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HEALTH SCIENCES

Antioxidants and cardioprotective effects of ethyl acetate fraction of *Canavalia rosea* leaves in myocardial ischemia-reperfusion injury

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Abstract: Different degrees in the biological activities of *Canavalia rosea* had been previously reported . In this study, our group assessed the cardioprotective effects of the ethyl acetate fraction (EAcF) of the *Canavalia rosea* leaves. Firstly, it was confirmed, by *in vitro* approach, that the EAcF has high antioxidant properties due to the presence of important secondary metabolites, as flavonoids. In order to explore their potential protector against cardiovascular disorders, hearts were previously perfused with EAcF (300 μg.mL^{-1}) and submitted to the global ischemia followed by reperfusion in Langendorff system. The present findings have demonstrated that EAcF restored the left ventricular developed pressure and decreased the arrhythmias severity index. Furthermore, EAcF significantly increased the glutathiones peroxidase activity with decreased malondialdehyde and creatine kinase levels. EAcF was effective upon neither the superoxide dismutase, glutationes reductase nor the catalase activities. In addition, the Western blot analysis revealed that ischemia-reperfusion injury significantly upregulates caspase 3 protein expression, while EAcF abolishes this effect. These results provide evidence that the EAcF reestablishes the cardiac contractility and prevents arrhythmias; it is suggested that EAcF could be used to reduce injury caused by cardiac reperfusion. However more clinical studies should be performed, before applying it in the clinic.

Key words: enzymes, antioxidant effect, cardiac contractility, *Canavalia rosea*.

INTRODUCTION

Myocardial ischemia-reperfusion (I/R) injury is still, increasingly, cardiovascular disease, which causes morbidity and mortality worldwide and, occurs by partial or complete reduction of oxygen levels in coronary microcirculation followed by the reestablishment of blood flow and physiological restoration of oxygen and nutrients in the myocardium (Draginic et al. 2022, Murphy & Steenbergen 2008). The attenuation of the damage during myocardial reperfusion is a critical step to reduce cardiac injury. In several studies, it has been demonstrated that during cardiac reperfusion, there is an impaired cellular metabolism characterized by calcium overload and exacerbated oxidative stress, which ultimately activate signaling pathways of cell death driving to damage of myocardial

contractility (Minamino 2012, Rodrigo et al. 2021, Sahna et al. 2002).

Evidences suggest that the activation of endogenous antioxidant enzymes represents one of the most important mechanisms involved in the cardioprotective effects of natural products (Yu et al. 2011, Yuan et al. 2015). Acute beneficial effects of flavonoids have been widely found and shown to reduce I/R injury in hearts through an intracellular mechanism that involves increased activity of antioxidant enzymes and/or reacting directly scavenging reactive oxygen species (ROS) (Kanaan & Harper 2017, Scholz et al. 2010, Yu et al. 2011).

Thus, novel alternative therapies employing natural products appear to be promising to present many chemical compounds that act in synergism by potentialize the antioxidant effects. Among the various parts of the plant *Canavalia rosea*, Costa et al. (2008), demonstrated that the leaves of *Canavalia rosea* present potential antioxidant effect suggesting beneficial properties in disorders related to oxidative stress. *C. rosea* (Beach bean or bay bean, *C. maritima*, *C. obtusifolia*, Fabaceae), is a plant commonly found on coastal areas and fixed to the sand dunes, forming dense ground covers (Gonçalves et al. 2008, Kitajima et al. 2008).

Other pharmacological activities have been related to the compounds of *C. rosea;* among these are the anti-inflammatory and vasorelaxant effects (Assreuy et al. 2009, Bezerra et al. 2007, Costa et al. 2008, Pattamadilok et al. 2008). The purpose of this study was to investigate the possible cardioprotection conferred by the ethyl acetate fraction (EAcF) of *C. rosea* leaves through its antioxidant properties in post-ischemic reperfused hearts.

MATERIAL AND METHODS

Plant material and extraction of the ethyl acetate fraction

Canavalia rosea leaves were obtained on the coast of the beach from Northeastern Brazil's region (Sergipe State, NE, 10º South latitude and 37º West longitude) in February of 2012 and were identified by a taxonomist at the Herbarium of the Department of Biology, Federal University of Sergipe. The voucher specimen (#24061) has been deposited at the host university. The leaves (2.2 kg) were washed, dried and reduced to powder, which was subjected to extraction with methanol immersion for 14 days (Argolo et al. 2004). After this period, the extract was concentrated (442.93 g) through solvent evaporation in a rotary evaporator (Laborota 4000, Heidolph Instruments GmbH & Co., Baviera, Germany) under reduced pressure at 45°C (Costa et al. 2008, Nakao et al. 1998). The concentrate was successively submitted to liquid-liquid extraction with hexane (174.81 g), chloroform (90.08 g) and ethyl acetate (2.78 g) to yield their respective fractions (Argolo et al. 2004, Sridhar & Seena 2006). The EAcF was then selected for further studies due to its high levels of flavonoids and recognized antioxidant effect (Costa et al. 2008, Nakao et al. 1998).

The stock solution of EAcF (60 $mg.mL^{-1}$) was prepared with 0.5% DMSO and used for *in vitro* tests. For *ex vivo* heart model tests, the end concentration of 300 $\mu g.mL^{-1}$ it was used, prepared in Krebs solution (in $mmol.L^{-1}$: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.8, glucose 11.1, KH_2PO_4 1.2; pH was adjusted to 7.4) added with 0.5% DMSO (Vehicle solution). This concentration of EAcF was chosen for the antioxidant effect by EC_{50} upon the dose response in atrium curve (data not shown).

EAcF phytochemical screening

The qualitative identification of the main classes of the secondary metabolites present in the EAcF (Table I) was made by using the colorimetric methods as proposed by Matos (2009).

Determination of total phenolic and flavonoid contents

Total phenolic contents (TPC) of EAcF were determined by using the Folin-Ciocalteu assay. Briefly, aliquots of 10 mg of EAcF was dissolved in 10 mL methanol. This solution (0.5 mL) was diluted to methanol (180 µg/mL) and mixed with 2.25 mL of Folin-Ciocalteu solution, 1.75 mL of 7.5% sodium carbonate solution, and 0.5 mL of distilled water. After incubation at 45° C for 20 min, the reaction mixture absorbance was measured at 765 nm using a UV–vis spectrophotometer (721 G visible spectrophotometer). TPC was calculated using Gallic acid standard curve ranging from 5 to140 µg/mL with ten points (y= 0.0103x + 0.0737; R^2= 0.9946). The data were expressed as mg of gallic acid equivalents (GAE)/g of dried EAcF (Barbosa et al. 2019).

The aluminum nitrate colorimetric method was used to determine the total flavonoid contents (TFC) of EAcF. Briefly, 0.5mL (180 µg/mL) sample was mixed with 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1M potassium acetate and 4.3 mL of methanol. Then, the mixture was incubated at room temperature for 10 min in the dark. The reaction mixture absorbance was read at 425 nm using 721 G visible spectrophotometer. TFC was calculated using Rutin standard curve ranging from 5 to140 µg/mL with ten points (y= 0.0036x - 0.0115; R^2= 0.9901). The data were then coverted into mg of Rutin equivalents (RE)/g of dried EAcF (Barbosa et al. 2019).

Reduction of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) *in vitro*

Total antioxidant activity of EAcF was evaluated using $DPPH^{\cdot}$ assay according to Paixão et al. (2013). Gallic acid (1 – 10 µg.mL^{-1}) and EAcF (5, 10, 15, 20, 30, 40 and 60 µg.mL^{-1}) were added to a solution of $DPPH^{\cdot}$ radical (40 µg.mL^{-1} in methanol). The absorbance of the reaction mixtures was determined at 515 nm in the first, fifth and tenth minutes, and then every 10 min up to 60 min. The results were expressed as the inhibition percentage (IP), while the antioxidant amount necessary to decrease the $DPPH^{\cdot}$ concentration in 50% (IC_{50}) was calculated by plotting the percentage of $DPPH^{\cdot}$ remaining (%$DPPH^{\cdot}$ Rem)

Table I. Phytochemical screening of ethyl acetate fraction of *Canavalia rosea* (EAcF).

Classes of metabolites	Assays	EAcF
Flavones, flavonols, flavanones and xanthones	Hydrochloric acid/magnesium	+
Leucoantocianidine	pH change	+
Catechins	pH change	+
Condensed tannins	Ferric chloride	+
Pyrogallic tannins	Ferric chloride	—
Pentacyclic triterpenoids	Anysaldehyde/Sulfuric acid	—
Saponins	Chloroform	+
Free steroids	Lieberman–Bouchard’s reagent	—
Resins	Turbidity	—
Alkaloids	Dragendorff reagent	+

after 60 min versus extract concentrations. Antioxidant activity index (AAI) was calculated as AAI = [DPPH˙ stock concentration (μg.mL^{-1})]/[IC_{50} (μg.mL^{-1})] (Scherer & Godoy 2009). The antioxidant activity is classified according to the AAI and was classified as poor (less than 0.5), moderate (between 0.5 and 1.0), strong (between 1.0 and 2.0) and very strong (higher than 2.0).

Measurement of lipid peroxidation *in vitro*

Evaluation of thiobarbituric acid-reactive substances (TBARS) was used to determine the potential of EAcF to reduce lipid peroxidation *in vitro* (Budni et al. 2007). Briefly, egg yolk homogenate (1% w/v, 1 mL) in phosphate buffer (pH 7.4) was sonicated (10 s) and mixed with freshly prepared solutions of the controls and EAcF at 0.1; 1; 10 and 100 μg.mL^{-1}. Lipid peroxidation was induced by adding ferrous sulphate ($FeSO_4$, 0.17 mol.L^{-1}). NAC (N-acetyl-L-cysteine) at 12.5, 25, 50 and 100 μg.mL^{-1} was used as a positive control, while the negative control was the vehicle (methanol P.A.). The mixture was incubated at 37°C for 30 min. Upon cooling, samples were centrifuged with 15% trichloroacetic acid (0.5 mL) at 1,200 rpm for 10 min. Supernatant was taken (0.5 mL), mixed with 0.67% thiobarbituric acid, incubated at 95°C for 60 min and, after cooling, the formation of TBARS was measured by reading the supernatant absorbance at 532 nm. Results were expressed as inhibition percentage of malondialdehyde (MDA) formation.

Animals and *ex vivo* experimental procedures

Forty-eight male *Wistar* rats (200 – 300 g) at 8-12 weeks of age were kept under controlled temperature (22 ± 1°C) and 12 h light/dark cycle with free access to standard rodent feed and water. The rats were randomly distributed into 4 groups (n = 12/each group): Sham, Vehicle, EAcF and NAC. Half of these animals were used for *in vivo* testing and the other 24 for *ex vivo* testing. The test solutions were perfused before I/R by 10 min, as described in detail in Figure 1. All experimental protocols were performed in accordance with the guidelines for the human use of laboratory animals and were approved by the Ethics Committee for Animal Research of the Federal University of Sergipe (Protocol #18/2012).

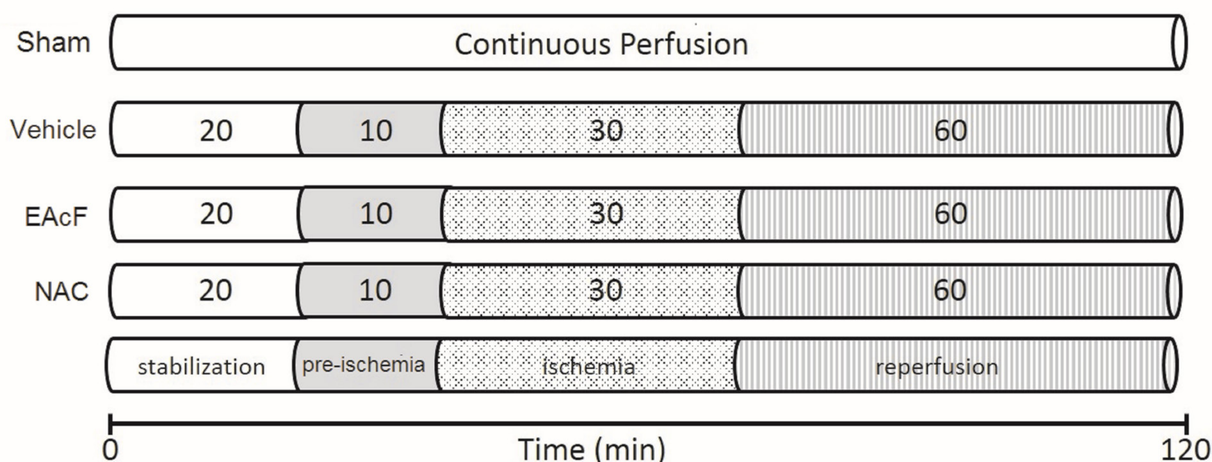


Figure 1. Experimental groups: Sham – not ischemic; Vehicle – previously perfused with Krebs' solution for 10 min enriched with 0.5% DMSO; EAcF – previously perfused with ethyl acetate fraction *C. rosea* (300 μg/mL) in Krebs' solution and NAC – previously perfused with NAC (24 μmol/L) in Krebs'. The hearts were submitted to 30 min global ischemia and 60 min reperfusion with Krebs' solution.

To verify the effect of EAcF, *ex vivo*, twenty-four animals, randomly distributed into 4 groups, were heparinized (800 I.U., i.p., 15 min), euthanized by guillotine and hearts were carefully removed and mounted in an aortic perfusion system (Langendorff) on a constant pressure, previously calibrated using a mercury column (15 cmHg). The hearts were perfused with Krebs solution, previously filtered through a cellulose acetate membrane (0.45 µm) to prevent microembolic events, oxygenated (95% O_2 + 5% CO_2) and kept at 37 ± 0.1°C (Haake F3, Berlin, Germany).

After a 20 min stabilization period with only Krebs solution, the hearts were perfused with vehicle solution (Krebs solution added with 0.5% DMSO) or EAcF (diluted in Krebs solution added with 0.5% DMSO) solution for 10 min, subjected to 30 min global ischemia, through the complete interruption of the nutrient solution flow, and reperfused with vehicle solution for 60 min.

Left ventricular developed pressure (LVDP) and heart rate (HR) measurements

To measure LVDP, the left atrium was removed for the introduction of a balloon in the left ventricular cavity. This device was coupled to a pressure transducer (HP1290A, Hewlett-Packard®, Illinois, USA). Signals were amplified (HP7754A, HP7754B, Hewlett-Packard® Illinois, USA), digitized (DI-710, Dataq Instruments®, Ohio, USA) and stored for further analysis. Using the software Windaq pro (Dataq Instruments®, Ohio, USA), it was possible to determine the RR intervals and convert them into HR. The data were then stored in a computer for processing.

Arrhythmias severity index (ASI)

All experimental procedures to obtain the LVDP were recorded and used to calculate the ASI. The presence of ventricular tachycardia and/or ventricular fibrillation after the reperfusion was identified, as previously described by Bernauer and Ferreira (Bernauer & Ernenputsch 1988, Ferreira et al. 2001).

Creatine kinase (CK) measurements

Creatine kinase levels were measured in perfused Krebs solution samples from the coronary circulation by enzymatic assay according to the manufacturer's recommendation (CK-NAC Liquiform - Labtest®, Minas Gerais, BRA), the results were expressed in $U.L^{-1}$.

Measurement of lipid peroxidation *ex vivo*

Lipid peroxidation was evaluated on ventricle samples homogenized in potassium phosphate buffer (50 $mmol.L^{-1}$, pH 7.4) containing butylated hydroxytoluene (12.6 $mmol.L^{-1}$). Measurements were performed in homogenates incubated during 45 min at 90ºC with 15% trichloroacetic acid, 0.37% thiobarbituric acid and 0.25 $mol.L^{-1}$ hydrochloric acid. Then, the homogenates were centrifuged for 5 min at 14,000 rpm, N-butanol and saturated NaCl solutions were added into the supernatant and then centrifuged (2 min). The supernatant absorbance was measured at 535 nm and 572 nm and expressed as nmol of $MDA.mg^{-1}$ of tissue (Dhalla et al. 2000, Lascano et al. 2013, Sadeghi et al. 2015).

Antioxidant enzyme activity

The ventricles were homogenized in ice-cold sodium phosphate buffer (pH 7.2) and homogenates were centrifuged for 5 min at 12,000 rpm. The supernatant was used for the determination of catalase (CAT), superoxide dismutase (SOD), glutathiones peroxidase (GPx) and reductase (GR) activities. All experiments were normalized by total protein content and measured according to Lowry et al. (1951).

CAT activity was determined according to Nelson & Kiesow (1972) by measuring the consumption of hydrogen peroxide (H_2O_2) (0.3

mol.L^{-1}) in buffer potassium phosphate (PBS - 50 mmol.L^{-1}, pH 7.0) using a spectrophotometer at 240 nm. CAT activity was expressed as the extinction of H_2O_2 during 1 min at 25°C (ΔE.min^{-1}.mg^{-1} protein).

SOD activity was evaluated according to Madesh & Balasubramanian (1998). The colorimetric assay involves the generation of superoxide anion radical ($O_2^{\cdot-}$) by pyrogallol autoxidation. The inhibition of $O_2^{\cdot-}$ production was assessed by the reduction of tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] and the SOD activity was measured at 570 nm. SOD activity was expressed as units *per* mg of protein (U.mg^{-1} protein).

GPx activity was assessed according to Paglia & Valentine (1967). Ventricles were homogenized in PBS (50 mmol.L^{-1}) plus KCl (140 mmol.L^{-1}, pH 7.4) and were then added to the supernatant, glutathione reductase (10 U.mg^{-1} protein), reduced glutathione (0.15 mmol.L^{-1}), NADPH (8.4 μmol.L^{-1}) and H_2O_2 (2.2 mmol.L^{-1}). Oxidation of NADPH to NAD^+ was monitored at 340 nm during 8 min, which represents GPx activity (nmol NADPH min^{-1}.mL^{-1}).

GR activity was determined according to the method of Carlberg & Mannervik (1985). In brief, the sample was homogenized in PBS (0.2 M, pH 7.5) containing EDTA (6.3 mmol.L^{-1}) supplemented with leupeptin solution (5 mg.mL^{-1} in water) and PMSF solution (100 mmol.L^{-1} in isopropanol). Albumin solution was added to homogenate (0.5 mg.mL^{-1} in buffer) and GSSH (10 mmol.L^{-1} in buffer). The reaction was started by adding NADPH (1.2 mg.mL^{-1} bicarbonate 0.5% (w/v)). The monitoring was done at 340 nm, 37°C for 8 minutes in one-minute intervals. The activity was expressed as nmol.min^{-1}.mg^{-1} protein.

Measurement of $O_2^{\cdot-}$ production in myocardium

Briefly, hearts were collected, and optimal cutting temperature compound (OCT compound) is used to embed tissue samples prior to frozen sectioning. Sections, 20 μm, were rehydrated with PBS in heated plate, 37ºC, incubated with the fluoroprobe dihydroethidium (DHE, 5 μM; Molecular Probes, Eugene, OR) for 5 min in the dark, and imaged by microscopy (Nikon eclipse C*i*) (Hingtgen et al. 2010).

Western blot analysis

Western blots were performed as previously described by Mota et al. 2017 (Mota et al.2017). Hearts were homogenized in ice-cold lysis buffer (in mmol.L^{-1}): 150 NaCl, 50 Tris-HCl, 5 EDTA.2Na, and 1 $MgCl_2$ containing 1% Triton X-100 and 0.5% SDS enriched with protease and phosphatase inhibitor cocktail (Sigma FAST, Sigma®, Missouri, USA). Protein samples (50 μg) were denatured and separated using 12% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (0.22 μm, Merck-Millipore®, Massachusetts, USA). Membranes were blocked at room temperature with 5% non-fat dry milk in TBS plus 0.1% tween 20 before incubation with anti-Caspase 3 and anti-GAPDH antibodies (1:1000; Santa Cruz Biotechnology®, Texas, USA). Immunodetection was carried out using enhanced chemiluminescence (Luminata strong™ - Western HRP substrate, Merck-Millipore®, Massachusetts, USA). Digitalized images were analyzed with the densitometry ImageJ software (NIH, Public Domain). GAPDH was used as a control for any variation in protein loading.

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed with the GraphPad Prism 5.1. Significant differences between groups were determined using one-way ANOVA for reduction

of DPPH, lipid peroxidation induced by FeSO4, LVDP, BPM, ASI and CK.

Antioxidant enzymes activities, CAT, TBARS and SOD were determined using one-way ANOVA and two-way ANOVA for GPx enzymes, followed both by post-hoc Bonferroni and values of $p<0.05$ were considered statistically significant.

RESULTS

Phytochemical screening of EAcF

The phytochemical screening of EAcF revealed the presence of important secondary metabolites, as flavonoids, xanthones, leucoantocianidina, catechins, tannins and saponins, all with antioxidant effects already reported (Table I).

Total flavonoids and other phenolic compounds assay

The methanol extraction for 14 days produced an extract dried which was successively submitted to liquid-liquid extraction with hexane, chloroform, and ethyl acetate to obtain an ethyl acetate fraction (EAcF) more concentrated in bioactive compounds. This separation process of biocompounds from vegetal matrices provided total flavonoids and total phenolic compounds values estimated to be 536.84 ± 23.15 (RE)/g of dried EAcF, and 600.05 ± 32.16 (GAE)/g of dried EAcF, respectively.

Antioxidant properties of EAcF *in vitro*

The *in vitro* assay shows that EAcF antioxidant properties justified by the performance in scavenging DPPH$^{\cdot}$ (IC_{50} 20.86 ± 0.34 $\mu g.mL^{-1}$; $p<0.0001$; Figure 2a) and AAI were considered as strong (AAI = 1.4). Reinforcing these results, EAcF significantly decreased the lipid

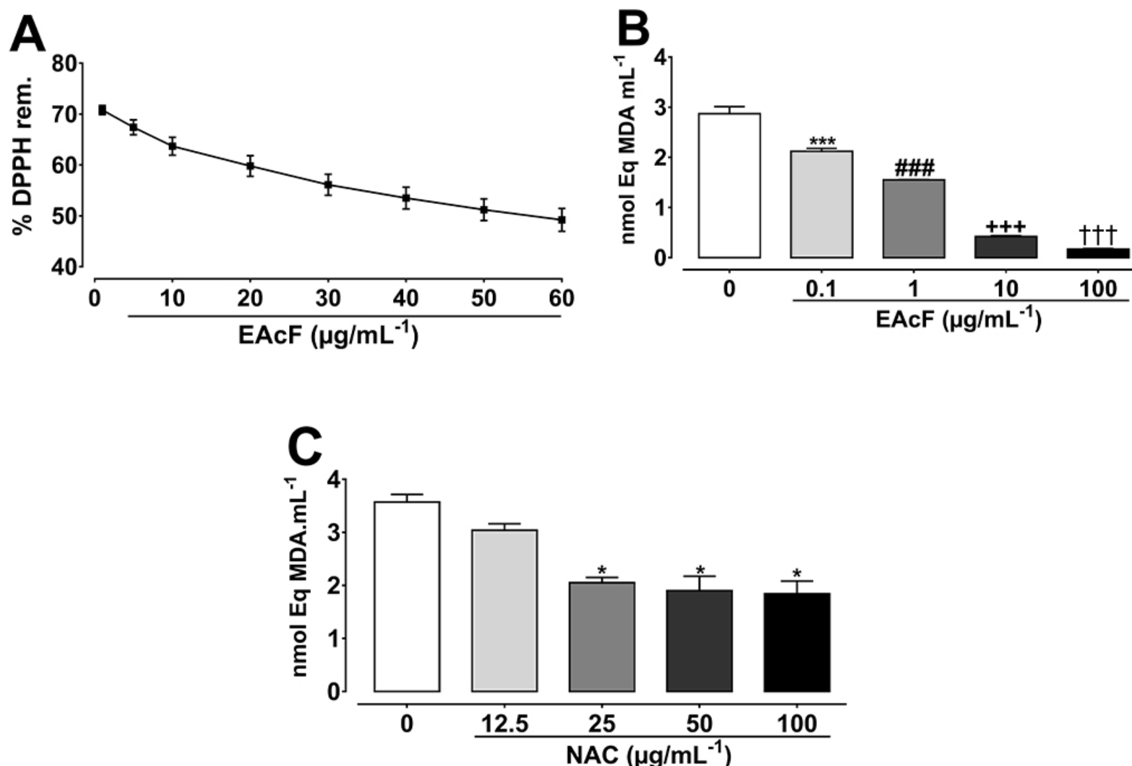


Figure 2. Effect of ethyl acetate fraction of *Canavalia rosea* (EAcF) and NAC on lipid peroxidation. (a) Kinetics of the reduction of DPPH; (b) lipid peroxidation induced by $FeSO_4$; (c) positive control of lipid peroxidation induced by $FeSO^4$. $^{***, ###, +++, †††}p<0.001$ compared with the previous concentration, $^{*}p<0.05$ compared with 12.5 concentration of NAC.

peroxidation induced by radicals generated from $FeSO_4$ ($p<0.0001$; Figure 2b). Comparing the percentage of inhibition using a positive control (NAC, Figure 2c), EAcF shows a concentration-dependent effect against oxidative damage in lipids, promoting inhibition of lipid peroxidation already at low concentration (0.1 $\mu g.mL^{-1}$). The MDA formation was almost abolished in high concentration (100 $\mu g.mL^{-1}$).

EAcF prevents reduction of cardiac contractility and lesion induced by I/R injury

The results revealed that myocardial I/R injury reduced significantly LVDP. However, the previous perfusion of EAcF (300 $\mu g.mL^{-1}$) was able to prevent the reduction of LVDP (Figure 3a) as well as the HR (Figure 3b) induced by myocardial I/R injury. The improvement of this performance induced EACF to be higher than that promoted by NAC. Furthermore, there was a reduction of ASI (arrhythmia severity index) in the hearts previously perfused with EAcF (Figure 3c) when compared to the Vehicle group, revealing reduced arrhythmia. These arrhythmic events were not observed in the Sham group. CK is an indicator of the severity of I/R-induced damage of the myocardial membrane and cellular injury. We observed that CK release was markedly augmented in Vehicle, which was fully inhibited by EAcF ($p=0.004$; Figure 3d).

EAcF prevents oxidative damage induced by I/R injury

The EAcF effects upon the oxidative stress after the injury induced by I/R were shown in Table II. Lipid peroxidation and enzymatic antioxidant activity (SOD, CAT, GPx and GR) were evaluated in hearts subjected to I/R injury. EAcF was able

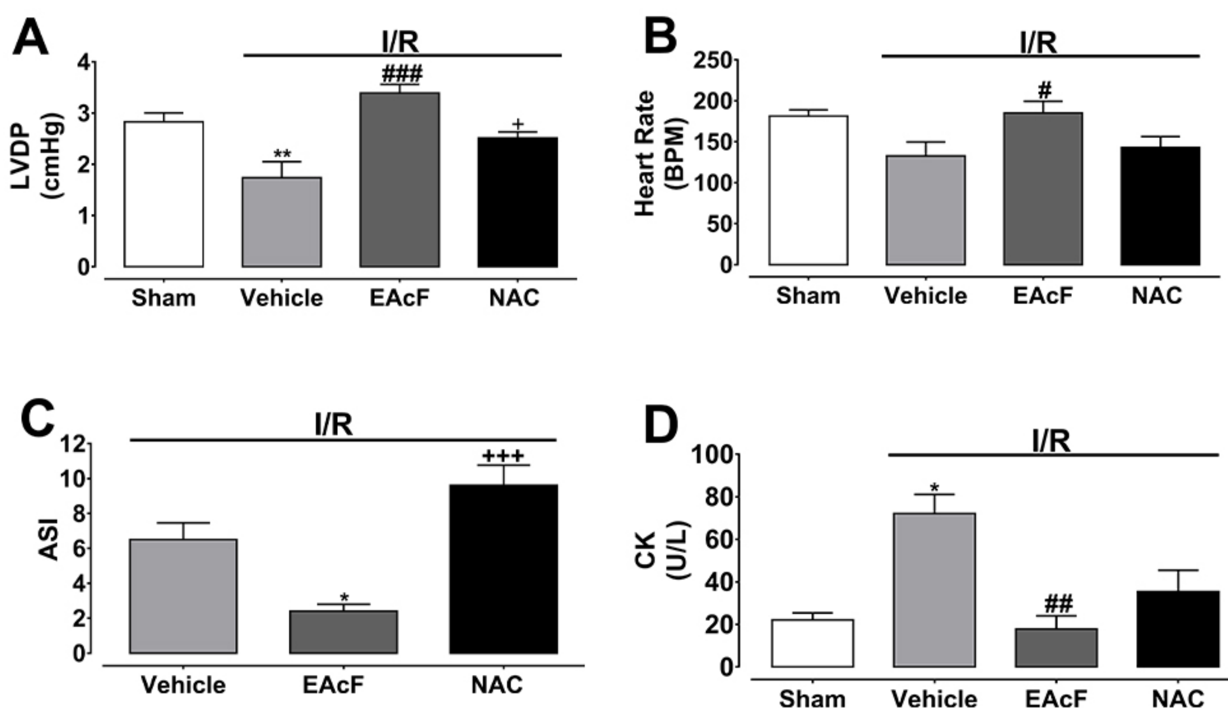


Figure 3. Ethyl acetate fraction of *Canavalia rosea* (EAcF) reduces myocardial ischemia-reperfusion injury. Effect of EAcF on the developed left ventricular pressure (a), heart rate (b) and arrhythmia severity index (c) in isolated heart; (d) Creatine Kinase (CK) levels in perfusate of isolated heart, all data made in hearts were submitted to ischemia and reperfusion. *p<0.05 or **p<0.01 compared to the sham group, #p<0.05 or ###p<0.001 compared to the Vehicle group, +p<0.5 or +++p<0.001 when compared to group EAcF (n = 6).

to prevent lipid peroxidation when compared to the Vehicle, just as did the NAC.

Furthermore, GPx activity was significantly increased and GR activity was reduced in the EAcF and NAC groups when compared to Vehicle group. However, there was no significant change in CAT and SOD activities in the EAcF group. SOD activity was reduced, and the CAT activity was increased in the NAC group compared to the Vehicle, indicating different mechanisms of action of these antioxidant substances.

EAcF inhibits ischemic-induced increases in myocardial O_2^-.

As shown in the representative photomicrographs and summary data in Figure 3, EAcF caused a marked decrease in O_2^- production, as indicated by the 4.5-fold increase in ethidium fluorescence in Vehicle + I/R compared with vehicle-treated rat.

EAcF prevents apoptosis in ischemic-reperfused hearts

To further evaluate whether EAcF provides cardioprotection against apoptosis, we evaluated caspase 3 protein expression. The caspase 3 expression was significantly downregulated in EAcF group when compared to Vehicle group. Similar effects were seen in the NAC group (Figure 4).

DISCUSSION

The limitations of injury caused by I/R may offer new alternative therapeutics. Previous studies based on plants, especially those rich in flavonoids, and other phenolic compounds, have verified their beneficial effect in several tissues, including in hearts submitted to I/R injury (Draginic et al. 2022, Yuan et al. 2015).

This antioxidant effect was confirmed *in vitro* and may be due to the presence of flavonoids, saponins, xanthones, condensed tannins (polymers formed by the condensation of flavans) detected in its composition. These results were in accordance by Sridhar & Seena (2006), who studied the seed processing of *Canavalia spp*.

Flavonoids may prevent propagation of oxidative reactions, reinforcing cellular antioxidant capacity besides possessing anti-inflammatory and anti-platelet aggregation effects, resulting in lower oxidant production by scavenging ROS and better re-establishment of blood in the ischemic zone (Kanaan & Harper 2017). In addition, catechins, derived from flavonoids, originate the various structural types of the class of condensed tannins to be scavengers of active oxygen such as the superoxide anion radical, singlet oxygen and hydroxyl radical (Nakao et al. 1998).

The antioxidant potential of EAcF was confirmed through the DDPH$^{\cdot}$ assay. The IC_{50} and

Table II. Enhanced endogenous antioxidant enzymes activities in ischemic-reperfused hearts previously with ethyl acetate fraction of *Canavalia rosea* (EAcF).

	TBARS (MDA nmol·mg^{-1} tissue)	SOD (U·µg^{-1} protein)	CAT (ΔE·min^{-1}·µg^{-1} protein)	GPx (NAPDH nmol ·min^{-1}·µg $^{-1}$ protein)
Sham	0.07 ± 0.003	5.76 ± 0.95	0.03 ± 0.001	0.62 ± 0.005
Vehicle + I/R	0.20 ± 0.020***	9.00 ± 1.05	0.03 ± 0.004	0.51 ± 0.008
EAcF + I/R	0.04 ± 0.001$^{\#\#\#}$	11.99 ± 0.83*	0.01 ± 0.004	0.99 ± 0.004$^{***,\#\#\#}$
NAC + I/R	0.07 ± 0.010$^{\#\#\#}$	6.36 ± 0.36*	0.14 ± 0.034$^{\Psi}$	3.41 ± 0.063$^{***;\#\#\#}$

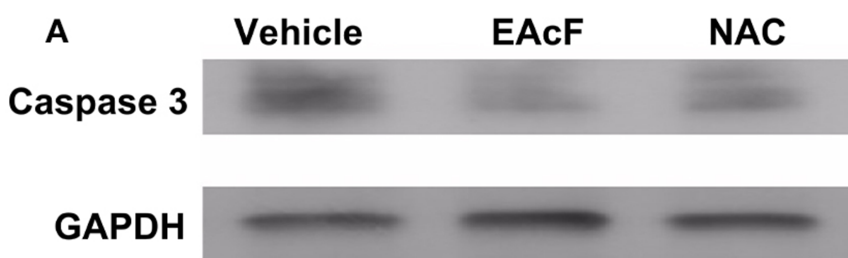
Compared to *Sham:* *p<0.05, **p<0.01,*p<0.001. Compared to Vehicle + I/R: $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001. Compared to Vehicle + I/R and EAcF + I/R: $^{\Psi}$p<0.01.**

IP parameters, for mixed compounds, indicate plant extracts which are more suitable as sources of pure antioxidant compounds (Argolo et al. 2004). In the present study, it was demonstrated that EAcF of *C. rosea* leaves have the antioxidant potential to stabilize the free radical DDPH$^{\cdot}$, similar to that found in other studies (20.86 ± 0.34 *vs* 32.2 ± 2.02 $\mu g.mL^{-1}$) (Panda & Naik 2008, Sun et al. 2002).

Likewise, free iron, released from heme proteins, also has the potential to form complex radical, which remove hydrogen atoms of polyunsaturated fatty acids (Lu et al. 2011) and cause lipid peroxidation. The significant increase in the Fe^{2+} chelating capacity of *C. maritima* related by Niveditha & Sridhar (2012), can thus prevent the formation of iron-catalyzed free radicals. It is likely that has happened in our study due to the reduced detection of MDA as the product in the ischemic tissue samples.

During ischemia, the ATP production is impaired due to the disruption of electron transport chain. The cell was depleted from high energy phosphates. Following these injuries, pyruvate oxidation decreases, and glycolysis, anaerobic metabolism, intracellular acidosis due to hydrogen ion (H^+) accumulation and death cell occur (Rodrigo et al. 2021). As a result, contractile function declines and Na^+ and Ca^{2+} ions increase in the cytosol. During recovery, the blood supply of the tissues trigger the production of oxygen free radicals and cause more damage (Kanaan & Harper 2017; Zhou et al. 2015). It should be pointed out that global ischemia (30 min) followed by reperfusion (60 min) in isolated rat hearts was associated with depressed contractile function as indicated by decreased LVDP (Ali et al. 2015).

Infusion of EAcF in hearts was able to improve LVDP, reduce arrhythmias and CK level in perfusated after I/R when compared to the Vehicle and NAC groups. Similarly, other plant derivates as *Bacopa monnieri and C. intybus* extract improved post-ischemic contractile function as compared with pre-ischemic status (Sadeghi et al. 2015, Srimachai et al. 2017). These improvements can be related to the above-mentioned antioxidant effects of *C. rosea*. The NAC attenuated changes in LVDP due to ischemia also in other studies (Dhalla et al. 2000).



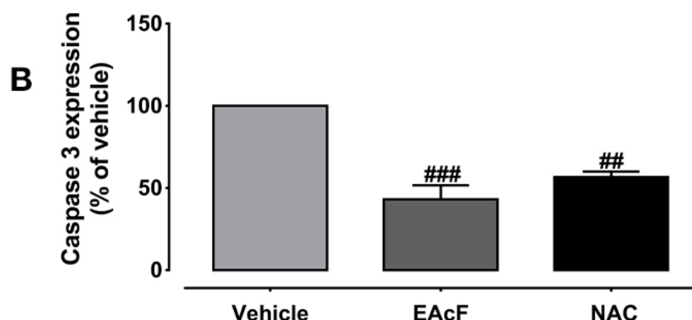


Figure 4. Ethyl acetate fraction of *Canavalia rosea* (EAcF) reduces expression of Caspase 3 in ischemic hearts. Top, representative image of western blot. Bottom, quantitative analysis. ##p<0.01 or ###p<0.001 compared to the Vehicle group (n = 6).

In different animal models, the return to normal pH after a period of acidosis is particularly prone to arrhythmias, which can evolve to ventricular tachycardia and fibrillation (Lascano et al. 2013, Roever et al. 2017). The evaluation of ASI showed a significant reduction in the group previously perfused with the EAcF, being significantly better than the group previously perfused with the Vehicle or NAC. The data suggest that the EAcF could prevent arrhythmias arising from the post-ischemic reperfusion, corroborating these results, which showed a significant decrease in the levels of CK in the perfusate of hearts submitted to I/R injury that had been previously perfused with EAcF (Figure 3).

During the reperfusion, the production of exacerbated reactive oxygen species triggers the reduction of cardiac function and increased arrhythmias. Thus, the enzymatic antioxidant system is the main mechanism defense in cardiomyocytes exposed to an oxidant environment (Münzel et al. 2017, Paixão et al. 2013, Scherer & Godoy 2009). The peroxidation of lipidic membrane causes structural damage that results in leakage of myocardial enzymes (Zhou et al. 2015). These events are reduced by catalase, superoxide dismutase and glutathione peroxidase (Budni et al. 2007, Ighodaro & Akinloye 2017), and are responsible for the maintenance of cellular integrity in different pathophysiological conditions (Ferreira et al. 2001, Kurutas 2016, Manoli et al. 2000, Paixão et al. 2013), such as ischemic and reperfusion cardiac.

It was observed that the EAcF increased GPx and decreased GR activities (Table II), which can have contributed to the reduction of lipid peroxidation (Table II) in cardiac tissue. We showed, also, a reduction in caspase 3 expression (Figure 4). Other plant extracts exhibit effects under the antioxidant enzymatic cellular system, the *S. miltiorrhiza* ethanol extract caused a significant increase in activities, in the myocardium, SOD, GPx and CAT and decreased MDA level (Grau et al. 2000, Jentzsch et al. 1996). Yet, the treatment of animals with *Ginkgo biloba* increased SOD and GPx activities, enhanced total glutathione levels and reduced MDA preventing ischemia and myocardial reperfusion lesions (Panda & Naik 2008). Also, the pre-treatment showed anti-apoptotic property (Qiao et al. 2014).

However, neither all plant extracts with antioxidant action have the ability to reduce lipid peroxidation, as it was seen with the administration of *Desmodium gangeticum* extract that did not reduce MDA in myocardium, but enhanced the recovery of antioxidant enzymes from the aggression of I/R injury (Kurian et al. 2010).

In conclusion, our data show that the previous perfusion of the heart with the EAcF of *Canavalia rosea* leaves prevents the cardiac arrhythmias and impaired left ventricular contractility caused by ischemia-reperfusion injury. These effects may be caused through two mechanisms with independent or synergetic actions. These mechanisms can include antioxidant activity of its components, sequestering directly radicals, as seen in some flavonoids, and due to an enhanced antioxidant enzyme GPx activity attenuating the oxidative stress and reducing cellular damage and cell death. However, additional studies such as the identification of compounds responsible for the cardioprotective and antioxidant activity of the EAcF of *Canavalia rosea* leaves should be carried out before the clinical application of the extract.

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Author contributions

MBJF: Participated in all stages of the work. Left ventricular developed pressure (LVDP) and heart rate (HR) measurements, Arrhythmias severity index (ASI), Creatine kinase (CK) measurements, Measurement of lipid peroxidation ex vivo, Antioxidant enzyme activity and Measurement of O2·- production in myocardium. ALBSB, SSA, and CSE: Plant collection, extraction and phytochemical screening. AMB: Determination of total phenolic and flavonoid contents. LAS and GKMA: Reduction of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) and Measurement of lipid peroxidation and data analysis. TRRB and RMS: Western blot analysis. CRG and CMLV Data analysis and interpretation. ERM and RGA: Writing of the manuscript. EAC and SLS: Conception, design of the study and Critical revision of the text.