are likely to be responsible for its effect on cardiomyocyte integrity, confirming that *S. cumini* is a promising plant source of bioactive compounds for the treatment of ischemic syndromes.

Keywords: Medicinal Plant, Bioprospecting, Natural products, Flavonols, Plant Extract.



BACKGROUND

Syzygium cumini (L.) Skeels (Myrtaceae), popularly known in Brazil as Jambolão, is extensively used to treat obesity, diabetes, and hypertension (Migliato *et al.*, 2006). Studies indicate the presence of myricetin, myricitrin, quercetin, kaempferol, and simple phenols such as ellagic acid, ferulic acid and gallic acid (Ruan; Zhang; Lin, 2008; Ayyanar and Subash-Babu, 2012; Sanches *et al.*, 2016).

1. *cumini* possesses antihyperglycemic (Villasenor and Lamadrid, 2006; Sharma *et al.*, 2011), antioxidant, antiatherosclerotic and antihyperlipidemic properties (Ruan; Zhang; Lin, 2008; Tanwar *et al.*, 2011; Sharma *et al.*, 2012), and cardioprotective activity reported for the fruits (Shukla *et al.*, 2014) and seeds (Mastan *et al.*, 2009; Atale *et al.*, 2017).

In recent years, our laboratory has been conducting toxicological and pharmacological tests in animals with a standardized hydroalcoholic extract of *S. cumini* leaves (HESc). The toxicological analysis in rodents revealed that the extract did not exhibit toxicity (Silva *et al.*, 2012). In the pharmacological tests, we demonstrate a hypotensive, antihypertensive, vasorelaxant and antispasmodic effect, and a voltage-dependent block of calcium channel current (Ribeiro, 2007; Monteiro *et al.*, 2018).

In this study, we evaluated the preventive effect of HESc on IHD in an experimental model of isoproterenol-induced myocardial ischemia in rats. The findings of HESc on electrocardiography, estimation of cardiac hypertrophy, and biochemical analyze of troponin I of the animals contribute to the scientific validation of *S. cumini* leaves as complementary or alternative therapy in the treatment or prevention of ischemic cardiovascular disorders.



METHODS

PLANT MATERIAL AND PREPARATION OF EXTRACT

Leaves were collected as previously described by Ribeiro *et al.* (2018), and a voucher specimen was deposited under No. 1069 (herbarium of the 'Prof. Dr. Berta Lange de Morretes "Medicinal Plant Garden, UFMA). The leaves were dried at room temperature, followed by maceration of the leaf powder (300 g) in 70% ethanol (1:3 w/v), concentration in a rotary evaporator under reduced pressure at a temperature below 60°C and lyophilized, with a dry weight of 49.8 g and yield 16.6% (Ribeiro *et al.*, 2018).

DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC) AND TOTAL FLAVONOID CONCENTRATION (TFC)

The TPC of all samples was determined with the Folin–Ciocalteu reagent and 20% sodium carbonate, method described by Dutra *et al.* (2014). Gallic acid (GA) was used as standard, and results expressed as GA equivalents per 100 g dry extract (GAE/100 g). In addition, the TFC was determined by the aluminium chloride colorimetric method described by Dutra *et al.* (2008). Quercetin (QUE) was used as standard, and results expressed as QUE equivalents per 100 g dry extract (QUE/100 g). All experiments were done in triplicate.

DETERMINATION OF DPPH•SCAVENGING ASSAY

The antioxidant activity of the extracts was measured based on the scavenging activity of the stable 1, 1- diphenyl 2-picrylhyorazyl (DPPH) free radical according to the method described by Brand-Williams, Cuvelier and Berset *et al.* (1995) with slight modifications. Gallic acid was used as a positive control. DPPH free radical scavenging ability (%) was calculated by using the:

$$\text{DPPH scavenging activity (\%)} = 100 - [(A_{\text{sample}} - A_{\text{blank}}) \times 100 / A_{\text{control}}]$$



where A_{sample} = absorbance of the sample after 30 min of reaction, A_{blank} = absorbance of the blank, and A_{control} = absorbance of the control.

EXPERIMENTAL ANIMALS

Male 08-week-old Wistar rats (*Rattus norvegicus*), weighing 200 to 250 g, obtained from the Animal House of UFMA were used. The animals were housed under controlled conditions of temperature (21 ± 2 °C) under a 12 h light-dark cycle and humidity from 55 - 65%. The experimental protocols were submitted to and approved by the Animal Research Ethics Committee from State University of Maranhão, Brazil, under license number 23115.013098/2020-73.

EXPERIMENTAL PROTOCOLS

Before starting the experiments, the animals were kept in the laboratory of Pharmacology of the UFMA by a period of one week for adaptation and monitored before and during the experimentation. After, the animals were randomly divided into the different groups and were treated by gavage (oral administration, gavage) with water (controls) or HESc dissolved in water, receiving 0.1 mL/100 g (rats).

Forty Wistar rats were divided into four groups of 10 animals each: healthy Control (0.1 ml water/100 g), ISO (0.1 ml water/100 g + ISO), high-dose HESc (500 mg/kg/day + ISO), and low-dose HESc (250 mg/kg/day + ISO). The animals were pretreated with HESc for 15 consecutive days. Acute myocardial infarction was induced on days 14 and 15 by subcutaneous injection of ISO diluted in 2 mL saline (0.9% NaCl; 85 mg/kg) (Rajadurai and Prince, 2007; Hassan *et al.*, 2015), in all groups, except for the healthy Control group that received only saline by the same route. Clinical signs of toxicity or mortality were recorded daily. At the end of the experiment (day 16), the animals were anesthetized (50 mg/kg ketamine and 10 mg/kg xylazine) for the subsequent experimental procedures.

The doses for HESc evaluated in this study were based on previous results obtained for Wistar in toxicity assay (Silva *et al.*, 2012), and spontaneously hypertensive (SHR)



rats, whose dose 500 mg/kg/day was able to promote changes in the animals' heart rate (Ribeiro *et al.*, 2014). Furthermore, this dose promoted a reduction in the weight of the left and right ventricles in hypertensive animals treated for 08 weeks (unpublished data), which motivated us to evaluate it in a shorter treatment time (15 days), together with a lower dose in cardiac ischemic injury model, to establish a time-dependent and dose-dependent effect relationship, respectively.

Electrocardiography

Electrocardiograms (ECG) were obtained from the anesthetized animals using a veterinary electrocardiograph (DL 600, Delta Life®, São Paulo, Brazil). The QTc interval was calculated using Bazzet's equation: $QTc = QT_{interval} / \sqrt{R-R_{interval}}$.

Estimation of cardiac hypertrophy and Biochemical analyze.

The heart was removed, and washed with ice-cold saline, weighed to obtain the heart weight-to-body weight ratio adapted by Yousefi *et al.* (2014). The heart was then cut to remove the left ventricle (LV) and the LV weight-to-body weight ratio was calculated to assess the degree of congestion.

After ECG, blood was collected from the abdominal artery of anesthetized rats into tubes without anticoagulant and centrifuged at 3,500 rpm for 10 min. Serum was separated, and Troponin I was measured by a rapid immunochromatographic assay (WAMA Diagnóstica®).

STATISTICAL ANALYSIS

The results were expressed as the mean \pm standard error of the mean (S.E.M.). One-way analysis of variance (ANOVA) followed by the Tukey post-test was used for multiple comparisons and the Student *t*-test for comparison of unpaired data. A *p* value <0.05 was considered significant. Statistical analysis was performed using the GraphPad Prism® 9.0 program.



RESULTS

TPC AND TFC OF HESC

The content of total phenols and flavonoids in HESc was 300.7 ± 3.9 GAE/100 g dry weight of extract and 83.5 ± 2.4 QUE/100 g dry weight of extract, respectively. These results suggest a high content of phenolic compounds, particularly flavonoids, in leaves of *S. cumini*.

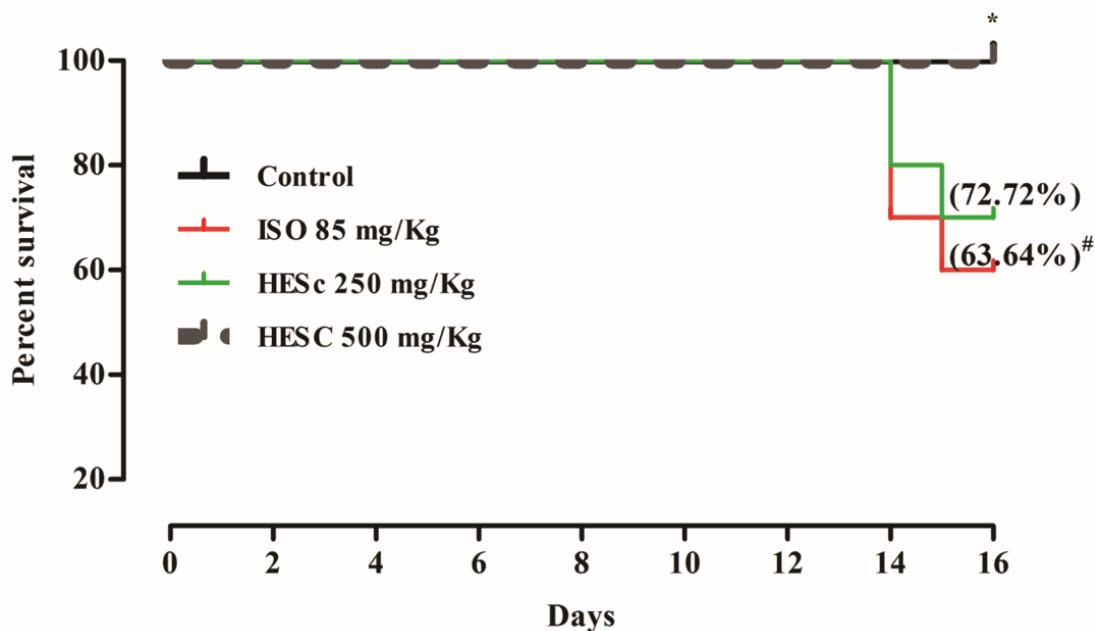
DPPH RADICAL SCAVENGING ACTIVITY OF HESC

HESc showed the highest DPPH scavenging activity, at 91.73 ± 0.30 % with an IC_{50} value of 4.02 ± 0.13 $\mu\text{g/mL}$. IC_{50} value of the positive control Gallic Acid was 1.55 ± 0.44 $\mu\text{g/mL}$ (92.30 ± 0.30 %).

HESC ENHANCES THE SURVIVAL OF RATS WITH ISO-INDUCED ISCHEMIC INJURY

The survival rate of animals that received the low-dose (250 mg/kg/day) and water (ISO group) was 72.72% and 63.64%, respectively (Fig. 1), and with the highest dose of HESc (500 mg/kg/day) was 100%. These results suggest that a HESc dose of 500 mg/kg/day may effectively prevent death and increase the chance of survival of animals with ischemic injury.

Fig 1. *Syzygium cumini* leaves increases chances of survival after ISO-induced ischemic injury



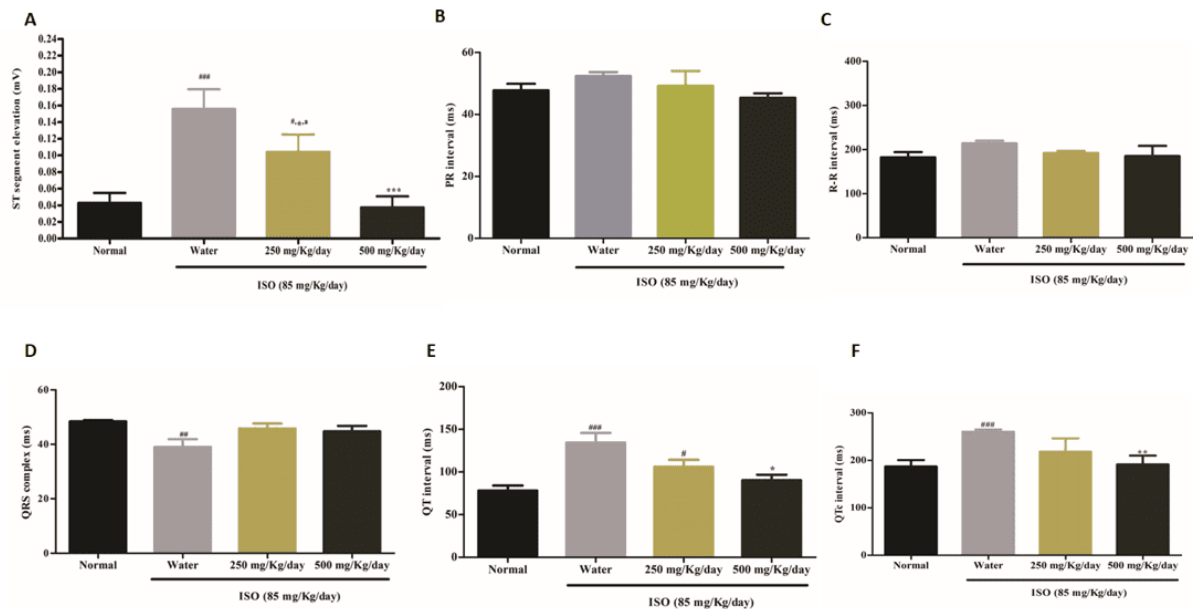
Source: Elaborated by the authors.

The survival rate of ISO-injected animal treated with Water and 250 mg/kg/d and 500 mg/kg/day of HESc. # $p < 0.05$ vs. Control group; * $p < 0.05$ vs. Water + ISO.

EFFECT OF HESC ON ELECTROCARDIOGRAPHIC PATTERN AND PARAMETERS

The data regarding ST and PR segments, QRS complex, QT and QTc intervals and R-R interval are shown in Figs. 2 and 3. The ECG recording of healthy control animals indicates a regular and uniform sinus rhythm (Fig. 3). ISO-induced animals exhibited significant ($p < 0.001$) ST segment elevation when compared to the Control group (Figs. 2A and 2B). Pretreatment with HESc (500 mg/kg/day) caused significant ($p < 0.001$) cardioprotection, with a more than 50% decline in ST segment elevation compared to the ISO-induced group and values like the Control group (Fig. 2A).

Fig 2. HESc prevents ISO-induced cardiac electrical changes of animals after ISO-induced myocardial injury

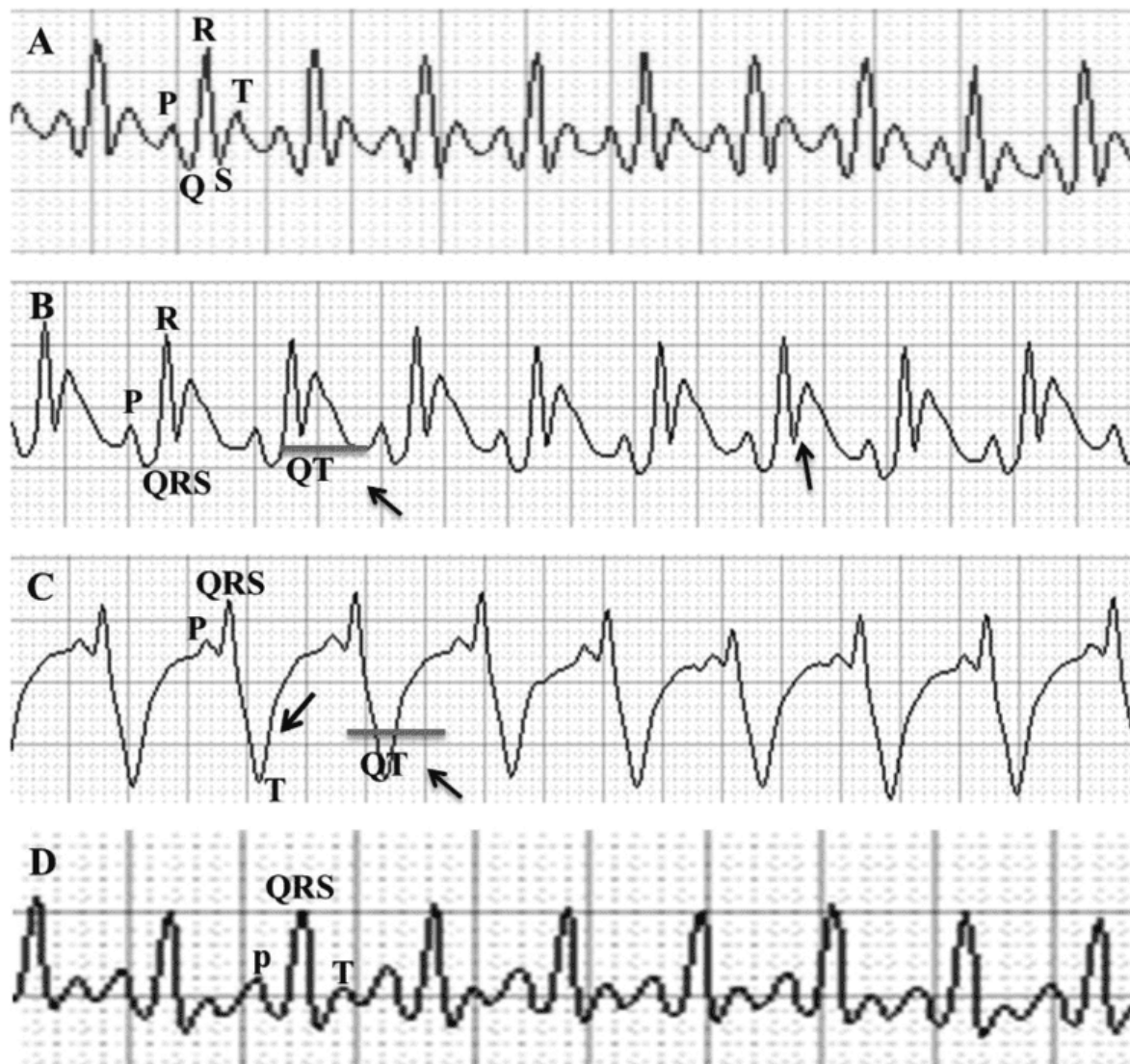


Source: Elaborated by the authors.

(A) ST segment. (B) PR segment. (C) R-R interval. (D) QRS complex. (E) QT interval. (F) QTc interval. Results are expressed as mean \pm S.E.M. (n = 6-10). ### p < 0.001 vs Control group; ## p < 0.01 vs Control group; *** p < 0.001 vs Water + ISO; # p < 0.05 vs HESc 500 mg/kg + ISO (ANOVA, Tukey).

In the ISO-induced group, a significant decline (p < 0.01) in the QRS (Fig. 2D) complex and an increase in the QT (p < 0.001) and QTc intervals (p < 0.001) were observed when compared to the control group (Figs 2E, 2F and 3B) with no significant alterations in the PR or R-R interval (Figs 2B and 2C). Pretreatment with HESc (500 mg/kg/day) resulted in significant cardioprotection, with restoration of the QT and QTc intervals when compared to the ISO-induced group (p < 0.05 and p < 0.01, respectively). This effect was more pronounced when compared to the lower dose, thus suggesting a dose-dependent cardioprotective effect of *S. cumini* leaves (Figs 3C and 3D).

Fig 3. Representative image of the electrocardiogram of animals after ISO-induced myocardial injury



Source: Elaborated by the authors.

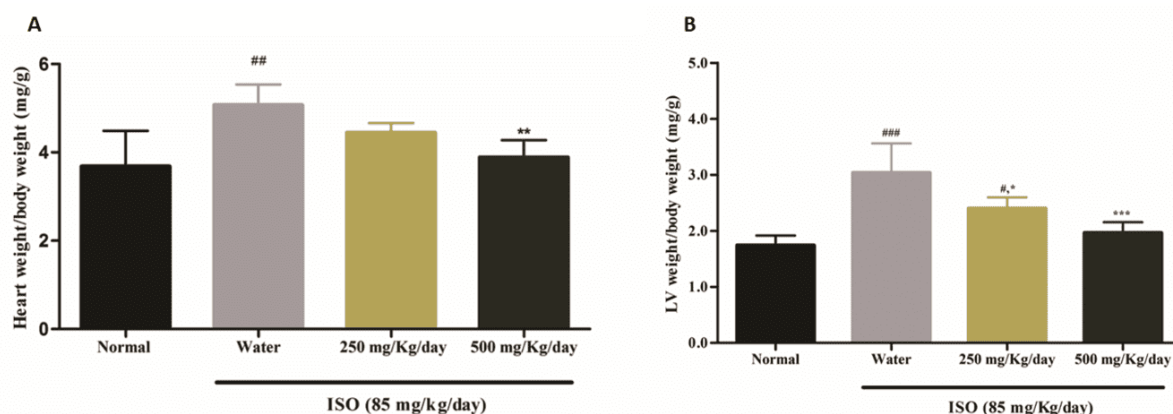
(A) Normal control group showing with electrocardiographic recording without changes. (B) Water + ISO showing ST segment elevation and QT interval prolongation. (C) HESc 250 mg/Kg/day + ISO group, presenting myocardial ischemia and QT interval prolongation. (D) HESc 500 mg/Kg/day + ISO, presenting a sinus rhythm with a slight increase in amplitude of the P wave.

EFFECT OF HESc ON THE ESTIMATION OF HYPERTROPHY AND TROPONIN I

A significant increase was observed in the heart weight/body weight ratio (Fig. 4A) and LV weight/body weight ratio (Fig.4B) in animals of the ISO-induced group. Pretreatment with the

highest dose of HESc for 15 days reduced the heart weight ($p < 0.001$) and LV weight ratios ($p < 0.001$) when compared to ISO-induced animals. In contrast, the lower dose reduced the LV weight/body weight ratio ($p < 0.05$), but the ratio differed from the Normal Control group ($p < 0.05$).

Fig 4. HESc reduced ISO-induced ventricular hypertrophy



Source: Elaborated by the authors.

(A) Heart weight/body weight (mg/g). (B) LV weight/body weight (mg/g). All values are indicated as mean \pm S.E.M. ($n=5$). $###p < 0,0001$ vs. Normal Control, $**p < 0,001$ vs. Water + ISO, $****p < 0,0001$ vs. Water + ISO (ANOVA, Tukey).

Troponin I was positive in all animals of the ISO-induced heart injury group and absent in serum of all animals pretreated with the high dose of HESc (500 mg/kg/day) or Control group (Table 1).



Table 1. Effect of *Syzygium cumini* on serum troponin I levels measured by immunochromatographic assay in animals submitted to acute myocardial infarction

Animal	Groups			
	Normal Control	Water + ISO	HESc 250 mg/Kg + ISO	HESc 500 mg/Kg + ISO
1	n	p	p	n
2	n	p	p	n
3	n	p	p	n
4	n	p	n	p
5	n	p	p	n

Source: Elaborated by the authors.

n: absence of troponin I; *p*: presence of troponin I. Troponin I was measured with the WAMA Diagnóstica® rapid immunochromatographic assay (*n* = 5).

DISCUSSION

This is the first study that evidences the therapeutic cardiopreventive potential of *Syzygium cumini* leaves, observed in ECG analysis or normalization of Troponin I, and probably is attributed to the high content of phenolic and flavonols compounds, and include the simple preparation of the extract and short treatment time.

One of the mechanisms supposedly involved in irreversible ISO-induced myocardial ischemic injury is calcium overload (Yousefi *et al.*, 2014). Responses include an increase in cardiac contractility and pathological alterations such as hypertrophy and cell apoptosis (Yousefi *et al.*, 2014; Bloom and Davis, 1972). The main route of entry of calcium into cardiomyocytes is through L-type calcium channels. In this respect, the blockage of these channels has been suggested to play a preventive role in ischemic injury. Previous results obtained by our group for HESc have shown a possible effect of the leaf extract of *S. cumini* on the inhibition of L-type calcium channels (Ribeiro *et al.*, 2018), in agreement with the present study in which HESc prevented ventricular remodeling in a dose-dependent manner.

IHD in general are associated with changes in the electric properties of the heart and ECG is used as a diagnostic tool of cardiomyopathies (Patel *et al.*, 2010). ST segment elevation is a sensitive marker of acute myocardial infarction and is related to the difference in membrane potential between ischemic and non-ischemic areas, with a



consequent loss of cell membrane function ((Patel *et al.*, 2010; Zhou *et al.* 2008). ST segment elevation has been observed in patients with myocardial ischemia (Peacock *et al.*, 2007) and in rats with ISO-induced myocardial injury (Kela; Reddy; Thombre, 1980; Rajadurai and Prince, 2007). Some authors suggest ST segment abnormalities to be an indicator of morbidity and mortality due to ischemic heart disease (Achari *et al.*, 2008; Mozos and Caraba, 2015). Our results showed that ISO-induced ischemic injury promoted changes in the ECG recording and ST segment elevation was suppressed by pretreatment with the highest dose of HESc.

Other ECG alterations are expected for the experimental model used, such as a reduction of the R-R interval and prolongation of the QT interval (Mikušová *et al.*, 2009). No differences in the R-R interval were observed in the present study. These intervals remained elevated in the groups even after the administration of ISO, suggesting bradycardia of the animals. This fact might be explained using ketamine and xylazine as anesthetic combination, which is known to cause bradycardia in rats (Sano *et al.*, 2016).

Prolongation of the QT interval is commonly associated with cardiac hypertrophy, more specifically LV hypertrophy, whose physiopathological relevance involves the development of mechanical dysfunction, pump failure and sudden death (Jose and Gupta, 2004; Davey *et al.*, 1994). In the present study, HESc restored the duration of the QT interval to normal levels, corroborating the dose-dependent effect of *S. cumini* leaves on cardiac remodeling. Additionally, the Troponin I biomarker evaluated in this study is used as a laboratory tool for diagnosis of acute myocardial infarction in humans (Kim *et al.*, 2014; Fathil *et al.*, 2015). The results showed a dose-dependent effect for HESc to prevent the plasma elevation of troponin I.

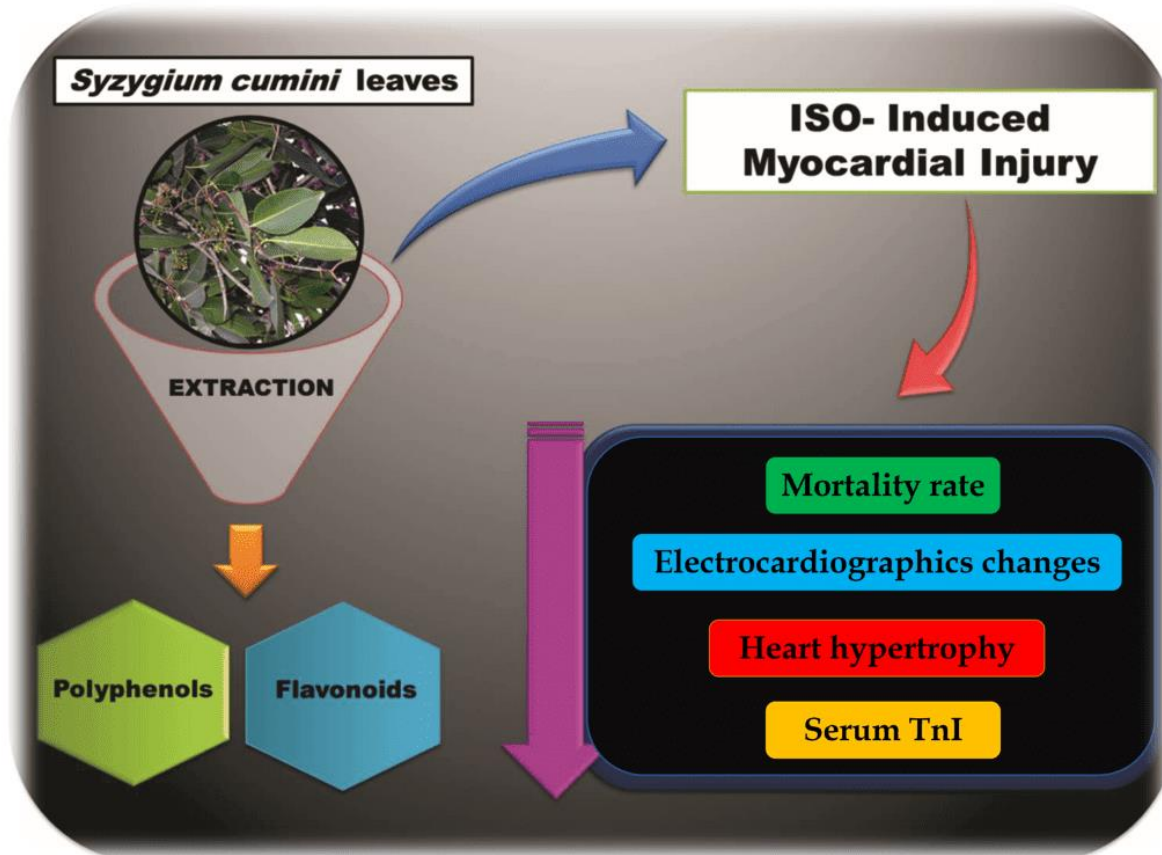
With respect to chemical composition, our results indicate that HESc presented higher content of polyphenols compared to leaf extract obtained by (Sanches *et al.*, 2016), which identified the presence of simple phenols like gallic acid and myricetin-derived flavonols in leaf extract. Ryu *et al.* (2016) showed that gallic acid prevents LV hypertrophy and fibrosis ISO-induced in mice. Zern *et al.* (2005) observed that supplementation of patients with an extract containing myricetin confers



cardioprotection against oxidative damage after consumption for 30 days. This finding was corroborated by Tiwari et al. (2009) who demonstrated the protective role of myricetin from *Vitis vinifera* in reducing the ISO-induced cardiac toxicity in rats. We may therefore suggest that the cardiopreventive activity of HESc against ischemic injury observed in this study is related in part to the presence of these compounds (Figure 5).

Detection of the antioxidant potential of plant extracts can be evaluated by a relatively simple spectrophotometric method through the DPPH assay. This method consists in evaluating the antioxidant capacity through the sequestering activity of the free radical 2,2-diphenyl-1-picryl-hydrazine-DPPH. The DPPH radical by the action of an antioxidant is reduced to form diphenyl-picryl hydrazine, yellow staining (Rao *et al.*, 2010; Lee *et al.*, 2007).

The results indicate a high percentage of inhibition for HESc positively correlated with phenolic and total flavonoid content. The antioxidant potential of *S. cumini* leaves was evidenced in other studies that corroborate our results (Ruan; Zhang; Lin, 2008; Sanches *et al.*, 2016; Eshwarappa *et al.*, 2014).

Fig 5. Proposed for the Cardiopreventive Action of *Syzygium cumini*

Source: Elaborated by the authors.

The large amount of polyphenols and the high content of flavonoids contribute to the high antioxidant potential of the plant extract, whose previous oral treatment for 15 days was able to reduce mortality, reverse electrical alterations, prevent cardiac hypertrophy, suppress heart failure, and elevation of TnI levels.

CONCLUSIONS

In conclusion, the results characterize a cardioprotective effect of *S. cumini* leaves in animals with ischemia by ISO. Further studies are needed to elucidate the mechanism underlying the preventive effect of *S. cumini* on the myocardial ischemic injury. Thus, it is hoped to contribute to the scientific validation of this species as a promising plant source of bioactive compounds in the treatment of ischemic heart diseases.



COMPETING INTERESTS

The authors declare that they have no competing interests.

Funding:

This work was supported by Fundação de Amparo à Pesquisa do Estado do Maranhão, Brasil (FAPEMA) project 2012–2014 (Reference nº: APP-UNIVERSAL-00354/12) and 2014-2016 (Reference nº: UNIVERSAL-00788/14). This study was conducted at the Federal University of Maranhão (UFMA) in cooperation with CEUMA University and State University of Maranhão.

ACKNOWLEDGEMENTS

This study it was conducted in cooperation with Universidade Federal do Maranhão (UFMA), Instituto Federal do Maranhão (IFMA) and Universidade Estadual do Maranhão (UEMA).

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Sent: March 17, 2023.

Approved: June 22, 2023.

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