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Protective Effect of Quercetin on Renal Tubular Cells and the Involvement with the Renin-Angiotensin-Aldosterone Axis

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HIGHLIGHTS

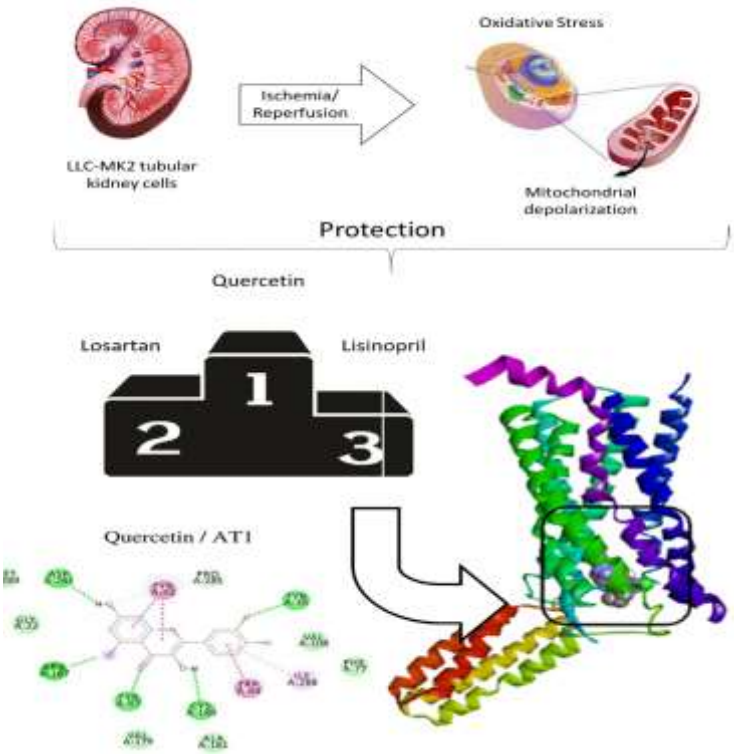
- Ischemia-reperfusion triggers acute kidney injury due to reactive oxygen species.
- Quercetin is an antioxidant flavonoid that modulate the Rennin-Angiotensin System.
- Quercetin prevents tubular cell death, oxidation, and mitochondrial depolarization.
- Quercetin interacts with the angiotensin AT1 receptor.

Abstract: Ischemia-reperfusion (I/R) plays an important role in the process of acute kidney injury (AKI) due to the generation of reactive oxygen species (ROS). Substances of natural origin have been studied in the prevention of oxidative damage related to I/R. Quercetin is a flavonoid with antioxidant potential and modulate enzymes, such the inhibition of the Rennin-Angiotensin System (RAS). The aim of this study is to evaluate the nephroprotective effect of quercetin against the I/R and analyze the inhibition of RAS. Rhesus monkey Kidney Epithelial Cells (LLC-MK2 line) were submitted to an *in vitro* ischemia/reperfusion model. After the reperfusion cells were treated with quercetin, the cell viability was accessed by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. Tubular cell damage was assessed by the Kidney Injury Molecule-1 (KIM-1) measurement. Oxidative stress was evaluated through Thiobarbituric Acid

Reactive Substances (TBARS) and reduced glutathione (GSH). The evaluation of cell death and the mitochondrial depolarization were analyzed by flow cytometry. Quercetin prevents cell death reducing oxidative stress and preventing mitochondrial membrane depolarization. Molecular docking showed that quercetin prevents cell damage better than losartan and lisinopril, inhibitors of RAS. Quercetin has a potential to interact with type 1 angiotensin II receptor (AT1) with greater affinity through the formation of five hydrogen bonds of strong intensity.

Keywords: Flavonoids; Acute Kidney Injury; Oxidative stress; Angiotensin's receptor; KIM-1.

GRAPHICAL ABSTRACT



INTRODUCTION

Acute kidney injury (AKI) is recognized as a public health problem, affecting around 13.3 million people per year [1]. Ischemia-reperfusion (I/R) plays an important role in the process of AKI leading to acute tubular injury, because when the blood supply is restored after prolonged ischemia, vascular endothelial and epithelial cells are activated, enhancing the generation of reactive oxygen species (ROS). This leads to many inflammatory consequences and apoptosis of tubular epithelial cells [2].

Several strategies are described to prevent or reverse the tissue damage established by oxidative stress and inflammatory status in the kidneys after the occurrence of I/R. Hemodynamic strategies such as the prescription of vasodilators and hydration, in addition to the administration of steroids and other anti-inflammatories, as well as, the use of antioxidants that improve mitochondrial metabolism, have been highlighted in clinical research [3]. In this context, substances of natural origin, mainly phenolic compounds, have been the object of study in the prevention and treatment of oxidative damage related to I/R, especially on the tubular tissue [4].

Quercetin (302.236 g/mol) is a polyphenolic flavonoid substance, chemically known as 3, 3′, 4′, 5, 7-pentahydroxyflavone (C15H10O7). It is naturally present in many vegetables and fruits such as onions, apples, capers, cilantro, and cranberries, as well as in black tea and red wine. Many biological studies on quercetin have shown different biological activities such as anti-hypertensive, antiobesity, anticancer vasodilator, anti-inflammatory, neuroprotective, antiatherosclerosis and hipocholesterolemic [5].

In the past, the benefits attributed to quercetin were due to its effect in oxidative stress. Thus, the binding of quercetin with several enzymatic and non-enzymatic targets were studied, being the inhibition of the Rennin-Angiotensin System (RAS) one of the most important [6]. Thus, the aim of this study was to evaluate the effect of quercetin as a nephroprotective molecule against the injury of ischemia followed by reperfusion (I/R) process and theoretically analyze the ability to inhibit angiotensin-converting enzyme (ACE) or antagonize type 1 angiotensin II receptors (AT1).

MATERIAL AND METHODS

In vitro ischemia/reperfusion model

Rhesus monkey Kidney Epithelial Cells (LLC-MK2 line) (ATCC CCL-7) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA), supplemented with 10% fetal bovine serum (FBS) in standard conditions (37 °C in a humidified atmosphere with 5% CO_2) until confluence.

In vitro ischemia/reperfusion (I/R) was induced by the method of the anaerobic chamber [7]. For ischemia induction, the normal growth culture medium was replaced by glucose, pyruvate and FBS-deprived DMEM and the plates were incubated for 12 hours in an anaerobic chamber at CO_2 95%. After this period, the reperfusion was performed by reoxygenation, with the addition of a complete culture medium and the oxygenated atmosphere for three hours.

Treatment and cell viability

After reperfusion, cells were treated using either quercetin, losartan, or lisinopril. Quercetin (Sigma-Aldrich, St. Louis, MO, USA) was previously diluted in dimethyl sulfoxide (DMSO) with two-fold dilution to ensure that DMSO concentration would never exceed 0.5%. So, quercetin was diluted in phosphate buffer (PBS) in different concentrations (200, 100, 50, 25, 12.5, 6.25 µg/mL, corresponding respectively to 6.62×10^{-1}, 3.31×10^{-1}, 1.65×10^{-1}, 8.27×10^{-2}, 4.13×10^{-2} and 2.07×10^{-2} µM) and cells were treated for 12 hours. DMSO 0.5% solution was used as negative control. Losartan (422.91 g/mol) and lisinopril (405.49 g/mol) (Sigma-Aldrich, St. Louis, MO, USA) were diluted in PBS in different concentrations (1×10^{-2}, 1×10^{-1}, 1, 1×10^{1} and 1×10^{2} µM) for 12 hours.

For all experiments, cells not submitted to I/R condition and then treated with PBS were considered negative control (CT-). Additionally, positive control (CT+) was defined as cells which were exposed to I/R but treated only with PBS.

Cell viability was accessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St Louis, MO, USA) assay. MTT assays were performed to assess the range or cytotoxicity of quercetin in LLC-MK2 cells. Then, MTT was used to measure the percentage of death after the protocol of ischemia/reoxygenation and the cell recovery ability in presence of quercetin.

Determination of KIM-1 levels

KIM-1 (Kidney Injury Molecule -1) levels were determined in the LLC-MK2 supernatant and cells homogenate using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc – Cat. DY1750, Minneapolis, MN). KIM-1 concentration was calculated based on the standard curve and expressed in absolute terms in ng/mg of protein.

Balance reduction-oxidation (redox) evaluation

Cells were collected, washed with ice-cold PBS, and suspended in 1 mL of 0.1 M potassium phosphate buffer added with 5 mM EDTA (ethylenediaminetetraacetic acid), 0.1% Triton X-100 and 0.6% sulfosalicylic acid (pH 7.5). The suspension was sonicated in ice water for 10 minutes. The suspension was centrifuged at 3000 G for 4 minutes and the supernatant was stored in a freezer at −80 °C. Protein content of the lysate was measured using the Bradford methodology.

For determination of thiobarbituric acid reactive substances (TBARS), 100 µL of the supernatant were mixed in 100 µL of 40% trichloroacetic acid and 400 µL of 60% thiobarbituric acid. The mixture was incubated at 96 °C for 30 minutes, then kept in an ice bath. To stop the reaction, 200 µL of glacial acetic acid were added to the suspension which was centrifuged (1700 g, 30 minutes). The obtained supernatant was read in a spectrophotometer (UV ASYS 340, Biochrom, Cambridge, UK) on a 530 nm reader. TBARS concentrations were then calculated using a calibration curve using malondialdehyde (MDA) as the standard and expressed in ng of TBARS/µg of cell proteins.

For the determination of reduced glutathione (γ-glutamil-L-cisteinilglicin, GSH), 400 µLof cell lysate were added to 800 µLof Tris-HCL buffer (0.4 M, pH 8.9) and 20 µLof the DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)) chromogen, also called Ellman's reagent (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was measured by spectrophotometry at 412 nm (UV ASYS 340, Biochrom, Cambridge, UK), using a GSH standard as calibration curve. The results were expressed as nmol of GSH/mg of protein.

Flow cytometry assays

After 12 hours of treatment with quercetin, LLC-MK2 cells were submitted to flow cytometry analysis to determine cell death mechanisms involved in ischemia/reoxygenation injury and the protective effect of quercetin. The cells were dislocated from the plate, centrifuged, and washed twice with binding buffer (10mM Hepes, 140mM NaCl, 2.5mM CaCl2, pH 7.4) to allow the labeling. Groups were analyzed in a FACSCalibur flow cytometry device (BD Biosciences, New Jersey, USA) using CellQuest Pro software.

Annexin V/7-amino-actinomycin (7-AAD) staining was used to differentiate necrosis and apoptosis. When entering early apoptosis process, cells express phosphatidylserine (PS) on the outer leaflet of the plasma membrane. Annexin V labeled stains PS. Necrotic cells lose their membrane integrity and are permeable to 7-AAD (DNA intercalator). LLC-MK2 cells were labeled with annexin V-PE and 7-AAD for 15 minutes and the fluorescence was detected by cytometry.

Mitochondrial depolarization was evaluated using rhodamine 123 (Rho123) (Sigma-Aldrich, St. Louis, MO, USA). Rho123 is a fluorochrome that selectively stains mitochondria in living cells, and its uptake is positively correlated with ΔΨm. Cells were washed with PBS and stained with Rho123 (10µg/mL) for 30 minutes. Then, cells were analyzed in the flow cytometer to measure the decrease in Rho123 accumulation in cell mitochondria. ΔΨm was determined by considering the fold change in the geometric mean of FL2 signal intensity in ratio with the control, considering weight 1 to control.

Molecular docking assay

The crystallographic structure of Angiotensin Converting Enzyme (ACE - PDB ID: 1O86), in addition to the AT1 receptor angiotensin (PDB ID: 4YAY), were obtained from Protein Data Bank [8]. The ACE structure was registered co-crystallized with Enalapril. AT1 receptor was co-crystallized with the theoretical inhibitor ZD7 (5,7-diethyl-1-{[2'-(1H-tetrazol-5-yl) biphenyl-4-yl] methyl}-3,4-dihydro-1,6-naphthyridin-2(1H)-one).

The molecular structure of quercetin (PUBCHEM CID 5280343) and the control ligands, ACE inhibitor Enalapril (PUBCHEM CID 5388962) and AT1 receptor inhibitor Losartan (PUBCHEM CID 3961), were designed using the MarvinSketch® academic licensed software version 20.13 of the ChemAxon© software package Marvin [9-10].

Molecular coupling simulations were performed using AutoDockVina Version 1.1.2. For each analysis, 100 cycles of 10 independent simulations were performed using the Lamarkian Genetic algorithm. Non-protein molecules were removed, and polar hydrogen was added by using AutoDock Tools 1.5.6 software. The adjustment results were grouped with Mean Square Root Deviation (RMSD) less ten 2.0 Å. The conformations were analyzed with the UCSF Chimera 1.8 pack and Discovery visualizer. Hydrogen bonds were classified as strong when the distance between the atoms was < 3.1 Å; as intermediate when the distance was between 3.1 and 3.5 Å; and as weak when the distance between the atoms was > 3.5 Å [11].

Statistical analysis

All the experiments were performed in triplicate (n=3), and the results were expressed as mean ± standard error mean (SEM). Gaussian distribution was verified by Shapiro-Wilk's normality test, and the comparison between groups was assessed using one-way ANOVA with Bonferroni's post-test. The significance criterium was $p<0.05$. All statistical analysis were performed using GraphPad Prism version 6.0.

RESULTS

Cell viability assays

Initially, aiming to establish the possible cytotoxicity of quercetin on LLC-MK2 cells, the MTT assay was performed. Considering the tested concentrations, quercetin did not cause significantly decrease in cell viability (Figure1A). In addition, cells submitted to the I/R procedure reduced approximately 50% of their viability when compared to the control (Figure1B). Nevertheless, cells that were undergone the I/R procedure but were later treated with quercetin showed a protective effect, indicated by maintenance, or increase of the viability, especially at the three highest concentrations (200, 100 and 50µg/mL or 6.62×10^{-1}, 3.31×10^{-1} and

1.65×10^{-1} µM respectively), showing 73.9, 63.9 and 60.2 % of viability, respectively, when compared to I/R group after 12 hours.

Losartan or lisinopril did not cause toxicity on LLC-MK2 cells at used concentrations (Figure1C). When cells exposed to I/R protocol were treated with these drugs, the treatment with lisinopril was not able to increase the cell viability. Losartan increased the viability only at the higher concentration (1×10^{2} µM) (Figure1D).

Kidney Injury Molecule-1 (KIM-1) releasing

Tubular cells damage was evaluated through the KIM-1 measurement. The I/R protocol increased KIM-1 releasing to cell supernatant in comparison with the control (140.5±1.2 vs. 5.2 ± 0.1 ng/mg of protein). Losartan and quercetin were able to reduce the cell damage (120.2 ± 3.7 ng/mg of protein and 30.5 ± 0.7 ng/mg of protein respectively). Quercetin presented comparatively better results, once quercetin, losartan and lisinopril were used at the same concentration (100 µg/mL) (Figure2A).

Aiming to better understand the protective effect of quercetin on tubular LLC-MK2 cells, KIM-1 was measured at the homogenate of cells treated with different concentrations of quercetin. In the homogenate, I/R increased the KIM-1 levels to 448.6 ± 25.9 ng/mg of protein, while the control presented 10.6 ± 0.7 ng/mg of protein. All group of cells treated with quercetin presented decreased KIM-1 on homogenate, but the 200 µg/mL treatment presented a better result, presenting KIM-1 equal to 100.1 ± 3.1 ng/mg of protein (Figure2B).

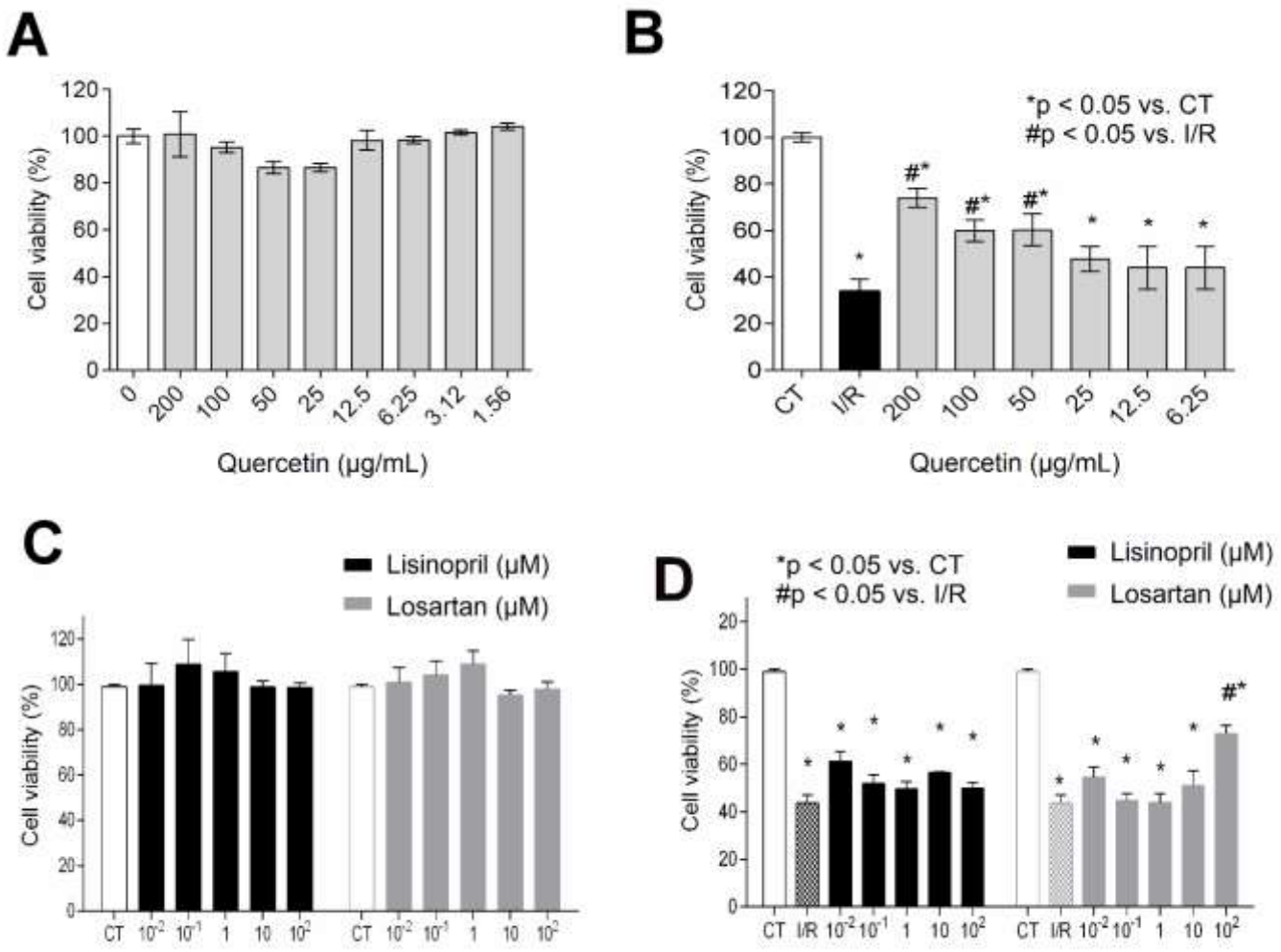


Figure 1. Cell viability in LLC-MK2 cells by MTT assay. (A) Toxicity range of Quercetin on LLC-MK2 cells; (B) Cell viability of LLC-MK2 cells submitted to ischemia and reoxygenation (I/R) and treated with quercetin; (C) Toxicity range of Lisinopril and Losartan on LLC-MK2 cells; (D) Cell viability of LLC-MK2 cells submitted to ischemia and reoxygenation (I/R) and treated with Lisinopril and Losartan. Results are shown as mean ± SEM. *P < 0.05 vs control group (CT); #P < 0.05 vs. I/R group.

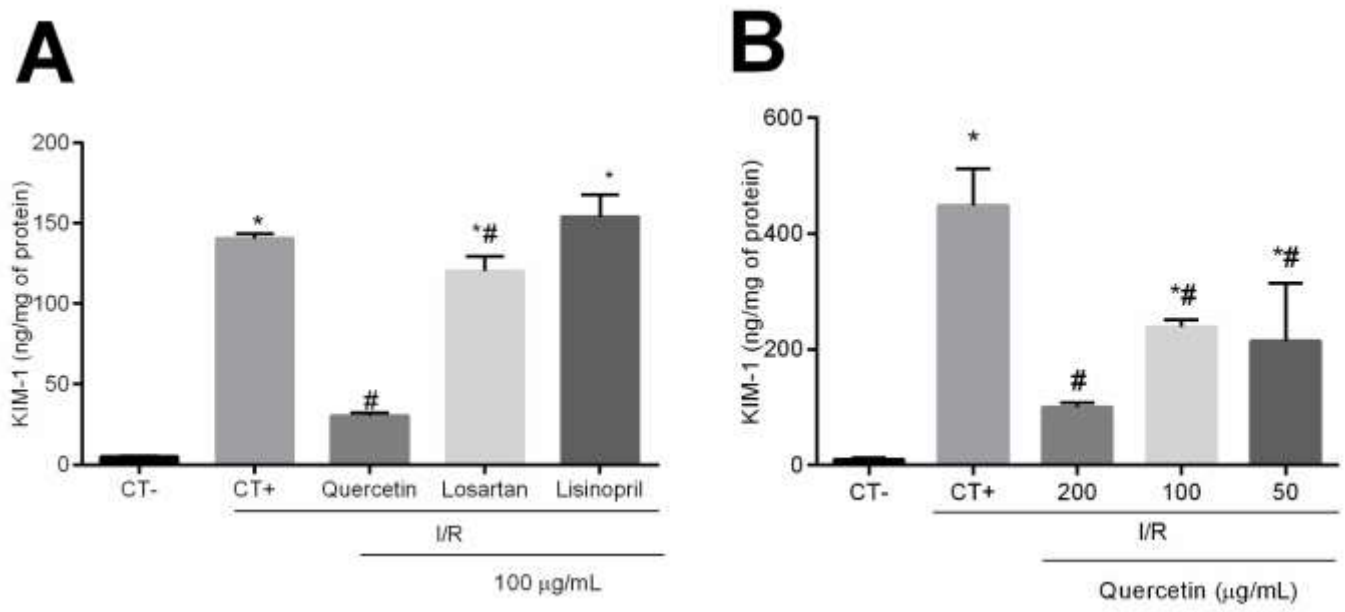


Figure 2. Kidney Injury Molecule-1 (KIM-1) concentrations in LLC-MK2 cells supernatant (A) and cells homogenate (B) CT-: Negative control; CT+: Positive control; I/R: Ischemia/Reperfusion. Results are shown as mean ± SEM. *P < 0.05 vs. negative control; #P < 0.05 vs. positive control.

Redox balance evaluation

The I/R protocol induced lipid peroxidation, represented by TBARS accumulation in comparison with the control (28.6 ± 0.6 vs. 15.5± 1.6 ng/mg of protein), as shown in Figure 3A. The treatment with 100 and 200 µg/mL quercetin reduced TBARS accumulation, although that happened partially. Additionally, I/R protocol stimulated the glutathione oxidation, decreasing the levels of reduced GSH (12.5 ± 0.6 nmol/mg of protein) in comparison with the control (43.1 ± 1.2 nmol/mg of protein). All the used concentration increased the amount of GSH, highlighting the 100 µg/mL (23.1 ± 0.8 nmol/mg of protein) (Figure3B).

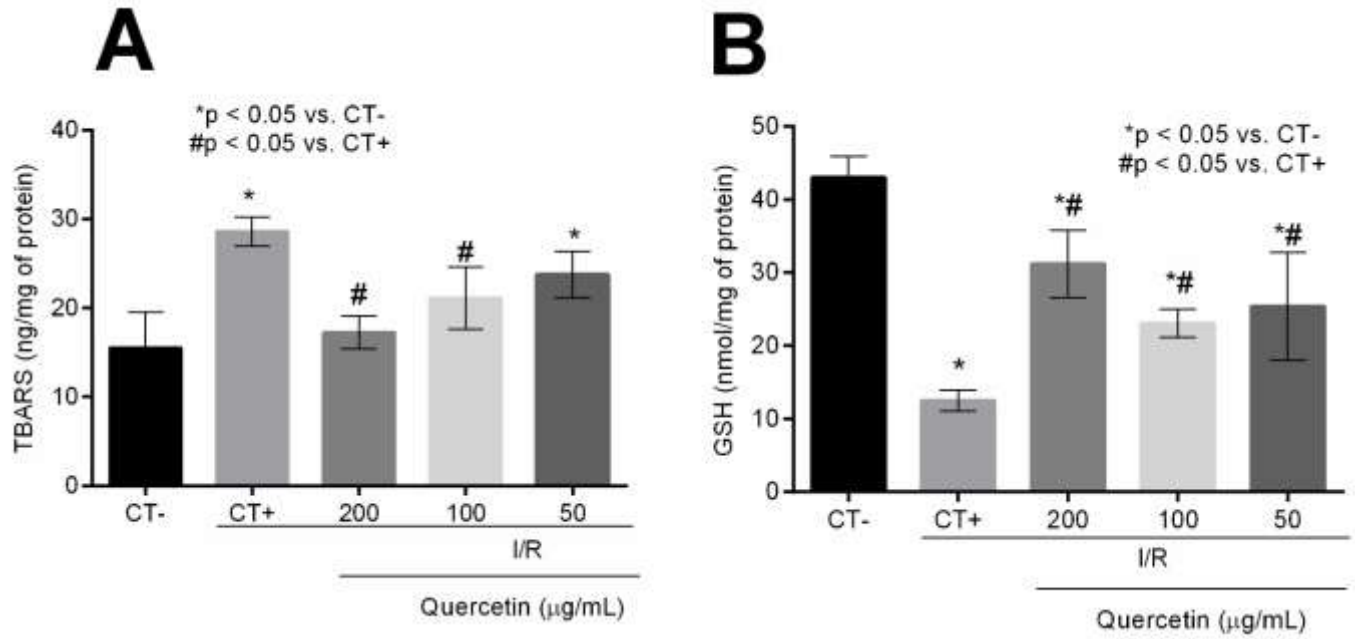


Figure 3. Oxidative stress evaluation. (A) Thiobarbituric acid reactive substances (TBARS) concentrations in LLC-MK2 cells homogenate; (B) Reduced glutathione (GSH) concentrations in LLC-MK2 cells homogenate. CT-: Negative control; CT+: Positive control; I/R: Ischemia/Reperfusion. Results are shown as mean ± SEM. *P < 0.05 vs. negative control; #P < 0.05 vs. positive control.

Flow cytometry assays

Flow cytometry was performed using 7AAD/Annexin-V staining, aiming to evaluate cell death mechanism in I/R (Figure4A). The figure 4C shows that cells that underwent I/R procedure presented significant labeling by both markers (23.4% of the events), indicating late apoptosis or secondary necrosis, evidenced by the cells marked by 7AAD +/Anx +, represented in the upper right quadrant of the dot plot. The comparative density plot indicating results is showed in Figure4B-F. In treated groups, quercetin (200, 100 and 50 µg/mL) was able to significantly reduce cell death by reducing double-labeled events. In the group treated with quercetin 200µg/mL, the double-labeled population was reduced to 8.7% of the events (Figure 4D).

Changes in mitochondrial transmembrane potential were studied to evaluate the respiratory impairment using the Rhodamine 123 dye (Figure 5). The I/R group has shown a decreased in the mitochondrial transmembrane potential, with approximately 20% of the control fluorescence intensity. After the quercetin treatment, there was a Δψm increase, being the fluorescence intensity of the 200 µg/mL quercetin group risen more than the double of the control.

Molecular docking analysis

After conducting the molecular docking simulations, the results were obtained regarding the regions of interactions, the affinity energies and the distances adjusted by the Root Mean Square Deviation (RMSD) of quercetin with the ACE and Angiotensin AT1 receptor, as well as their respective known inhibitors, enalapril and losartan. Aiming to assess the quality of the interactions as satisfactory, results with higher binding enthalpy were considered; that is, a satisfactory interaction energy would be the one that results in a greater loss of energy. The reference value for a satisfactory interaction energy is from -6.0 Kcal/mol. As for the RMSD, values of < 2.0 Å were adopted as acceptance criteria.

When the molecular docking simulations with ACE were performed, it was observed that quercetin showed the potential to bind to the same target as enalapril and lisinopril (Fig 6 A-C) but interacting with different amino acids. Thus, it was possible to observe that the interactions between quercetin and enalapril occur with the same affinity energy (-7.4 Kcal / mol), differing only in RMSD values, in which quercetin was 1.884 Å, while the RMSD for enalapril was 1.078 Å, according to Table 1.

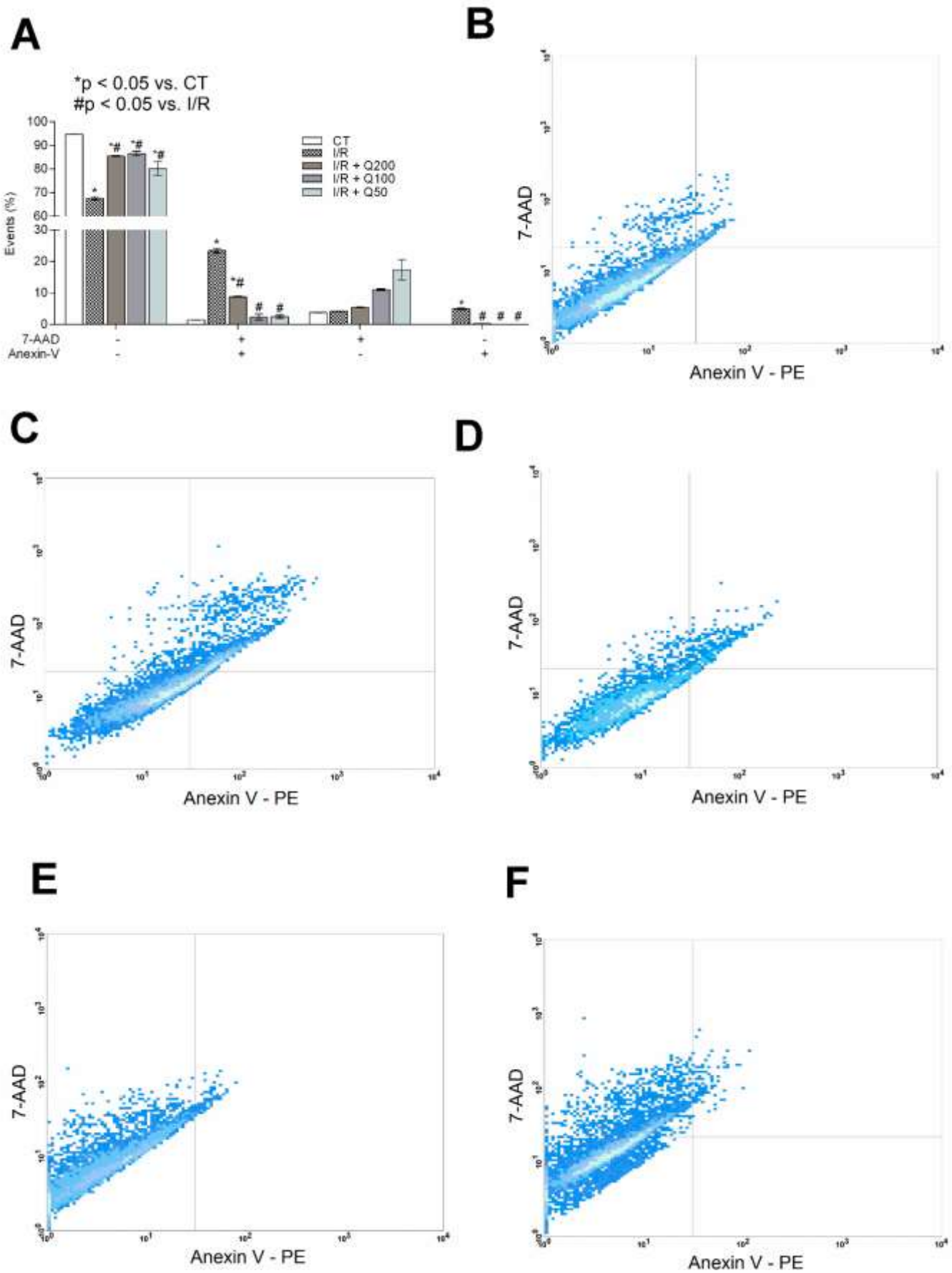


Figure 4. Cell death pathway assay. Necrotic cells were labeled by 7-AAD (7-amino-actinomycin), while apoptotic cells were labeled by annexin V (Anx). (A) Statistical analysis and percentage of distribution of events (cells); (B) Dot plot representation of events in control group (CT), (C) Ischemia/Reoxygenation (I/R) group. (D) Cells treated with Quercetin at 200 µM – Q200. (E) Cells treated with Quercetin at 100 µM – Q100; (E) Cells treated with Quercetin at 50 µM – Q50. Results are shown as mean ± SEM. *P < 0.05 vs. control; #P < 0.05 vs. I/R group.

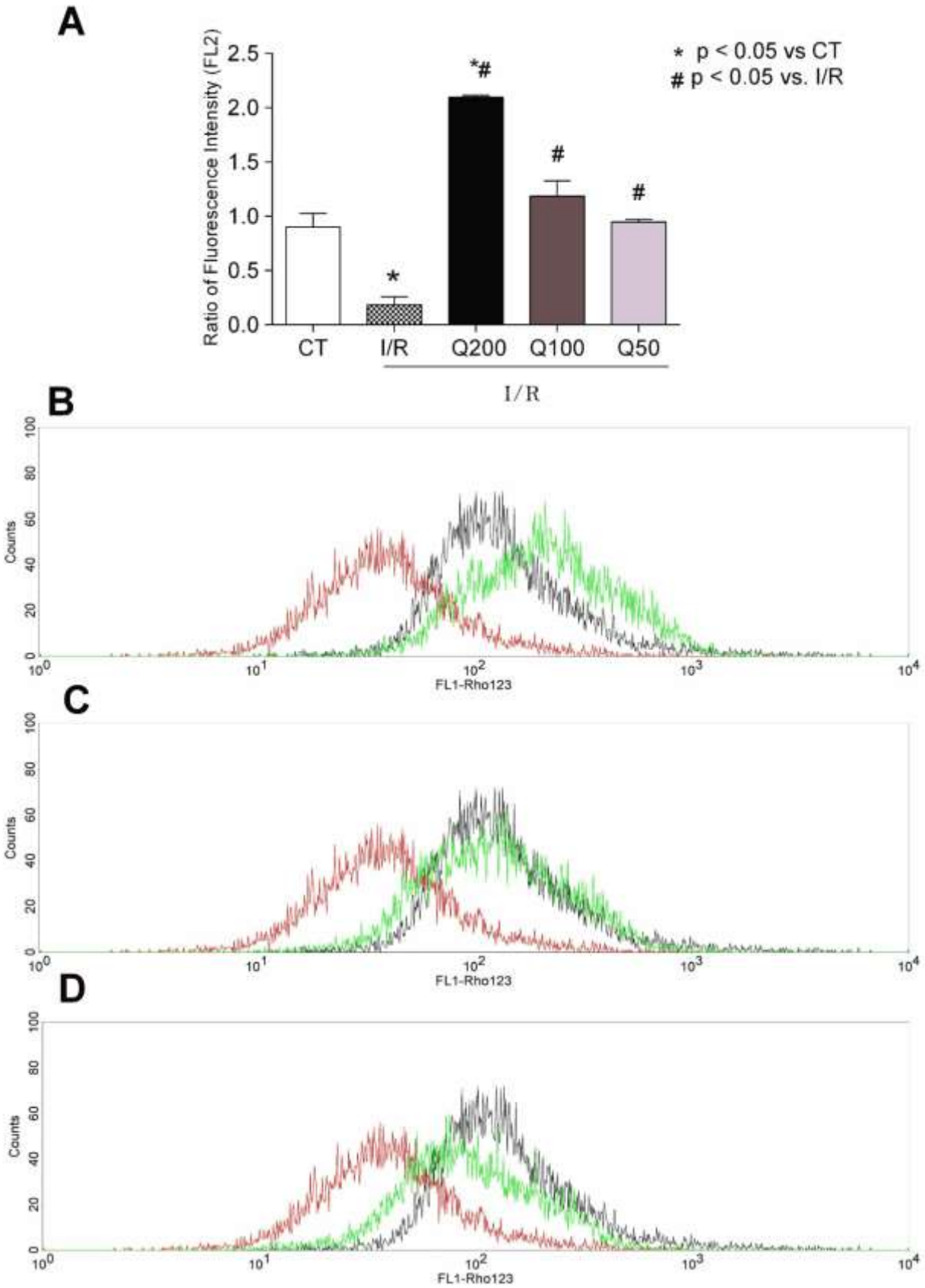


Figure 5. Analysis of mitochondrial transmembrane potential by flow cytometry. (A) Statistical analysis of the ratio of fluorescence in comparison to the control (CT); (B to D) Histogram analysis of representative mitochondrial transmembrane potential in cell population exposed to ischemia/reperfusion (I/R) treated with Quercetin at 200, 100 and 50 μM (Q200, Q100 and Q50 respectively). Data are expressed as fluorescence ratio relative to control ± SEM. *P < 0.05 vs. control; #P < 0.05 vs. I/R group.

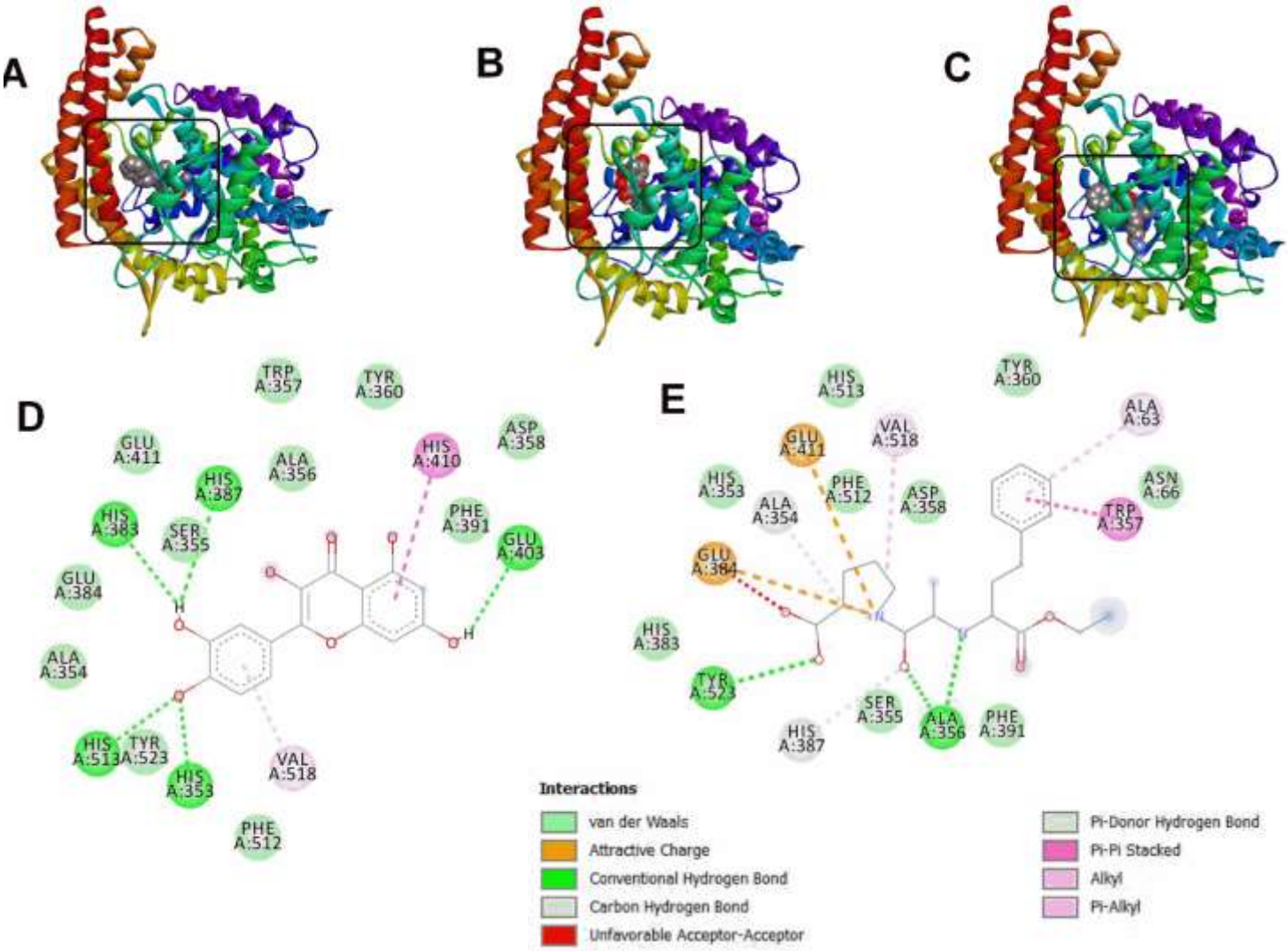


Figure 6. Representation of the interaction of ligands with the Angiotensin-Converting Enzyme (ACE) by molecular docking. (A) Enalapril; (B) Quercetin and (C) Lisinopril. 2-D maps representing the interactions of (D) Quercetin and (E) Enalapril with the ACE.

Table 1. Energetic affinity of ligands with Angiotensin-Converting Enzyme (ACE).

Inhibitor	Affinity (Kcal/mol)	RMSD (Å)
Enalapril	-7,4	1,078
Quercetin	-7,4	1,884
Lisinopril (redock)	-7,2	1,915

When analyzing specific interactions of the compounds with the amino acid residues, quercetin demonstrated to form interactions with ACE, mostly strong hydrogen bonds with histidine (HIS) 353 and 513, serine (SER) 355 and glutamate (GLU) 403, and hydrophobic interactions with phenylalanine residue (PHE) 391 (Table 2). There are still other pi-pi T shaped interactions with the histidine residue 410 and a pi-alkyl one with the valine residue (VAL) 518, as showed in the 2D-maps in Figure 6 (D-E).

Table 2. Types of interactions and distances (Å) between the ligands and the amino acid residues of the Angiotensin-Converting Enzyme (ACE).

Quercetin / ACE			Enalapril / ACE		
Residue	AA	Distance (Å)	Residue	AA	Distance (Å)
Hydrogen Bonds			Hydrophobic Interactions		
353 A	HIS	1.92	1 A	ALA	3.70
355 A	SER	3.67	357 A	TRP	3.68
355 A	SER	3.38	357 A	TRP	3.92
403 A	GLU	2.38	360 A	TYR	3.89
513 A	HIS	2.44	518 A	VAL	3.80
Hydrophobic Interactions					
391 A	PHE	3.62			

Enalapril mainly presents hydrophobic interactions with alanine (ALA) 1, tryptophan (357), tyrosine (TYR) 360 and valine (VAL) 518 residues. In addition, other charge attraction interactions with glutamate residues 384 and 411, π-π stacking with the tryptophan residue (357), and π-alkyl with the alanine (ALA) 63 and valine (VAL) 518 residues which do not significantly contribute to the molecule's affinity with the receptor (Figure 6).

In molecular docking simulations for the AT1 receptor, quercetin was compared to two other substances with known ability to inhibit AT1 receptors: losartan, and a theoretical enzyme inhibitor, Z7D. In the analysis, it was demonstrated that all substances bind to the same region in the molecule (Figure 7 A-C). Analyzing the energies of the interactions and the RMSD values according to Table 3, quercetin showed a satisfactory affinity energy, as well as losartan of -8.1 Kcal/mol, indicating stability in the interaction. In comparison, the theoretical inhibitor ZD7 showed a higher energy, -10.4 Kcal/mol. As for RMSD, quercetin had a lower value compared to the others (1.524 Å), indicating a more specific interaction.

Table 3. Energetic affinity of ligands with the Angiotensin AT1 receptor.

Inhibitor	Affinity (Kcal/mol)	RMSD (Å)
ZD7 (redock)	-10,4	1,843
Losartan	-8,1	1,900
Quercetin	-8,1	1,524

Figure 7 (D-E) shows the 2-D maps representing the binding site of quercetin with the enzyme, showing the main residues involved. Quercetin showed two main types of interactions, most of which are strong hydrogen bonds with tyrosine residues (TYR) 35; 87, arginine (ARG) 167, cysteine (CYS) 180 and aspartate (ASP) 281, and π - π stacking interactions with tryptophan (TRP) 84 and tyrosine (TYR) 92 (Table 4).

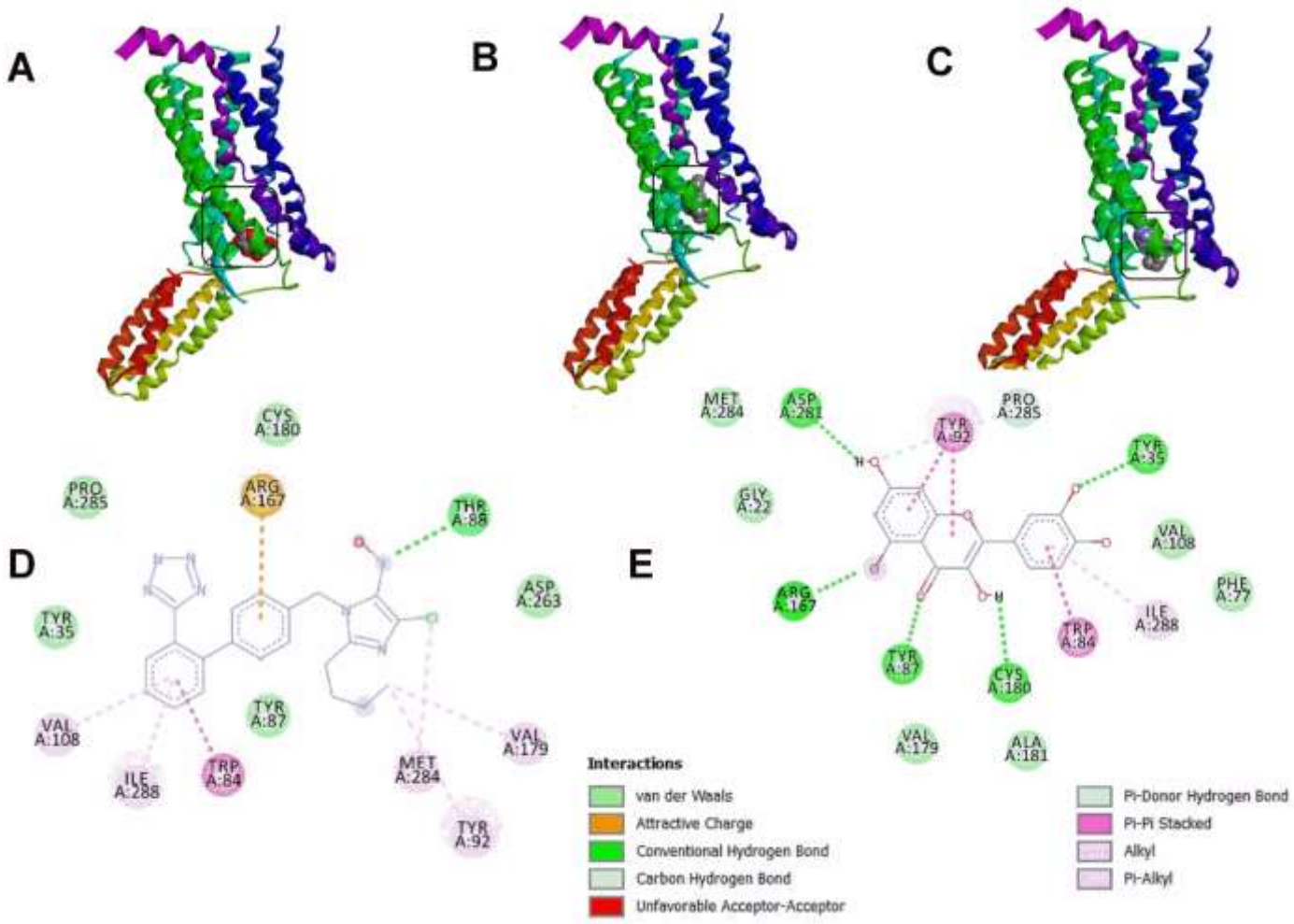


Figure 7. Representation of the interaction of ligands with the Angiotensin AT1 receptor by molecular docking. (A) Quercetin; (B) ZD7 and (C) Losartan. 2-D maps representing the interactions of (D) Losartan and (E) Quercetin with the ACE.

DISCUSSION

The present work showed that quercetin was able to prevent the oxidative damage induced in tubular kidney cells after ischemic events. This potential was related to the capacity of preventing the cell death, protecting mitochondria against depolarization. Ischemia decreases on the oxygen offering. Since the oxygen is the final acceptor of electrons on the cellular respiratory chain, the reperfusion, consequently, leads to ROS formation and oxidative stress. Though necessary, the reperfusion, must happen in a controlled environment, avoiding the ROS accumulation and, even to damage to cell lipids, nucleic acids, and functional and structural proteins. Therefore, several strategies have been studied aiming to promote a secure reperfusion; antioxidants substances are the major candidates to this pharmacological approach. However, it is necessary to prevent the ROS formation and, subsequently, the direct, biochemical, and vascular events associated to this, instead of removing the pre-formed ROS. Thus, enzymatic targets are hardly studied to establish nephroprotective tools [12].

Table 4. Interactions and distances (Å) between the ligands and the amino acid residues of the AT1 receptor.

Losartan / AT1						Quercetin / AT1		
Residue	AA	Distance	Residue	AA	Distance	Residue	AA	Distance
Hydrophobic Interactions			Hydrogen Bonds			Hydrogen Bonds		
84A	TRP	3.76	88A	THR	2.50	35A	TYR	2.02
84A	TRP	3.45	π-Stacking			87A	TYR	1.98
87A	TYR	3.63	84A	TRP	4.13	167A	ARG	2.97
92A	TYR	3.64	84A	TRP	4.16	180A	CYS	2.40
92A	TYR	3.86	π-Cation Interactions			281A	ASP	2.19
92A	TYR	3.70	167A	ARG	5.07	π-Stacking		
108A	VAL	3.51	Halogen Bonds			84A	TRP	4.07
179A	VAL	3.85	263A	ASP	3.41	92A	TYR	4.06

Mitochondrial dysfunction plays an important role in the progression of acute renal injury, being considered an initial event in several forms of AKI, mainly because they are responsible for more than 90% percent of the energy production by oxidative phosphorylation, responding to stimuli with ordered processes including mitochondrial dynamics (fission, fusion) and mitophagy. Thus, a disorder of mitochondrial dynamics after I/R conditions contributes to apoptosis increased oxidative stress and inflammation. Imbalance in mitochondrial fusion and fission processes and suppression of mitophagia promote ischemia-mediated kidney damage [13].

In the present study, quercetin improved cell viability, decreased the lipid and glutathione oxidation and prevented cell death after I/R process. Moreover, quercetin was able to reduce the number of cells marked by annexin V and 7AAD, indicating a reduction in the cell death process in a dose-depending manner. That corroborated with the recovering of mitochondrial metabolism by the increase of Rho123 fluorescence signal. Flavonoids such quercetin and other phenolic compounds are widely used in the literature as antioxidant molecules and, consequently, stand out for their potential to protect against oxidative and inflammatory injuries in various tissues. Recently, it was showed that the gallic acid protected on cisplatin-induced mitochondrial dysfunction and inflammation in rat kidney, leading to the decreased mitochondrial ROS formation and lipid peroxidation represented by TBARS, increased mitochondrial glutathione, superoxide dismutase, glutathione peroxidase and catalase improving mitochondrial transmembrane potential, which reduced the inflammation and tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) release [14].

Quercetin, specifically, is a well-studied antioxidant molecule that shows potential prevention against hemodynamic and vasculature injuries. In previous research, it was showed that quercetin inhibited endothelial dysfunction and atherosclerosis due to this enzymatic inhibition of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase and decreasing of heme oxygenase-1 siRNA expression [15]. Beyond the vascular events, direct injury to the tubular cells, and consequent apoptosis, are key events of the pathophysiological alterations occurring in I/R. Oxidative stress plays a fundamental role in the process of renal apoptosis. Studies showed that, either by signal transduction molecules or by causing cellular damage, ROS can activate apoptosis process [16].

It is known that quercetin alleviates kidney fibrosis by reducing renal tubular epithelial cell senescence inhibiting the mitophagy. Several authors associate these effects to the fact that phenolic compounds, such as flavonoids, could inhibit the epithelial-mesenchymal transition, preventing the occurrence of irreversible dysfunctions resulting from the establishment of acute kidney injury. It has previously been shown that quercetin presented protective effects against oxidative toxicity in human embryonic kidney (HEK-293), preventing oxidative stress [17]. Therefore, it is necessary to find pharmacological tools that allow the association of hemodynamic improvements with direct protective effects on renal tubules, the most affected

tissues by ischemic injuries, focusing mainly on enzymatic targets and hemodynamic events related to hypoxia and oxidative stress.

In fact, quercetin has been extensively studied, mainly due to its compatibility with animal biological receptors and transporters, especially humans, interfering in the process of establishing various pathologies. For example, this flavonoid demonstrated an inhibitory potential on the epithelial to mesenchymal transition of the renal tubular system stimulated by TGF-β1 (Transforming Growth Factor-beta 1), an important event in the establishment of renal fibrosis, indicating an alternative in the prevention of irreversible tissue damage associated with renal injury. Hence, these events result in the inhibition of E-cadherin transcription, which inhibits the proliferation and invasion processes associated with renal fibrosis [18].

These results are relevant because quercetin has demonstrated the nephroprotective potential in models of obstructive kidney injury. This protection is due to its antioxidant and, as a result, anti-inflammatory potential, acting in an immunomodulatory manner. For instance, quercetin has been shown to improve tubulointerstitial kidney damage by modulating the polarization of M1/M2 macrophages, in addition to reducing levels of iNOS (Inducible Nitric Oxide Synthase) and IL-12. Evidence indicates that the pharmacological mechanism involved in this cascade of events involves significant changes in protein translation, since the effects of quercetin were associated with negatively regulated activities of NF-κB (Nuclear Factor Kappa light chain enhancer of activated B cells) p65 [19].

In this sense, in the present work, quercetin was able to decrease the KIM-1 release to cell's supernatant. KIM-1 is a transmembrane protein present in proximal tubular cells, which is cleaved by metalloproteinases after injury. The ectodomain is detectable in urine, while the transmembrane domain acts like a phosphatidylserine receptor, signaling cell death, promoting phagocytosis of apoptotic bodies and oxidized lipids. Previous work showed that quercetin decreased $HgCl_2$-induced nephrotoxicity in Sprague-Dawley rats, decreasing urinary KIM-1 and tissue inhibitor of metalloproteinases 1 (TIMP-1). Quercetin also reduced the vascular activation, decreasing urinary vascular endothelial growth factor (VEGF); and inhibited phagocytosis, decreasing urinary monocyte chemoattractant protein-1 (MCP-1) [20].

In an experimental study carried out on Wistar rats, it was observed that quercetin reduced cisplatin-induced nephrotoxicity due to its antioxidant, vascular, anti-inflammatory, and anti-apoptotic effects. The levels of caspase-3 cleaved in the renal tissue were reduced after treatment with quercetin, all associated with the inhibition of NF-κB activation, inhibition of apoptosis, and a consequent reduction in the levels of TNF-α, iNOS and inhibition of neutrophilic infiltration in the renal tissue. In addition, these effects are tissue and hemodynamic, since quercetin also has the potential to increase cortical blood flow, improving perfusion in the nephrons [21]. It is a constant challenge to slow the progression of renal fibrosis and maintain tissue functionality, particularly in the case of tubular epithelial cells. An *in vitro* study showed that quercetin relieves renal fibrosis by reducing angiotensin II-induced senescence in renal tubular epithelial cells. These conclusions were associated with the inhibition of fragmentation, the accumulation of mitochondrial mass and the modulation of mitophagy, an autophagic process of mitochondria [22].

Ergo, when observing a cascade of cellular events involved with the nephroprotective potential of quercetin, a promising perspective is outlined, since specialists in the field of pharmacological nephroprotection describe that antioxidant substances of natural origin are candidates for experimental and even clinical studies. Nonetheless, consistent results were obtained when substances with a potential enzyme inhibitor or antagonists of biochemical pathways which generate oxidative stress were used. Antioxidant substances of the scavenger type, which reduces the preformed reactive species instead of inhibiting their formation, do not usually generate a significant protective effect or cause chronic repercussions [23].

In the current study, the nephroprotective effects of quercetin were compared to lisinopril and losartan, inhibitors of RAS. Lisinopril, an ACE inhibitor, did not present protective effects against I/R-induced AKI in the used model. Moreover, losartan, an angiotensin receptor blocker (ARB), presented a moderated effect when compared to quercetin. A study showed that irbesartan, an AT1 blocker, reduced intestinal inflammation and stress-induced ROS accumulation in mice via AT1 signaling and ACE-dependent mechanism in mice. It is known that AT1 inhibition, overloads ACE, which activates the antioxidant and anti-inflammatory Angiotensin (1-7) pathway, corroborating with the findings of this work [24].

In this context, the present work used molecular docking simulations to correlate the antioxidant potential of quercetin with its possible protective effect on renal tubular epithelial cells. According to the thermodynamic properties of the interactions, it was observed that quercetin has a potential to interact with the angiotensin AT1 receptor with greater affinity, presenting a stable bond characterized by an enthalpy of -8.1 Kcal/mol. In fact, this interaction occurs through the formation of five hydrogen bonds of strong intensity, in addition to

non-covalent attractions between π-π stacking aromatic rings, which promotes the stacking of molecular structures and, consequently, greater stability for the interaction. Comparatively, the interaction simulation with the AT1 receptor did not show hydrophobic interactions, unlike what happened with ACE, indicating possible interactions of less stability between these enzymes.

Literature data indicate a possible pharmacological effect of quercetin on the renin-angiotensin-aldosterone axis, generating a protective effect. For example, quercetin has been proven to attenuate ethanol-induced iron deposition and the consequent damage to the myocardium caused by alcoholism, by regulating the L-type Ca^{2+} voltage dependent on angiotensin with effects comparable to those of nifedipine, an inhibitor Ca^{2+} channels, or losartan [25].

In addition to it, studies demonstrate that excess intracellular angiotensin II induces apoptosis through the mitochondrial pathway in a variety of cells, including endothelial and epithelial cells. A study carried out with human umbilical vein endothelial cells demonstrated that quercetin inhibits angiotensin II-induced apoptosis through the mitochondrial pathway in a concentration and time-dependent manner. Moreover, loss of mitochondrial membrane potential, positive regulation of cytochrome c and Bax, negative regulation of Bcl-2 and activation of caspase-9 and caspase-3 caused by angiotensin II were also recovered after treatment with quercetin [26].

Thus, there are sufficiently consistent studies to state that quercetin improves redox imbalance, especially with involvement of angiotensin II. Literature data demonstrate that quercetin has a hypotensive, cardioprotective and nephroprotective effect associated with the reduction of mRNA transcription for NADPH-oxidase and, subsequently, with the levels of nitric oxide and superoxide induced by angiotensin II [27].

These findings are compatible with the fact that flavonoids such as quercetin exert modulation of apoptotic and inflammatory genes and, consequently, angiotensin II antagonism. Models of urethral obstruction justify these statements when observing that quercetin inhibits the expression of monocyte-1 chemotactic protein, as well as enalapril and losartan. However, the expression of the Fas surface receptor gene is inhibited by quercetin, but not by enalapril or losartan, indicating an anti-apoptotic potential of quercetin [28].

The association of silico studies with practical experimental models can help to better understand the mechanisms involved in the beneficial effects already described for quercetin. The results of this study indicate the involvement of the possible blockade of the renin-angiotensin-aldosterone axis in the nephroprotection and antioxidant effect associated with quercetin. About this theme, a study carried out through chronic treatment with quercetin demonstrated that this flavonoid does not inhibit the activity of the angiotensin converting enzyme in vivo or *in vitro*, despite having a hypotensive effect even after the administration of angiotensin I and II, indicating that the inhibition could happen by another route of the axis [29]. Thus, the main strands reinforce the hypothesis that quercetin has modulatory effects on AT1 receptors and the consequent calcium-dependent signaling, corroborating the findings of the present study.

Altogether, our results suggest that quercetin inhibits the oxidative stress on the mitochondria of tubular cells, preventing the cell death. Thus, more specific studies must be performed to improve approaches in this issue.

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