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GINKGO BILOBA NEPHROPROTECTIVE EFFECTS IN ANIMAL MODELS WITH VANCOMYCIN-INDUCED NEPHROTOXICITY

EFECTE NEFROPROTECTOARE ALE GINGKO BILOBA PE MODELE DE ANIMALE CU NEFROTOXICITATE INDUSĂ DE VANCOMICINĂ

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ABSTRACT | REZUMAT

Ginkgo biloba has been used in traditional Chinese medicine for thousands of years. Clinically, the biological effects of Gingko biloba include the elimination of free radicals, antiapoptotic effect, anti-inflammatory and antioxidant activities. Our study consisted of the evaluation of the nephroprotective effects of Gingko biloba in an experimental model of nephrotoxicity with vancomycin. Eighteen male Wistar rats were divided into 3 groups: CONTROL, VANCO and VANCO+GBI, (6 mice/batch). The compounds were administered daily for 10 days by i.p. Gingko biloba prevented renal impairment which was demonstrated by the urinary N-acetyl-β-D-glucosaminidase activity index and Cystatin C. The significant decrease of the biochemical parameters in the VANCO+GBI group compared to the VANCO group, demonstrates the nephroprotective effect of GBI, this being in accordance with other scientific studies.

Keywords: Gingko biloba, vancomycin, acute kidney injury, nephroprotection

Ginkgo biloba este folosit în medicina tradițională chineză de mii de ani. Din punct de vedere clinic efectele biologice ale Ginkgo biloba includ eliminarea radicalilor liberi, efect antiapoptotic activitate antiinflamatorie și antioxidantă. Studiul nostru a constat în evaluarea efectelor nefroprotectoare ale Gingko biloba într-un model experimental de nefrotoxicitate cu vancomicină. Optsprezece șobolani masculi Wistar au fost împărțiți în 3 loturi: CONTROL, VANCO și VANCO+GBI, (6 șoareci/lot). Compușii au fost administrați zilnic timp de 10 zile prin i.p. Ginkgo biloba a prevenit insuficiența renală, care a fost demonstrată de indicele de activitate N-acetil-β-D-glucozaminidază urinară și de Cistatina C. Scăderea semnificativă a parametrilor biochimici în grupul VANCO+GBI în comparație cu grupul VANCO, demonstrează efectul nefroprotector al GBI, acest lucru fiind în concordanță cu alte studii științifice.

Cuvinte cheie: Gingko biloba, vancomicină, injurie acută renală, nefroprotecție

Acute kidney injury (AKI) can resolve completely or cause chronic kidney damage. The degree of kidney damage is influenced by the onset of high blood pressure, local inflammation and ongoing nephrotoxic medication (6).

Vancomycin (VAN) is a commonly used tricyclic glycopeptide antibiotic, which is considered the antibiotic of choice primarily for infections involving methicillin-resistant *Staphylococcus aureus* (MRSA). In the past, clinicians were reluctant to prescribe VAN due to the product's impurities, with many doctors calling it "mud from Mississippi." These impurities have been associated with adverse effects, including AKI. Although current preparations purified by high performance liquid chromatography are 90-95% pure, even today, generic VAN formulations present an increased risk of AKI (7). Unlike serum creatinine, new markers of kidney damage may provide insight into the location, severity, and aetiology of the lesion (16). Recently, several biomarkers have been discovered that can detect tubular lesions early, and their elevated values should indicate proximal tubular lesions in the case of N-acetyl-β-D glycosaminidase (urinary NAG) and cystatin C (Cys C) (13).

Ginkgo biloba (GBI) has been used in traditional Chinese medicine for thousands of years, and clinically, it was originally prescribed for the treatment of Alzheimer's disease and cognitive deficits. GBI contains on average 27% polyphenols isorhamnetin, kaempferol quercetin and about 6% lactone terpenes (ginkgolides A, B, C, J and M), and bilobalide (4). Most studies often use standardized GBI extract 76 (10).

Thus, this article presents an effective protocol for inducing AKI on Winstar rat models using VAN, and after installation the neuroprotective effects of GBI were evaluated by measuring kidney function (urea, creati-

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nine) and the use of new urinary biomarkers in clinical trials Cys C, urinary NAG, and urinary NAG index.

MATERIALS AND METHODS

The substances used in the study are VAN (Vancomycin Kabi® 500 mg, powder for concentrate for intravenous solution) which was obtained from FRESENIUS KABI Romania; Tramadol (Tramadol® 50 mg/ml, solution for injection) supplied by KRKA Romania; xylazine (Xylazin Bio® 100 mg/ml, solution for injection) obtained from Bioveta; saline (0.9% NaCl, infusion solution) obtained from Braun Romania; and isoflurane (Anesteran® liquid for inhalation vapors) provided by Rompharm Romania.

Eighteen adults male Wistar rats, clinically healthy, with a body weight of 300±50g where used. The safety and experimental procedures were in accordance with Directive 2010/63/EU and the national legislation (law 43/2014). Also, the experimental procedures were approved within the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca (USAMV CN) by the Bioethics Commission.

The animals were kept in adequate zoo hygienic conditions, within the Biobase within the Faculty of Veterinary Medicine of Cluj-Napoca. The rats were housed in cages made of polypropylene, where an optimal space / animal head was ensured, with environmental improvements ("enviromental enrichment"), namely the introduction into the accommodation of various objects, plastic tubes, burrows from wood and plastic, etc. The environmental parameters were: humidity 40-60%, temperature: 22±1°C, artificial 12/12 hours light -dark cycle, standardized food quantity (purchased from the Cantacuzino-Bucharest Institute) and drinking water from the public network "*ad libitum* ".

An automatic haematology analyser Abacus Junior Vet (Diatron Messtechnik®, Hungary) was used to perform the complete blood count. Serum urea and serum creatinine were measured for kidney function using the spectrophotometric kinetic method and the Jaffe reaction, in the case of serum creatinine, by means of the spectrophotometer (Screen Master Touch, Hospitex Diagnostics®, Italy). The unit of measurement of the obtained values was expressed in mg/dl.

Serum calcium and phosphorus dosing was measured using the FRESENIUS® 3-EHF ionometer and the unit of measurement was mg/dl.

Cys C was determined using the enzyme-linked immunosorbent assay (ELISA), and this was determined using a commercial ELISA kit (R&D Systems, MN, USA) according to the manufacturer's instructions. The principle of the method consists in immobilizing the proteins on a solid support, by capturing it by a specific antibody, and subsequently adding a detection antibody bound to an enzyme, thus forming the „sandwich" reaction. The reaction that is obtained produces a detectable signal, most often a color change the results obtained was expressed in pg/ml.

The spectrophotometer (Chema Diagnostica®, Italy), calibrated at a wavelength of 515 nm, was used to determine the urinary concentration of creatinine.

Urinary creatinine was determined using a commercial kit with two reagents: Reagent 1: Picric Acid and Reagent 2: Sodium Hydroxide and Disodium Phosphate. This was determined by a colorimetric reaction, the method based on the Jaffe reaction, and consists in quantifying the rate of formation of the colour complex between creatinine and alkaline picrate, at 37 ºC. The results obtained were expressed in g/l.

The determination of the urinary enzymatic activity of NAG was performed using a spectrophotometer (Screen Master Touch, Hospitex Diagnostics®, Italy) set at a wavelength of 505 nm. The analysis was performed at 37 °C, using a commercially available kit (Diazyme Lab, CA, USA). The commercial kit contains three reagents: Reagent 1: MNP-G1cNAc and HCl, Reagent 2: Citric Acid and Potassium Phosphate, and Reagent 3: Sodium Carbonate (buffer solution). The principle of the method is based on the ability of NAG to hydrolyse 2-metoxy-4- (2'nitrovinyl) -phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (MNP-GlcNAc) to 2 methoxy-4- (2 'nitro vinyl) -phenol. The unit of measurement used for urinary NAG was expressed in U/l .

The rats were divided into 3 experimental lots, 6 random individuals per lot. Our study started on 24.03.2020 and took place over a period of 10 days. The treatment and the route of administration are shown in Table 1.

As oral administration of the substances is a stressful operation for rodents, sedation by intramuscular injection with Xylazine (Xylazin Bio® 100 mg/ml) 5 mg/kg was performed to facilitate oral administration

Table 1

Batches of rats

Name	Treatment	Route of administration
CONTROL	1mL NaCl 0.9%	Per os, through gavage
VANCO	200mg/kg/day	Subcutaneous
VANCO+GBI	VAN 200mg/kg/day GBI (Tanakan® 40 mg/ml) 100 mg/kg/day.	Subcutaneous Through gavage

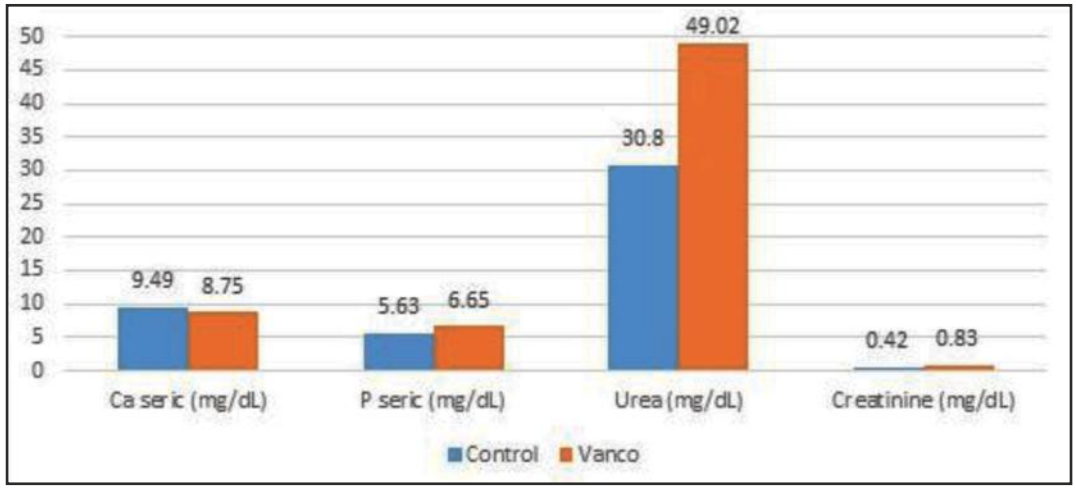

Fig. 1. Usual blood and urinary biochemical parameters from the 11th day of the study (lot average values), from the CONTROL and VANCO lots

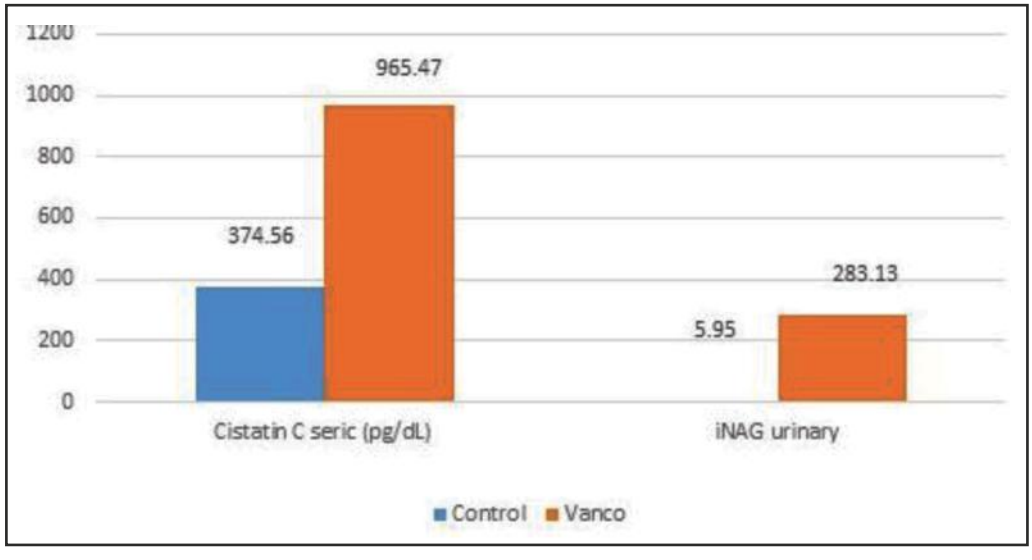

Fig. 2. Blood and urinary biochemical parameters from the 11th day of the study (lot average values), from the CONTROL, VANCO lots

and reduce stress. Measures have also been taken to limit the suffering of rats by analgesia, namely subcutaneous administration of Tramadol (Tramadol® 50 mg/ml) 20 mg/kg/day.

In the 11th day, the last day of study, all rodents were euthanized by prolonged narcosis with Isoflurane (Anesteran®) using the anaesthesia chamber, followed by biological sampling and incineration of the corpses in the USAMV crematorium.

In this study, biological samples were collected from blood and urine. Before being euthanized, whole blood samples were taken from the infraorbital sinus, in tubes with EDTA, from which a complete haematological examination was performed. Whole blood samples were also collected in heparinized tubes, which were processed by centrifugation (3000/rpm for 5 minutes at 4ºC) to obtain the plasma needed to dose creatinine, urea Cys C and electrolytes dosing, represented by serum Ca and P. Serum urea concentration was measured using a kinetic spectrophotometric method, and serum creatinine concentration was measured using a kinetic spectrophotometric method, based on the Jaffe reaction. Cys C was measured by a colorimetric, quantitative method.

After the rats were euthanized, urine samples were taken by cystocentesis, following laparotomy. These were collected in sterile Eppendorf tubes. The tubes were centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant was used for the determination of creatinine and urinary NAG and the subsequent determination of NAG index activity. Thus, urinary creatinine concentration was determined by a kinetic spectrophotometric reaction using the Jaffe method and NAG was measured using an end point spectrophoto-

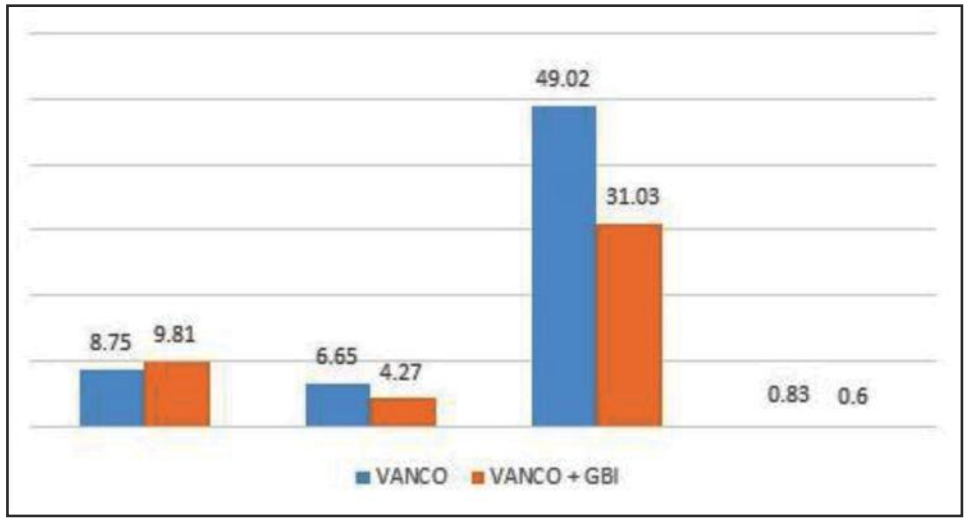

Fig. 3. Blood and urinary biochemical parameters from the 11th day of the study (lot average values), within the VANCO, VANCO+GBI

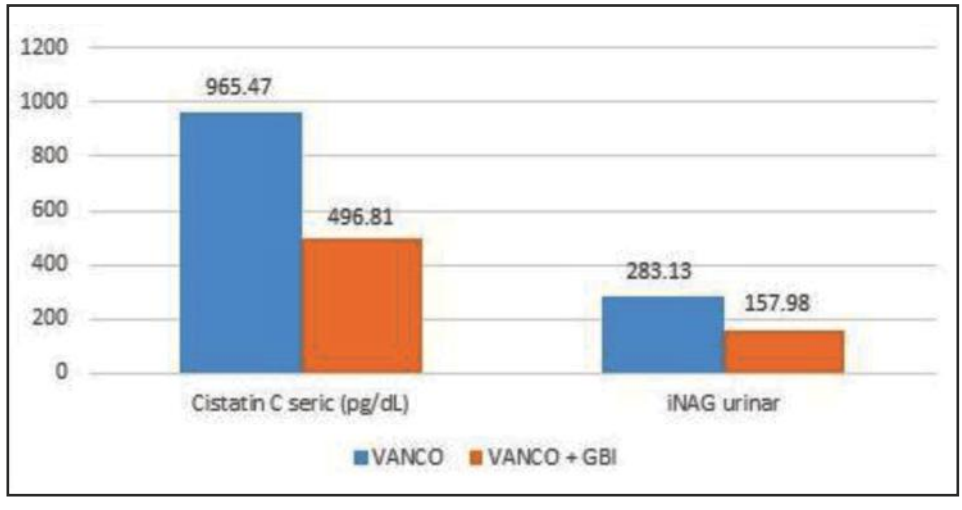

Fig. 4. Blood and urinary biochemical parameters from the 11th day of the study (lot average values), within the VANCO, VANCO+GBI

metric reaction. Urinary NAG index (urinary iNAG) was calculated based on the mathematical formula NAG (U/L)/ Creatinine (g/l) (3). The determinations were made less than six hours after the sampling.

The results of the biological parameters were processed with the Excel program Microsoft Office 2019, where the arithmetic mean was used. The batches (VANCO and VANCO+GBI) were compared with the control lot and between them for each biological parameter taken into account.

RESULTS AND DISCUSSION

All rats subjected to VAN toxicity tests remained alive until the end of the experiment. The body weight of the treated rats did not decrease significantly compared to the control lot. The results that were obtained at the end of the experiment are represented by haematological and biochemical parameters. The haematological parameters of the haemoleukogram were within normal limits in all lots, similar to those found in the control lot. Commonly used biochemical markers can be seen in Fig. 1, where it was shown that representative of the diagnosis of AKI is the serum concentration of urea and creatinine. The urea concentration, mediated on the whole VANCO lot, dosed from the blood, showed an increase of 1.59 times compared to the average urea concentration of the CONTROL lot, and the serum creatinine, shows an increase of 1.97 in the VANCO lot compared to the CONTROL lot.

The concentration of serum Ca and serum P did not show a statistically significant variation for the two lot. So, we can summarize that from the usual biochemical markers, those that can certify the presence of

AKI are urea and creatinine. These two biomarkers are extremely sensitive to changes in the balance of kidney function. By comparing these lots, the aim was to obtain an experimental model of acute kidney injury, induced by the administration of VAN.

Rats in the CONTROL lot were given 0.9% oral NaCl. The variation of the parameters within this lot p is small, the lot being homogeneous. The rats in the VANCO lot received vancomycin.

The new generation biomarkers used for the early diagnosis of AKI are represented in Fig. 2. Serum-mediated Cys C on the whole VANCO group showed an increase of 2.57 times compared to the CONTROL group, and iNAG on the whole VANCO group showed an increase of 47.58 compared to the average concentration in the CONTROL group.

The usual biomarkers used for AKI prediction are represented in Fig. 3, where we notice that the urea value decreased by 1.57 times and creatinine by 1.38 times in the VANCO+GBI group. Serum Ca levels increased significantly in the VANCO+GBI group compared to the VANCO group. In contrast, serum phosphorus activity was significantly lower in the VANCO +GBI group compared to the VANCO group.

Lot III, VANCO+GBI, consisted of 6 rats that received VANCO and GBI. The values of the new biomarkers, from group II (VANCO), group III (VANCO+ GBI) are shown in Fig. 4, where the nephroprotective effect of GBI was compared.

Cys C and urinary iNAG values in the VANCO and VANCO+GBI group where Cys C decreased almost 1.94 times in the VANCO+GBI group compared to the VANCO group. INAG levels were significantly lower in the VANCO+GBI group compared to the VANCO group, decreasing 1.79 times. This shows that the GBI extract had a nephroprotective effect.

The VANCO+GBI group received vancomycin and GBI standardized extract, Tanakan® product.

Serum calcium levels were significantly increased in the VANCO+GBI lot compared to VANCO lot and serum phosphorus levels, on the other hand, were significantly lower in the VANCO+GBI lot compared to the VANCO lot. The values of the biochemical parameters in the VANCO group were significantly increased compared to the values in the CONTROL group where the urinary iNAG increased approximately 47 times after VAN administration and is consistent with other experimental studies where there were increases in urinary iNAG. They have been reported in dogs with various conditions such as CKD, leishmaniasis and pyometra, as well as dogs treated with aminoglycosides, various antibiotics, glucocorticoids and some nonsteroidal anti-inflammatory drugs (5), NAG/creatinine index (iNAG) representing the ratio of NAG urinary and urinary creatinine. The concentrations of the two urinary markers are compared, which ensures good urinary excretion within 24 hours. This comparison is used to avoid influencing the specific urinary weight (5). This significant increase may support the hypothesis that iNAG is a specific marker of kidney distress, namely tubular kidney distress, which has the ability to change its values early compared to traditional markers, urea and creatinine. Also, by this sudden increase of the iNAG, the strong damage of the proximal convoluted tubes is noticed, due to the nephrotoxicity of the VAN, thus demonstrating the obtaining of an efficient and predictable experimental model of AKI.

Cys C recorded increased values after the administration of NPV and by this significant increase we can say that it is a specific marker of renal distress, which changes its values early, compared to urea and creatinine (2). In children and adolescents, Cys C has a more accurate estimate of eGFR than creatinine clearance, and the diagnosis of CKD is more accurate, so Cys C may be more effective in detecting acute changes in eGFR, and new markers used may be used more precisely to replace creatinine. Clearance (8).

VAN nephrotoxicity varies depending on the number of nephrotoxic drugs administered or if the patient is older, the minimum concentration of 15 μg/ml, has an incidence of 8.1% and 16.3% for concomitant piperacillin-tazobactam therapy.

In vitro experimental *studies* have shown the pathophysiological principles underlying VAN nephrotoxicity, demonstrating that it is based on inducing mitochondrial membrane depolarization with the production of mitochondrial reactive oxygen species and peroxidation of mitochondrial cardiolipin phospholipid in the proximal tube (11). Studies indicating the molecular mechanisms of VAN nephrotoxicity have shown that induced kidney tubular lesions have been ameliorated by the use of antioxidants, however, researchers have not yet reached a final consensus. Thus, the development of strategies to avoid this unwanted side effect is of the utmost importance, however (12). The levels of biochemical parameters decreased significantly in the VANCO+GBI lot, following the administration of GBI extract, so these data could be compared with data from the literature where Akdere et al. (2014) demonstrated the nephroprotective effects of GBI extract against kidney ischemia-reper-fusion lesions in rats, GBI being administered for 14 days and 2 hours before causing kidney ischemic lesions. Differences in malonaldehyde were observed between Control group and the GBI group (1).

Also, Sherif et al. (2019) obtained an AKI model using Methotrexate, where there was a significant increase in serum creatinine of 140% and BUN urea by 168.8% compared to the control group, and the group in which GBI was administered showed a marked decrease in serum creatinine (40%) and urea BUN

(46%) compared to the group treated with Methotrexate ($p < 0.05$), thus confirming its nephroprotective effect (14).The biological effects of GBI extract include the elimination of free radicals, antiapoptotic, anti-inflammatory and antioxidant activities (15), effects previously demonstrated in various experimental studies, that may represent a treatment option in AKI, induced by Methotrexate (14), or Gentamicin (9).

GBI treatment in our study significantly improved kidney function caused by VAN by measuring kidney biomarkers, showing the presence of a high degree of nephroprotection against drug nephrotoxicity, however in future studies remains to study the mechanism of action of GBI. The advantages of the present study are the encouragement of the use of phytomedicines as adjuvant therapy in the treatment of nephropathy, having a nephroprotective effect, and the replacement of non-specific markers of kidney distress with specific markers to detect early kidney damage. As for disadvantages of this study, we can say that the small number of rats used, the short duration of the experiment, 10 days.

CONCLUSIONS

We can conclude that AKI, following the nephrotoxic effect induced by VAN, was successfully installed in this experimental model. Cys C and iNAG recorded significantly higher values in the VANCO group compared to the CONTROL group. The low values of Cys C and iNAG in the VANCO+GBI lot thus demonstrate the ability to change their values in case of nephrotoxicity and nephroprotection, this biomarker being a specific marker of kidney tubular distress compared to urea and creatinine. The significant decrease of the biochemical parameters in the VANCO+GBI group compared to the VANCO group, demonstrates the nephroprotective effect of GBI, this being in accordance with other scientific studies.

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