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ABSENCE OF NEPHROPROTECTIVE EFFECT: EVALUATION OF AN AQUEOUS EXTRACT OF *CLINOPODIUM VIMINEUM* (L.) KUNTZE (LAMIACEAE) USING A RENAL FAILURE MODEL WITH FEMALE *WISTAR* RATS

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ABSTRACT

Clinopodium vimineum, popular in Costa Rica as *menta de palo*, is an aromatic shrub native to Central America and the Caribbean. It is employed in traditional medicine to relieve gastrointestinal symptoms (carminative and antispasmodic properties). Thus, it is reported as an antimicrobial, sedative, analgesic, anti-inflammatory, healing, and antioxidant. Therefore, the nephroprotective effect of an aqueous extract of this plant was tested using a model of renal damage induced through potassium chromate in female *Wistar* rats. The plant material extract was prepared, and a test was carried out with four groups of five experimental subjects each. In addition to the control groups (positive and negative), one group was treated with the extract. Another was also given a pretreatment with this material prior to the induction of acute kidney injury. Each animal's renal function parameters were evaluated. They include creatinine, sodium, potassium, and glucose concentrations in serum and creatinine, sodium, potassium, glucose, and protein concentrations in urine. Urinary flow was obtained too. Furthermore, histopathological analysis of the kidneys was performed. The information obtained concluded that the aqueous extract of *C. vimineum* does not have a nephroprotective effect in the model of kidney damage induced with potassium chromate in female *Wistar* rats at the dose tested.

KEYWORDS: *Clinopodium vimineum* (L.) Kuntze, aqueous extract, acute kidney injury, *Wistar* rats, renal function parameters, histopathological analysis.

INTRODUCTION

Acute kidney injury (AKI) is a potentially reversible pathology. It is characterized by reduced glomerular filtration rate (GFR), proteinuria, accumulation of circulating nitrogenous compounds, and kidney inability to maintain electrolyte balance.[1, 2, 3] According to its origin, it can be classified as prerenal, intrinsic, and postrenal.[3, 4]

Culturally, indigenous populations have implemented the use of natural products as an alternative or complementary treatment for kidney diseases. About 40 % of the general population in Costa Rica consumes them for medicinal purposes.[5] *Clinopodium vimineum* (known in Costa Rica as *menta de palo*) is an aromatic shrub native to Central America and the Caribbean. It is described as a plant with woody stems of vertical growth, multiple ramifications, and a height between 15 and 20 cm, leaves in an oval and opposite shape, with toothed margins and a lime green upper side, engraved veins covered by a villus, and small tubular white (or with pink/purple shades) flowers.[6, 7]

It has been utilized to relieve gastrointestinal symptoms because of its carminative and antispasmodic properties. Additionally, it has been described as an antimicrobial, sedative, analgesic, anti-inflammatory, and cicatrizing agent.[6, 7] As a complement, some investigations have found oxidative activity for different plant extracts of the *Clinopodium* genus, usually attributed to their phenolic compounds.[8, 9]

Regarding the AKI induction model with $K_2Cr_2O_4$, it is based on generating large amounts of free radicals by

chromium reduction (Cr^{6+} to Cr^{3+}). As a result, it damages the brush border membrane of the proximal convoluted tubule (PCT) and distal convoluted tubule (DCT) cells.[10, 11] The accumulation of oxidizing agents alters the membranes, modifying various substances' reabsorption and secretion processes, leading to renal function alterations.[11, 12]

Therefore, this research aimed to evaluate the potential nephroprotective effect of an aqueous extract of *C. vimineum* with presumed antioxidant activity in a model of renal damage induced with potassium chromate in female *Wistar* rats. This evaluation has already been done with other Costa Rican traditional medicine plants, such as *Cymbopogon citratus*[13] and *Passiflora biflora*.[14]

MATERIALS AND METHODS

Vegetal material

C. vimineum collected at the School of Agriculture of the Humid Tropical Region (EARTH University) (10°13'09" N 83°35'33" W) located in *Guácimo, Limón*, was used. The sample was identified and deposited in the Dr. Luis A. Fournier Origgi Herbarium of the School of Biology of the Universidad de Costa Rica (UCR, for its Spanish acronym). The plant material consisting of the flowers, buds, and stems was dried at room temperature and subsequently pulverized with a Willey® Mill No. 0.3 until a fine material was obtained for conservation.

Preparation of the aqueous extract of the plant material

An infusion was prepared with 45 g of powdered plant material, adding boiling water. The mixture was allowed to stand for 30 minutes in a container with a lid. Subsequently, the mixture was diminished by reduced pressure at 50 °C employing a Büchi® R-134 rotavapor (water bath B-480 and Büchi® B-721 vacuum controller). To obtain the fine powder that would facilitate the extract dosage, it was lyophilized for four days (LABCONCO® FreeZone 6) and stored at -80 °C.

Experimental animals

Adult female *Wistar* rats (HsdBrlHan: WIST) (207.80 ± 3.33 g of body weight), between eight and nine weeks of age, from the Laboratory of Biological Assays (LEBi, for its Spanish acronym) of the UCR were employed. The choice of sex was because creatinine is actively secreted in male rats, ceasing to be a marker for glomerular filtration.[15, 16]

A total of 20 subjects were kept in polycarbonate boxes in the laboratory for *in vivo* work at the Phytopharmacology and Pharmaceutical and Cosmetic Technology Laboratory (LAFITEC, for its Spanish acronym) of the INIFAR under the following conditions: temperature: 23.2 °C; relative humidity: 73 %, and periods of light and darkness: 12 hours. Bottled drinking water (Members Selection®) and rodent food (Aguilar & Solís®) were supplied *ad libitum*. They were kept in the laboratory for seven days without manipulation to allow their adaptation. The handling and care of the animals were performed under the provisions of the Animal Welfare Law (No. 7451) and the guidelines stipulated in the Guide for the Care and Use of Laboratory Animals (Decree No. 26668). The experimental design of the model was reviewed and approved by the Institutional Committee for the Care and Use of Animals (CICUA, for its Spanish acronym) of the UCR, document CICUA-018-16.

Experimental design

It was developed by modifying a procedure from a previous investigation.[12] Random block modeling was carried out with a total of four groups of five experimental subjects each: negative control group (group 1, saline solution was injected instead of $K_2Cr_2O_4$, and water was administered as a replacement for the extract), positive control group (group 2, AKI was induced using a single dose of 20 mg/kg $K_2Cr_2O_4$ and did not receive any dose of the extract, only water), pretreatment group (group 3, was given a previous dose of the aqueous extract of 150 mg/kg orally by intragastric cannula and three hours later AKI was induced using the same dose of $K_2Cr_2O_4$ utilized in group 2) and treatment group (group 4, AKI was induced using a single dose of $K_2Cr_2O_4$ as for group 2). After the induction of AKI, the last two groups received a daily dose of the aqueous plant extract (150 mg/kg by intragastric cannula) for four consecutive days.

The trial lasted five days, ensuring that the animals had access to food and drinking water *ad libitum*. After the last dose administration, the animals were placed individually in metabolic cages for eight hours on the fifth day. Total urine was collected, separated into fractions of approximately 500 µl (Eppendorf tubes), and stored at -80 °C (Thermo ScientificTM RevcoTM UxF refrigerator) until analysis. Finally, each animal was euthanized by decapitation to obtain total blood, and a general necropsy was made to collect kidney tissue. Plasma samples were obtained by centrifugation at 3000 rpm for 10 minutes (HettichZentrigugen® model Universal 320 R) at LAFITEC.

Evaluation of renal function parameters

The following renal function markers were defined: creatinine, sodium, potassium, and glucose serum concentrations, plus creatinine, sodium, potassium, glucose, and protein urine concentrations. With the value of urinary flow, the following renal function parameters were determined:

1. Creatinine concentration ($Cn_{creatinine}$): it was measured in plasma and urine utilizing the CreatinineRandox® kit. All samples were processed following the manufacturer's specifications. $Cn_{creatinine}$ was spectrophotometrically quantified at a maximum wavelength (λ_{max}) of 505 nm through an ELISA plate reader (BioTekSynergy® HT coupled to Gen5TM Software) from LAFITEC.

2. Electrolyte balance: a pool of plasma and urine samples was sent to the Clinical Analysis Laboratory of the Faculty of Microbiology of the UCR, where sodium and potassium concentrations were quantified with Roche Diagnostics® 9180 Electrolyte Analyzer, based on the ion-selective electrode potentiometry. The ions' purification was calculated from the concentrations obtained, and the electrolyte balance was determined.
3. Glucose concentration ($Cn_{glucose}$): it was established in plasma and urine samples using the GLUC-HK Randox® kit based on an enzymatic spectrophotometric method. All samples were handled according to the manufacturer's specifications. Glucose quantification in the samples was performed with a λ_{max} of 340 nm for reading with an ELISA plate reader (BioTek Synergy HT coupled to Gen5TM Software). The $Cn_{glucose}$ of the biological samples was determined from the reference standard absorbance.
4. Urinary protein excretion: total protein concentration was quantified in urine samples through the Sigma-Aldrich kit based on the Bradford method.[17, 18] Total protein quantification was determined spectrophotometrically in an ELISA plate reader (BioTekSynergy HT coupled to Gen5TM Software) with a λ_{max} of 495 nm 20 minutes after the colored complex formed. Protein concentration (Cn_{prot}) and urinary protein excretion (EU_{prot}) were calculated.

Histopathological analysis of kidneys

The harvested organs were fixed with 10 % buffered formalin. Tissues were then dehydrated with increasing concentrations of ethanol, cleared with xylene, and embedded in paraffin. A rotary microtome (Leica RM2245) was used to make 4 µm sections. Then, they were stained with hematoxylin-eosin. The interpretation followed for the staining was: intense bluish coloration for the nucleus, red-pink coloration for the cytoplasm, matrix, and collagen fibers, and bright red for the erythrocytes.[19]

After staining with hematoxylin-eosin, each renal section was given a general description to determine the severity degrees in the nephron sections, such as glomerulus, PCT, and DCT. Its morphology and cellular structure (nuclei and appearance of its chromatin) were evaluated.[20, 21]

Statistical analysis

The results were expressed as the mean together with its relative standard deviation. The difference between groups was executed by univariate statistical analysis (ANOVA), and the comparison between the means of the groups was done utilizing Tukey's multiple comparison analysis with a significance level of 95 % ($p < 0.05$), using the software GraphPad Prism® version 6.01 for Windows.

RESULTS AND DISCUSSION

Evaluation of renal function parameters

Creatinine is the most widely considered marker to establish glomerular function. It is an endogenous substance, 100 % eliminated by this renal excretion mechanism. By determining the substance purification, it is possible to estimate the process alteration and indicate the level of protection by the xenobiotics administration.[22, 23, 24]

From the urine volumes obtained for each experimental animal in the metabolic cages for approximately eight hours, it was possible to quantify the urinary flow of every subject. GFR was calculated through plasma and urinary creatinine concentrations and urinary flow. **Table 1** shows a significant increase in serum $Cn_{creatinine}$ in the *C. vimineum* pretreatment group relative to the negative control ($p < 0.05$).

Table 1: Temporal variation in urinary and serum $Cn_{creatinine}$ in the *in vivo* model of renal toxicity using an aqueous extract of *C. vimineum*. The data were expressed as the mean and its respective relative standard error. Concentrations were compared to negative control, n = 5 animals per group, * $p < 0.05$.

Experimental group	Urine $Cn_{creatinine}$ (µg/ml)	Serum $Cn_{creatinine}$ (µg/ml)
Negative control	205.5 ± 16.3	14.25 ± 0.86
Positive control	138.9 ± 10.1	19.19 ± 1.19
C. vimineum pretreatment	165.6 ± 9.6	24.13 ± 1.79*
C. vimineum treatment	161.0 ± 18.6	20.97 ± 3.00

The $Cn_{creatinine}$ and urinary flow defined the GFR through creatinine clearance ($Cl_{creatinine}$) for the different experimental groups (**Figure 1A**). A significant reduction was observed for the two experimental groups and the positive control concerning the negative control group ($p < 0.05$). It should be noted that the most remarkable change was between the group with *C. vimineum* pretreatment and the negative control.

The results obtained from serum $Cn_{creatinine}$ and their clearance revealed the existence of damage at the glomerular level. It is reflected in the ability loss to filter this substance adequately and, therefore, in the blood plasma accumulation.[25] Furthermore, the rise in creatinine values for the experimental groups to which the aqueous extract was administered suggests the absence of protection at the glomerular level. The results for the pretreatment and treatment groups behaved

equally to those from the positive control group.

Under normal conditions, it is not common to find glucose in the urine, and although freely filtered, it is reabsorbed by active transport in 99.9 %. As a complement, under normal physiological conditions, the glomerulus cannot filter substances with a particle size greater than 1000 Da. For example, most proteins are unable to cross the glomerular capillary. Only those with a low molecular weight are filtered at the tubular level and then returned to the plasma by passive diffusion.[26, 27, 28] These substances were considered biomarkers in the model to determine the functioning of glomerular filtration and tubular reabsorption.

According to the data acquired, glucose levels in urine increased significantly in the experimental groups concerning the negative control ($p < 0.05$) (**Figure 1B**). They behaved similarly to the positive control group. Moreover, the total protein excretion was obtained from the urinary flow for all experimental groups exposed to the extract of the *in vivo* model. For both, a significant increase in EU_{prot} is shown when compared with the group without AKI ($p < 0.05$) (**Figure 1C**), observing a behavior like the group with AKI. The group with the most significant difference in these parameters compared to the negative control was the one that received a pretreatment prior to the AKI induction. Together with $Cl_{creatinine}$, these results suggest an aggravation in glomerular damage in subjects pretreated with the extract.

The loss of the mechanisms of glomerular filtration and tubular reabsorption, with the consequent proteinuria and glycosuria, are characteristics of nephrotoxicity with chromium which the administration of *C. vimineum* did not efficiently reverse. Such parameters suggest a lack of kidney protection from the aqueous extract of the plant.

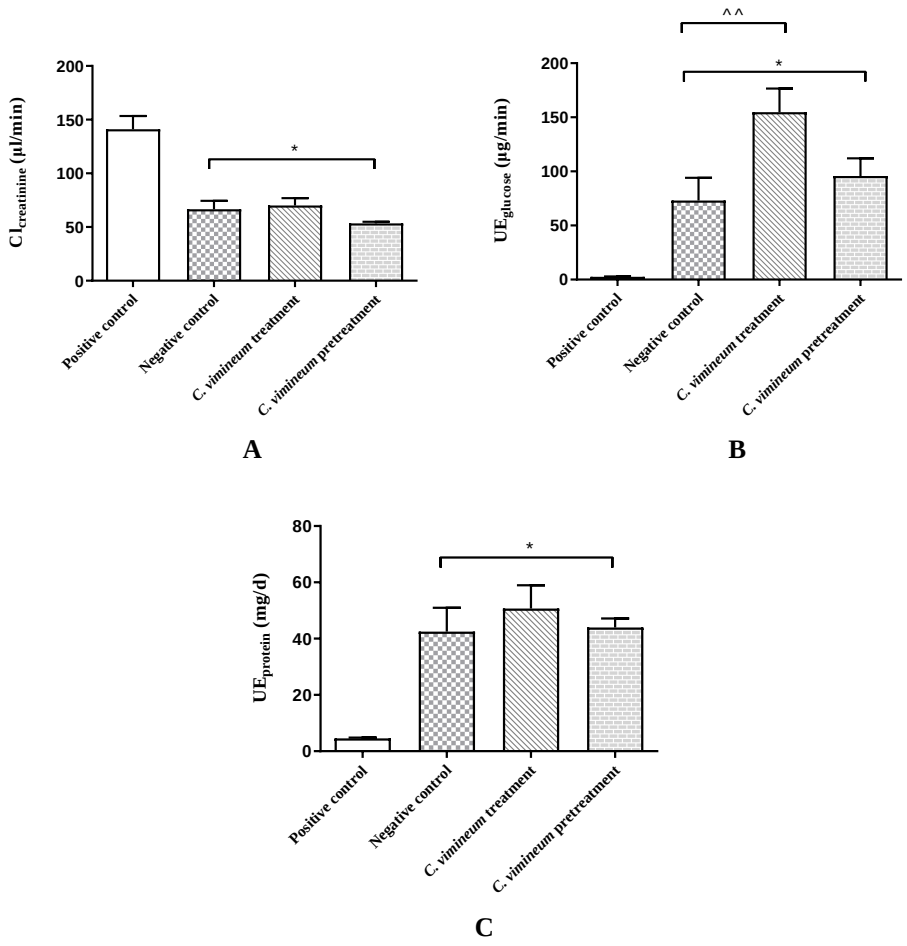


Figure 1: Determination of renal parameters in a potassium chromate model of AKI using female *Wistar* rats. A. Creatinine clearance ($Cl_{creatinine}$). B. Urinary excretion of glucose ($UE_{glucose}$). C. Urinary excretion of proteins. Groups of 5 subjects were treated with potassium chromate for AKI induction. The quantification of $Cn_{creatinine}$ in biological samples was determined by Jaffé's *in vitro* colorimetric method, glucose by GodPad, and protein by Bradford. Data are expressed as the mean plus the standard error of the mean. * $p < 0.05$ regarding the group without AKI (negative control).

On the other hand, sodium and potassium ions are reabsorbed mainly by transporters located in the brush border cells of the renal tubules. Therefore, they serve as indicative parameters of damage at the cellular level of these structures. In some pathophysiological conditions, the damage is generated in the tubular epithelial cells, altering their integrity. This problem is reflected in the mechanisms involved in the reabsorption processes. Therefore, its values are expected to be elevated in urine when there is AKI.[3, 28]

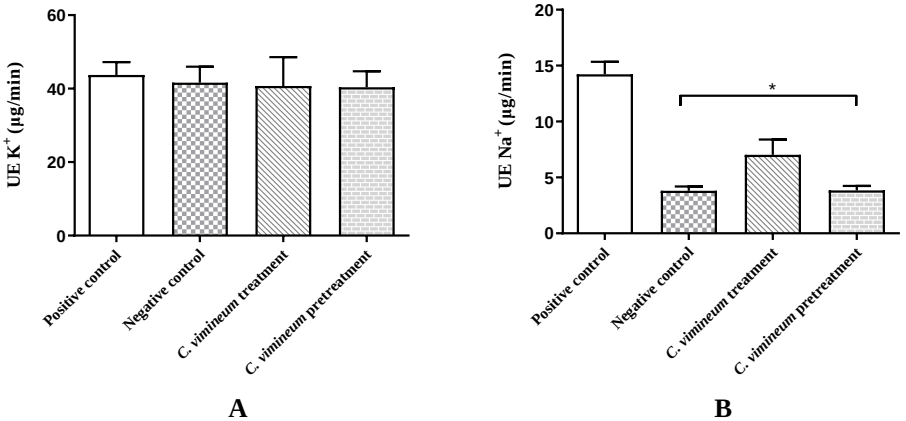
For sodium and potassium quantification, biochemical variables were established from the serum and urine samples (**Table 2**). The electrolyte balance of the diverse experimental groups was evaluated as a renal function parameter. According to the results obtained from the clearance of both electrolytes, there was only a significant reduction in sodium clearance in all experimental groups compared to the negative control ($p < 0.05$). Also, although the values of potassium clearance and fractional excretion of both electrolytes showed differences with the group without AKI, these were not statistically significant.

Table 2: Serum and urinary biochemical parameters to determine electrolyte balance at the end of the *in vivo* model of renal toxicity using an aqueous extract of *C. vimineum*. The data were expressed as the mean and its relative standard error. Concentrations were compared to the negative control, n = 5 animals per group, * $p < 0.05$.

Experimental group	Potassium clearance (µl/min)	Sodium clearance (µl/min)	Fractional excretion of potassium (%)	Fractional excretion of sodium (%)
Negative control	93.96 ± 7.39	2.05 ± 0.16	66.60 ± 3.73	1.46 ± 0.08
Positive control	90.27 ± 9.38	0.53 ± 0.06*	138.40 ± 12.77	0.82 ± 0.08
C. vimineum pretreatment	83.32 ± 8.94	0.56 ± 0.06*	156.40 ± 17.10	1.04 ± 0.11
C. vimineum treatment	96.48 ± 18.62	1.06 ± 0.20*	152.70 ± 48.80	1.67 ± 0.53

Thus, no significant differences were observed in urinary potassium excretion (UE K^+) compared to the group without AKI (**Figure 2A**). In contrast, urinary sodium excretion (UE Na^+) did exhibit a significant reduction among all experimental groups in relation to the negative control ($p < 0.05$).

As shown in **Figure 2B**, when relating the differences between the groups with *C. vimineum* treatment and pretreatment concerning the negative control, the most significant sodium depletion occurred in the AKI group with pretreatment, with an average value of 3.84 + 0.41 µg/min. The treatment group with *C. vimineum* showed an average value closer to the negative control group (7.03 ± 1.36 µg/min). The previous could suggest some protection at the tubular level, but this should be studied further.



A **B**

Figure 2: Urinary electrolyte excretion in a potassium chromate model of AKI using female *Wistar* rats. A. UE Na^+. B. UE K^+. Groups of 5 subjects were treated with potassium chromate for the AKI induction. UE is a parameter that correlates urinary flow with each electrolyte clearance. The sodium and potassium clearance determinations were performed from each electrolyte's serum and urinary concentrations, quantified by the ion-selective electrode potentiometry. Data are expressed as the mean plus the standard error of the mean. * $p < 0.05$ in relation to the group without AKI (negative control).

These data show alterations in sodium elimination in the groups with *C. vimineum* pretreatment and treatment in the presence of AKI. The findings point to a lack of renal protection in the brush border cells located in the renal tubules because of the imbalance in sodium and potassium in the active homeostasis, closely linked to the alteration of transport mechanisms of reabsorption triggered by the nephrotoxic action of chromium.[1, 29]

Histopathological analysis of kidneys

For the histopathological analysis of the four experimental groups (**Figure 3**), the morphological changes at the level of the PCT and DCT, the loss of tissue coloration, and the discovery of cell necrosis are noted.

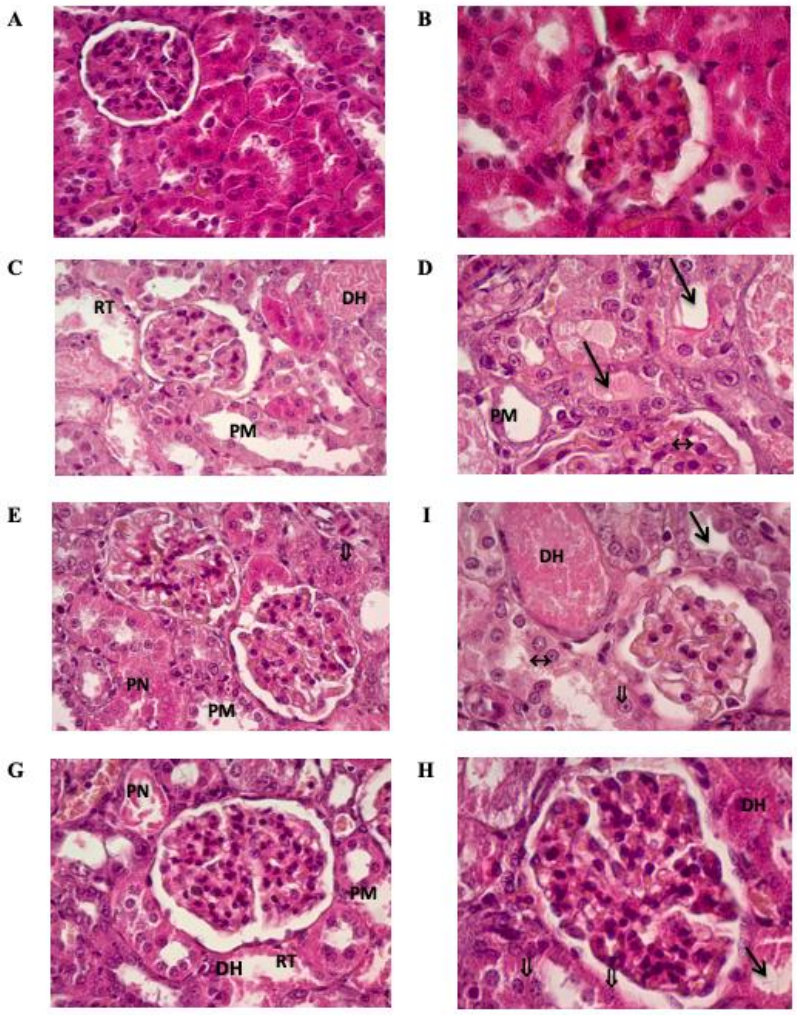


Figure 3: Sagittal section of a kidney extracted from female *Wistar* rats in an *in vivo* model of nephrotoxicity. A and B. Group without AKI. C and D. AKI group without treatment. E and F. AKI group plus treatment with *C. vimineum*. G and H. AKI group plus pretreatment with *C. vimineum*. DH: hyaline deposition. RT: cell size reduction. PM: loss of microvilli in PCT and DCT. PN: loss of cell nuclei. Simple arrow: cellular vacuolized. Down arrow: pyknotic nuclei. Bidirectional arrow: chromatin condensation in cell nuclei. Hematoxylin and eosin staining. Objectives: 40X and 63X.

The above outcomes coincide with a picture of parenchymal AKI. Kidney damage alters the integrity of the renal parenchyma with the irreversible restoration of intraglomerular hemodynamics, inducing acute tubular necrosis (ATN).[3, 30] Besides, in ATN, there is a process of tubular cell desquamation, degeneration, occlusion of the tubular lumen, and tubular injury. This situation could simultaneously lead to apoptosis and necrosis, generating an abrupt cytoskeleton rupture, microvilli loss, and cell polarity. The appreciated histological analysis confirms the results of the biomarkers since readily appreciable alterations were observed in AKI groups. The primary evidence is chromatin retraction of the cell nuclei, pyknotic nuclei presence, partial loss of cell nuclei, cell vacuolization, plasmatic membranes rupture, appreciable change in the expected size of renal epithelium cells, and a high number of hyaline depositions, mainly at the level of the PCT and DCT.[31]

Moreover, no calcification was observed in any experimental group. Such a situation could be justified by the short duration of animal exposure to potassium chromate.[19] It should be noted that *C. vimineum* pretreatment showed a slight improvement in renal morphological changes. Previous administration with the aqueous extract could slightly reduce the condition induced by this substance.

Some studies have associated hyaline depositions with high salts concentrations at the tubular level, which, together with a decrease in pH due to ischemia conditions, causes protein origin residues to precipitate at the tubular level. This scenario originates a certain degree of occlusion with loss of adequate renal function.[1, 32, 33]

In the case of AKI symptoms with ATN manifestation, the first line of treatment suggested is drugs or substances with antioxidant potential to favor the elimination of ischemic and nephrotoxic triggers.[29] In the case of the *C. vimineum* plant, it has polyphenolic compounds associated mainly with phenolic acids and flavonoids. According to *in vitro* and *in vivo* studies, its antioxidant activity has been demonstrated. Several authors have described protective effects on cell injuries, inhibiting tumor growth, activating hepatic detoxification systems, and blocking metabolic pathways associated with carcinogenesis.[34, 35, 36] However, despite the plant's chemical composition, the results for the biomarkers of renal function and the ATN evidences in the histopathological analysis proposed that *C. vimineum* has a reduced capacity to neutralize reactive oxygen species generated in response to the nephrotoxic mechanism of chromium.

Regarding secondary prevention suggested for AKI, some measures favor renal regeneration processes. Among the strategies to limit renal aggression are those that improve hemodynamics and renal perfusion to reverse the nephrotoxic process. The utilization of diuretics in toxic ATN has not shown benefit, and there is controversy regarding its consideration in critically ill patients with AKI. Its mechanism could generate forced diuresis, causing protein accumulation at the tubular level, which in hypoxic conditions precipitates and generates obstructive-type damage.[1, 29, 37] Given that *C. vimineum* has a diuretic potential comparable to other drugs such as hydrochlorothiazide,[7] UE of proteins, glucose, and electrolytes results justified, reinforcing the lack of renal protection of the said plant.

CONCLUSIONS

The *C. vimineum* treatment and pretreatment groups showed an increase in the following renal function biomarkers: serum $Cn_{creatinine}$, urinary glucose excretion, urinary total protein excretion, and fractional excretion of sodium and potassium, in comparison with negative controls. Likewise, there was a reduction in the renal function biomarkers referring to urinary $Cn_{creatinine}$, $Cl_{creatinine}$, sodium clearance, and UE Na^+.

The histopathological analysis of the renal sections for the two groups to which the aqueous extract was administered presented various findings associated with ATN, such as brush border loss at the PCT and DCT, microvilli reduction, hyaline depositions, pyknotic nuclei appearance, chromatin condensation in cell nuclei, cell vacuolization, and cell size reduction.

All these discoveries establish that the aqueous extract of *C. vimineum* does not have a nephroprotective effect in the model of kidney damage induced with potassium chromate in female *Wistar* rats at the dose studied.

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