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Article

# Nephroprotective effects of *Lippia sidoides* ethanolic extract against ischemia/ reperfusion-induced acute kidney injury

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Ischemia/reperfusion injury (I/R) is commonly related to acute kidney injury (AKI) and oxidative stress. Antioxidant agents are used to treat this condition. *Lippia sidoides* is a brazillian shrub with anti-inflammatory and anti-oxidative properties. Thus, the aim of this study is to evaluate the effect of *Lippia sidoides* ethanolic extract (LSEE) on *in vivo* and *in vitro* models of AKI induced by I/R. Male Wistar rats were submitted to unilateral nephrectomy and ischemia on contralateral kidney for 60 min via clamping followed by reperfusion for 48 h. They were divided into four groups: Sham, LSEE (sham-operated rats pre-treated with LSEE), I/R (rats submitted to ischemia) and I/R-LSEE (rats treated with LSEE before ischemia). Kidney tissues homogenates were used to determine stress parameters and nephrin expression. Plasma and urine samples were collected for biochemical analysis. I/R i*n vitro* assays were evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and flow cytometry assays in Rhesus Monkey Kidney Epithelial Cells (LLC-MK2). The LSEE treatment prevented biochemical and nephrin expression alterations, as well as oxidative stress parameters. In the *in vitro* assay, LSEE protected against cell death, reduced the reactive oxygen species and increased mitochondrial transmembrane potential. LSEE showed biotechnological potential for a new phytomedicine as a nephroprotective agent.

**Keywords:** Alecrim-pimenta. Acute kidney injury. Nephrin. Thymol.

## INTRODUCTION

Renal Ischemia Reperfusion (I/R) injury is linked to a variety of clinical disorders, such as those observed in patients under renal transplantation, heminephrectomy, sepsis and other hypoxic insults, culminating with a sudden decrease in renal function and clinical acute kidney injury (AKI) (Li, Burdmann, Mehta, 2013). The mechanisms of I/R injury are primarily related but not limited to inflammatory response, including oxidative stress through the release of reactive oxygen species (ROS)

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and metabolic disorders (Ponticelli, 2014; Schröppel, Legendre, 2014). Several strategies are studied in order to prevent or minimize AKI, including antioxidants agents (Palli *et al*., 2017).

In this line, herbal species have been studied to promote the oxidoreductive balance and protect the renal tissue (Pascual *et al*., 2001; Zhao *et al*., 2019). *Lippia sidoides* is a *Verbenaceae* plant known in Brazil as "alecrim-pimenta", originally found in northeastern Brazil, but cultivated in several Brazilian states due its use in folk medicine as antiseptic and anti-inflammatory (Matos, Oliveira, 1998; Monteiro *et al*., 2007). The therapeutic effect of *L. sidoides* is attributed mainly to the presence of thymol, which is the major component of the plant's essential oil and it is also found in ethanolic extracts ( Matos, Oliveira, 1998). The extract is easier to prepare, and it has lower costs compared with the essential oil (Aparecida Braga *et al.* 2019), besides the higher stability and practicality of a powder. Thus, the aim of the present study was to evaluate the nephroprotective effect of *Lippia sidoides* ethanolic extract (LSEE) against I/R-induced AKI.

## METHODS

### Collection of *Lippia sidoides* and preparation of ethanolic extract

The leaves of *Lippia sidoides* were collected from the Medicinal Plants Garden Prof. Francisco José Abreu Matos of the Federal University of Ceará (UFC). The identification of the plant was carried out through *exsiccatae,* registered (n° 25149) at the Prisco Bezerra Herbarium of UFC.

The *L. sidoides* Ethanolic Extract (LSEE) was obtained from the Laboratory of Phytochemistry of Medicinal Plants located at the UFC's Pici Campus. The leaves were washed with distilled water and oven dried (72 hours at 35 °C). The dried leaves were then milled in a Wiley mill and the obtained powder was submitted to extraction in 70 % ethanol at room temperature, by static maceration. The obtained extract was filtered and the supernatant was collected, then, the supernatant was subjected to solvent removal on a rotary evaporator (at 45 °C), frozen, and lyophilized. The lyophilized extract was then dissolved in water before the experiment.

### Phytochemical Screening of Lippia sidoides Ethanolic extract

The powder obtained from ethanolic extracts of *L. sidoides* leaves was submitted for phytochemical screening. According to previously described pharmacognostic techniques for each chemical group identification, specific reagents were used to identify the presence of each class of substances (Matos, 1996a, 1996b). The Medicinal Plants Garden Prof. Francisco José Abreu Matos is a germplasm bank, therefore, the aim was to verify the preserved genetic characteristics of the plant. The thin-layer chromatography (TLC) method on silica was performed to characterize thymol and quercetin in the extract. Commercially analytical grade dichloromethane and hexane (6:4, v/v) (St. Louis, MO, USA) were used as the mobile phase. LSEE was analyzed by Gas Chromatography coupled to Mass Spectrometry (Shimadzu GCMS QP2010s). The identification of the constituents was performed by interpreting the respective mass spectra by computerized comparison of the fragmentation records (m/z) with the spectra available in the NIST (National Institute of Standards and Technology) database.

### Experimental groups

The experimental protocol, which was approved by the Ethical Committee on Animal Research of UFC (no. 106/2017), was in accordance with the ethical guidelines. Male Wistar rats were obtained from the central bioterium of UFC, weighing approximately 200 g. They were kept under controlled conditions (25 ± 2 °C room temperature, 12 h shift of the light-dark cycle). Food and water were provided *ad libitum*. Oral gavage of all animals was performed 3 days prior to the experiment and 1 h before the surgical procedure. The animals were divided into four experimental groups (n = 6):

SHAM: vehicle treated group; submitted to SHAM surgery;

SHAM-LSEE: SHAM group, treated with LSEE (150 mg/kg of weight), and submitted to SHAM surgery.
Ischemia/ Reperfusion (I/R): vehicle treated group; submitted to I/R.
I/R-LSEE: group treated with LSEE (150 mg/kg of weight) and submitted to I/R.

### Surgical procedure

Animals were anesthetized with intraperitoneal 90 mg/kg of ketamine (Ketalar®, Pfizer) and 10 mg/kg xylazine (Cristália Pharmaceutical and Chemical, São Paulo, Brazil). A laparotomy incision was performed. A right nephrectomy followed by an ischemia in the left kidney by clamping the renal artery for 60 min, was performed. Then, the procedure was followed by a 48-h of reperfusion (Hauet *et al*., 2000; Sampaio *et al*., 2016; Tillet *et al*., 2015).

During surgery, the body temperature of the animals was maintained between 36.5 and 37 °C. Temperature, heart rate, and respiratory frequency were checked every half an hour. Sham operations without the clamping of the renal artery were performed.

After surgery, the animals were observed for a 48-h of reperfusion period. During the last 24h, urine samples were obtained by a metabolic cage. At the end of this period, blood samples were collected. Additionally, left kidneys for homogenate obtention were collected.

### Measurement of biochemical parameters

Blood samples were collected in tubes containing heparin, and they were centrifuged (2500 RCF for 10 min) to collect the plasma. Urinary and plasma concentrations of protein, albumin and urea were measured by using an automatic analyzer (Roche Diagnostics Limited, Rotkreuz Switzerland). Creatinine clearance (CrCl) was calculated by using the formula: $CrCl = (V\,min \times uCr)/pCr$, in which *V min* is the urinary volume per minute, and *uCr* and *pCr* are urinary and plasmatic creatinine levels respectively, as estimated glomerular filtration rate (GFR) (Darling, Morris, 1991). The albumin/creatinine ratio was also calculated. Furthermore, plasmatic and urinary levels of sodium ($Na^+$), potassium ($K^+$) and chloride ($Cl^-$) were measured by using an ion selective electrode (Roche Diagnostics Limited, Rotkreuz Switzer-land). The fractional excretion of such electrolytes (%FE) was calculated as $\%FE = ([U]/[P])/(uCr)/pCr) \times 100$, in which [U] and[P] are, respectively, the urinary and plasmatic concentrations of each electrolyte. Urinary Kidney Injury Molecule (KIM-1) and Neutrophil gelatinase-associated lipocalin (NGAL) were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, IncCat. RKM100).

### Western blotting analysis

To quantify the nephrin protein, a kidney tissue was homogenized using by Tissue Lysis (Thermo Scientific, USA) in lysis buffer (RIPA; 25 mM Tris-HCl, pH 7.6; 150 mM NaCl; ethylenediaminetetraacetic acid (EDTA) 5 mM, 1% NP40, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS) and protease inhibitor (1 mL inhibitor: 100 mL RIPA). The lysed solution was centrifuged (8,000 g, 10 min, 4 °C) and the supernatant was collected. The protein concentration was determined by the acid assay employing the Pierce TM bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, USA). Laemmli buffer with β-mercaptoethanol was added to the samples (40 μg) and denatured at 98 °C for 5 min. Then, proteins were separated though electrophoresis (2h at 100 V) on a Sodium Dodecyl Sulfate (SDS) polyacrylamide gel (8%) and transferred to the nitrocellulose membrane (120 V). The membranes were blocked in 5% bovine serum albumin for 1.5 h and they were incubated overnight with the primary antibodyin rabbits: anti-Nefrin (1:1,000) and anti-β-actin (1:500) (Novus Biologicals, USA). Chemiluminescent detection was performed with the Clarity Western Enhanced Chemiluminescence (ECL) Substrate kit (Bio-Rad) after incubation with the secondary anti-rabbit antibody (1:1,000) (Bio-Rad) for 1.5h, using the ChemiDoc system (Bio-Rad). The densitometric quantification of the bands was performed using the ImageLab software, version 6.0 (Bio-Rad).

### Determination of oxidative stress

The left kidney was removed and was used to produce the homogenate for oxidative stress analysis. The samples were weighed and kept in an ice bath and the homogenization was performed in 1 mL of 0.1 M potassium phosphate buffer with 5 mM EDTA, 0.1% Triton X-100 and 0.6% sulfosalicylic acid (pH 7.5). Then, the obtained suspension was centrifuged at 3,000 g for 4 min.

The malondialdehyde (MDA) concentration in the kidney tissue was estimated by the Thiobarbituric Acid Reactive Substances (TBARS) method as an indicator of lipid peroxidation (Gyurászová *et al*., 2019). Thiobarbituric acid (0.6%) was added to homogenate, the mixture was incubated at 96 °C for 20 min. The absorbance was measured by spectrophotometry at 532 nm (Asys UV 340, Biochrom, Cambridge, UK). The results were expressed in µg/ g tissue.

The glutathione (GSH) levels were measured according to the 5,5-dithiobis (2-nitrobenzoic acid DTNB) method. DTNB reacts with reduced GSH on the homogenate to form a yellow product. The optical density of the samples and their pattern were spectrophotometrically measured at 412 nm (Asys UV 340, Biochrom, Cambridge, UK). The results were expressed in µg/ g tissue.

### Tubular kidney cells culture and hypoxia/ reoxygenation assay

The proximal renal tubular cells LLC-MK2 were donated by the Department of Biochemistry of the University of São Paulo and cultured in Dulbecco's modified Eagle's medium (DMEM, invitrogen, USA), supplemented with 10% fetal bovine serum (FBS) in standard conditions. To determine non-toxic LSEE concentrations, LLC-MK2 cells were treated with LSEE at several concentrations (1000; 500; 250; 125; 62.5 and 31.25 µg/mL). To guarantee that DMSO concentrations in the experimental groups never exceeded 0.5%, two-fold dilutions were performed. After 24h, the cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St Louis, MO, USA) assay (Vanden Berghe *et al*., 2013).

To induce acute damage *in vitro,* cells ($1 \times 10^5$ cell/ mL) were cultivated in 96-well plates for 24 h to allow cell adhesion and proliferation. To induce hypoxia, the normal growth culture medium was replaced by glucose, pyruvate, and FBS deprived DMEM. The plates were also incubated within an anaerobic chamber during 24h. Reoxygenation was performed by adding a complete culture medium as previously described (Kurian, Pemaih, 2014; Sampaio *et al*., 2019). After 3h of re-oxygenation, the plates were treated with non-toxic LSEE concentrations, in order to observe recovery.

### Flow cytometry assays

The LLC-MK2 were submitted to hypoxia-reoxygenation protocol, treated with LSEE 62.5 µg/mL (lower concentration, which generates protection), and incubated for 24 h. Those cells submitted to I/R were used as positive control and the not submitted to I/R were used as negative control. After that, the cells were labeled with specific probes and submitted to flow cytometry analysis.

To evaluate the production of cytoplasmic ROS, the 2',7'- dichlorofluorescein diacetate (DCFH-DA) was used. Cytosolic ROS oxidizes DCFH and forms a fluorescent product. The cells were washed with Phosphate buffer saline (PBS), labeled with DCFH-DA (100µM) and analyzed in the flow cytometer, to verify the increase of green fluorescence. ROS production was determined by considering the ratio of the geometric mean of FL1 signal intensity in comparison with the control (O'Connor *et al*., 1998).

The rhodamine 123 (Rho123) (Sigma-Aldrich, St. Louis, MO, EUA) is a fluorochrome that stains mitochondrial matrix, seeking to evaluate the mitochondrial transmembrane potential. LLC-MK2 cells were washed with PBS and stained with Rho123 (10 µg/ mL) for half an hour. After that, the cells were analyzed in the flow cytometer to measure the decrease in Rho123 accumulation in mitochondria. The transmembrane mitochondrial potential ($\Delta\Psi$m) was determined by considering the ratio of the geometric mean of FL2 signal intensity in comparison with the control.

### Statistical analysis

All data were expressed as mean ±standard error of mean (SEM). For statistical comparisons between the groups, one-way and/or two-way ANOVA was used, followed by Bonferroni test. Significance was set at $p < 0.05$. Statistical analyses were performed using GraphPad Prism 6.0 (USA).

## RESULTS

### Phytochemical Screening of *Lippia sidoides* Ethanolic extract

According to previously described pharmacognostic techniques, the presence of flavonoids, catechin tannins, anthraquinones and steroids were detected in LSEE - Table I. The presence of thymol and quercetin, having Rf 0.62 and Rf 0.48, respectively, were confirmed by TLC analyses.

**TABLE I -** Phytochemical Screening of *Lippia sidoides* Ethanolic extract by pharmacognostic techniques

| Secondary metabolites | Result |
|---|---|
| Alkaloids | Absence |
| Anthocyanidins | Absence |
| Anthraquinones | Presence |
| Coumarins | Absence |
| Cyanogenic Heterosides | Absence |
| Digitalis Hetorosides | Absence |
| Flavonoids | Presence |
| Steroids | Presence |
| Tannins | Presence (catechin tannins) |

Gas Chromatography Mass Spectrometry (GC/MS) analyses of LSEE showed the main constituents with the respective retention times displayed in Figure 1: thymol (63.25%), beta-caryophyllene (12.47%), and p-cymene (10.09%). Moreover, we identified alpha-phellandrene (1.35%), myrcene (2.58%), alpha-terpinene (1.63%), eucalyptol (1.26%), gamma-terpinene (4.84%), byciclogermacrene (1.76%) and alloaromadendrene (0.78%).

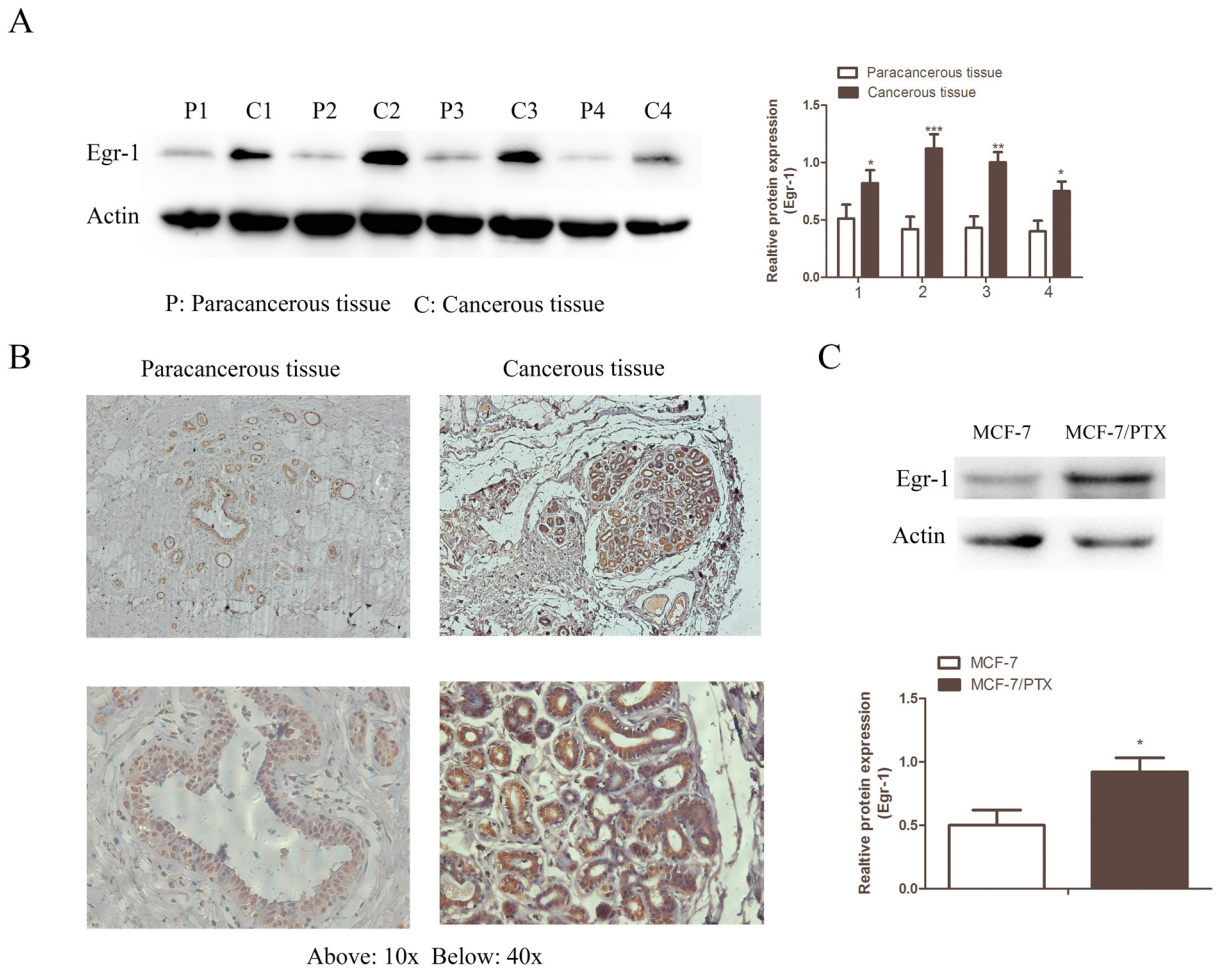


**FIGURE 1 -** Chromatogram of GC-MS from *Lippia sidoides* Ethanolic extract.

### Effect of LSEE in I/R-induced AKI

As expected, I/R kidney injury caused an intense reduction in GFR, evidenced by an increment in pCr, pUr and a marked reduction in CrCl. Also, we evidenced an increase in the urinary albumin/creatinine ratio. Previous treatment with LSEE partially protected all renal alterations induced by I/R injury.

In relation to markers of tubular injury, the I/R group has an increment in the EF of sodium, potassium and chloride, besides a marked increment in the new biomarkers urinary KIM-1 and NGAL. All these markers were significantly attenuated by the LSEE pre-treatment – Table II and Figure 2.

**TABLE II -** Analysis of traditional biochemical parameters after I/R injury in urine and blood samples

| | SHAM | SHAM-LSEE | I/R | I/R-LSEE |
|---|---|---|---|---|
| pCr | 0.311 ± 0.013 | 0.303 ± 0.012 | 1.08 ± 0.06 * | 0.55 ± 0.02 *# |
| pUR | 30.67 ± 1.14 | 34.67 ± 2.18 | 149.8 ± 10.31 * | 56.80 ± 1.80 *# |

**TABLE II -** Analysis of traditional biochemical parameters after I/R injury in urine and blood samples

| | SHAM | SHAM-LSEE | I/R | I/R-LSEE |
|---|---|---|---|---|
| ACR | 19.27 ± 0.92 | 16.40 ± 0.54 | 128.4 ± 16.45 * | 43.49 ± 4.70 # |
| CrCl | 1.53 ± 0.06 | 1.62 ± 0.09 | 0.13 ± 0.02 * | 0.66 ± 0.04 *# |
| FENa+ | 0.12 ± 0.009 | 0.108 ± 0.006 | 1.10 ± 0.12 * | 0.37 ± 0.05 # |
| FEK+ | 13.25 ± 0.56 | 11.42 ± 0.85 | 89.96 ± 8.02 * | 27.04 ± 2.94 *# |
| FECl- | 0.38 ± 0.02 | 0.42 ± 0.03 | 4.00 ± 0.30 * | 0.94 ± 0.05 # |

pCr – Plasmatic creatinine (mg/dL); pUR– Plasmatic urea (mg/dL); CrCl – Creatinine clearance (mL/min); $FENa^{+}$–Fractional excretion of sodium (%); $FEK^{+}$–Fractional excretion of potassium (%); $FECl^{-}$ – Fractional excretion of chloride (%); ACR – Albumin/ Creatinine ratio (mg/g-Cr). *p < 0.05 vs. Sham, #p< 0.05 vs. I/R.

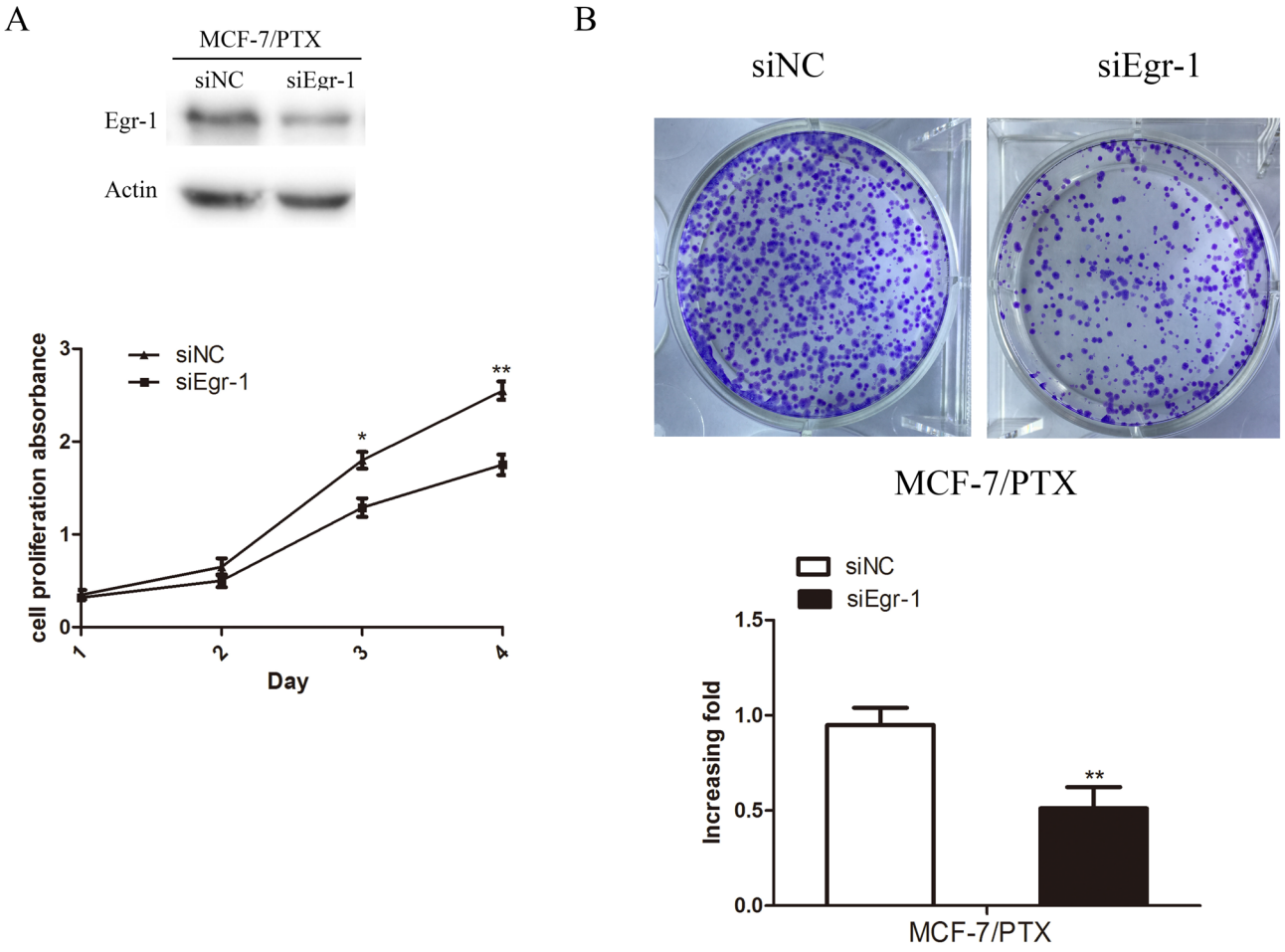


**FIGURE 2 -** The effect of Lippia sidoides on KIM-1 (A) and NGAL (B). Values shown are mean ± SE. Mean of 6 rats for each group. *p < 0.05 vs. Sham, #P < 0.05.

### Analysis of Nephrin by Western blotting

In order to investigate the glomerular damage after I/R, and the protection exerted by the LSEE in this tissue, the expression of nephrin after I/R and LSEE treatment was evaluated by Western blot. Animals in the I/R group had a mean reduction of 54% in the nephrin expression. LSEE was able to prevent the down regulation of nephrin in comparison with the I/R group – Figure 3.

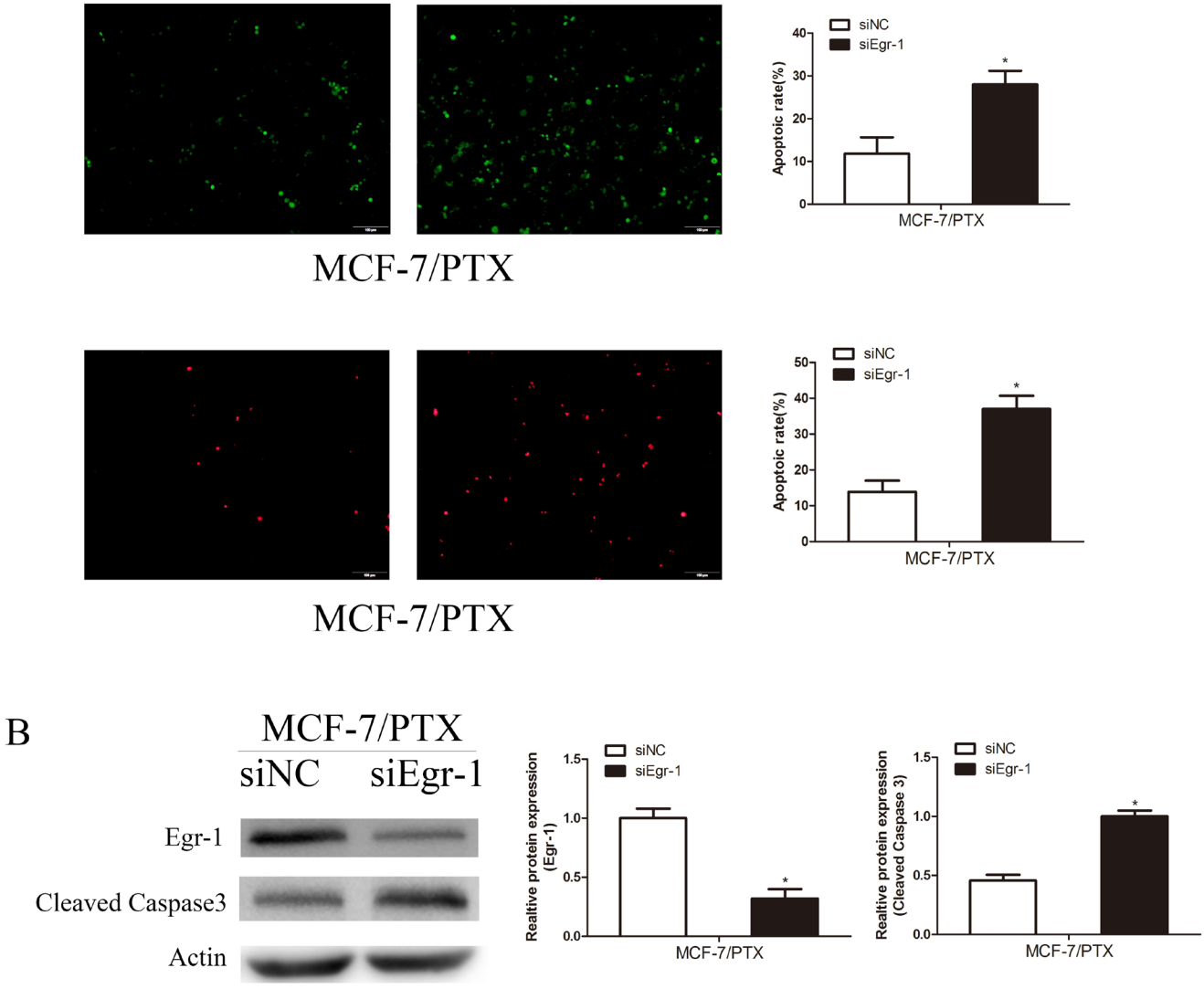


**FIGURE 3 -** Nephrin expression. (A) graphical representation after actin housekeeping normalization; n = 6/group; (B) representative image of Western blot analysis of Nephrin and actin in renal tissue according to each group. *p < 0.05 vs. Sham, #p< 0.05 vs. I/R.

### TBARS and GSH levels

The REDOX balance associated with I/R-induced AKI is shown in Figure 4. Oxidative stress in the I/R group was assessed by detecting the MDA increase and GSH decrease after the experiments. The LSEE pre-treatment protected the increasing in the renal MDA levels and the decreasing in the GSH levels, when compared to the I/R group.

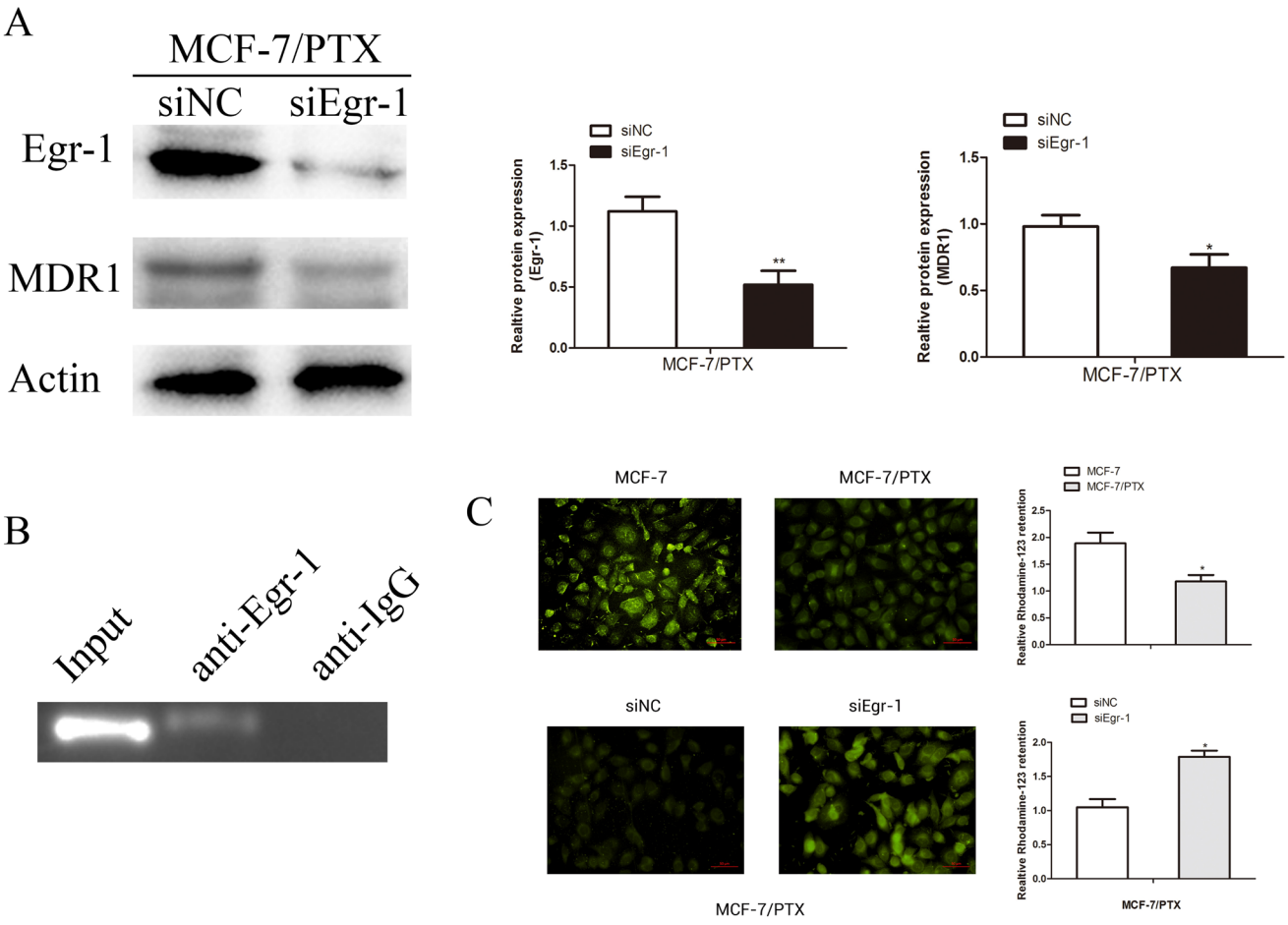


**FIGURE 4 -** *Lippia sidoides* treatment was effective in inhibiting oxidative stress and improving the antioxidant enzymatic activities. (A) Malondialdehyde (MDA) determination. (B) Reduced glutathione (GSH) determination. Values shown are mean ± SEM. Mean of 6 rats for each group. *p < 0.05 vs. Sham, #p < 0.05 vs. I/R.

### Cell viability assay

Among the studied concentrations, the LSEE presented cytotoxicity on LLC-MK2 cells at 500 and 1000 µg/mL, as shown in Figure 5A. Subsequently, to evaluate the protective effect, only non-toxic concentrations were chosen. Additionally, Figure 5B showed that I/R was able to reduce the viability by around 50% when compared to the control group. There was an increase in cell viability in the groups treated with LSEE, especially at 62.5 µg/mL (83.98% ± 1.38) and 125 µg/mL (71.37% ± 1.19) (Figure 5B).

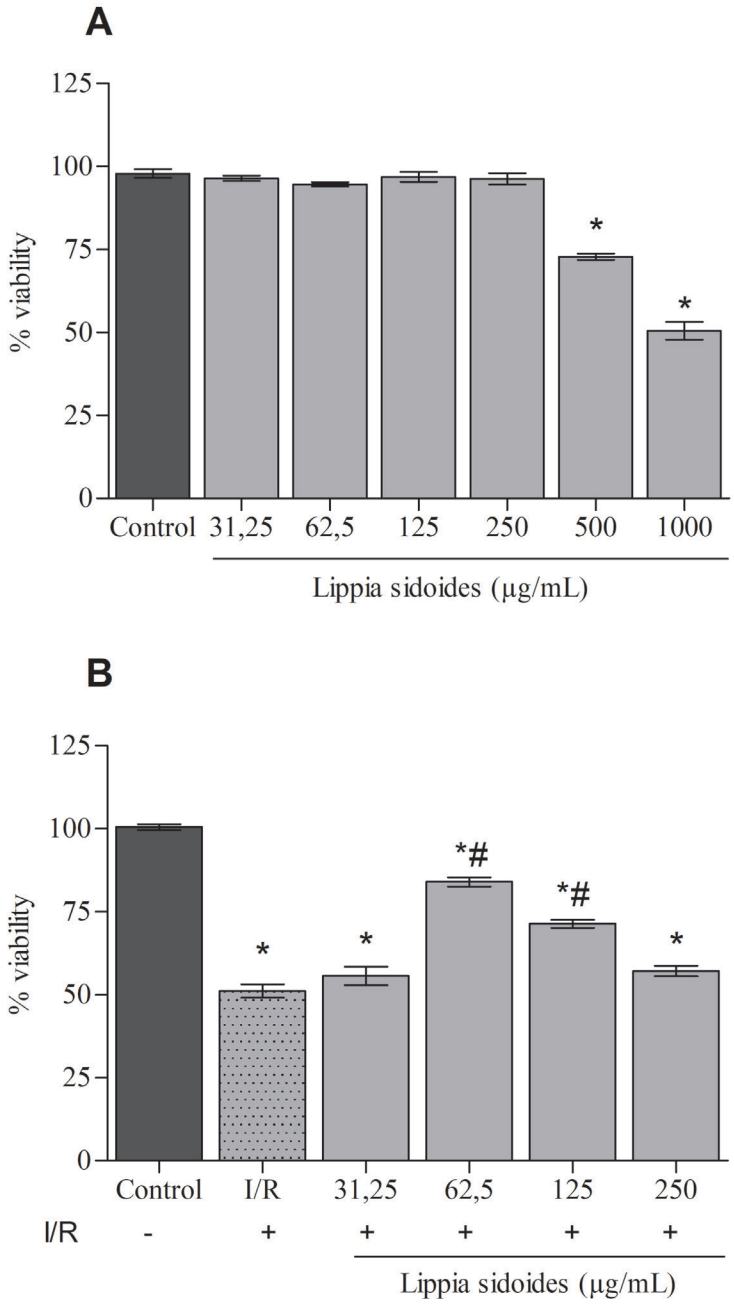


**FIGURE 5 -** *Lippia sidoides* treatment was able to improve cell viability culture assay. (A) Evaluation of cytotoxicity on LLC-MK2 cells on aerobic conditions. (B) Effect of *Lippia sidoides* on I/R. Values shown are mean ± SEM. *p < 0.05 vs. control, #p < 0.05 vs. I/R.

### Flow cytometry assays

In order to analyze cytoplasmic ROS production, DCFH-DA assay was performed in cells submitted to hypoxia and reoxygenation and treated with LSEE 62.5 µg/mL. Non-treated cells submitted to an aerobic atmosphere were used as a control group. The LSEE treatment reduced the ratio of relative fluorescence intensity to (1.02 ± 0.03 vs. 1.5 ± 0.03) indicating an antioxidant effect.

The analysis of mitochondrial transmembrane potential using Rhodamine 123 showed a reduction of 54 % in the relative fluorescence intensity in the I/R group

when compared to the control. The treatment with LSEE was able to increase Rho 123 accumulation, increasing the ratio (0.98 ± 0.05 vs. 0.46 ± 0.03), indicating that LSEE protects against mitochondrial depolarization (Figure 6).

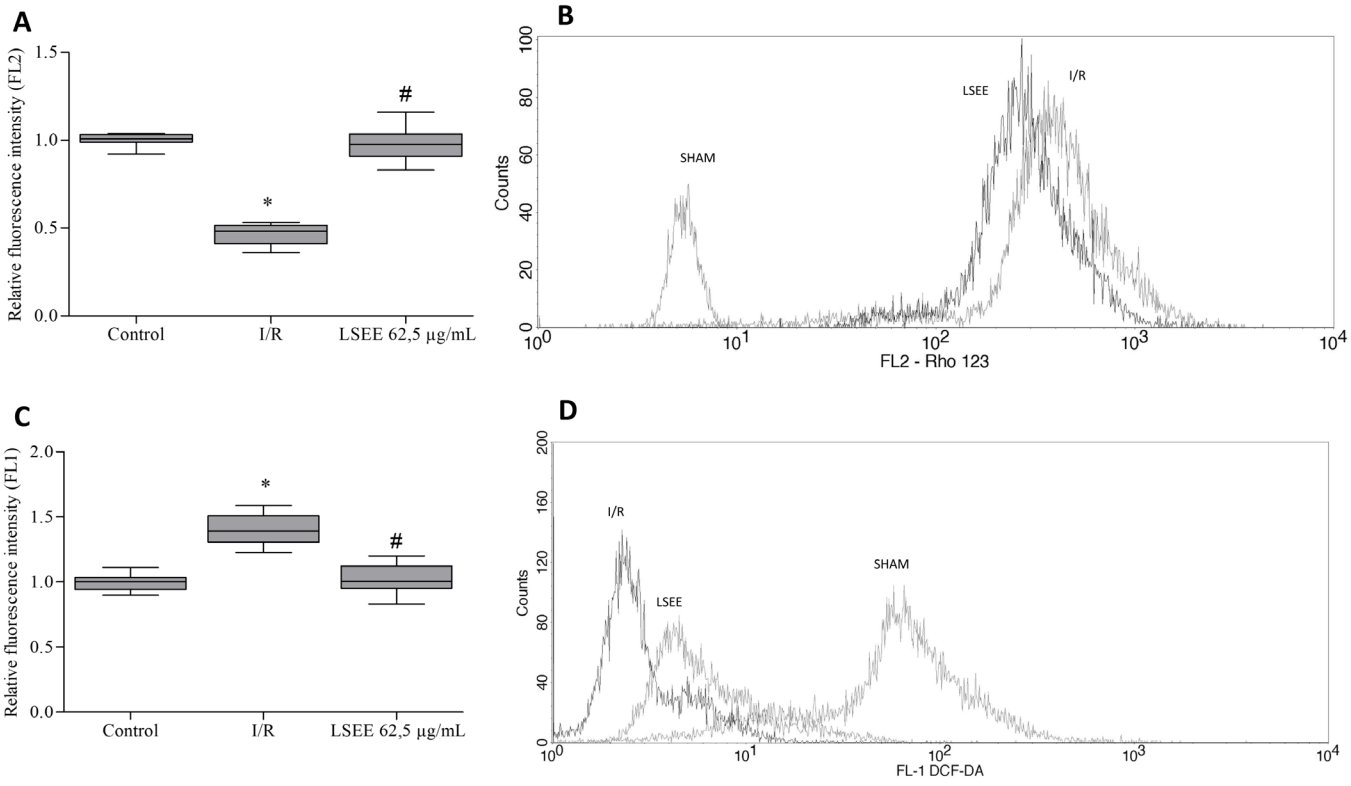


**FIGURE 6 -** Analysis of cellular respiration by flow cytometry. (A) Mitochondrial transmembrane potential analysis by rhodamine 123 labeling. (B) Histogram analysis of representative mitochondrial transmembrane potential in cell population treated with *Lippia sidoides* 62.5 µg/mL. (C) Production of ROS by DCFH-DA assay. (D) Histogram analysis of representative ROS in cell population treated with Lippia sidoides 62.5 µg/mL. The data expressed as fluorescence ratio relative to control ± SEM. * $p < 0.05$ vs. Sham, #$p< 0.05$ vs. I/R.

## DISCUSSION

Renal I/R injury is related to the reduction of renal blood flow, accumulation of ROS, and depletion of intracellular ATP. These events result in mitochondrial depolarization and disruption of the electron transport chain, causing cell death. Several therapeutics approaches are practiced in order to minimize the damage caused by oxidative stress, highlighting the administration of antioxidant substances (Parikh *et al.*, 2020; Weidenbusch *et al.*, 2019). In this context, natural products are cheap alternatives used in order to prevent oxidative damages related to I/R (Najafi *et al.*, 2014; Sampaio *et al.*, 2016).

*Lippia sidoides* is rich in phenolic compounds such as monoterpene thymol and flavonoid quercetin, both of which have antioxidant activity. These compounds prevent lipid peroxidation due to the presence of one hydroxyl group linked to an aromatic ring and protect against ROS formation (Games *et al.*, 2016). In previous study, anti-inflammatory and antioxidant effects have been demonstrated in *"in vivo"* assay using 100mg/kg doses of thymol (Golbahari, Abtahi Froushani, 2019). In the knowledge of 63.25% of this extract is composed of thymol, the doses of 150 mg/mL of extract contain approximately 100mg of thymol, being ideal for this study.

Animal models of renal I/R are important for the development of translational understanding about

treatment and mechanism-related to AKI. Additionally, *in vitro* models are well-established tools that allow to analyze cellular alterations, as well correlating cellular events with systemic intercurrences (Weng *et al.*, 2018). In this study, we showed that LSEE could protect against I/R-induced AKI. For this purpose, biochemical, molecular, oxidative and cellular parameters of kidney injury were evaluated.

Furthermore, beyond traditional biomarkers, early markers of AKI were used as NGAL and KIM-1, which are detectable at 24 to 48 hours prior to the rise in creatinine (McCullough *et al.*, 2013). NGAL allows an evaluation of GFR and tubular dysfunctions (Arakawa *et al.*, 2019) and KIM-1 is a biomarker for renal proximal tubule injury, undetectable in normal kidneys, but it is found elevated in AKI (Brooks *et al.*, 2015; Lahoud *et al.*, 2015). The present study showed that markers of renal tubular injury, such as urinary KIM-1 and fractional electrolyte excretion, were altered in the I/R-group. The I/R group also increased albumin-creatinine ratio and urinary NGAL, which are markers of glomerular damage. The treatment with LSEE was able to protect against tubular and glomerular damage.

In the glomerulus, podocytes comprise a triple-layer structure with vascular endothelial cells and the basement membrane, forming a critical barrier for the filtration of large molecules such as proteins (Wada *et al.*, 2016). In this context, nephrin is a structural molecule of the podocytes that provides architectural support to cells via crosslinking with the actin cytoskeleton (Liu *et al.*, 2015). The progression of AKI is related to urinary glomerular nephrin loss, resulting in lower levels of this protein in the glomeruli. Consequently, this dysfunction might contribute to reduced renal function. Additionally, nephrin could be used as a marker of glomerular-specific renal damage in several settings and can be observed prior to the development of albuminuria (Chen *et al.*, 2019). In the present study, I/R caused a reduction of nephrin expression and the pre-treatment with LSEE was able to reverse this.

Functional alterations found in AKI might be related to the fact that the kidney tissue is sensitive to hypoxia, although reperfusion plays an important role in ischemic tissue regeneration. I/R has been associated with injury exacerbation caused by the production of reactive oxygen and nitrogen species (Devarajan, 2006; Rovcanin *et al.*, 2016). Excessive ROS production and the unbalance of enzymatic and non-enzymatic antioxidant systems results in the I/R-induced renal deterioration. TBARS are the classical indicator of oxidative stress and GSH is an endogenous antioxidant and a marker of the oxidative stress levels (Finaud, Lac, Filaire, 2006; Wu *et al.*, 2004). This study showed that LSEE treatment protected the reduction in MDA levels and increase GSH levels observed I/R induced AKI, demonstrating a reduction in oxidative stress and an increase in antioxidant capacity.

ROS production is initially triggered by the mitochondrial respiratory chain dysfunction in the ischemic phase and exacerbated in the reperfusion phase, which can cause cell death by directly impairing DNA, proteins, and lipids (Kalogeris, Bao, Korthuis, 2014). Thus, in order to study renal tubules as targets of I/R injury, the present work used a well-stablished model based on ischemia and reoxygenation using renal tubular cell lines (LLC-MK2). The LSEE promoted a recuperation in the cell viability, which can be explained because the tubular epithelium has a potent capacity to proliferate and replace lost cells after ischemic or toxic insults (Brooks *et al.*, 2015). This observation is ratified by the reduction in intracellular ROS accumulation after I/R and LSEE treatment observed in the DCFH assay, consequently increasing mitochondrial transmembrane potential and protecting against cell death, since the mitochondria is a primary site of intracellular ROS production (Li *et al.*, 2017).

Indeed, natural substances, especially phenolic ones are described as antioxidant and have a protective effect on renal functions, mainly after ischemic events (Da Costa *et al.*, 2015; Liu *et al.*, 2011; Najafi *et al.*, 2014; Sampaio *et al.*, 2016). Altogether, our results indicate that LSEE was able to protect against injury through direct mechanisms in tubular cells and demonstrates an antioxidant effect, as well as a protection against damage on glomerulus. Moreover, the improvement in biochemical parameters indicate a beneficial effect of LSEE in renal I/R-induced injury. Thus, this extract has biotechnological potential for the application in the prevention of AKI induced by I/R, being a cheap and practical base for further studies.

## CONCLUSION

In conclusion, this study provides data of the nephroprotective effects of LSEE in I/R-induced injury, being able to reverse the increase in NGAL and KIM-1 levels and the down regulation of nephrin, indicating a protection in renal tubules and glomerulus. This protection can be attributed to its antioxidant effect observed by redox balance assays. Thus, our results suggest the potential benefits of LSEE to protect the kidney against acute ischemic renal injury, but stability studies are necessary to prove its biotechnological potential as herbal medicine.

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## DECLARATION OF COMPETING OF INTERESTS

The present paper does not present conflict of interest.

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