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Bacopa Monnieri Protects the Directly Affected Organ as Well as Distant Organs Against I/R Injury by Modulating Anti-Inflammatory and Anti-Nitrosative Pathways in A Rat Model for Infra-Renal Aortic Occlusion

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Bacopa Monnieri Protects the Directly Affected Organ as Well as Distant Organs Against I/R Injury by Modulating Anti-Inflammatory and Anti-Nitrosative Pathways in A Rat Model for Infra-Renal Aortic Occlusion

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ABSTRACT

Objective: To investigate the protective effect and underlying mechanisms of *B. monnieri*, a medicinal plant, on kidney and skeletal muscle injury induced by infra-renal abdominal aorta clamping for 2-hours (ischemia) and following removal of the clamp (reperfusion, 2-hours).

Methods: Rats were divided into four groups (n = 6): (I) animals given only saline (sham-control); (II) animals given *B. monnieri* extract for 10-days (300 mg/kg/day) (*Bacopa*-treated sham); (III) animals subjected to ischemia/reperfusion (I/R); (IV) animals given *B. monnieri* extract and then subjected to I/R. Kidneys and lower extremity muscles were examined for GPx, CAT, iNOS, 3-NT, IL-1β and TNF-α. Apoptosis and injury were evaluated by TUNEL and H&E staining, respectively.

Results: I/R resulted in TUNEL positive cells, periarterial edema and glomerular capillary dilatation, decreased GPx activity, unchanged CAT, iNOS, 3-NT, IL-1β and TNF-α in kidney. *B. monnieri* minimized renal remote reperfusion injury, and Group IV showed a lower degree of renal histopathology score when compared to the others. *B. monnieri* mitigated muscle I/R injury, decreased muscle hypertrophy, myofibril abnormalities and apoptosis. Muscle 3-NT and cytokine levels were increased by I/R, and *B. monnieri* inhibited iNOS and 3-NT both in sham-control and I/R groups. Muscle GPx unaffected by I/R or *B. monnieri, but* CAT was inhibited only in *B. monnieri*-treated I/R group. Muscle iNOS, 3-NT, IL-1β, TNF-α levels and CAT activity of *B. monnieri*-treated I/R rats were lower than those in sham-control or *Bacopa*-treated sham.

Conclusions: *B. monnieri* can protect the directly affected organ as well as distant organs against I/R injury by modulating anti-inflammatory and anti-nitrosative pathways.

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Bacopa monnieri; ischemia–reperfusion; infra-renal aortic occlusion; nitrosative stress; inflammation; apoptosis

Introduction

Ischemia is defined as a loss of blood supply due to a reduction in the arterial flow within a tissue, and reperfusion is the term used to define the reestablishment of flow after a period of ischemia, which may aggravate the ischemic cellular damage, leading to failure of multiple organs [1]. Ischemia-reperfusion (I/R) is a well-known pathological condition and its complications are mainly characterized by a profound inflammatory response and oxidative injury occurring in distant organs [2, 3]. During ischemia, the inflammatory mediators released into the systemic blood circulation and the activity of the antioxidant enzymes is diminished, leading to an increase in the formation of free oxygen radicals and nitrogen species and oxidative-nitrosative stress at reperfusion [3, 4]. Reperfusion is accompanied by other reactive oxygen species, which impair mitochondrial Ca^{2+} homeostasis, and to further cytochrome C release and initiation of apoptosis [5]. Targeting involved pathologies by standard treatment individually produces improved recoveries, but they fail in translation [6]. For instance, clinical applications targeting mitochondria have encountered certain limited positive outcomes [7]. A large number of experimental studies have proposed the use of different substances for the purpose of avoiding or minimizing the deleterious effects secondary to I/R. Therefore, at present, several multi-functional plant biologicals, such as polyphenols [8], the triterpenoids [9] and saponins [10] may have the potential to fulfill such a role.

B. monnieri is an important medicinal plant that has been used in traditional Ayurvedic medicine for centuries [11]. In pharmacologic studies, the *B. monnieri* extracts (e.g., Brahmi) has been attributed with various medicinal properties including anti-inflammatory, antioxidant, cardiotonic, anti-hypercholesterolemic and nerve tonic, and these have been correlated to the presence of steroidal saponins called bacosides [12–15]. There is evidence that *B. monnieri* improves systemic vascular function and relaxes a wide range of arteries [16]. It regulates endothelial NO release and inhibits Ca^{2+} influx and sarcoplasmic reticulum Ca^{2+} release in vasculature [16]. Furthermore, *B. monnieri*

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improves myocardial function following I/R injury through recovery of coronary blood flow, contractile force and decrease in infarct size as *in vivo*[3] and *in vitro* [15]. The mechanism of action might be mainly related to its antioxidant and anti-inflammatory properties [14, 17]. Various extracts of *B. monnieri* were found to significantly inhibit many inflammatory enzyme cascades and the release of IL-6, TNF-α, MMP-3, and caspase 1 and 3 [14]. In addition to Bacosides, indole compounds and phenolic acids in this plant have also been reported to be responsible for anti-inflammatory activity by reducing COX-2 and cPGES expression [18]. These observations suggest that *B. monnieri* extract may be a promising agent for the management of deleterious effects secondary to I/R in distant organs.

Therefore, we aim to determine the effects of short-term (10 days) treatment with *B. monnieri* extract in remote injury and inflammation in lower extremity muscle and kidney following infra-renal aortic occlusion in rats.

Materials and methods

Animals

Twenty-four adult, age-matched (10–12 weeks old) and weight-matched (170–222 g) Wistar albino male rats were used in the study. The rats were obtained from Gazi University Laboratory Animal Breeding and Experimental Research Center, where they were housed with free feed and water during the pretesting and experiments. Compliance of the protocols of our experimental research with the relevant principles was approved by Gazi University Animal Experiments Local Ethics Committee (Protocol No: G.Ü.ET-17.078). In accordance with the guidelines, humanitarian care was provided for all of the animals; Guidelines for the Principles of Laboratory Animal Care prepared by the National Scientific Research Association, The Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. All experimental procedures were performed in accordance with the circadian rhythm of rats (12 hours of daylight, 12 hours of darkness).

Experimental groups and treatment

The experimental animals were divided into four groups. The first was Group I; Sham-control group (n = 6). This group received only physiologic saline (vehicle) by oral gavage once daily for 10 days. After 10 days of maintenance, dissection was performed by laparotomy without an aortic clamp. The next was Group II; Bacopa-treated sham group (n = 6). *B. monnieri* extract was applied by dissolving in physiological saline (300 mg/kg/day p.o) for 10 days [19]. After 10 days of maintenance, dissection was performed by laparotomy without an aortic clamp. The third was Group III; Ischemia/Reperfusion (I/R) group (n = 6). The rats in this group were given vehicle during 10 days of care. Laparotomy was performed after 10 days of care and two hours of ischemia was created by untraumatized cross-clamping from the infra-renal region of the abdominal aorta. After this, reperfusion was achieved by removing the clamp from the abdominal aorta for two hours. The last was Group IV; I/R group which was under treatment with *B. monnieri* extract (n = 6).

B. monnieri extract (a powder extract from *B. monnieri*) was obtained from Akses Swiss GmbH, Switzerland. According to the data sheet, the extract mixture includes mainly triterpenoid saponins as bacopasaponins (particularly bacoside A and bacopaside I), and the principal flavonoids (luteolin and apigenin). *B. monnieri* extract was dissolved in 0.5 ml of physiologic saline and was given to the rats in Groups II and IV by oral gavage once a day for 10 days (300 mg/kg/day).

Surgical procedure, protocol of ischemia-reperfusion (I/R) by aortic occlusion

Laparotomy was performed after ten days of care and 120 minutes of ischemia was created by untraumatized cross-clamping from the infra-renal region of the abdominal aorta. After this, reperfusion was achieved by removing the clamp from the abdominal aorta for 120 minutes. All surgical procedures performed during the experiment were performed under sterile conditions with ketamine and xylazine anesthesia.

General anesthesia was applied according to the protocol before the surgical procedure. Two hours of fasting took place before the anesthesia and rats were prevented from drinking water 60 minutes prior to the procedure. All of the animals were treated with ketamine and xylazine 30 min prior to the surgery (Ketamine hydrochloride, (Ketalar, 45 mg/kg rat i.m; Parke-Davis Eczacibasi, Istanbul, Turkey) and Xylazine hydrochloride (Alfazyne, 2%, 5 mg/kg rat, i.m; Ege Vet, Izmir, Turkey). The depth of anesthesia was monitored during the perioperative period. Reflex monitoring, muscle tone, breathing pattern, heart rate and rhythm were evaluated periodically. Spontaneous breathing was achieved in all animals during the anesthesia. In order to prevent heat loss, a perioperative lower body heating blanket was used. In this period, periodic intraperitoneal physiologic saline was administered to ensure fluid-electrolyte stability. Surgical procedures were performed under a heating lamp while the rats were in a supine position. The abdominal areas of the rats were shaved and the skin was prepared aseptically. A skin-subcutaneous incision was made from the abdominal midline and, after this, respectively, the peritoneum was opened, the abdominal wall was lateralized, and the intestines were lateralized over a sterile damp gauze moistened with saline outside the intestinal region. The abdominal aorta was exposed through a midline abdominal incision, and the infrarenal aorta was cross-clamped for two hours followed by two hours of reperfusion [20]. A bulldog clamp was used for infrarenal aortic occlusion. The occlusion was monitored periodically by palpating the distal aorta and both femoral arteries. Occlusion and ischemia were confirmed with pulsation loss in the examined pulses. In accordance with the model of distal ischemia, the

application of the aortic clamp was continued for two hours, and then distal circulation was achieved again when the aortic clamp was removed. Reperfusion was monitored periodically by palpating the distal aorta and both femoral arteries. Circulation and reperfusion were confirmed by the presence of pulses in the examined areas. Application for the distal reperfusion model was continued for two hours. After I/R, both the kidneys and the muscles of the lower extremities were harvested.

ELISA analysis

Saline-flushed tissues from each rat were minced into small pieces and washed three times with PBS, and then lysed with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.25% Na-deoxycholate, 2% Triton-X100, 1 mM PMSF, 2 µM leupeptin). The protein concentration of each lysate was measured by BCA protein assay Kit (Pierce, Rockford, Illinois, USA). ELISAs for glutathione peroxidase (GPx), catalase (CAT), inducible nitric oxide synthase (iNOS), 3-nitrotyrosine (3-NT) (Cusabio Technology LLC Houston, USA) and pro-inflamatory cyokines IL-1β, TNf-α (Boster Biological Technology, Valley Ave Pleasanton, USA) were performed according to the manufacturer's instructions. In brief, 100 µl of tissue lysates dispended in antibody-embedded 96 well plates and after all of the steps absorbance was read at 450 nm by a microplate reader (PowerWave XS2; BioTek Instruments Inc., USA). All of the calculations were made according to kits own standard-curve graphs and normalized with their protein concentrations. All the samples were tested in duplicate.

Hematoxylin-eosin (H&E) staining

Tissue samples of suitable size were collected and rinsed with a phosphate buffer solution. After being fixed in 10% neutral formaldehyde for 72 hours, the tissues were embedded in paraffin. Sections of 4 µm thickness were obtained from paraffin blocks on slides using a microtome (Leica SM 2000, Germany). The sections were deparaffinized using xylene and rehydrated using a graded ethanol series. The rehydrated sections were then stained with hematoxylin and eosin and the slides were examined histologically using a computerized photo-light microscope (Leica DM4000B, Germany).

TUNEL staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to detect DNA fragmentation and apoptotic cell death in the tissues. The TUNEL assay was performed using an in situ cell death detection kit (Chemicon, USA cat#S7101). Paraffin sections of four µm thickness were obtained from paraffin blocks on polylysine-coated slides. After endogenous peroxidase was inactivated in 3% hydrogen peroxide, the tissues were equilibrated and applied with TdT enzyme in a humidified chamber at 37 °C for one hour. Reactions were stopped by incubating tissues in stop/wash buffer for ten minutes before proceeding anti-digoxignenin conjugate in a humidified chamber for thirty minutes. The sections were applied with peroxidase substrate 3,3′-diaminobenzidine (DAB) (Invitrogen, Carlsbad, CA cat#00-2020) to detect TUNEL positive cells and were counterstained with methyl green. After dehydration with 100% n-butanol, the slides were closed using mounting medium and examined under a computerized photo-light microscope (Leica DM4000B, Germany).

Statistical analysis

The results of the ELISA assays were analyzed using an analysis of variance (ANOVA) test with Dunnet's post hoc test. The results were expressed as mean ± SEM and values of $p < 0.050$ were considered as statistically significant.

The kidney sections were scored over ten fields assessing changes in the following morphological variables; glomerular capillary dilatation, peritubular capillary dilatation, tubular dilation, desquamation of tubular cells and periarterial edema.

The skeletal muscle sections were scored over ten fields, assessing changes in the following morphological variables; nuclear migration, amitotic division, disrupted myofibril organization and hypertrophy.

Samples were semi-quantitatively scored from 0 to 3 according to the level of damage: 0, normal; 1, mild; 2, moderate; and 3, severe morphological changes. Histopathological evaluations were conducted by two independent histologists, who were blind to the treatment protocol. Statistical analyses were held with a Chi-squared test using GraphPad Prism 7 software.

Statistical analyses for TUNEL staining were performed using a Statistical Package for the Social Sciences (SPSS) statistical software (SPSS for Windows, version 20.0). All of the data was expressed as means ± standard deviation (SD). TUNEL-positive cells in lower extremity muscle and kidney tissues were counted out in five different fields in cross-sections from each group. A Kruskal-Wallis test was used for nonparametric data and comparisons between groups were performed using a Mann-Whitney U test; p values of less than 0.05 were accepted as statistically significant.

Results

GPx, CAT, iNOS, 3-NT, IL-1β and TNf-α

I/R led to a significant decrease in glutathione peroxidase activity (GPx) in the kidneys of rats. Ten days pretreatment with *B. monnieri* extract (1 x 300 mg/kg p.o.) resulted in a significant amelioration in renal GPx (Figure 1). No statistical difference was observed in the skeletal muscle GPx among the experimental groups (Figure 1).

Neither I/R nor *B. monnieri* affected catalase activity (CAT) in the rat kidney. However, extremity muscle CAT significantly decreased in the *B. monnieri*-treated I/R group compared to both sham control and I/R groups (Figure 1).

No significant change was observed in the iNOS activity (iNOS) of kidney tissues. iNOS was significantly decreased

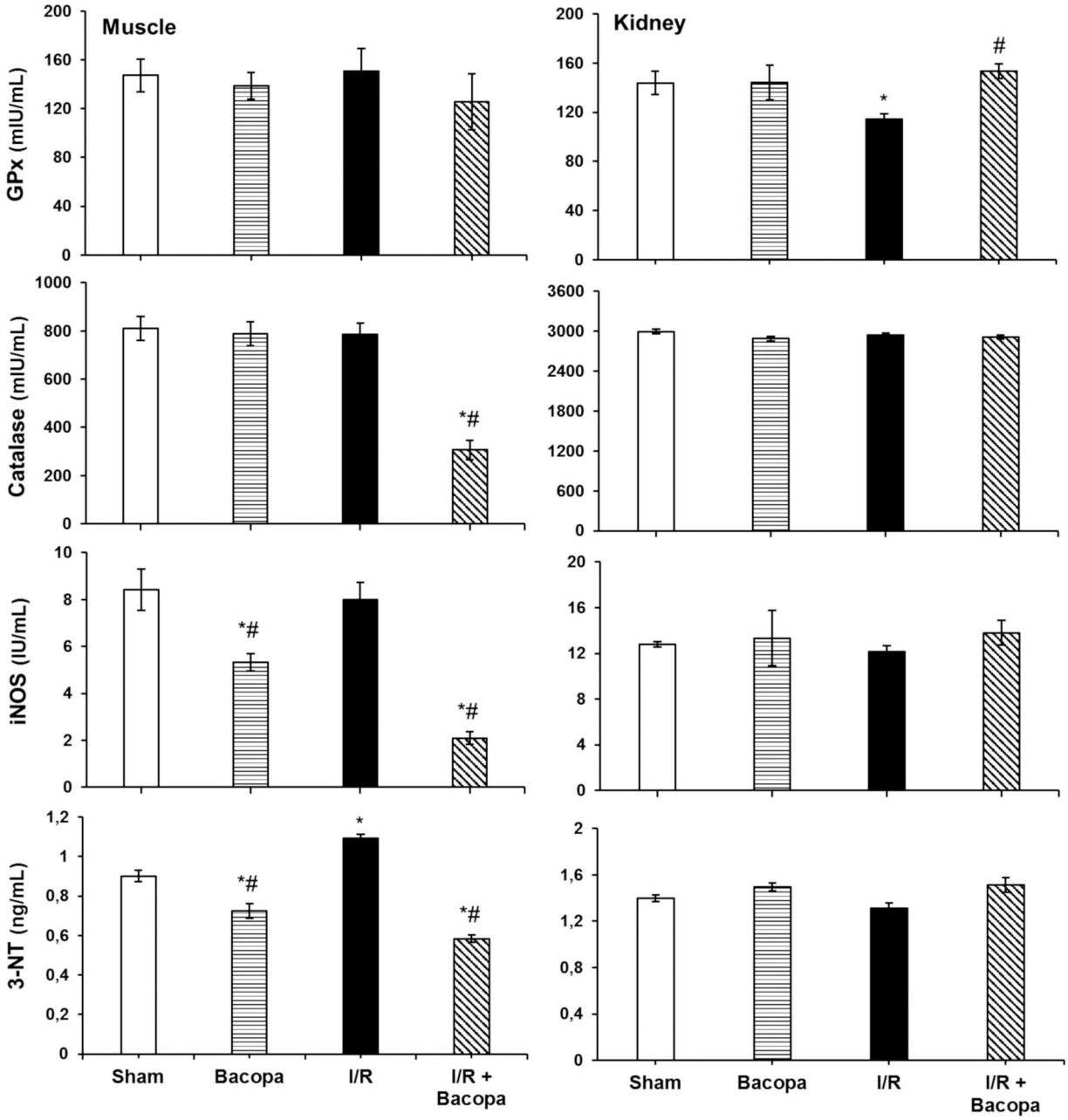


Figure 1. Glutathione peroxidase (GPx), catalase, inducible nitric oxide synthase (iNOS), 3-nitrotyrosine (3-NT) levels of skeletal muscle (Muscle) and kidney (Kidney) of rats obtained from sham control (Sham), Bacopa-treated sham (Bacopa), Ischemia/Reperfusion (I/R) and Bacopa-treated I/R (I/R + Bacopa) groups. *$p < 0.05$ vs sham-control (Sham); #$p < 0.05$ vs Ischemia/Reperfusion (I/R).

by *B. monnieri* treatment in the sham and I/R rats. The iNOS of *B. monnieri*-treated rats was significantly lower than those obtained in the sham-control or I/R groups (Figure 1).

3-Nitrotyrosine levels (3-NT) of kidneys were not significantly different in the experimental groups studied. In the extremity muscle, I/R significantly increased 3-NT. The *B. monnieri* treatment inhibited 3-NT of the extremity muscles of rats with or without I/R (Figure 1).

There was no significant difference in kidney IL-1β levels among the experimental groups. However, IL-1β levels in the lower extremity muscle were significantly higher in I/R group when compared with the sham-control group. The extremity muscle IL-1β levels were decreased in the *B. monnieri*-treated I/R group compared to the sham-control or I/R groups (Figure 2).

In the kidney, TNF-α levels were found to be in similar degrees among the experimental groups. I/R significantly increased the lower extremity muscle TNF-α levels. However, *B. monnieri* treatment prevented I/R-induced increase in TNF-α levels in the lower extremity muscle, and TNF-α levels *B. monnieri*-treated group was found to be significantly less than the sham-control or I/R group (Figure 2).

H&E staining of the lower extremity muscle

In the H&E staining of the sham control and *B. monnieri*-treated groups, the skeletal muscle was normal in appearance

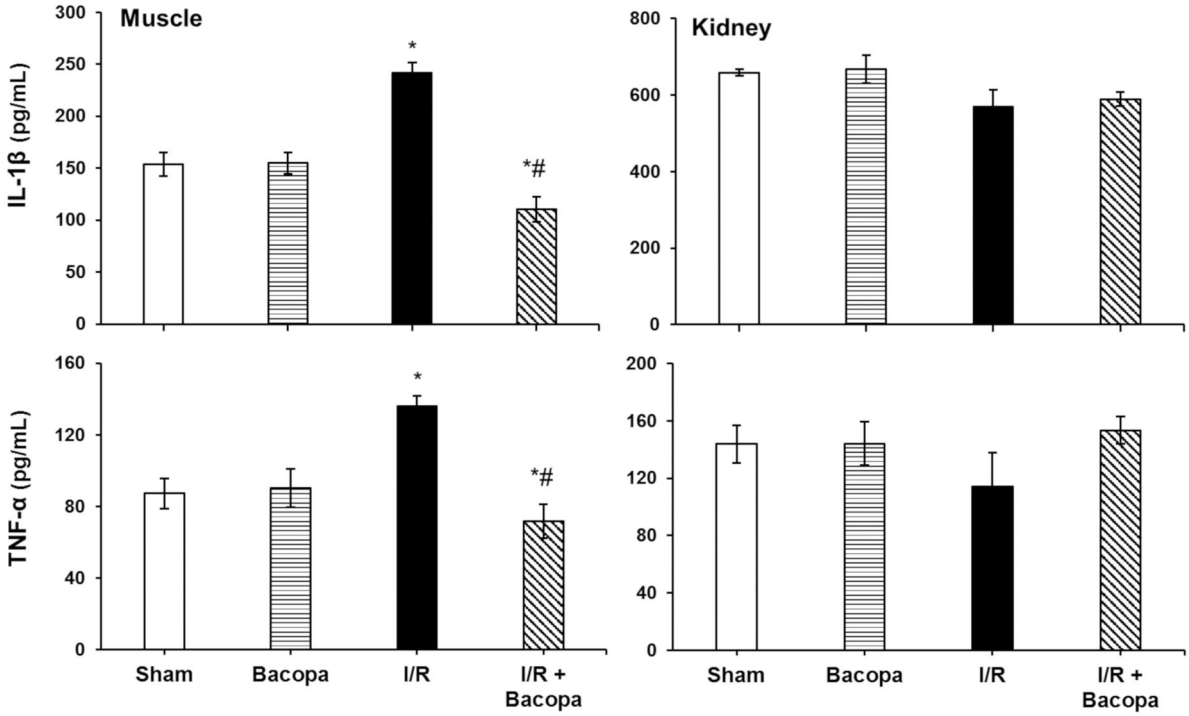


Figure 2. IL-1β and TNF-α levels of skeletal muscle (Muscle) and kidney (Kidney) of rats obtained from sham-control (Sham), Bacopa-treated sham (Bacopa), Ischemia/Reperfusion (I/R) and Bacopa-treated I/R (I/R + Bacopa) groups. *$p < 0.05$ vs sham control (Sham); #$p < 0.05$ vs Ischemia/Reperfusion (I/R).

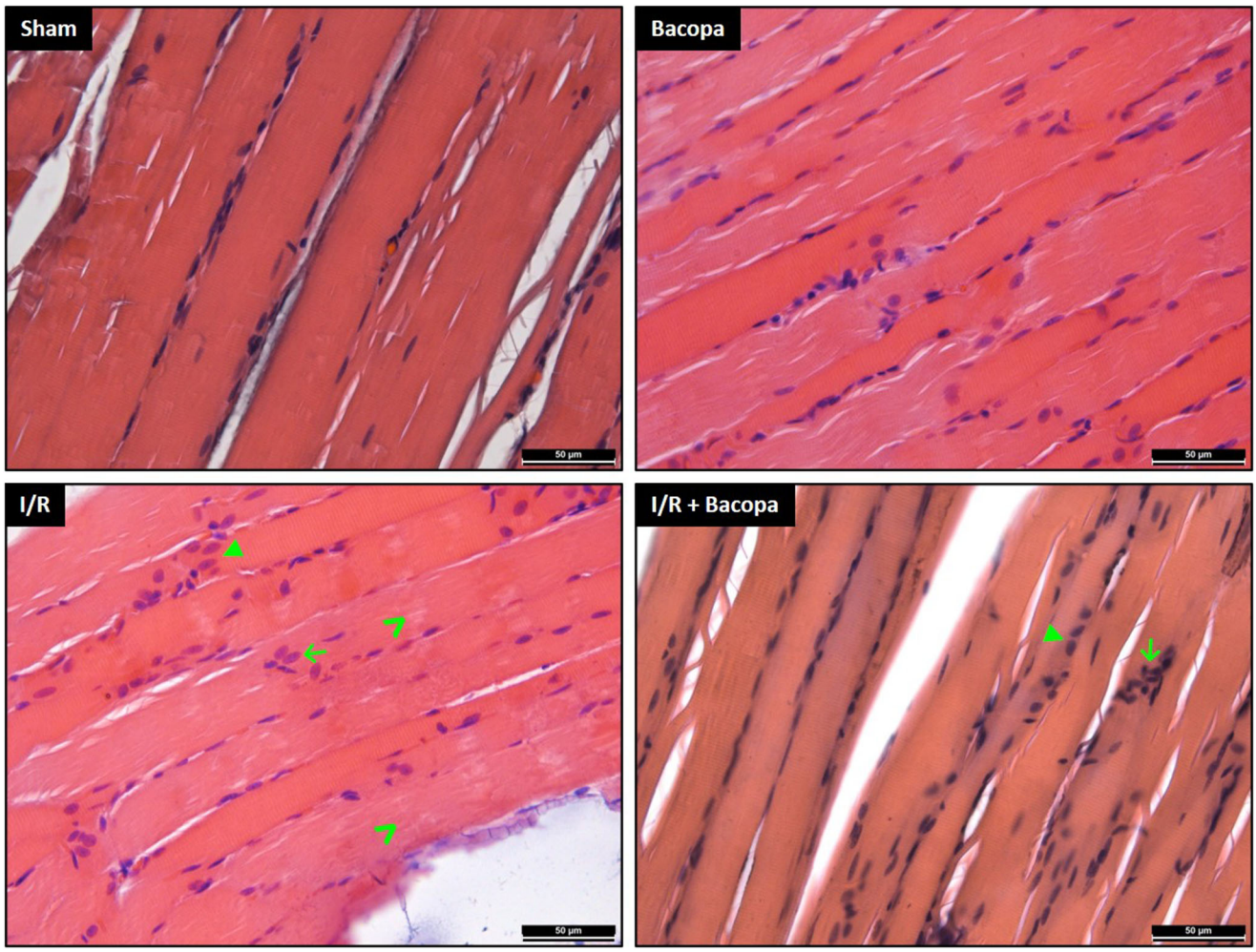


Figure 3. Representative photomicrographs of Hematoxylin and eosin stained sections from the lower extremity muscle. Sham-control (Sham) and Bacopa-treated sham (Bacopa) rats exhibited normal structures of skeletal muscle tissue. I/R rats (I/R) showed nuclear migration to the central position (↑), amitotic nuclear division (▼) and disrupted myofibril organization (>). Bacopa-treated I/R rats (I/R + Bacopa) showed nuclear migration to the central position (↑) and amitotic nuclear division (▼). Magnification, ×400.

with the nuclei located on the periphery directly under the sarcolemma and regular striations. The blood vessels were clear in the endomysium which surrounded muscle fibers (Figure 3). In I/R group, the most striking findings were nuclear migration to the central position and amitotic nuclear division. Myofibril organization was disrupted and there were no striations, particularly in the fibers with nuclear migration (Figure 3). No change in endomysium was observed. Nuclear migration and amitotic division were also detected in the *B. monnieri*-treated I/R group. However, myofibril organization and striations were more regular than in I/R group (Figure 3). Histological scores for severity of injury from muscle tissues are shown in Table 1.

Table 1. Injury severity score from lower extremity muscle histology.

	0	1	2	3
Sham control	6	0	0	0
Bacopa	5	1	0	0
I/R	0	0	0	6
I/R + Bacopa	0	2	3	1

Sections were scored 0-3 (0, normal; 1, mild; 2, moderate; 3, severe) over 10 fields assessing changes in the following morphological variables: nuclear migration, amitotic division and disrupted myofibril organization. With the sham group (normal histology), there was a significant difference between I/R group (chi-squared test, $P = 0.005$). Moreover, the I/R group treated with Bacopa monnieri showed a significant decline from severe to mild injury (chi-squared test, $p = 0.0138$).

H&E staining of kidney

In the sham control group, H&E staining of all the structures was normal in appearance in the kidney cortex and medulla (Figures 4 and 5). In the *B. monnieri*-treated group, the most typical finding in the cortex was the relative dilatation of glomerular capillaries. Hyalinization of the urinary space was also prominent, and squamous epithelial cells forming the parietal layer of the Bowman's capsule could not be observed clearly. Distinct dilatation was present in the peritubular capillaries as in the glomeruli. The proximal and distal tubules with macula densa were observed with their normal structures (Figure 4). There was dilatation of medullary capillaries while the thin loop of Henle and collecting ducts were normal in appearance in this group (Figure 5). Dilatation of glomerular capillary network was also seen in I/R group. The parietal layer of the Bowman's capsule was clear with its squamous epithelial cells. Distal tubules and macula densa structures were normal, but there was desquamation of proximal tubular epithelial cells

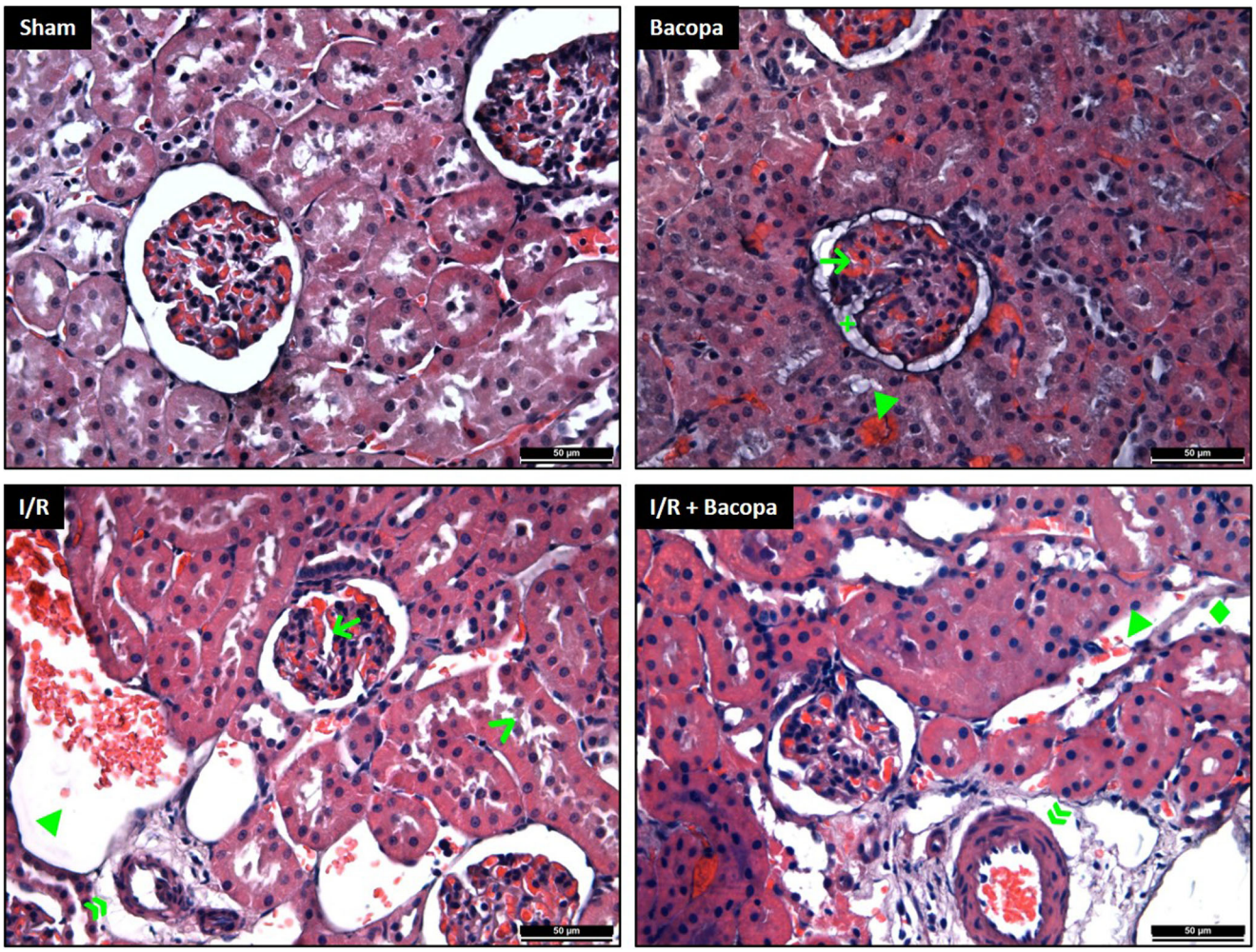


Figure 4. Representative photomicrographs of Hematoxylin and eosin stained sections of renal cortex. Structures of cortex in sham-control rats (Sham) were normal in their appearance. Bacopa-treated sham rats (Bacopa) showed dilatation of glomerular capillaries (↑), hyalinization of urinary space (+) and dilatation of peritubular capillaries (▼). In the I/R rats (I/R) there were dilatation of glomerular capillaries (↑), dilatation of peritubular capillaries (▼), desquamation of proximal tubular epithelial cells into lumen (>) and periarterial edema (»). Bacopa-treated I/R rats (I/R + Bacopa) showed decreased periarterial edema (»), dilatation of distal tubules (◆) and dilatation of peritubular capillaries (▼). Magnification, ×400.

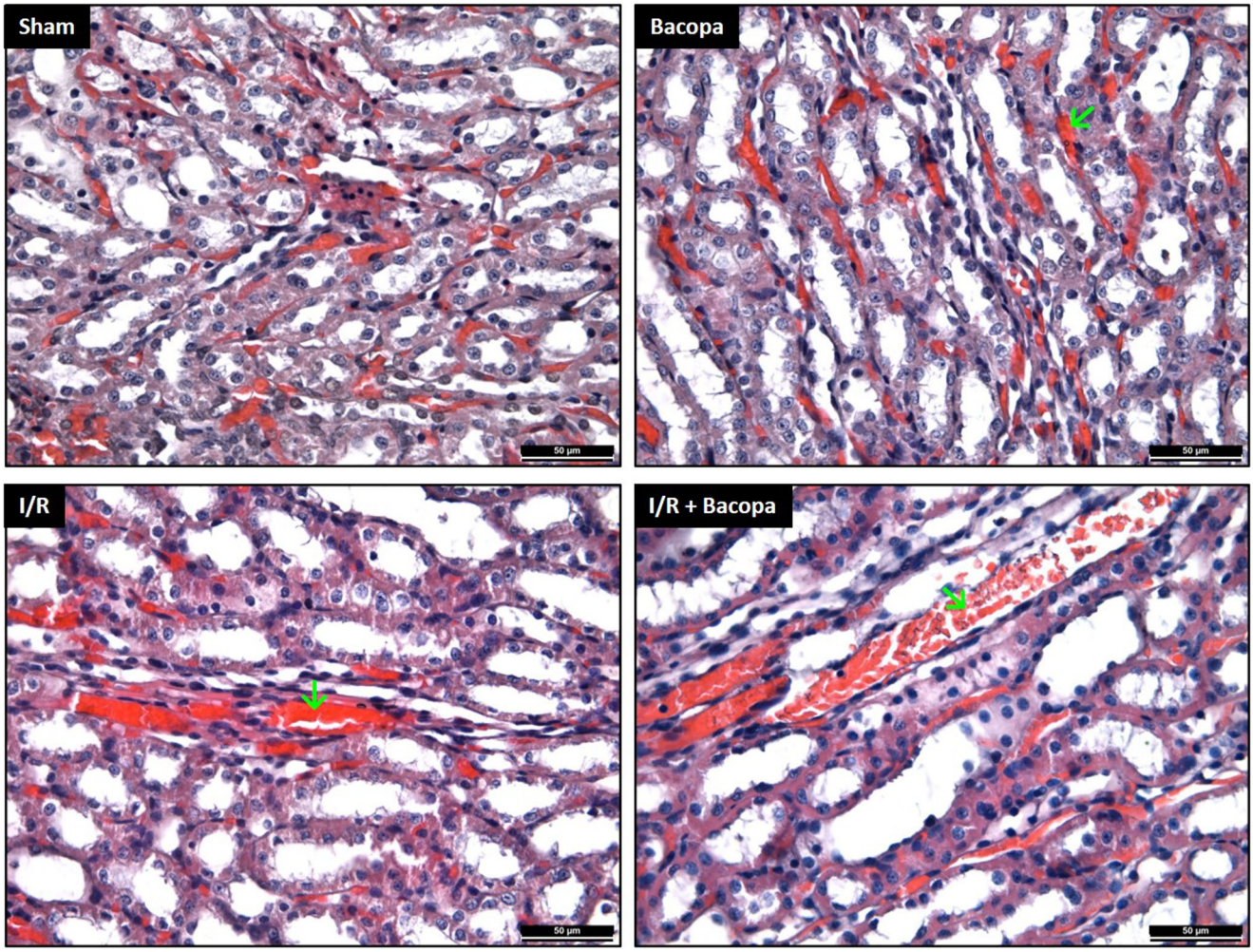


Figure 5. Representative photomicrographs of Hematoxylin and eosin stained sections of renal medulla. Structures of medulla in sham-control rats (Sham) were normal in their appearance. Bacopa-treated sham rats (Bacopa), I/R rats (I/R) and Bacopa-treated I/R rats (I/R + Bacopa) showed dilatation of medullary capillaries (↑). Magnification, ×400.

into the lumen. The most remarkable feature of this group was very intensive and common dilatation of peritubular capillaries. Particular periarterial edema was also observed in the cortex (Figure 4). In the medulla, there were dilated capillaries, but the thin loop of Henle and collecting ducts were normal in appearance (Figure 5). Glomerular capillaries, the parietal layer of the Bowman capsule, macula densa and proximal tubules were normal in the kidneys of the *B. monnieri*-treated I/R group. There was no dilatation of the glomerular capillaries or desquamation of proximal tubular epithelial cells. Periarterial edema was decreased compared to I/R group. However, dilatation of distal tubules and intensive dilatation of peritubular capillaries continued to be seen (Figure 4). Dilated capillaries were present in the medulla while the thin loop of Henle and collecting ducts were normal in appearance (Figure 5). Histological scores for severity of injury from kidney tissues are shown in Table 2.

Table 2. Injury severity score from renal histology.

	0	1	2	3
Sham control	6	0	0	0
Bacopa	0	3	3	0
I/R	0	0	1	5
I/R + Bacopa	0	3	2	1

Sections were scored 0-3 (0, normal; 1, mild; 2, moderate; 3, severe) over 10 fields assessing changes in the following morphological variables: glomerular capillary dilatation, peritubular capillary dilatation, tubular dilation, desquamation of tubular cells and periarterial edema. With the sham group (normal histology), there was a significant difference between I/R group (chi-squared test, $P = 0.0025$). Moreover, the I/R group treated with Bacopa monnieri showed a slightly significant decline from severe to mild injury (chi-squared test, $p = 0.0498$).

TUNEL staining of the lower extremity muscle and kidney

In order to evaluate the apoptosis in skeletal muscle and kidney tissues, we performed TUNEL staining. No differences were found in the apoptotic rate of tissues between the sham-control and *B. monnieri*-treated groups (Figures 6 and 7). TUNEL staining revealed that the proportions of apoptotic cells in both skeletal muscle and renal cortex tissues of I/R group rats markedly increased compared with those of sham-control and *Bacopa monnieri*-treated groups. The increased muscular and renal tubular cell death induced by I/R was significantly reduced in Bacopa *monnieri*-treated I/R rats (Figures 6 and 7).

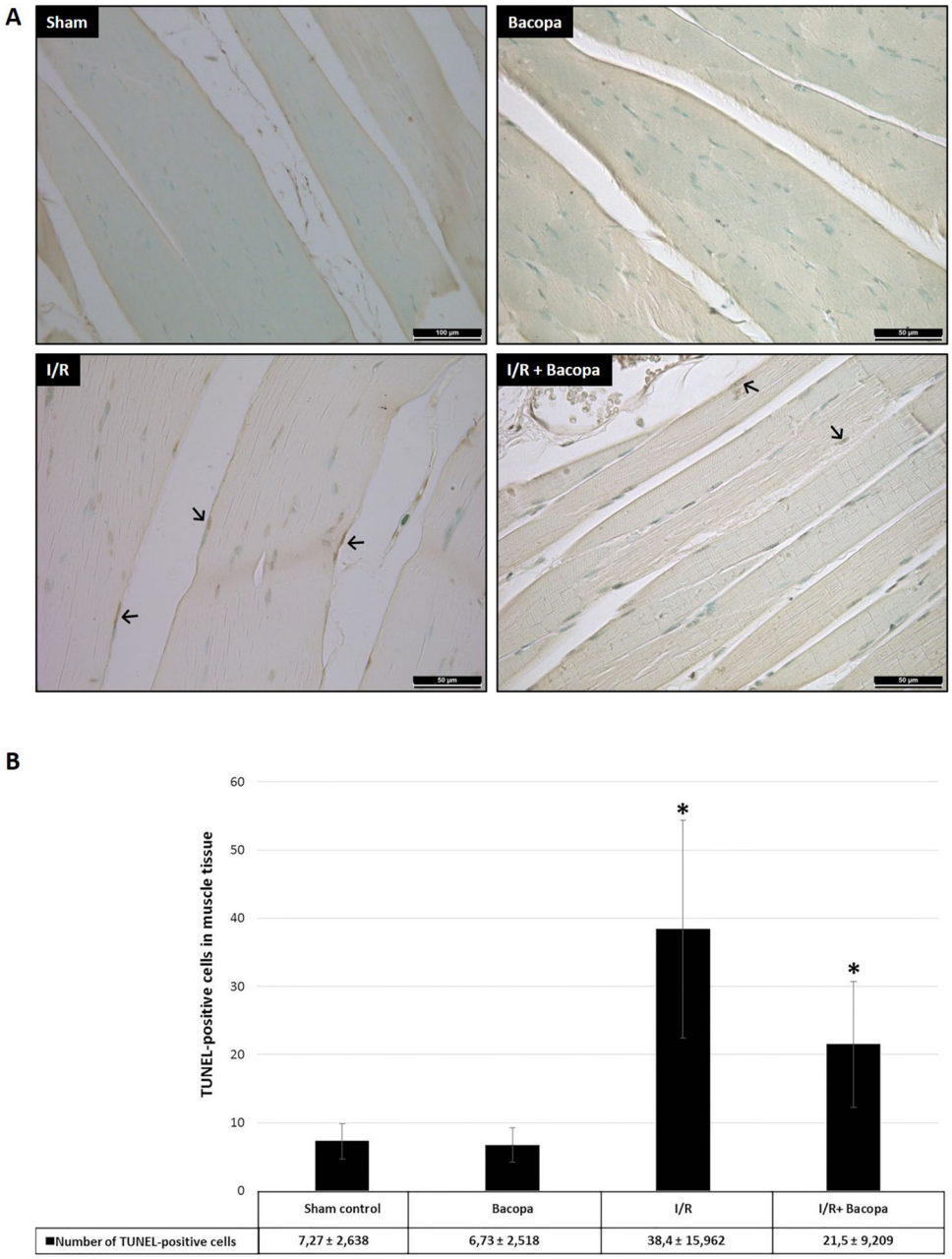


Figure 6. Effect of Bacopa treatment on lower extremity muscle cell apoptosis. (A) Representative photomicrograph of TUNEL staining from muscle tissues of rats. Arrows show TUNEL-positive skeletal muscle nuclei. Magnification, ×400 (B) Quantitative analysis of TUNEL-positive stained skeletal muscle cells. (*: mean number of TUNEL-positive cells were statistically significantly higher in I/R and I/R-Bacopa groups as compared to the other groups, $p < 0.05$). Sham, Sham-control; Bacopa, Bacopa-treated sham; I/R, Ischemia/Reperfusion; I/R + Bacopa, Bacopa-treated I/R.

Discussion

This study demonstrated that *B. monnieri* can directly protect lower extremity muscles against I/R injury thus minimizes renal remote reperfusion injury by mainly controlling cell death pathways, inhibiting inflammatory response and suppressing nitrosative stress.

Reperfusion is a fundamental step in the treatment of ischemia. However, clinical and experimental evidences show that the main events leading to cell and tissue dysfunction are related to reperfusion [2, 4, 5]. Much has been studied about the pathophysiology of reperfusion injury, and some mechanisms have already been well evidenced such as the role of proinflammatory factors, vascular endothelial dysfunction, increased protein S-nitrosylation and oxidative stress [1, 4–6]. Ischemia followed by reperfusion may induce apoptosis and an inflammatory response that affects tissue repair [1, 6]. In the current study, the skeletal muscle was directly affected from ischemia and an advanced redox disturbances was occurred in skeletal muscle after reperfusion compared to those in the kidneys; that is, 3-NT, a marker of nitrosative stress, increased in skeletal muscle of I/R rats, while CAT, GPx and iNOS remained unchanged. As is well known, nitric oxide (NO) reacts with superoxide to form peroxynitrite, which attacks proteins and produces 3-nitrotyrosine (3-NT) [21]. Therefore, in the context of our findings, it can be argued that superoxide increased by I/R in skeletal muscle reacts with NO, contributing to 3-NT

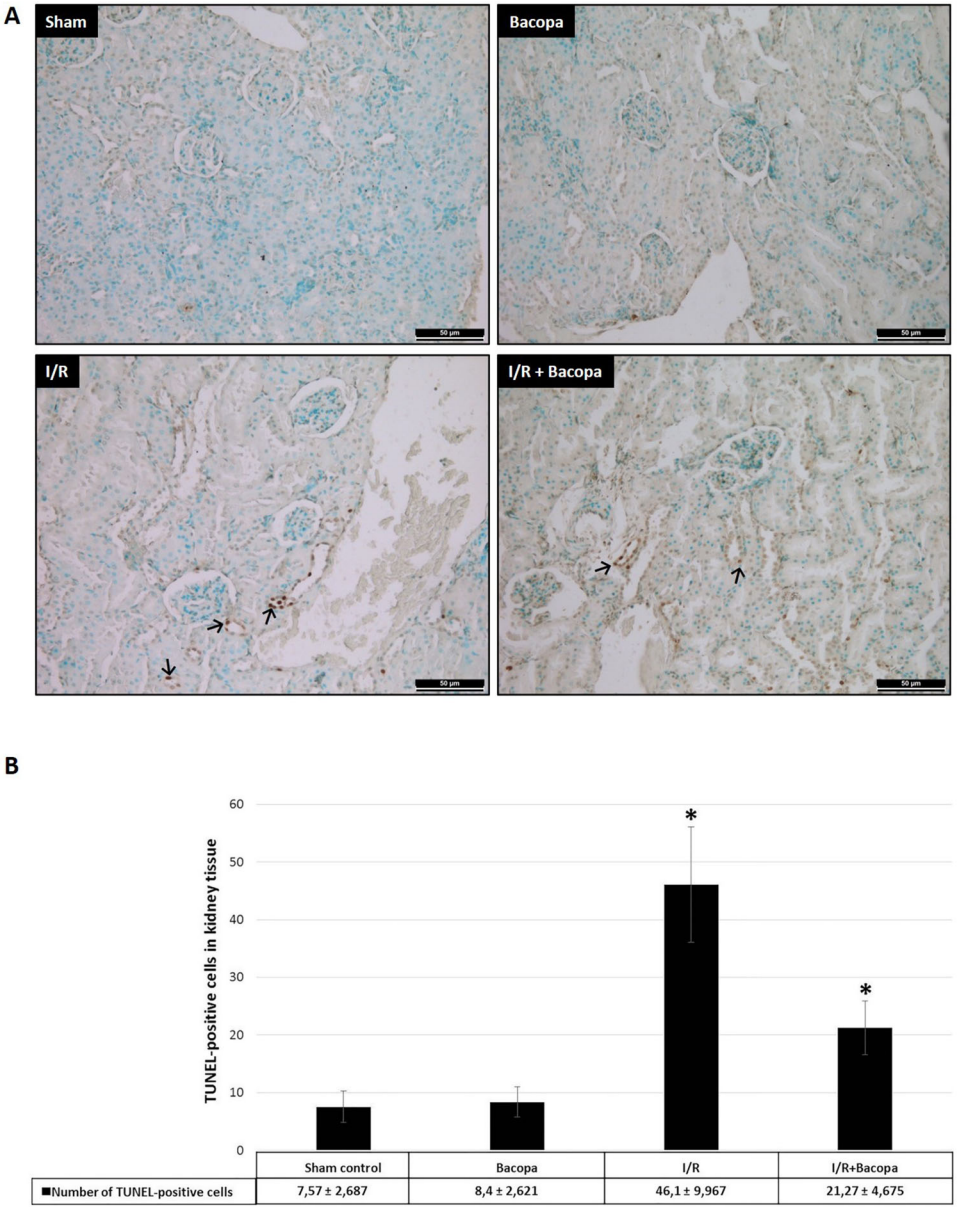


Figure 7. Effect of Bacopa treatment on renal tubular cell apoptosis. (A) Representative photomicrograph of TUNEL staining from kidney cortex tissues of rats. Arrows indicate TUNEL-positive renal tubular cells. Magnification, ×400 (B) Quantitative analysis of TUNEL-positive stained renal tubular cells in kidney cortex. (*: mean number of TUNEL-positive cells were statistically significantly higher in I/R and I/R-Bacopa groups as compared to the other groups, $p < 0.05$). Sham, Sham-control; Bacopa, Bacopa-treated sham; I/R, Ischemia/Reperfusion; I/R + Bacopa, Bacopa-treated I/R.

increase and nitrosative stress that plays a role in I/R injury in skeletal muscle [22]. In support of this, we found that increased 3-NT levels contributes to elevated proinflammatory cytokine levels (IL-1β and TNF-α) in the skeletal muscle of I/R rats. In addition, a significant number of TUNEL positive cell nuclei are associated with impaired myofibril organization, amitotic nuclear division and migration in the skeletal muscles of I/R rats.

Clinically, I/R contributes to acute kidney injury, which is a serious clinical syndrome with high mortality rates [23]. Many of experimental studies performed a direct ischemia by clamping renal hilum [24]. In our model, the kidney, in fact, has not been subjected to ischemia as compared to the skeletal muscle. Although the kidney is indirectly affected by ischemia, even in this case, ROS, which was once produced especially during reperfusion, spreads throughout the body and can cause local and distant injuries, so that a method capable of controlling ROS production can protect the affected organ as well as the distal organs directly. The study of the remote reperfusion injury on the kidney is a relatively new subject; therefore, there are very few publications that allow us to compare with the present results. Marques Dos Santos et al. [25] observed a clear efficacy of ischemic post conditioning on renal protection against renal local or remote I/R. Other studies demonstrated that additional infrarenal aortic-clamping leads to increased renal damage and oxidative stress, despite adequate perfusion of kidneys after suprarenal aortic-clamping [26]. These kind of exposures may lead to a disruption on cellular polarity, cytoskeleton and integrity due to impaired redox signaling, excess protein oxidation, lipid peroxidation and DNA breakdown, finally may results in renal cell death and loss of renal

function [1, 4, 27]. During reperfusion, cell damage is worsened to an extent greater than ischemia itself, due to the production of ROS, which increase excessively beyond the antioxidant capacity of the renal cells [23]. Accordingly, the results of this study showed that there was an increasing number of TUNEL positive cells in the kidneys of I/R rats, while proinflammatory cytokine, CAT, iNOS and 3-NT were unaffected and only GPx was decreased. Also, I/R rats had vascular dilatation of the glomerular and peritubular capillaries, and peripheral edema and cell loss in the proximal tubules. Nonetheless, it is also possible to argue that there may be different cellular mechanisms contributing to the histopathology observed in the kidneys of rats exposed to I/R injury. For instance, activated calcineurin-NFATc signaling pathway in response to Ca^{2+}-overload [28] and deranged histone deacetylase activity [29] are likely to exacerbate remote kidney damage in the presence of I/R. In addition, continuous activation of Wnt/β-catenin and autophagy signaling along the I/R have been shown to trigger maladaptive tissue responses and cause disruptive outcomes [30, 31].

Recent studies have confirmed the beneficial role of certain plant-derived products in I/R pathogenesis [11, 27]. This plant-based approach has shown promising results in regulating I/R injury and provides the impetus for the search for novel agents from plant origins. *B. monnieri*, a medicinal Ayurvedic plant, has been extensively studied for its beneficial effects against many disorders because of its strong anti-oxidant and anti-inflammatory properties [12, 13, 15, 16]. According to this, the present study demonstrated that a ten-day pretreatment of rats with *B. monnieri* prior to I/R results in a reduction in periarterial edema, tissue loss and apoptosis in the renal tubules. In addition, glomerular capillaries, parietal layer of the Bowman capsule, macula densa and proximal tubules were found to be less damaged in the kidneys of *B. monnieri*-treated I/R group. The histopathological scores showed that a reduced remote I/R injury in the kidney *B. monnieri* treated I/R group. The first possible mechanism of action by which *B. monnieri* could attenuate remote reno-degeneration is the protection of an antioxidant status and consequently controlling oxidative stress. A ten-day pretreatment with *B. monnieri* probably preconditioned the cellular redox pathways leading to mitigation in renal oxidative stress and apoptosis. The normalization of the activity of kidney GPx antioxidant system by *B. monnieri*, consistent with low histopathology score obtained from *B. monnieri* treated I/R rats, supports our notion. Another possible mechanism targeted by *B. monnieri* to overcome I/R injury is the inflammation [15, 16, 18]. However, our results showed that there was no statistically significant difference in IL-1β or TNF-α levels between I/R rats and *B. monnieri* treated I/R rats.

It has been reported that skeletal muscle I/R injury often result in loss of contractile function and in severe cases may cause disability, limb amputation and even death [32]. The present study showed a striking and directly damage to skeletal muscle of I/R rats, manifested by an impaired myofibril organization, abnormal nuclear activity and increased apoptosis. However, pretreatment with *B. monnieri* prevented I/R-induced damage to the skeletal muscle, which was evidenced by an improvement in the histopathology score. Because skeletal muscle IL-1β and TNF-α levels were decreased by *B. monnieri* treatment, the regulation of inflammatory signaling seems to be an important mechanism contributing. As supportive, a recent study has shown that *B. monnieri* extract reduces pro-inflammatory cytokine expression in the brain of an animal model for neurotoxicity, even when administered at 50 mg/kg body weight per day [33]. Interestingly, we found that the levels of IL-1 and TNF-α detected in the skeletal muscle of I/R group treated with *B. monnieri* are lower than those obtained in the sham control group. Although we do not know the reason of why *B. monnieri* inhibited the cytokine levels in I/R rats more than sham-control or *B. monnieri*-treated sham, some of the bioactive substances of the plant, like triterpenoids, bacosides and/or flovanoids might activate a number of negative regulatory mechanisms that may govern cytokine expression and secretion in the presence of I/R [34]. Although, the effects of *B. monnieri* have not been previously reported in skeletal muscle and in infrarenal aortic occlusion model, here, we found that *B. monnieri* extract also interacts with nitrergic system in the skeletal muscle. This interaction was not only intended to protect the skeletal muscle against I/R injury, but also directly inhibited iNOS and 3-NT levels. This study provided evidence to reveal the anti-nitrosive activity of *B. monnieri*, which is thought to be associated with the prevention of I/R injury and may also explain the increased negative regulatory activity of *B. monnieri* on cytokine levels in the presence of I/R. According to this, *B. monnieri* has been shown to down regulate pro-inflammatory cytokines (IL-6, TNF-α), chemokine (MCP-1) and iNOS expressions, thereby inhibiting NO generation and HO-1 expression in a different model for oxidative injury [33].

Saponins (ginsenosides), one of the major bioactive compounds of the *B. monnieri*, has been shown to inhibit liver 3-NT in an animal model for acetaminophen toxicity [35], and to reduce cytokine mixture-induced NO production and iNOS expression in a cell culture model [36]. *B. Monnieri* saponins are predominantly bacopaside I and bacoside A, a mixture of bacoside A3, bacopaside II, jujubogenin isomer of bacopasaponin C, and bacopasaponin C [37, 38]. Certain microRNAs (miR-367, miR-222, and miR-29), which are probably influenced by *B. monnieri* bioactive substances, saponins [39], have been reported to inhibit cytokine secretion in skeletal muscle cells indirectly by altering the activity of NOS and signal transducers and activators of transcription (STAT) proteins [32, 40]. Furthermore, bacoside A components, bacoside A3 and bacopaside II have been shown to provide a high cyto-(neuronal) protection via decreasing intracellular ROS. Other than saponins, flavonoids such as luteolin and apigenin are important redox regulating bioactive substances of *B. monnieri* [41] that might reduce nitrosative stress and inflammatory response of skeletal muscle, at least via inhibition of superoxide production [33]. However, how bioactive compounds of *B. monnieri* affect the skeletal muscle nitrergic system, the underlying

mechanisms and their relationship to inflammatory pathways require extensive experimental research. Another interesting finding of the study is that *B. monnieri* inhibits catalase (CAT) activity in skeletal muscle of I/R rats, but does not affect CAT activity when administered to sham rats. CAT is a heme enzyme that efficiently scavenges H_2O_2 when it is present in high concentrations [42]. CAT also converts superoxide to water and molecular oxygen, largely in peroxisomes, and is linked metabolically to pentose phosphate pathway because NADPH is tightly bound to CAT in mammals. The oxidation of bound NADPH to bound NADP + results in the accumulation of an inactive form of CAT (compound II) [43, 44]. The ROS generated by I/R has been reported to be via NADPH oxidases, and I/R-induced ROS production via NADPH oxidases may lead to insufficient NADPH to continue normal CAT activity. Although the presence of low NADPH concentrations by secondary to G6PD deficiency seems to be reasonable to measure a low CAT activity, the inhibited CAT activity may, at least, be partially due to the formation of hydrogen bonds between the CAT and the flovanoid components of *B. monnieri*. In the presence of certain flavonoids, the formation of unreactive CAT, compound-II has been detected [45]. Nevertheless, the involvement of Hsp70, Wnt/β-catenin signaling, and autophagy in the mechanism of the action of *B. monnieri* on I/R pathology should not be overlooked because I/R has complex processes involving multiple cellular and molecular pathways, and *B. monnieri* modulates the activity of Hsp70 against oxidative stress [31, 33, 46]. It is imperative that future research on the action mechanisms of *B. monnieri* in I/R-induced skeletal muscle injury also explores the role of HIF-1α and mitophagy, as well as the other key mechanisms of cellular stress [47]. Accordingly, *B. monnieri* alleviates oxidative stress-induced cellular toxicity by inhibiting JNK-mediated apoptosis through improved mitochondrial function, Nrf_2 expression and redox stabilization [17, 45, 46].

To the best of our knowledge, the present study is the first to document that *B. monnieri* protects kidney and skeletal muscle against infra-renal aortic occlusion and reperfusion injury in rats. In this experimental model, although the cellular redox modulatory bioactives of *B. monnieri* appears to be generally responsible for overall beneficial effects, the contribution of newly identified bioactive compounds (i.e. jujubogenin and pseudojujubogenin) in the plant [38] and related cellular mechanisms should be investigated. For instance, the monnieracids as lipid peroxidation inhibiting compounds, have recently been identified in *B. monnieri* [48]. Nonetheless, although there are some limitations such as the use of an extract with unknown amounts of different compounds that may have an additive or antagonistic effect among them, the study may lead to further research to clarify these limitations based on the good results of *B. monnieri* shown herein.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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