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Nephroprotective Effects of *Imperata cylindrica* Root Aqueous Extract (ICRAE) on Sprague-Dawley Rats with Gentamicin-induced Acute Kidney Injury

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RESEARCH ARTICLE

Abstract

Background & Objective: Acute kidney injury (AKI) refers to a sudden impairment of kidney function and represents a significant medical condition in developing countries. Despite the current measures for preventing AKI, there is still a great need to search for plant-based prevention and treatment. This study aimed to evaluate the nephroprotective effects of cogon (*Imperata cylindrica*) root aqueous extract (ICRAE) in Sprague-Dawley rats with gentamicin-induced Acute Kidney Injury (AKI).

Methodology: Fifteen Sprague-Dawley rats were randomly assigned to five groups: Control (*per orem* NSS, intraperitoneal NSS), Gentamicin (p.o. NSS, i.p. gentamicin), and three treatment groups ICRAE 100, 500 and 1000 (p.o. 100, 500 or 1000 mg/kg ICRAE, i.p. gentamicin). ICRAE and NSS were administered at days 1-17 while gentamicin at days 8-17. Kidney weight to body weight ratio (KWBWR), biochemical, and histological parameters were evaluated and statistically analyzed.

Results: There was an observed trend of decreasing kidney weight as extract concentration increased. A significant decrease (p= 0.0466) in serum creatinine was observed in ICRAE 100 and 1000. Furthermore, a trend of decreasing BUN as extract concentration increased was also observed (p= 0.23142). Histopathology analysis showed similar damages in the tubules and interstitium across all groups. Distal tubule hyaline casts were present in Gentamicin, ICRAE 100, and ICRAE 500 but absent in ICRAE 1000.

Conclusion: The results signify a potential nephroprotective effect of the extract especially in the early stages of AKI. This effect is mainly attributed to the flavonoids and reducing substances in ICRAE, which exhibit antioxidant and anti-inflammatory properties, as confirmed by the phytochemical analysis performed.

Keywords: *Imperata cylindrica, acute kidney injury, gentamicin, Cogon*

Introduction

Acute kidney injury (AKI) refers to the sudden impairment of kidney function characterized by diagnostic features, which include an increase in Blood Urea Nitrogen (BUN) concentration and/or increase in plasma or serum creatinine, both often associated with a reduction in urine volume. It is a significant medical complication in developing countries, the incidence of which varies with geographic, ethnic and socio-economic factors. AKI may require hospitalization associated with significantly increased length of stay, mortality, and healthcare costs [1].

Currently, there are non-pharmacologic and pharmacologic measures for preventing AKI. The former includes the estimation of several risk factors for AKI, and the avoidance or minimization of using nephrotoxic agents (i.e. amphotericin B, aminoglycosides) [2,3]. However, avoidance of these nephrotoxic drugs is not entirely possible since they remain the drug of choice for the treatment of gram-negative bacilli infections, septicemia, and endocarditis [4]. The pharmacologic interventions that have been used include fluids, inotropes, and vasopressors for resuscitation, especially in critically-ill patients. However, these are only supportive measures [2,3]. Thus,

there is still a great need to improve on the current measures for prevention and treatment of AKI.

Studies have looked into the compounds found in medicinal plants and their ability to target specific mechanisms underlying AKI. Inflammation is thought to have an important role in the pathophysiology of acute kidney disease. One of the mechanisms by which the inflammatory response contributes to renal cell injury is the generation of reactive oxygen species (ROS) [5,6,7]. ROS generation has been implicated in renal injury following ischemia, sepsis, and nephrotoxicity [8]. Accordingly, antioxidants that are able to sequester the ROS could prevent AKI.

Cogon (*Imperata cylindrica*), also known as spear grass, or locally as *kugon*, is said to comprise around 50% of the total grazing area in the Philippines, making it easily accessible [9, 10]. Cogon is used by many Filipinos in making roofs for houses and as raw material for household items [11,12,13]. In addition, the roots or rhizomes of cogon are used by several ethnolinguistic groups throughout the country as decoctions to relieve kidney problems [12,14,15]. The root has been shown to exhibit antioxidant activities due in part to its phenolic and serotonin content [16,17]. In another study by Chan *et al.* (2008), *I. cylindrica* rhizomes were shown to possess 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities comparable to those found in the leaves and stems [18]. There are also active constituents in the *I. cylindrica* rhizomes, such as cylindrene, which inhibit vascular smooth muscle contractions by inhibiting 5-lipoxygenase [19]. Based on these data, *I. cylindrica* has antioxidant and anti-inflammatory effects that may be nephroprotective. These mechanisms merit further study to be able to support or dispute the folkloric use of cogon root.

Methodology

The procedures involving animal care and use were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) - National Institutes of Health [20].

Procurement and Preparation of ICRAE

Dried roots of cogon were harvested in Naic, Cavite. The roots were cleaned using tap water and were air-dried for 24 hours. The identity of the roots was authenticated by a botanist from the Botany Section of the National Museum of the Philippines. A decoction was prepared as recommended for use: 250 g of dried roots boiled in 1 L of distilled water. The resulting preparation was freeze-dried into 26.8 g of powdered lyophilate at the UP-PGH Tissue Bank.

Phytochemical Screening

The lyophilate was reconstituted with distilled water to obtain the original concentration of the decoction. This was subjected to the following qualitative chemical tests to determine the different phytochemicals present in the extract: pH, presence of tannins, glycosides, reducing substances, alkaloids, plant acids, and saponins. Differentiation tests for saponins and flavonoids were also performed [21].

Experimental Animals

Twenty-two four-month-old Sprague-Dawley albino rats, weighing between 135 g to 175 g at the start of the study, were used [22]. Acclimatization to such conditions was done for two weeks prior to the experiment proper.

Pre-testing the Induction of Acute Kidney Injury

Seven male Sprague-Dawley albino rats were used for the pre-test, which was done two weeks prior to the experiment proper. Six rats were injected i.p. with gentamicin sulfate (80 mg/kg/day) daily. The sacrifice of rats was done on days 5, 6, 7, 8, 9 and 10. One control rat was injected with 0.9 NSS (normal saline solution) daily and was sacrificed at day 5. Both the biochemical and histopathology profiles of the rats were used as parameters to determine the earliest time these changes can be seen. This was the basis for the duration of AKI induction in the experiment proper.

Experiment Proper

The experiment proper consisted of 18 days from the initial administration of the treatments until the collection of specimens. *Per orem* (p.o.) administration of treatments was done from days 1 to 17. Induction of AKI was done via i.p. administration of gentamicin sulfate on days 8 to 17 [22, 23]. On day 18, the rats were anesthetized and sacrificed. Both the right and left kidneys of each rat were harvested and blood samples were collected. For the whole duration of the experiment, the rats were observed and weighed daily.

Randomization, Allocation, and Treatment of Rats

Fifteen male Sprague-Dawley albino rats were randomly divided into five groups (two control groups and three

treatment groups). The treatment and control groups consisted of Control (NSS p.o., NSS i.p.), Gentamicin Sulfate (NSS p.o., gentamicin sulfate i.p.), ICRAE 100 (100 mg/kg *I. cylindrica* p.o., gentamicin sulfate i.p.), ICRAE 500 (500 mg/kg *I. cylindrica* p.o., gentamicin sulfate i.p.), and ICRAE 1000 (1000 mg/kg *I. cylindrica* p.o., gentamicin sulfate i.p.).

Prior to treatment administration, the rats in each group were weighed and the mean weights of the five groups were compared using ANOVA to ascertain a homogenized randomization of the rats in the five groups.

For the control groups (Control and Gentamicin Sulfate), 0.9 NSS was administered p.o. daily from day 1 to day 17. For the treatment groups (ICRAE 100, ICRAE 500 and ICRAE 1000) were administered p.o. daily from day 1 to day 17. Treatments and NSS were administered only at a specific time prior to feeding (12:00 pm).

Induction of AKI and Sacrifice of Rats

Acute kidney injury was induced for Gentamicin Sulfate group, ICRAE 100, ICRAE 500, and ICRAE 1000. To induce the AKI, gentamicin sulfate (80 mg/kg/day) was administered daily for 10 days from day 8 to day 17 [22,23]. It was administered i.p. on the right hemiabdomen [22]. For Control, 0.9 NSS was administered i.p. also on the right hemiabdomen. This was done daily for 10 days from day 8 to day 17, as well.

On the 18th day, the rats were sacrificed through cervical dislocation after anesthetization with an intramuscular injection of Zoletil (30 mg/kg). The rats were dissected to collect blood samples via cardiac puncture. The left and right kidneys were harvested for the assessment of renal function and pathology.

Assessment of Renal Tubular Function, Structure, and Pathology

Blood (at least 2 mL) was collected via cardiac puncture using a 3 mL syringe and was placed in 5 mL plain sterile Falcon tubes [22,24]. The blood was allowed to clot for 3-4 hours and was centrifuged at 2000 rpm for 15 minutes [24]. The sera were collected and placed in 2.5 mL microcentrifuge tubes for storage at 4^{oC} prior to serum creatinine (mg/dL) and blood urea nitrogen (mg/dL) analysis.

The left and right kidneys were collected by opening the peritoneal cavity and dissecting the retroperitoneum. The renal vessels were first clamped and then cut at the hilum. The kidneys were blotted dry using filter paper and weighed using an analytical balance [22]. The kidneys were preserved in 10% neutral buffered formalin and processed for histological preparation and staining [22,23]. Serial sections (2 µm cuts) of the cortex embedded in paraffin wax and stained with haematoxylin and eosin were requested for histological scoring [22]. The following histopathology features were scored: glomerular congestion, blood vessel congestion, interstitial edema, inflammatory cells, tubular necrosis, tubular hyaline casts and tubular RBC casts [23].

Statistical analysis

Outcome parameters were reported as mean ± SEM. Non-post hoc statistical analyses were done using the STATA 10 and R program statistical software at 80% power. Post hoc analyses were calculated manually [25]. A two-way analysis of variance with kidney laterality as blocking variable was used to analyze the mean KWBWR of both left and right kidneys of all groups. A Scheffe post-hoc analysis was then used for pairwise comparison. For the biochemical outcome parameters, the normality of the data was initially tested using the Shapiro-Wilk Test. Data homogeneity was also tested using Bartlett's test. The Kruskal-Wallis one-way analysis of variance by ranks was used to determine overall significant difference among the different sample groups. A Dunn post-hoc test was subsequently used for pairwise comparison between the groups. For the histopathology parameters, Kruskal-Wallis one-way analysis of variance by ranks was used to determine overall significant difference among the sample groups. A Dunn post-hoc test was consequently used for pair wise comparison. The p-values were compared to the critical values from the Kruskal-Wallis table of values with sample groups less than five. A probability value of $p < 0.05$ was considered statistically significant.

Results

General Animal and Laboratory Data

All animals were weighed before and after the experiment. The difference between these weights was expressed as body weight change and was not found statistically significant. Kidneys were also collected, weighed and expressed as percentage of total body weight. As shown in Table 1, a trend can be observed among the controls and among the treatment groups. As extract concentration increases, right and left kidney weights also decrease approaching normal values.

Table 1. *Effect of Gentamicin Sulfate and ICRAE on kidney weights and percent KWBWR*

Parameters	Control (NSS+NSS)	Gentamicin (NSS+Genta)	ICRAE 100 mg/kg	ICRAE 500 mg/kg	ICRAE 1000 mg/kg
Mean kidney weight (mg)	0.67 ± 0.05	0.82 ± 0.05	0.92 ± 0.06	0.78 ± 0.05	0.77 ± 0.02
Mean weight/ 100g body weight (mg)	0.38 ± 0.02*	0.46 ± 0.01	0.50 ± 0.02*	0.46 ± 0.02	0.44 ± 0.01

Values are mean ± SEM, n=6
*Significantly different at α=0.05

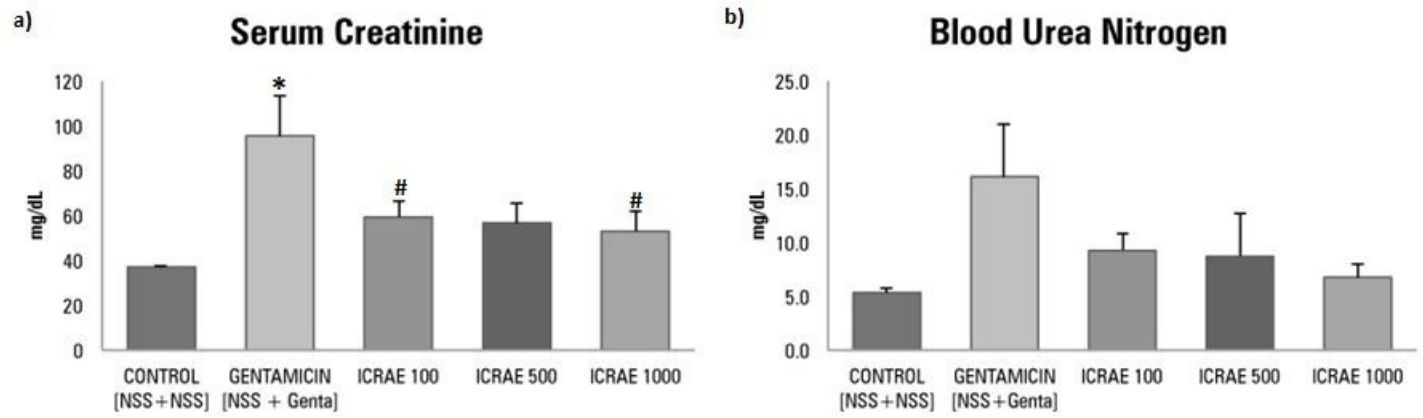

Figure 1. *Effect of Gentamicin Sulfate (NSS+Genta) and ICRAE (100, 500, 1000) on (a) serum creatinine and (b) BUN compared to control group (NSS+NSS).*

| Data expressed as mean ± SEM (n=3/group; n=2/group for NSS+Genta). Kruskal-Wallis, with post-hoc analysis. *p<0.05, versus the control group; # p<0.05, versus the NSS+Genta group.

Kidney Function Tests

As shown in Figure 1, the administration of gentamicin sulfate resulted in a significant increase (p = 0.0466) in serum creatinine (95.5 ± 17.5 mg/dL), but no significant increase (P = 0.23142) in BUN levels (16.1 ± 4.8) compared to the normal control group. ICRAE 100 and 1000 showed a significant decrease in serum creatinine compared to the gentamicin sulfate group (p = 0.0466). Kruskal-Wallis test revealed no significant difference among BUN levels across the treatment groups (p = 0.23142).

Despite the lack of statistical significance, a trend can be observed. As extract concentration increases, serum creatinine and BUN decreases approaching normal values.

Histopathology Analysis

Microscopic evaluation of all the kidney samples showed no interstitial edema. However, glomerular congestion, vascular congestion, interstitial infiltrates, tubular necrosis and tubular RBC casts were present in all treatment groups (Figures 2 to 6). Hyaline casts were present in distal tubules of the Gentamicin Sulfate, ICRAE 100 and ICRAE 500 groups (Figure 7).

The scores of the histopathology parameters for AKI are presented in Table 2. Tests for normality and homogeneity showed a non-normal and non-homogenous data set, resulting in the use of Kruskal-Wallis one-way ANOVA. The test on the individual parameters showed a significant difference in tubular hyaline casts (p = 0.046). Post hoc analysis using Dunn's least significant difference showed that the number of tubules with hyaline casts in the Gentamicin Sulfate group was significantly higher than Control (Rank Mean difference = 8.17), ICRAE 100 (Rank Mean difference = 6.00), ICRAE 500 (Rank Mean difference = 6.00), and ICRAE 1000 (Rank Mean difference = 8.17) with no significant difference observed between the Control, ICRAE 100, ICRAE 500, and ICRAE 1000 groups.

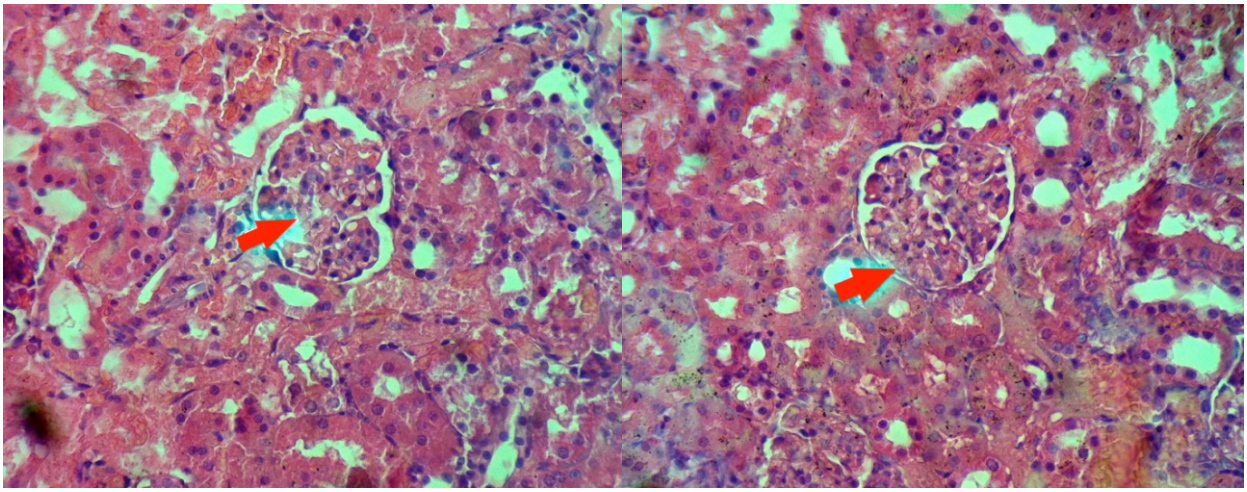

Figure 2. *Glomerular Congestion. Both arrows point to congested glomeruli with numerous RBCs present (40 X H&E)*

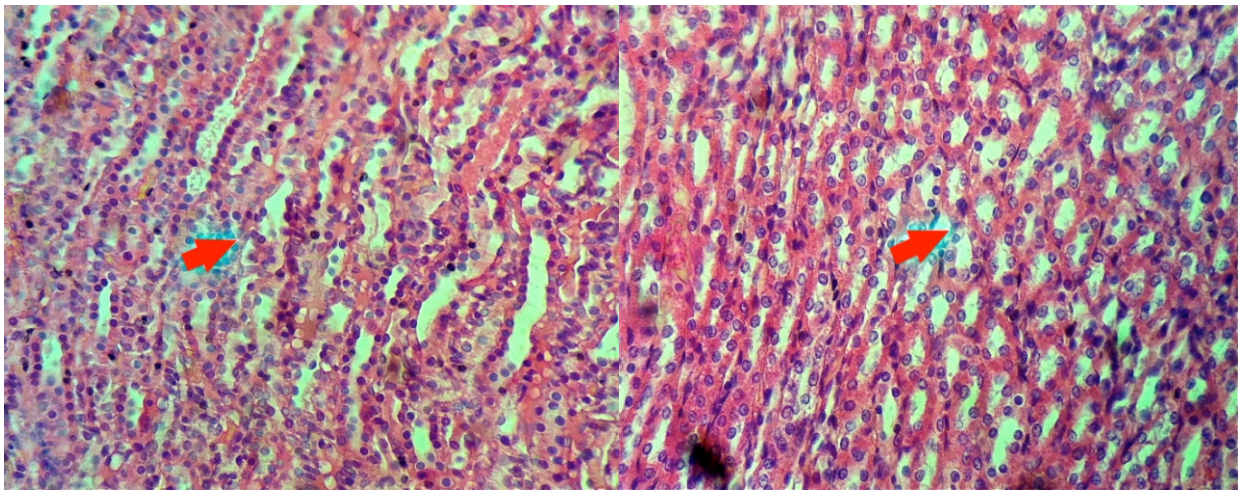

Figure 3. *Vascular Congestion. Both photos demonstrate vascular congestion showing presence of numerous RBCs in between the tubules (40 X H&E)*

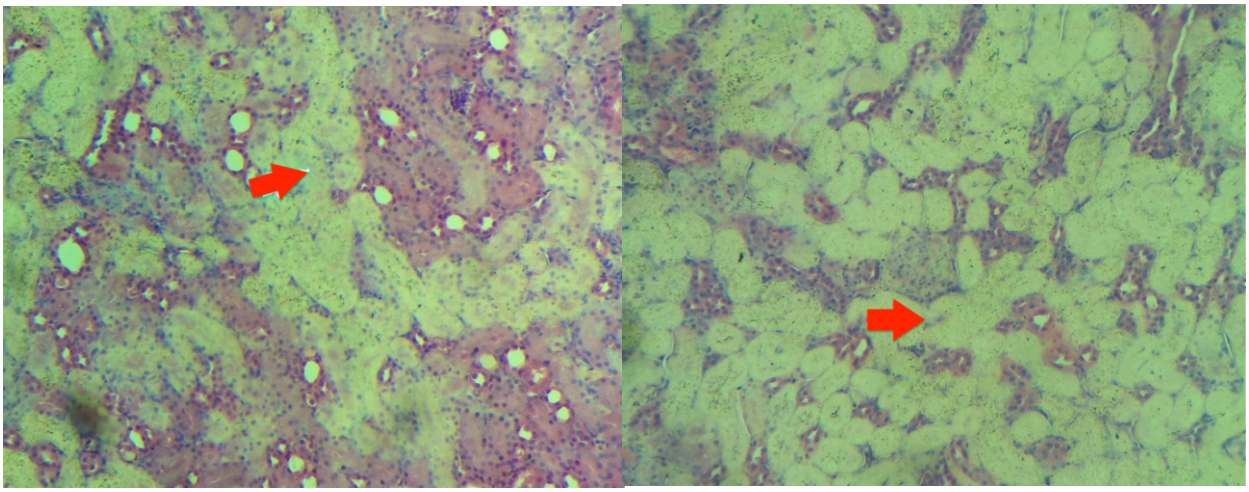

Figure 4. *Tubular Necrosis. The photos show evident necrosis of the tubules (40x H&E).*

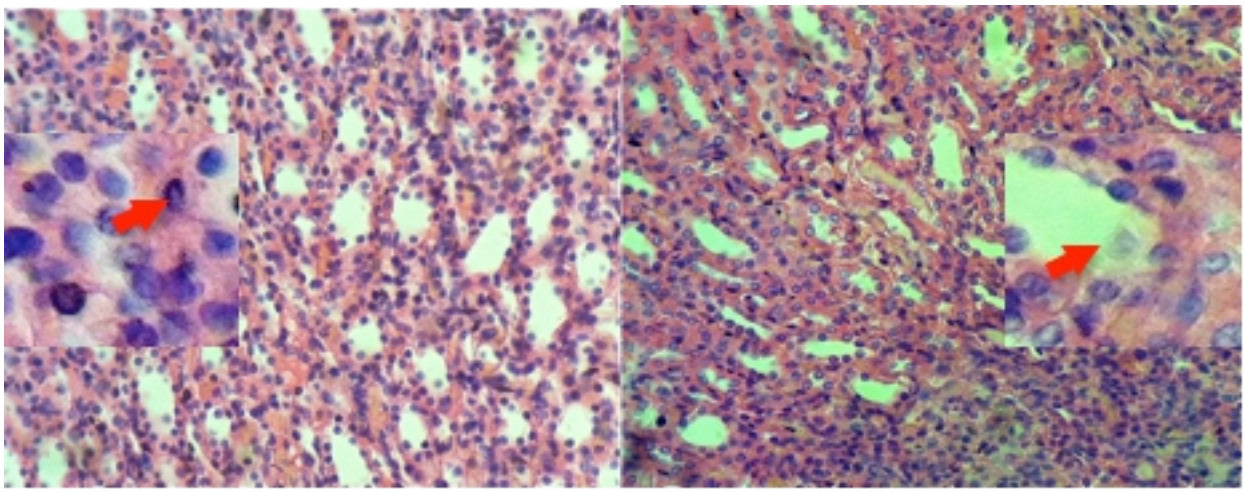

Figure 5. *Interstitial Infiltrates. Left inset: the arrow points to a neutrophil. Right inset: the arrow points to a monocyte (40x H&E).*

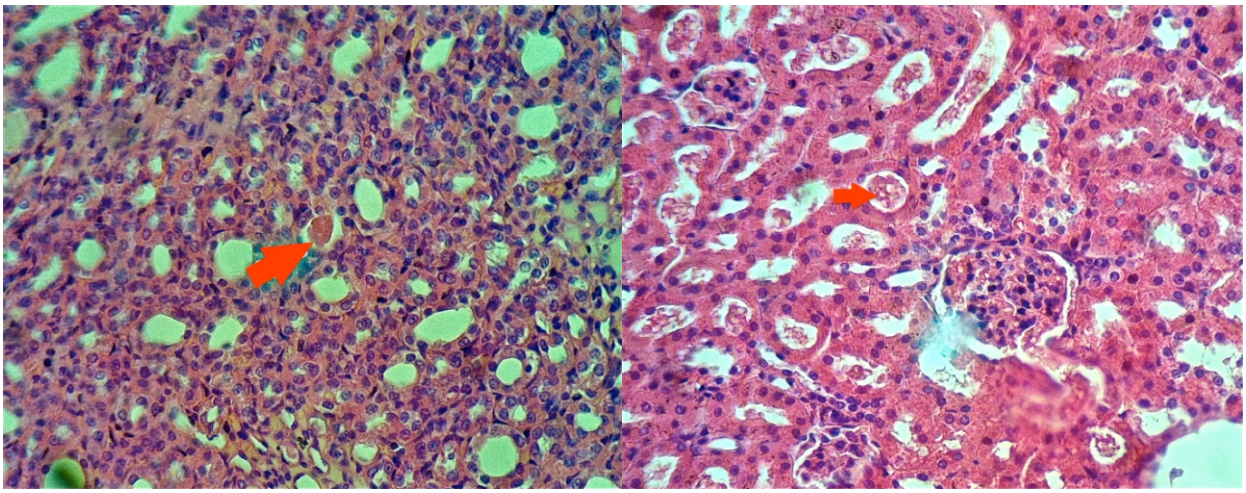

Figure 6. *RBC Casts. Black arrows point to RBC casts present in the distal tubules (40x H&E).*

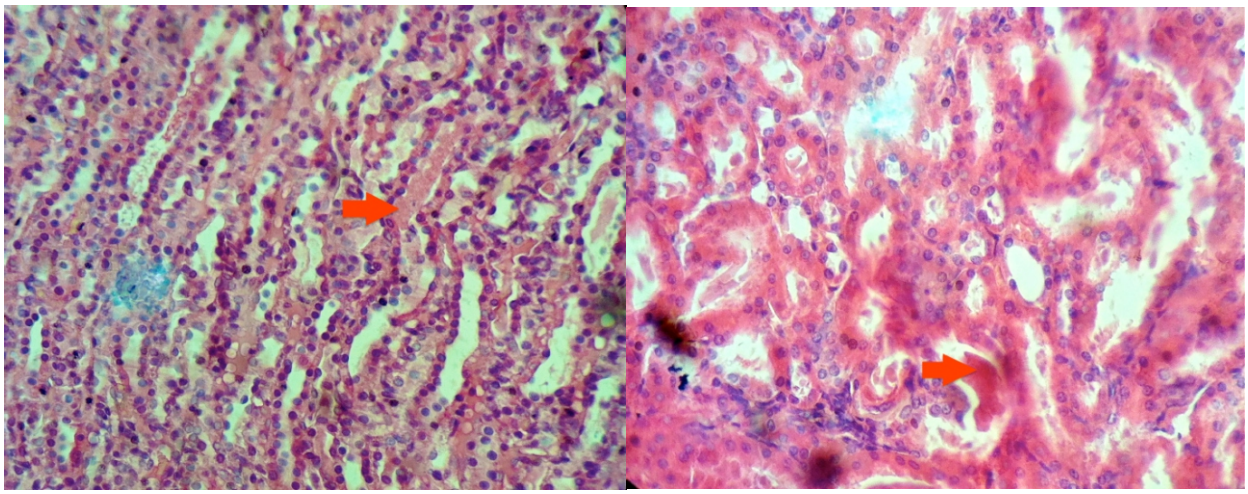

Figure 7. *Hyaline Casts. Black arrows point to hyaline casts present in the distal tubules (40x H&E).*

Table 2. *Histopathology scoring of the kidney tissue samples*

Histopathology changes	Control (NSS+NSS)	Gentamicin Sulfate (NSS+Genta)	ICRAE 100	ICRAE 500	ICRAE 1000
Glomerular congestion	+	++	++	+	++
Blood vessel congestion	+++	+++	++	++++	+++
Inflammatory cells	+	++	++	+	++
Interstitial edema	-	-	-	-	-
Tubular necrosis	++	++	++	++	+++
Tubular RBC casts	+	++	++	++	+
Tubular Hyaline casts	-	++	+	+	-
Average Score	1.33	1.76	1.43	1.54	1.52

Right kidney of one randomly selected subject per group
*p < 0.05
H > 8.333; this corrects for the small sample size per group

Phytochemical Screening

The yellow-brown colored solution had a pH of 5 (weakly acidic) and was found to have glycosides, reducing substances, and flavonoids. The solution was negative for tannins, alkaloids, plant acids, saponins, and flavones or flavonols.

Discussion

Effects of ICRAE on the biochemical parameters for AKI

Results showed that intraperitoneal administration of gentamicin sulfate for 10 days at a dose of 80 mg/kg significantly increased serum creatinine. BUN levels also increased although the difference was not statistically significant. Based on previous studies using the same concentration and duration of gentamicin sulfate administration, both serum creatinine and BUN was significantly increased [26]. However, the non-significant difference that was observed may be attributed to the low power of the statistical test mainly due to the small sample size used in this study. Concomitant treatment with ICRAE resulted in a significant decrease of serum creatinine levels only at 100 mg/kg and 1000 mg/kg with no significant difference in BUN levels. However, a dose-dependent decrease in serum creatinine and BUN has been observed in increasing ICRAE concentrations. This shows a potential nephroprotective effect by ICRAE in terms of biochemical markers.

It must be noted that the biochemical parameters were limited to serum creatinine and BUN levels since these are currently the ones included in the diagnosis of AKI, and these are the most accessible. However, these parameters are not specific for AKI since changes in serum creatinine and BUN may be normal responses to extracellular volume depletion or decreased renal blood flow [27]. Also, BUN and serum creatinine may not be sensitive enough to detect early and minor kidney injury, unlike histopathology, which is the gold standard [28,29]. Thus, it is important to correlate these outcomes with the histopathology results to confirm the nephroprotective effects observed.

Effects of ICRAE on the kidney weights in gentamicin-induced AKI

The weight of the kidney in relation to body weight differs significantly ($p < 0.01$) across treatments but is not significantly different bilaterally. Post-hoc analysis showed that the difference lies between Control Group and ICRAE 100. Although not statistically significant, an increase in kidney weights was observed in Gentamicin Sulfate groups compared

to the Control group, consistent with literature showing that AKI may manifest as an increase in kidney weight and may probably be caused by interstitial edema, post-injury triglyceride accumulation in renal cortex and proximal tubular cells, or an accumulation of ammonia which stimulates epithelial hypertrophy [30,31,32]. The significant increase in kidney weights suggests that the administration of the extract may have augmented the kidney injury-associated weight increase at a dose of 100 mg/kg.

Effects of ICRAE on the histopathology of gentamicin-induced AKI

Results from the study showed the significant decrease in tubular hyaline casts as the dose of *I. cylindrica* extract increased. The presence of casts in AKI is important especially in the early stages of AKI since it can be used as an effective early marker of renal tubular damage [33]. Normally, casts are formed from the precipitation of Tamm-Horsfall proteins in the renal tubules. This particular protein is a common matrix in all types of urinary casts. On the other hand, in the setting of AKI, hyaline casts can also be formed when the Tamm-Horsfall protein aggregates with light chain proteins, which are not reabsorbed by the proximal tubules due to direct damage to the proximal tubule cells [34]. Thus, preventing tubular damage would exert a protective effect on the formation of excess hyaline casts in the tubules via the preservation of the reabsorptive function of the proximal tubules. Through this mechanism, the concentration of light chain proteins in the filtrate may decrease and this results in lesser precipitation into casts.

The significant decrease in hyaline casts in ICRAE treated rats adds additional evidence to the potential nephroprotective effect of the extract especially in the early stages of AKI. This is consistent with the biochemical markers wherein serum creatinine and BUN decreased in ICRAE treated rats.

Nephroprotective effects of ICRAE based on Phytochemical Screening

In the phytochemical screening, ICRAE tested positive for glycosides. Glycosides are found in the plant kingdom in a wide variety due in part to the different stereochemical configurations of the sugar component. With hydrolysis, the sugar can be broken off, yielding aglycones, such as terpenoid, steroid, flavonoid, quinonoid, lignan, other simple phenolics, and isothiocyanate [35]. In this case, the specific glycoside in ICRAE may be classified under flavonoid glycosides, as suggested by the presence of flavonoids in the extract. One example of a dietary flavonoid is quercetin, which has been found to exhibit protective effects against gentamicin-induced nephrotoxicity by preventing a decrease in antioxidants, such as glutathione, superoxide dismutase and catalase, inhibiting lipid peroxidation, and preventing renal tubular necrosis [36]. Flavonoids also exhibit their antioxidant properties by modifying the lipid packing order and decreasing the fluidity of the membrane, thus hindering the diffusion of free radicals [36, 37]. The flavonoids present in ICRAE may possibly work in the same mechanism to produce a nephroprotective effect. However, it must be noted that not all flavonoids show a nephroprotective effect. This is attributed to the specific flavonoid's structure and its ability to incorporate into the lipid bilayer [37]. Therefore, the specific flavonoid present in ICRAE should be investigated further in future studies.

ICRAE tested positive for reducing substances, which could indicate the ability of ICRAE to sequester iron. This is in agreement with previous studies which showed the presence of ferritin in the different parts of *I. cylindrica* [38,39]. ICRAE represents exogenous ferritin, which is known to upregulate the synthesis of endogenous ferritin [40]. Endogenous ferritin, in turn, sequesters the iron that is released from the renal cortical mitochondria due to the administration of gentamicin sulfate. In effect, the catalysis of oxidative stress by iron is prevented.

Limitations in data analysis

Since the study involves samples taken from previously sacrificed animals, some autolytic changes may also have been present as a result of the spontaneous degradation and destruction of cells or tissues by autologous enzymes. These changes are particularly evident and more rapid in tissues that are rich in enzymes such as the kidney, liver, and brain. In a previous study, changes in the nucleus, such as separation of the external and internal membranes, as well as the degradation of the external membrane, were found to occur in proximal tubule epithelial cells of rats in as early as four hours post mortem, and even within the first hour at higher temperatures of 30^{oC} [41]. In our study, autolytic changes were observed even in the negative control group, which may have contributed to some unwanted damage in these samples. Although proper fixation in formalin using the standard and recommended concentration of 10% has been done, chilling of both the tissues and formalin can be considered for future studies to prevent such autolysis in tissues, such as the kidneys [42]. Increasing the concentration may not be preferred mainly because of the increase in

formic acid which may confer additional damage to the tissues. Furthermore, one limitation of the test is that it may fail to approximate a chi-square distribution when the number of groups is less than three (k<3) or when the number of samples in a group is less than five (N< 5) and this may make the computed p-value incorrect. This may lead to more false positive and false negative results that can be observed [44,45]. However, this was already corrected in the analysis by using the classical hypothesis testing using a special table for critical values for small samples instead of using the computed *p*-value as basis for significance [45].

Conclusion and Recommendations

In this study, the KWBWR, serum creatinine, and BUN levels, as well as the hyaline casts showed a dose-dependent decrease with increasing ICRAE concentration This shows a possible nephroprotective effect which could be explained by the presence of flavonoids and reducing substances in ICRAE. To further support and confirm the findings of this study, bioassay-guided fractionation should be performed to determine the active fraction of the extract, from which the identity of the possible active constituents can be elucidated. Aside from measuring kidney function and doing histological analysis, work on ICRAE may also involve measuring oxidative stress biomarkers, such as glutathione, superoxide dismutase, and catalase, as well as lipid peroxide levels (TBARS), such as those done in previous studies to confirm whether ICRAE has ROS scavenging properties [36]. Inactivation of the binding of gentamicin sulfate to the brush border and prevention of phospholipid overloading in lysosomes are other mechanisms of nephroprotection which can be investigated by including the measurement of urinary gentamicin sulfate excretion and examination of the gentamicin sulfate and ICRAE interaction on the brush borders [22]. The limitations of serum creatinine and BUN as biomarkers of AKI could be addressed in future studies with the use of more sensitive and more specific biomarkers (if available) such as urinary kidney injury molecule 1 (Kim-1) [29]. Urinalysis and urine output measurement may also be performed in addition to the methodologies in the study. This involves investigation of the physical, chemical and microscopic features of the urine that may correlate strongly with the presence of AKI.

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