



Original Research Article (Experimental)

## Preconditioning with *Azadirachta indica* ameliorates cardiorenal dysfunction through reduction in oxidative stress and extracellular signal regulated protein kinase signalling



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### ABSTRACT

**Background:** *Azadirachta indica* is widely distributed in Africa, Asia and other tropical parts of the world. *A. indica* (AI) is traditionally used for the treatment of several conditions including cancer, hypertension, heart diseases and skin disorders. Intestinal ischaemia-reperfusion is a common pathway for many diseases and may lead to multiple organ dysfunction syndrome and death.

**Objective:** In this study, we investigated the ameliorative effects of AI on intestinal ischaemia-reperfusion injury-induced cardiorenal dysfunction.

**Materials and methods:** Sixty rats were divided into 6 groups; each containing 10. Corn oil was orally administered to group A (control) rats for 7 days without intestinal ischaemia-reperfusion injury. Group B underwent intestinal ischaemia-reperfusion injury (IIRI) without any pre-treatment. Groups C, D, E and F were pre-treated orally for 7 days with 100 mg/kg AI (100 and 200 mg/kg) vitamin C (100 and 200 mg/kg) respectively and thereafter underwent IIRI on the 8th day.

**Results:** The cardiac and renal hydrogen peroxide increased significantly whereas serum xanthine oxidase and myeloperoxidase levels were significantly elevated ( $p < 0.05$ ) in IIRI only when compared to the control. The cardiac and renal reduced glutathione, glutathione peroxidase, protein thiol, non-protein thiol and serum nitric oxide (NO) decreased ( $p < 0.05$ ) significantly following IIRI. Immunohistochemical evaluation of cardiac and renal tissues showed reduced expressions of the extracellular signal regulated kinase (ERK1/2) in rats with IIRI only. However, pre-treatment with *A. indica* and vitamin C significantly reduced markers of oxidative stress and inflammation together with improvement in antioxidant status. Also, reduced serum NO level was normalised in rats pre-treated with *A. indica* and vitamin C with concomitant higher expressions of cardiac and renal ERK1/2.

**Conclusions:** Together, *A. indica* and vitamin C prevented IRI-induced cardiorenal dysfunction via reduction in oxidative stress, improvement in antioxidant defence system and increase in the ERK1/2 expressions. Therefore, *A. indica* can be a useful chemopreventive agent in the prevention and treatment of conditions associated with intestinal ischaemia-reperfusion injury.

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## 1. Introduction

Intestinal ischaemia results from any condition which leads to arterial occlusion by embolism or thrombi [1,2]. It may also be the sequelae of non-occlusive processes as is found in conditions causing low mesenteric blood flow like cardiac insufficiency and sepsis [3,4].

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However, important features of acute mesenteric ischaemia include bacterial translocation, systemic inflammatory response syndrome and reperfusion injury [5]. In order to prevent irreversible damage to an ischaemic organ, restoration of blood flow is essential, however; reperfusion may accentuate the injury produced by ischaemia alone [6–8]. Cellular damage caused by the reperfusion of a previously viable ischaemic tissue is defined as ischaemia-reperfusion injury [9]. This reperfusion injury exacerbates the ischaemic damage of the intestinal microcirculation together with a negative outcome [10,11]. Reperfusion of splanchnic arteries following occlusion may precipitate circulatory shock with the consequent activation and adhesion of polymorphonuclear neutrophils, release of proinflammatory substances and formation of both oxidative and nitrosative stress [12–14]. Intestinal ischaemia-reperfusion is a common pathway for many diseases and may lead to multiple organ dysfunction and death [15]. In humans, thrombosis of the mesenteric venous vessels can result in haemorrhagic infarction with acute mesenteric ischaemia and irreversible severe tissue pathology [16–18]. Complex interactions between the endothelium and several cell types can be provoked by ischaemia-reperfusion with resultant microvascular injury, cellular necrosis and/or apoptosis [19–21]. In severe conditions, resulting inflammatory responses from ischaemia-reperfusion injury may lead to systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) [22,23]. Therefore, I-R injury may extend beyond the ischaemic area at risk to cause injury of remote non-ischaemic organs [8].

*Azadirachta indica*, a plant belonging to the family Meliaceae and widely distributed in Africa, Asia and other tropical parts of the world has been extensively utilised in traditional medical practices. It has been reported that various parts of the plant have various medicinal and pharmacological properties [24–29]. The different components of *A. indica* have been indicated to possess antioxidant, anti-inflammatory, anti-proliferative and modulation of various signalling pathways [28,29]. These properties make *A. indica* a therapeutic candidate that can be traditionally used for the treatment of several conditions characterized by free radical generation, inflammatory reactions, cellular proliferations and dysregulation cellular signalling pathways such as in cancer, hypertension, heart diseases and skin disorders [31–34]. Intestinal ischaemia-reperfusion injury is a challenging and life-threatening clinical problem with diverse causes and high mortality rate. With the plethoric actions and possible beneficial effects of *A. indica*, we have evaluated the ameliorative effects and the possible mechanism of action of the methanol extract of *A. indica* and Vitamin C on IIRI- induced cardiorenal dysfunction and oxidative stress in rats.

## 2. Materials and methods

### 2.1. Extraction of plant material

Fresh leaves of *A. indica* were collected from the Botanical Garden, University of Ibadan and deposited in the herbarium with voucher number UIH-22527. The leaves were cleaned, air-dried and crushed into coarse powder using an electric blender. The powdered leaves were soaked in n-hexane for 72 h and agitated at intervals, then filtered and afterward soaked in methanol for 24 h and agitated at intervals. The mixture was filtered thereafter and filtrate was concentrated *in-vacuo* at 40 °C using a rotary evaporator to give a semi-solid methanol extract of *A. indica* that was finally used for this study.

### 2.2. Chemicals

Vitamin C, Sulphosalicylic acid, 2-dichloro-4-nitrobenzene (CDNB), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), trichloroacetic

acid (TCA), thiobarbituric acid (TBA), reduced glutathione (GSH), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), sodium hydroxide (NaOH) pellets, epinephrine, xylenol orange, Sorbitol, were purchased from Sigma Aldrich (USA). Normal goat serum, Biotinylated antibody and Horse Radish Peroxidase (HRP) System was purchased from (KPL, Inc., Gaithersburg, Maryland, USA). Extracellular signal regulated kinase (ERK) antibody was purchased from (Bioss Inc. Woburn, Massachusetts, USA) while 3, 3'-Diaminobenzidine (DAB) tablets were purchased from (AMRESO LLC, OHio, USA). All other chemicals used were of analytical grade and obtained from British Drug Houses (Poole, Dorset, UK).

### 2.3. Experimental animals

Sixty male Wistar rats were obtained from the experimental animal house of the Faculty of Veterinary Medicine, University of Ibadan and housed in well-ventilated cages. The rats were fed with commercial rat chow and water was provided *ad libitum*. The rats were subjected to natural photoperiod of about 12 h light and 12 h darkness daily. The animals were acclimatized for seven (7) days prior to the commencement of the experiment. The protocols used were in conformity with the guidelines of the National Institutes of Health (NIH) guidelines for laboratory animal care and use [35].

### 2.4. Experimental protocol

The animals were randomly divided into six (6) experimental groups with ten (10) animals in each group, and the treatment was as follows:

Group A: Administered with corn oil orally for seven days without intestinal ischaemia-reperfusion

Group B: Administered with corn oil orally for seven days with intestinal ischaemia-reperfusion on the 8th day

Group C: Administered with 100 mg/kg body weight of *A. indica* orally for seven days with intestinal ischaemia-reperfusion on the 8th day (AI1)

Group D: Administered with 200 mg/kg body weight *A. indica* orally for seven days with intestinal ischaemia-reperfusion on the 8th day (AI2).

Group E: Administered with 100 mg/kg body weight of vitamin C orally for seven days and intestinal ischaemia-reperfusion on the 8th day (Vit C<sub>1</sub>).

Group F: Administered with 200 mg/kg body weight of vitamin C orally for seven days and intestinal -reperfusion on the 8th day (Vit C<sub>2</sub>).

### 2.5. Surgical procedure for the induction of intestinal ischaemia-reperfusion injury

Rats were anaesthetized with Ketamine (90 mg/kg; i.m.) and Xylazine (10 mg/kg; i.m.). A ventral midline laparotomy was performed after shaving and local cleaning with antiseptic solution. To induce intestinal ischaemia, the superior mesenteric artery (SMA) was dissected out and carefully clamped with an atraumatic microvascular clip. Thereafter, the intestines were returned into the abdomen and the incision was closed temporarily. The clip was removed following 30 min of occlusion of the SMA and reperfusion was allowed for 45 min. The animals were thereafter sacrificed by cervical dislocation.

### 2.6. Serum collection

About 5 ml of blood was collected from the retro-orbital venous plexus into sterile plain tubes and left for about 30 min to clot. The

clotted blood was thereafter centrifuged at 4000 rpm for 10 min. Serum was decanted into eppendorf tubes and stored at  $-40^{\circ}\text{C}$  till the time of analysis.

### 2.7. Preparation of tissues for analysis

The blood samples were then centrifuged at 4,000 rpm for 10 min. The serum was collected and stored in the refrigerator at  $-4^{\circ}\text{C}$ . The heart and kidney tissues were removed, minced with scissors and homogenized in ice-cold 0.1 M phosphate buffer, pH 7.4. The resultant homogenates were centrifuged at 10,000 g at  $4^{\circ}\text{C}$  for 15 min. The post mitochondrial fractions were collected and processed for biochemical assays.

### 2.8. Biochemical assays

The post-mitochondrial fractions of the heart and kidneys were assayed for the estimation of reduced GSH at 412 nm according to the method by Beutler et al. [36]. The Glutathione-S-transferase (GST) was measured by the method of Habig et al. [37] and glutathione peroxidase (GPx) activity was determined as described by Rotruck et al. [38]. The sulfhydryl (total thiol) and non-protein thiol (NPT) content was determined as described by Ellman [39]. The activity of xanthine oxidase (XO) was determined according to method of Akaike et al. [40]. The serum myeloperoxidase (MPO) activity was determined according to the method of Xia and Zweier [41]. The malondialdehyde (MDA) level was calculated as described by Varshney and Kale [42]. Lipid peroxidation in  $\mu\text{mol}$  MDA formed/mg protein as a marker of oxidative stress was computed with a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$ . Hydrogen peroxide generation was estimated as described Wolff [43]. Nitric oxide (NO) in the serum was measured as earlier described [44,45]. The concentration of nitrite in the sample was determined from a sodium nitrite ( $\text{NaNO}_2$ ) standard curve and was expressed as  $\mu\text{mol/L}$ . Protein concentration was determined by Biuret method as described by Gornal et al. [46].

### 2.9. Immunohistochemistry of cardiac and renal extracellular signal regulated kinase (ERK)

Immunohistochemistry of paraffin embedded tissue of the heart and kidneys was performed after the tissues were obtained from buffered formalin perfused rats. Paraffin sections were melted at  $60^{\circ}\text{C}$  in the oven. Dewaxing of the samples in xylene was followed by passage through ethanol of decreasing concentration (100–80%). Peroxidase quenching in 3%  $\text{H}_2\text{O}_2$ /methanol was carried out with subsequent antigen retrieval performed by microwave heating in 0.01 M citrate buffer (pH 6.0) to boil. All the sections were blocked in normal goat serum (10%, HistoMark<sup>®</sup>, KPL, Gaithersburg MD, USA) and probed with ERK antibody (Bioss, San Diego, California, USA), 1:300 for 16 h in a refrigerator. Detection of bound antibody was carried out using biotinylated (goat anti-rabbit, 2.0  $\mu\text{g/ml}$ ) secondary antibody and subsequently streptavidin peroxidase (Horse Radish Peroxidase-streptavidin) according to manufacturer's protocol (HistoMark<sup>®</sup>, KPL, Gaithersburg, MD, USA). Reaction product was enhanced with diaminobenzidine (DAB, Amresco<sup>®</sup>, USA) for 6–10 min and counterstained with high definition haematoxylin (Enzo<sup>®</sup>, NY–USA), with subsequent dehydration in ethanol. The slides were covered with coverslips and sealed with resinous solution. The immunoreactive positive expression of ERK intensive regions were viewed starting from low magnification on each slice then with  $100\times$  magnifications using a photo microscope (Olympus) and a digital camera (Toupcam<sup>®</sup>, Touptek Photonics, Zhejiang, China).

### 2.10. Statistical analysis

All values are expressed as mean  $\pm$  standard deviation (SD). The test of significance between two groups was estimated by Student's t-test. One way Analysis of Variance (ANOVA) with Tukey's post-hoc test using GraphPad Prism 5.0 was also performed with  $p$ -values  $< 0.05$  considered statistically significant.

## 3. Results

### 3.1. Effect of *A. indica* and vitamin C on markers of oxidative stress markers and antioxidant defence system

In Table 1, there was a significant ( $p < 0.05$ ) increase in the cardiac and renal  $\text{H}_2\text{O}_2$  level of rats that underwent ischaemia-reperfusion injury only when compared to the control. However, pre-treatment with  $\text{Al}_1$  (100 mg/kg) and  $\text{Al}_2$  (200 mg/kg) caused a significant decrease ( $p < 0.05$ ) in the  $\text{H}_2\text{O}_2$  levels when compared with the rats which underwent IIRI only. Furthermore, a significant ( $p < 0.05$ ) reduction in the  $\text{H}_2\text{O}_2$  levels of the rats pre-treated with vit  $\text{C}_1$  (100 mg/kg) and vit  $\text{C}_2$  (200 mg/kg) was obtained when compared with the rats that underwent IIRI only (Table 1). Our study also shows that the rats which underwent ischaemia-reperfusion injury only, had significant ( $p < 0.05$ ) reduction in the content of cardiac and renal reduced glutathione (GSH) when compared to the control (Table 2). Further, pre-treatment with  $\text{Al}_1$  (100 mg/kg) and  $\text{Al}_2$  (200 mg/kg) caused a significant increase ( $p < 0.05$ ) in the GSH content when compared with the rats which underwent IIRI only. Similarly, there was a significant increase ( $p < 0.05$ ) in the GSH levels of the groups pre-treated with vit  $\text{C}_1$  and vit  $\text{C}_2$  when compared with the rats that underwent IIRI only (Table 2).

In the heart tissues, there was a significant decrease ( $p < 0.05$ ) in protein thiol level of rats that underwent IIRI only when compared with control, while there was a significant increase ( $p < 0.05$ ) in protein thiol levels of rats pre-treated with  $\text{Al}_1$  and Vit  $\text{C}_1$  when compared with IIRI only (Table 3). In the renal tissues, there was a significant decrease ( $p < 0.05$ ) in the level of protein thiol of rats that underwent IIRI only when compared with the control, however, there was a significant increase ( $p < 0.05$ ) in the rats pre-treated with  $\text{Al}_1$ ,  $\text{Al}_2$ , Vit  $\text{C}_1$  and Vit  $\text{C}_2$  when compared with the rats that underwent IIRI only (Table 3). In another experiment, there was a significant decrease ( $p < 0.05$ ) in non-protein thiol level in the heart and kidney tissues of rats that underwent IIRI only when compared to the control while there was a significant increase ( $p < 0.05$ ) in non-protein thiol level in the heart and kidney

**Table 1**

The effect of *A. indica* and vitamin C on the level of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) generated in cardiac and renal tissues of experimental rats with ischaemia-reperfusion injury.

Groups	Treatment (mg/Kg)	$\text{H}_2\text{O}_2$ (heart) ( $\mu\text{mole/mg}$ protein)	$\text{H}_2\text{O}_2$ (kidney) ( $\mu\text{mole/mg}$ protein)
A	Control	14.89 $\pm$ 0.38	15.29 $\pm$ 0.74
B	IRI only	16.83 $\pm$ 0.65 <sup>a</sup>	20.56 $\pm$ 0.14 <sup>a</sup>
C	IRI + $\text{Al}_1$	15.89 $\pm$ 0.58 <sup>a,b</sup>	18.68 $\pm$ 0.76 <sup>a,b</sup>
D	IRI + $\text{Al}_2$	16.14 $\pm$ 0.14 <sup>a,b</sup>	18.10 $\pm$ 0.25 <sup>a,b</sup>
E	IRI + Vit $\text{C}_1$	15.98 $\pm$ 0.25 <sup>a,b</sup>	17.04 $\pm$ 1.05 <sup>a,b</sup>
F	IRI + Vit $\text{C}_2$	15.06 $\pm$ 0.58 <sup>b</sup>	17.03 $\pm$ 0.69 <sup>a,b</sup>

The results above are shown as Mean  $\pm$  Standard deviation for each group of eight (8) rats per group.

IRI = Ischaemia-reperfusion injury;  $\text{Al}_1$  = *Azadirachta indica* (100 mg/kg);  $\text{Al}_2$  = *Azadirachta indica* (200 mg/kg); Vit  $\text{C}_1$  = Vitamin C (100 mg/kg); Vit  $\text{C}_2$  = Vitamin C (200 mg/kg).

<sup>a</sup>  $p < 0.05$  when compared with the corn oil control group.

<sup>b</sup>  $p < 0.05$  when compared with ischaemia-reperfusion injury group.

**Table 2**

The effect of *A. indica* and vitamin C on the level of reduced glutathione (GSH) in the cardiac and renal tissues of experimental rats with ischaemia-reperfusion injury.

Groups	Treatment (mg/Kg)	GSH (heart) (μmole/mg protein)	GSH (kidney) (μmole/mg protein)
A	Control	9.08 ± 1.04	56.33 ± 1.16
B	IRI only	7.94 ± 0.13 <sup>a</sup>	53.58 ± 1.26 <sup>a</sup>
C	IRI + AI <sub>1</sub>	9.19 ± 0.55 <sup>b</sup>	56.75 ± 1.06 <sup>b</sup>
D	IRI + AI <sub>2</sub>	8.65 ± 0.14 <sup>b</sup>	58.75 ± 1.26 <sup>a,b</sup>
E	IRI + Vit C <sub>1</sub>	8.75 ± 0.20 <sup>b</sup>	55.96 ± 0.51 <sup>b</sup>
F	IRI + Vit C <sub>2</sub>	9.00 ± 0.46 <sup>b</sup>	56.40 ± 0.55 <sup>b</sup>

The results above are shown as Mean ± Standard deviation for each group of eight (8) rats per group.

IRI = Ischaemia-reperfusion injury; AI<sub>1</sub> = *Azadirachta indica* (100 mg/kg); AI<sub>2</sub> = *Azadirachta indica* (200 mg/kg); Vit C<sub>1</sub> = Vitamin C (100 mg/kg); Vit C<sub>2</sub> = Vitamin C (200 mg/kg).

<sup>a</sup> *p* < 0.05 when compared with the corn oil control group.

<sup>b</sup> *p* < 0.05 when compared with ischaemia-reperfusion injury group.

**Table 3**

The effect of *A. indica* and vitamin C on the level of protein thiol (PT) in the cardiac and renal tissues of experimental rats with ischaemia-reperfusion injury.

Groups	Treatment (mg/Kg)	PT (heart) (nmole/mg protein)	PT (kidney) (nmole/mg protein)
A	Control	45.81 ± 3.71	62.19 ± 5.04
B	IRI only	34.83 ± 6.58 <sup>a</sup>	56.33 ± 4.40 <sup>a</sup>
C	IRI + AI <sub>1</sub>	65.20 ± 7.65 <sup>a,b</sup>	107.92 ± 3.09 <sup>a,b</sup>
D	IRI + AI <sub>2</sub>	38.54 ± 3.47 <sup>a</sup>	76.00 ± 6.97 <sup>a,b</sup>
E	IRI + Vit C <sub>1</sub>	42.00 ± 5.22 <sup>b</sup>	67.67 ± 6.78 <sup>b</sup>
F	IRI + Vit C <sub>2</sub>	38.96 ± 2.09 <sup>a</sup>	67.35 ± 6.80 <sup>b</sup>

The results above are shown as Mean ± Standard deviation for each group of eight (8) rats per group.

IRI = Ischaemia-reperfusion injury; AI<sub>1</sub> = *Azadirachta indica* (100 mg/kg); AI<sub>2</sub> = *Azadirachta indica* (200 mg/kg); Vit C<sub>1</sub> = Vitamin C (100 mg/kg); Vit C<sub>2</sub> = Vitamin C (200 mg/kg).

<sup>a</sup> *p* < 0.05 when compared with the corn oil control group.

<sup>b</sup> *p* < 0.05 when compared with ischaemia-reperfusion injury group.

tissues of rats that were pre-treated with AI<sub>1</sub>, AI<sub>2</sub>, Vit C<sub>1</sub> and Vit C<sub>2</sub> when compared with the rats that underwent IIRI only (Table 4).

The activity of renal and cardiac GPx decreased (*p* < 0.05) significantly in rats that underwent ischaemia-reperfusion injury only when compared to the control (Table 5). In contrary, pre-treated of rats with AI<sub>1</sub> and AI<sub>2</sub> and vit C<sub>1</sub> and vit C significantly increased (*p* < 0.05) the antioxidant activity of GPx when compared with the rats which underwent IIRI only (Table 5).

In the heart tissues, there was a significant increase (*p* < 0.05) in the GST level of rats which underwent IIRI only when compared with control, however, there was a significant decrease (*p* < 0.05) in

**Table 4**

The effect of *A. indica* and vitamin C on the level of non-protein thiol (NPT) in the cardiac and renal tissues of experimental rats with ischaemia-reperfusion injury.

Groups	Treatment (mg/Kg)	NPT (heart) (nmole/mg protein)	NPT (kidney) (nmole/mg protein)
A	Control	94.35 ± 2.50	116.95 ± 3.60
B	IRI only	85.15 ± 2.97 <sup>a</sup>	104.92 ± 0.72 <sup>a</sup>
C	IRI + AI <sub>1</sub>	94.35 ± 7.04 <sup>b</sup>	118.56 ± 2.18 <sup>b</sup>
D	IRI + AI <sub>2</sub>	89.14 ± 2.81 <sup>a,b</sup>	115.63 ± 5.13 <sup>b</sup>
E	IRI + Vit C <sub>1</sub>	104.58 ± 9.61 <sup>a,b</sup>	114.50 ± 3.67 <sup>b</sup>
F	IRI + Vit C <sub>2</sub>	110.63 ± 5.79 <sup>a,b</sup>	127.40 ± 2.04 <sup>a,b</sup>

The results above are shown as Mean ± Standard deviation for each group of eight (8) rats per group.

IRI = Ischaemia-reperfusion injury; AI<sub>1</sub> = *Azadirachta indica* (100 mg/kg); AI<sub>2</sub> = *Azadirachta indica* (200 mg/kg); Vit C<sub>1</sub> = Vitamin C (100 mg/kg); Vit C<sub>2</sub> = Vitamin C (200 mg/kg).

<sup>a</sup> *p* < 0.05 when compared with the corn oil control group.

<sup>b</sup> *p* < 0.05 when compared with ischaemia-reperfusion injury group.

**Table 5**

The effect of *A. indica* and vitamin C on the level of glutathione peroxidase (GPx) in the cardiac and renal tissues of experimental rats with ischaemia-reperfusion injury.

Groups	Treatment (mg/kg)	GPx (heart) (μmole of GSH/min/mg protein)	GPx (kidney) (μmole of GSH/min/mg protein)
A	Control	141.36 ± 4.77	98.47 ± 6.19
B	IRI only	122.47 ± 4.00 <sup>a</sup>	75.82 ± 6.35 <sup>a</sup>
C	IRI + AI <sub>1</sub>	133.20 ± 6.81 <sup>a,b</sup>	88.83 ± 4.97 <sup>a,b</sup>
D	IRI + AI <sub>2</sub>	132.33 ± 4.80 <sup>a,b</sup>	100.91 ± 4.03 <sup>b</sup>
E	IRI + Vit C <sub>1</sub>	136.17 ± 5.01 <sup>a,b</sup>	93.45 ± 2.06 <sup>a,b</sup>
F	IRI + Vit C <sub>2</sub>	139.55 ± 7.97 <sup>b</sup>	94.06 ± 4.21 <sup>b</sup>

The results above are shown as Mean ± Standard deviation for each group of eight (8) rats per group.

IRI = Ischaemia-reperfusion injury; AI<sub>1</sub> = *Azadirachta indica* (100 mg/kg); AI<sub>2</sub> = *Azadirachta indica* (200 mg/kg); Vit C<sub>1</sub> = Vitamin C (100 mg/kg); Vit C<sub>2</sub> = Vitamin C (200 mg/kg).

<sup>a</sup> *p* < 0.05 when compared with the corn oil control group.

<sup>b</sup> *p* < 0.05 when compared with ischaemia-reperfusion injury group.

the GST level of rats that were pre-treated with AI<sub>1</sub>, AI<sub>2</sub>, Vit C<sub>1</sub> and Vit C<sub>2</sub> when compared with the rats that underwent IIRI only (Table 6). In the kidney tissues, there was no significant difference (*p* > 0.05) in the GST level of rats that underwent IIRI only and control (Table 6). However, there was a significant (*p* < 0.05) increase in the rats pre-treated with AI<sub>1</sub>, AI<sub>2</sub> and Vit C<sub>1</sub> when compared with the rats that underwent IRI only whereas there was no significant difference (*p* > 0.05) in the rats that were pre-treated with Vit C<sub>2</sub> when compared with the rats that underwent IIRI only (Table 6).

### 3.2. Effect of *A. indica* and vitamin C on markers of inflammation and renal damage

The result from Table 7 shows that the rats which underwent ischaemia-reperfusion injury only had significantly (*p* < 0.05) increased serum level of xanthine oxidase (XO) when compared to the control. However, a significant decrease (*p* < 0.05) in serum level of xanthine oxidase (XO) was obtained in the rats pre-treated with AI<sub>1</sub> and AI<sub>2</sub> and vit C<sub>1</sub> and vit C<sub>2</sub> when compared with the rats which underwent IIRI only (Table 7). The serum level of myeloperoxidase (MPO) increased (*p* < 0.05) significantly in rats that underwent ischaemia-reperfusion injury only when compared to the control (Table 8) indicating inflammation, oxidative stress and cardiac damage. On the other hand, however, there was a significant (*p* < 0.05) decrease in the levels of myeloperoxidase (MPO) in the rats pre-treated with AI<sub>1</sub> and AI<sub>2</sub> and vit C<sub>1</sub> and vit C<sub>2</sub> when compared with the rats which underwent IIRI only (Table 8).

**Table 6**

The effect of *A. indica* and vitamin C on the level of glutathione-S-transferase (GST) in the cardiac and renal tissues of experimental rats with ischaemia-reperfusion injury.

Groups	Treatment (mg/Kg)	GST (heart) (μmol CDNB-GSH complex formed/min/mg protein)	GST (kidney) (μmol CDNB-GSH complex formed/min/mg protein)
A	Control	0.739 ± 0.060	1.001 ± 0.373
B	IRI only	2.347 ± 0.343 <sup>a</sup>	0.979 ± 0.374
C	IRI + AI <sub>1</sub>	0.643 ± 0.032 <sup>a,b</sup>	2.388 ± 0.078 <sup>a,b</sup>
D	IRI + AI <sub>2</sub>	0.696 ± 0.034 <sup>b</sup>	1.695 ± 0.648 <sup>a,b</sup>
E	IRI + Vit C <sub>1</sub>	0.605 ± 0.083 <sup>a,b</sup>	2.086 ± 0.111 <sup>a,b</sup>
F	IRI + Vit C <sub>2</sub>	2.010 ± 0.275 <sup>a,b</sup>	1.052 ± 0.438

The results above are shown as Mean ± Standard deviation for each group of eight (8) rats per group.

IRI = Ischaemia-reperfusion injury; AI<sub>1</sub> = *Azadirachta indica* (100 mg/kg); AI<sub>2</sub> = *Azadirachta indica* (200 mg/kg); Vit C<sub>1</sub> = Vitamin C (100 mg/kg); Vit C<sub>2</sub> = Vitamin C (200 mg/kg).

<sup>a</sup> *p* < 0.05 when compared with the corn oil control group.

<sup>b</sup> *p* < 0.05 when compared with ischaemia-reperfusion injury group.

**Table 7**

The effect of *A. indica* and vitamin C on the level of xanthine oxidase (XO) in the serum of experimental rats with ischaemia-reperfusion injury.

Groups	Treatment group (mg/kg)	Xanthine oxidase (serum) ( $\mu\text{mole/L}$ )
A	Control	0.184 $\pm$ 0.003
B	IRI only	0.224 $\pm$ 0.008 <sup>a</sup>
C	IRI + Al <sub>1</sub>	0.188 $\pm$ 0.012 <sup>b</sup>
D	IRI + Al <sub>2</sub>	0.197 $\pm$ 0.004 <sup>a,b</sup>
E	IRI + Vit. C <sub>1</sub>	0.218 $\pm$ 0.001 <sup>a,b</sup>
F	IRI + Vit C <sub>2</sub>	0.198 $\pm$ 0.007 <sup>a,b</sup>

The results above are shown as Mean  $\pm$  Standard deviation for each group of eight (8) rats per group.

IRI = Ischaemia-reperfusion injury; Al<sub>1</sub> = *Azadirachta indica* (100 mg/kg); Al<sub>2</sub> = *Azadirachta indica* (200 mg/kg); Vit C<sub>1</sub> = Vitamin C (100 mg/kg); Vit C<sub>2</sub> = Vitamin C (200 mg/kg).

<sup>a</sup>  $p < 0.05$  when compared with the corn oil control group.

<sup>b</sup>  $p < 0.05$  when compared with ischaemia-reperfusion injury group.

**Table 8**

The effect of *A. indica* and vitamin C on the level of myeloperoxidase (MPO) in the serum of experimental rats with ischaemia-reperfusion injury.

Groups	Treatment group (mg/kg)	Myeloperoxidase (serum) ( $\mu\text{mole/L}$ )
A	Control	11.68 $\pm$ 1.13
B	IRI only	23.23 $\pm$ 0.06 <sup>a</sup>
C	IRI + Al <sub>1</sub>	11.19 $\pm$ 0.56 <sup>b</sup>
D	IRI + Al <sub>2</sub>	7.57 $\pm$ 0.56 <sup>a,b</sup>
E	IRI + Vit. C <sub>1</sub>	9.76 $\pm$ 0.75 <sup>a,b</sup>
F	IRI + Vit C <sub>2</sub>	13.36 $\pm$ 0.77 <sup>a,b</sup>

The results above are shown as Mean  $\pm$  Standard deviation for each group of eight (8) rats per group.

IRI = Ischaemia-reperfusion injury; Al<sub>1</sub> = *Azadirachta indica* (100 mg/kg); Al<sub>2</sub> = *Azadirachta indica* (200 mg/kg); Vit C<sub>1</sub> = Vitamin C (100 mg/kg); Vit C<sub>2</sub> = Vitamin C (200 mg/kg).

<sup>a</sup>  $p < 0.05$  when compared with the corn oil control group.

<sup>b</sup>  $p < 0.05$  when compared with ischaemia-reperfusion injury group.

### 3.3. Effect of *A. indica* and vitamin C on serum nitric oxide (NO) bioavailability as a marker of hypertension

The bioavailability of serum nitric oxide (NO) was taken as a marker of hypertension in this study. The result shows a significant ( $p < 0.05$ ) reduction in serum NO level in rats that underwent IIRI only when compared to the control (Table 9). However, pre-treatment with Al<sub>1</sub> and Al<sub>2</sub> and vit C<sub>1</sub> and vit C<sub>2</sub> caused a significant improvement in NO level compared with rats which underwent IIRI only; which was suggestive of possible anti-hypertensive effect of Al and Vitamin C (Table 9).

**Table 9**

The effect of *A. indica* and vitamin C on the level of nitric oxide (NO) in the serum of experimental rats with ischaemia-reperfusion injury.

Groups	Treatment group (mg/kg)	Serum nitric oxide ( $\mu\text{mole/l}$ )
A	Control	0.036 $\pm$ 0.007
B	IRI only	0.025 $\pm$ 0.001 <sup>a</sup>
C	IRI + Al <sub>1</sub>	0.034 $\pm$ 0.004 <sup>a,b</sup>
D	IRI + Al <sub>2</sub>	0.036 $\pm$ 0.006 <sup>b</sup>
E	IRI + Vit. C <sub>1</sub>	0.042 $\pm$ 0.003 <sup>a,b</sup>
F	IRI + Vit C <sub>2</sub>	0.029 $\pm$ 0.006 <sup>a,b</sup>

The results above are shown as Mean  $\pm$  Standard deviation for each group of eight (8) rats per group.

IRI = Ischaemia-reperfusion injury; Al<sub>1</sub> = *Azadirachta indica* (100 mg/kg); Al<sub>2</sub> = *Azadirachta indica* (200 mg/kg); Vit C<sub>1</sub> = Vitamin C (100 mg/kg); Vit C<sub>2</sub> = Vitamin C (200 mg/kg).

<sup>a</sup>  $p < 0.05$  when compared with the corn oil control group.

<sup>b</sup>  $p < 0.05$  when compared with ischaemia-reperfusion injury group.

### 3.4. Immunohistochemistry of renal and cardiac extracellular signal regulated kinase (ERK 1/2)

Figs. 1 and 2 show lower expressions of renal and cardiac Extracellular Signal Regulated Kinase (ERK) expressions compared to IIRI only. However, higher expressions of ERK were obtained in rats pre-treated with Al<sub>1</sub> and Al<sub>2</sub> and vit C<sub>1</sub> and vit C<sub>2</sub> (Figs. 1 and 2) respectively.

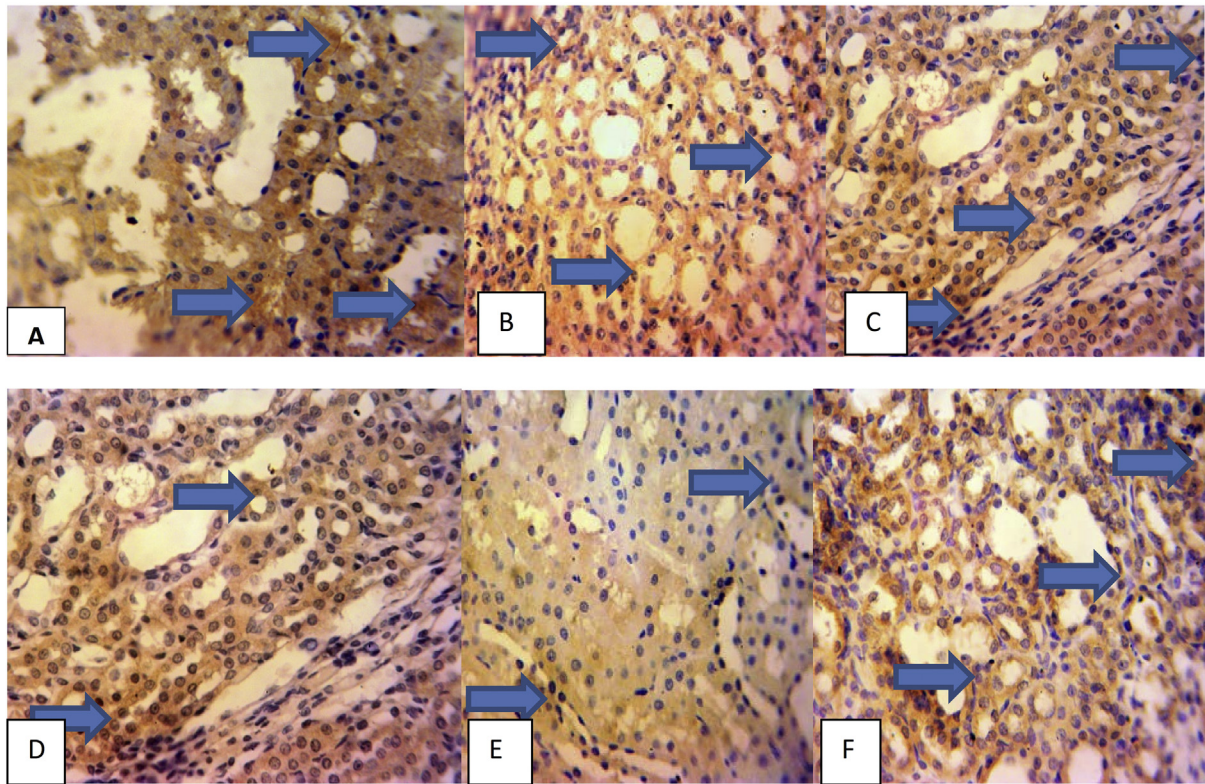
## 4. Discussion

Generation of reactive oxygen species (ROS) from oxygen molecules is one of the factors that causes injury after reperfusion [47]. ROS are derived from the electron transport chain of the mitochondria and xanthine oxidase during catabolism of purines [2]. These ROS can overwhelm the antioxidants' enzymes leading to oxidative stress which then cause damaging effects to the cells [48]. In this study, intestinal ischaemia-reperfusion caused a significant increase in H<sub>2</sub>O<sub>2</sub> and XO with a significant decrease in GSH levels in both the heart and kidneys. These effects were reversed in the rats treated with Al (100 and 200 mg/kg) as well as Vit C (100 and 200 mg/kg) when compared with the controls. This result indicates the antioxidant and the free radical scavenging activity property of Al and vitamin C consistent with earlier report of the antioxidant property of *A. indica* [49].

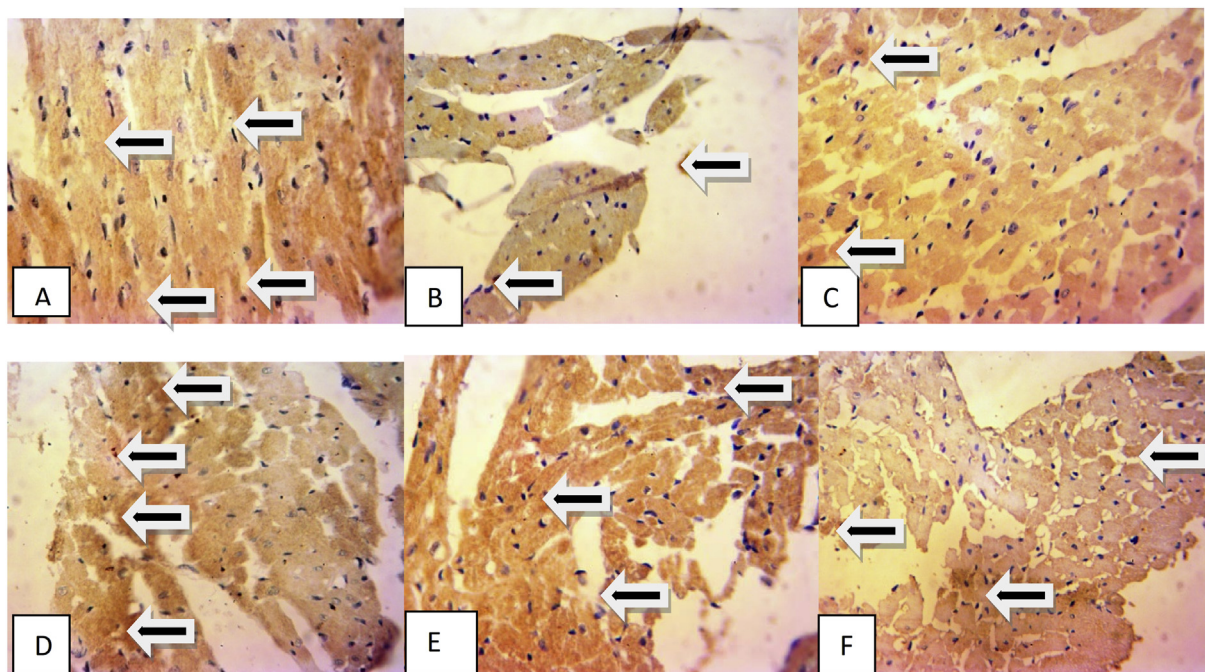
The antioxidant properties of Al and vitamin C were further demonstrated with the significant increase in the levels of GSH and GPx in the heart and kidneys of rats pre-treated with Al and vitamin C. The decreased level of GSH was observed during IIRI which was probably due to decreased ileal GSH synthesis contributing to its depletion [50]. The reduced glutathione (GSH) is a non-enzymatic intracellular antioxidant defence system that participates in detoxifying H<sub>2</sub>O<sub>2</sub>, when it donates its electron to H<sub>2</sub>O<sub>2</sub>, reducing it to H<sub>2</sub>O and O<sub>2</sub>. The antioxidant capacity of GSH resides in its sulfhydryl (SH) moiety as GSH also protects the cell from lipid peroxidation by acting as a substrate for GPx and GST [51]. The GPx catalyses the conversion of H<sub>2</sub>O<sub>2</sub> and other hydroperoxides to water and oxygen with the help of reduced glutathione (GSH) as a cofactor. The significant increase in the levels of GSH in the Al and vitamin C pre-treated rats shows the ability of Al and vitamin C to increase the level of GSH in order to mop up H<sub>2</sub>O<sub>2</sub>. Similarly, the altered levels of GPx in the heart and kidneys due to IIRI were restored with pre-treatment with Al and vitamin C to a level similar to that of the control. The alteration in the level of GPx was probably due to the reduced level of NADPH which is the principal intracellular reductant, however, pre-treatment with Al and vitamin C resulted in a significant restoration of the G-6-PD activity as previously reported [52].

Total thiol consists of intracellular and extracellular thiols which could be in the free form e.g. oxidized and reduced glutathione or bound to proteins e.g. albumin [53]. The thiol molecules have been reported to scavenge free radicals and also play a role in detoxification, signal transduction and apoptosis [54]. Therefore, decreased levels of thiols have been found in some diseases associated with renal, cardiovascular and several other organ dysfunction [55]. In this study, IIRI decreased the level of both protein and non-protein thiols in the kidneys and heart while Al and vitamin C pre-treatment improved the levels of the thiols in both the heart and kidneys. This result from the present study further shows the antioxidant activity of Al. This is consistent with the reported ability of both Al and vitamins C which help in the improvement of xenobiotic metabolism and detoxification [56].

In this present study, it was found out that similar to vitamin C, Al ameliorated oxidative stress induced by IIRI. This was



**Fig. 1.** Group A (control) shows higher expressions of renal Extracellular Signal Regulated Kinase (ERK 1/2) expressions in the renal tissues. Group B (Ischaemia reperfusion; IRI only) shows lower expressions of ERK than the control. Group C (IRI + *A. indica* (100 mg/kg)) shows higher expressions of ERK than Group B. Group D (IRI + *A. indica* (200 mg/kg)) shows higher expressions of ERK compared to Group B. Group E (IRI + Vitamin C (100 mg/kg)) shows higher expressions of ERK similar to that of Group A. Group F (IRI + Vitamin C (200 mg/kg)) shows higher expressions of ERK similar to that of Group A. The slides were counterstained with high definition haematoxylin and viewed objectives (magnification).



**Fig. 2.** Group A (control) shows higher expressions of Extracellular Signal regulated Kinase (ERK 1/2) expressions in the cardiac tissues. Group B (Ischaemia reperfusion; IRI only) shows lower expressions of ERK than the control. Group C (IRI + *A. indica* (100 mg/kg)) shows higher expressions of ERK than Group B. Group D (IRI + *A. indica* (200 mg/kg)) shows higher expressions of ERK compared to Group B. Group E (IRI + Vitamin C (100 mg/kg)) shows higher expressions of ERK similar to that of Group A. Group F (IRI + Vitamin C (200 mg/kg)) shows higher expressions of ERK similar to that of Group A. The slides were counterstained with high definition haematoxylin and viewed objectives (magnification).

consistent with the reduction in the level of serum xanthine oxidase levels in IRI which can contribute to the severe organ damage observed after reperfusion in ischaemic tissues [57]. In addition; xanthine oxidase degrades xanthine to uric acid. In the purine degradation pathway, it oxidizes nicotinamide adenine dinucleotide (NADH) to generate superoxide anion radical ( $O_2^-$ ) and  $H_2O_2$  during reperfusion. The increase in serum xanthine oxidase activity was directly proportional to the serum uric acid which has been implicated as a biomarker of oxidative stress in cardiorenal diseases and also a mediator of hypertension [58,59]. Hence, the level of xanthine oxidase in the serum was associated with IIRI. However, pre-treatment of rats with AI and vitamin C caused a significant reduction in the serum xanthine oxidase activity normalizing hyperuricaemia (high levels of uric acid in the blood) which is a diagnostic marker for renal damage and hypertension.

Similarly, AI and vitamin C also ameliorated the oxidative stress, inflammation and cardiac damage signified by the decreased level of myeloperoxidase in the serum of the rats pre-treated with AI and vitamin C. The MPO catalyses the cycle that produces oxidizing agents such as HOCl, oxidation of NO and reduction in NO bioavailability [60]. In patients with cardiovascular diseases, MPO is usually increased [61]. AI contains important bioactive compounds which include phytosterols (sitosterols, stigmasterol and campesterol) and flavonoids (rutin and quercetin), commonly known for their antioxidant, anti-inflammatory and antimicrobial activities [62]. Also, consistent with observation is an earlier study in which AI was reported to decrease the activity of colonic MPO [56]. Since, AI could normalize the aforementioned elevated MPO, suggesting AI as an antioxidant, anti-inflammatory and cardioprotective phytonutrient.

In this study, the level of serum NO was reduced following ischaemia-reperfusion injury. NO has been shown to be a mediator and/or protector of the vascular systems in several vital organs including the heart, liver, lungs and kidneys. These protective actions of nitric oxide in ischaemia-reperfusion injury are due to its potential as an antioxidant, anti-adhesion, and anti-inflammatory agent [63]. Also, normalization of nitric oxide bioavailability is an important factor in the amelioration of hypertension by preventing platelet aggregation, improving smooth muscle relaxation keeping blood vessels patent thereby lowering blood pressure. However, low levels of NO lead can to imbalance between dilation and vasoconstriction in favour of constriction increasing blood pressure with decreased flow leading to hypertension [64]. More importantly, NO may combine with superoxide anion radical to form peroxynitrite (ONOO<sup>-</sup>) which is a cytotoxic agent. The formation of peroxynitrite may also contribute significantly to the reduced bioavailability of NO and hence to the development of cardiovascular and renal dysfunction. In this study, AI and Vitamin C ameliorated ischaemia-reperfusion injury-induced reduction in NO levels in the serum especially in the rats pre-treated with AI (200 mg/kg) and Vit C (100 mg/kg). However, one of the limitations of the present study is that we could not take the blood pressure measurement as a clinical parameter for proper correlation of observable reduced bioavailability of NO as an indication of hypertensive state. Furthermore, we endeavour to take this into consideration the significance of this clinical parameter in our future study after the surgical procedure.

The results in the present study showed that IIRI crashed the levels of GSH and GPx activity in both the heart and kidneys. However, IIRI increased the level of GST in the heart. The increase might be attributable to adaptive responses of cells to oxidative stress, whereas the pre-treated rats had significantly decreased level of GST. At 200 mg/kg vitamin C, there was a significant

increase in the level of GST which was probably due to the ability of vitamin C to function as pro-oxidants at high concentration [65]. However, in the kidneys; IIRI did not reduce the levels of GST activity as the level was similar to that of the control without IIRI. Meanwhile, AI and vitamin C increased the levels of GST but at 200 mg/kg vitamin C, the level of GST was similar to that IRI only which might be suggestive of vitamin C as a pro-oxidant at high concentration. Hence, caution must be exercised in the use of synthetic antioxidant such as vitamin C and the interpretation of the results thereof.

The extracellular signal-regulated protein kinases 1 and 2 (ERK 1/2) signalling pathway is a cascade consisting of at least three families of protein kinases, including Raf (MAPKKK or MEKK), MAPKKs (MEK1 and MEK2), MAPK (ERK1 and ERK2 or p42/p44 MAPKs). The ERK pathway not only regulates a wide range of cellular behaviours, such as growth, proliferation, migration, differentiation, apoptosis and autophagy, but also mediates inflammatory responses [66,67]. The ERK pathway can be activated by a variety of extracellular stimuli such as growth factors, cytokines, mitogens, hormones and oxidative or heat stress [68]. It has been demonstrated that activation of ERK 1/2 mediated neuroprotection of dexmedetomidine, a potent and highly selective  $\alpha_2$ -adrenoceptor agonist in transient cerebral ischaemia-reperfusion [69]. The activation of the Reperfusion Injury Salvage Kinase (RISK) pathway, which incorporates phosphatidylinositol-3-OH kinase (PI3K), AKT/Protein Kinase B (PKB) and p44/42 Mitogen Activated Protein Kinase (MAPK) underlies protection against IIRI [70]. In the present study, IIRI reduced the activation of the RISK pathway thereby reducing the expression of ERK as shown by reduction in the immune-positive reaction IRI only group. However, AI and vitamin C ameliorated tissue damage following IIRI by increasing the expressions of ERK (a survival protein) as mediated in the RISK pathway.

The results in the present study have shown that intestinal ischaemia reperfusion injury does not only have deleterious effect on the intestines, but also on the heart and kidneys which was shown by the inhibition of both the enzymic and non-enzymic antioxidants and increased generation of ROS. However, AI was able to ameliorate these deleterious effects by increasing the *in vivo* antioxidant status, reduction in markers of oxidative stress, inflammation, cardiac and renal damage together with improvement in NO bioavailability. Tissue survival was also mediated via increase in the expressions of ERK.

## 5. Conclusion

Together, *A. indica* and vitamin C prevented IRI-induced cardiorenal dysfunction via reduction in oxidative stress, improvement in antioxidant defence system and increase in the ERK1/2 expressions. Therefore, *A. indica* can be a useful chemotherapeutic agent in the prevention and treatment of conditions induced by intestinal ischaemia-reperfusion injury.

## Conflict of interest

There is no conflict of interest (political, religious, academic or financial) whatsoever attached with this manuscript.

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