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Original Article

EFFECT OF METHANOLIC FRACTION OF *KALANCHOE CRENATA* ON RENAL MORPHOPHYSIOLOGY IN ADRIAMYCIN-INDUCED IMPAIRED KIDNEY IN RATS

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ABSTRACT

Objectives: The effect of methanolic extract of *Kalanchoe crenata* (MEKC) was investigated on renal morphology and function in adriamycin-induced kidney impairment in rats.

Methods: Ether anesthetized rats received three intravenous injections (days 0, 14, 28) of 2 mg/kg body weight of adriamycin. Repeated doses of the extract (0, 50 and 68 mg/kg bw) and losartan (10 mg/kg bw) were administered orally once daily, for 6 weeks, to adriamycin- nephropathic rats. Kidney functions were assessed through proteinuria, creatinemia and creatinuria, renal malondialdehyde (MDA) level, superoxide dismutase (SOD) activity and morphology analyses.

Results: The 50 and 68 mg/kg MEKC, as the losartan, decreased proteinuria: -63.74 % and -64.94 % respectively, significantly ($P<0.01$) increased the creatinuria and the creatinuria/creatinemia ratio, and also decreased the creatinemia in diseased rats. The plant extracts markedly ($P<0.05$) increased plasma sodium, and decreased ($P<0.01$) the urinary sodium and potassium levels. The MEKC has remarkably ($P<0.01$) decreased the level of the thiobarbituric acid reactive substances and increased the SOD level in nephropathic rats. The extract has improved the damage of kidney induced by adriamycin.

Conclusion: The results indicate that the treatment with the *K. crenata* methanolic extract may improve proteinuria and all the symptoms that breed from nephropathy, and could improve kidney morphology. Therefore, *K. crenata* could be promising for the development of a standardized phytomedicine for the treatment of kidney disease.

Keywords: Adriamycin, *Kalanchoe crenata*, Nephropathy, Antioxidant, Rat, Methanol extract.

INTRODUCTION

Chronic kidney disease is a worldwide global public health problem, and is characterized by glomerulosclerosis and tubulointerstitial fibrosis which are the final common pathways of progression [1,2]. Its prevalence is increasing as the result of an increased prevalence of diabetes, arterial hypertension and drugs toxicity [3,4]. Most chronic renal diseases are characterized by increased glomerular matrix accumulation and collapse of the capillary lumina. The structural alterations in the glomerulus are accompanied by sustained proteinuria, often of nephrotic range. Tubular atrophy and dilatation, interstitial inflammatory infiltrates, accumulation of interstitial fibroblasts, and increased matrix deposition are associated with progressive glomerulosclerosis which ultimately lead to interstitial fibrosis and loss of renal function [1,5].

The interstitial recruitment of macrophages and lymphocytes as a major source of inflammatory and profibrotic mediators plays an important role in chronic interstitial inflammation and fibrosis [2]. Therapeutic strategies to prevent or delay loss of organ function in chronic renal disease therefore target pathways in the fibrotic tissue remodeling. Conventional treatment of kidney diseases includes oral Enzyme conversion inhibitors (ECI) like losartan. In areas such as developing countries where safe modern drugs and health centres are lacked, the World Health Organisation (WHO) has suggested indigenous plants to be used as alternative medicine [6]. Alternative therapies could include plant products. Approximatively 80 % of rural African communities still use phytotherapy to control or treat many diseases. *Kalanchoe crenata* (Crassulaceae) is an herbaceous plant used in Cameroon Western Region, as anti-inflammatory and antidiabeticdrug [7,8]. The increasing morbidity and mortality of the chronic kidney disease have become an important research field and Adriamycin-induced nephropathy is the most prototypical and commonly used experimental model of this disease [2,5,9,10]. The adriamycin in nephropathy toxicity study in Wistar rats induced proteinuria, increased blood creatinine, decreased creatinuria [2, 11]. The present work was undertaken with the aim of assessing the effect of the methanolic fraction from methanol extract of *Kalanchoe crenata* on adriamycin-induced nephropathic disorder in rats.

MATERIALS AND METHODS

Plant extract

The whole plant of *Kalanchoe crenata* was collected from Batié (west Region of Cameroon) in January and March 2012, and was identified by the National Herbarium of Yaounde (Cameroon) were the voucher specimen (50103/YA) was kept. The whole plant was cleaned, shade-dried and powdered. The powder of *K. crenata* (2 kg) was macerated in 10 L of methanol for 72 h at room temperature. Removal of the solvent from the extract under reduced pressure yielded 113.6 g (5.68 %) of a dark green residue. This residue was put in the n-hexane to remove its hydro insoluble compounds. The final residue (not soluble in hexane) obtained after drying constituted the methanol extract of *K. crenata* (MEKC). The yield of the extract was 41.8 g (2.09 %). Prior to the administration to animals, the extract was solubilized in distilled water, with the volume of administration < 1 mL for each experimental animal.

Preliminary phytochemical tests

Phytochemical properties of the methanolic fraction of *Kalanchoe crenata* were tested by the standard methods described by Sofowora, using various reagents [12]: Mayer and Dragendoff's reagents for alkaloids; $FeCl_3$ for tannin; frothing test for saponin; magnesium turning and HCl for flavonoids; NaCl and Fehling's solutions for glycoside; diethyl ether, sulphuric acid and anhydride acetic for steroids; ether-chloroform and NaOH for anthraquinones and $FeCl_3$ and $K_3Fe(CN)_6$ for phenols and polyphenols.

Acute toxicity evaluation

The MEKC was tested for its acute toxicity in mice. Five groups of six mice each were orally administered one of the different doses of the extract: 0 (control group), 2, 4, 6, 8, 10 g/kg body weight. The animals were observed continuously for initial 2 h, intermittently for the next 6 h and then at 24 h and 48 h following drug administration for death and overt behavior: lethargy, jerkiness, sensitiveness to noise and touch, and respiratory rate. The lethal dose 50 (LD_{50}) was determined with the formulae [13].

$$DL_{50} = Xs - d(\Sigma p - ½)$$

Xs = lethal dose for 100% of mice; d = interval between the doses

p = proportion of death per group; Σ p = sum of death proportions

Induction of kidney disease

Male wistar albino rats (200-250 g) raised in the animal house of the Faculty of Science of the University of Yaoundé I, were maintained under natural laboratory conditions (temperature, and dark/light cycle) and allowed access to food and water *ad libitum*. Animal housing and experiments in vivo were done according to the guidelines of the European Union directive on Ethical Evaluation of Animal Experiments (CEE Council 86/609)[14] and approved by the Institutional Committee of the Ministry of Scientific Research and Innovation of Cameroon.

To induce renal impairment, the rats anesthetized with ether received three intravenous (penile vein) injections (days 0, 14, 28) of 2 mg/kg body weight Adriamycin (2 mg/mL doxorubicin hydrochloride: Pharmacia Italia, S. P. A., Italy) in 9 % NaCl [11, 15], when the Control group received normal saline. Rats with proteinuria >3 g/L, creatinemia >57 μmol/L and creatinuria < 10 mmol/L were used in our experiments as nephropathy rats.

Animal treatment

In the experiment, 5 normal and 20 nephropathic rats were used. The nephropathic rats were randomly divided into four groups of 5 animals each: one group of nephropathic control rats (NeC) received, as normal control rats (NC), distilled water (5 mL/kg); two groups received 50 mg/kg bw (NeK50) and 68 mg/kg bw (NeK68) *Kalanchoe crenata* extract respectively. The last group (positive control: NeL10) received 10 mg/kg bw losartan (losartan + hydrochlorothiazide, HYZAAR, Merck Sharp &Dohmet-Chibret, MSD, Paris). The extract doses were obtained from the tradipractitioner method. The drugs were orally administered daily for 6 weeks. The effect of the extract on the renal impairment was evaluated from blood and urinary Na^+, K^+, protein and creatinine, renal thiobarbituric acid-reactive substances (TBARS i. e. malondialdehyde MDA), superoxide dismutase (SOD), and morphology.

Serum, urine and homogenate samples

Urine and blood samples were obtained from each rat at day 0 and at 2 weeks interval thereafter until week 12. After six weeks of treatment, the rats were housed individually in the metabolic cage for 24 hours for urine collect. The xylol was put in the collection container to prevent the evaporation. The rats were fasted for 24 h, spot urine samples were collected for protein, creatinine, sodium and potassium level estimation. The biochemical assays were performed within the 24 hours after the collection.

The rats were then ether anesthetized, sacrificed and blood samples collected into normal tubes and were allowed to clot at room temperature. Serum was separated by centrifugation (3000 tr/min at 30ºC, 10 min), aliquoted and kept frozen at -20°C for the estimation of creatinine, sodium, potassium levels. The kidneys were removed, weighed and placed immediately in an ice-cold buffer (0.25 M sucrose, 10 mM Tris and 0.3 mM EDTA; pH7.4) and washed until bloodless. The organ was homogenized using a teflon homogenizer. Homogenate was centrifuged for 15 min at 15.000 rpm [14]. The whole supernatant was removed, aliquoted and frozen at -20°C pending subsequent tests.

Serum and urine analysis

Creatinine level was analyzed in serum using commercial diagnostic kits (Elitect Laboratories, SEPPIMS A. France). Sodium and potassium were analysed in blood and urine by a selective electrode ion auto-analyser (ILLYTE). Urinary creatinine was estimated spectrophotometrically with commercially available kits (Biodirect and Elitech). Assays with kits were carried on according to the manufacturers' recommendations. Urinary protein was quantified by precipitation turbidimetric test of protein in trichloroacetic acid (TCA 12 %) or sulfosalicylic acid [13].

Homogenate analysis

TBARS level (MDA activity)

To estimate TBARS, 0.4 mL of homogenate was added to 2 mL of glacial acetic in a test tube. To this mixture was added 2 mL of 1 % thiobarbituric acid in 0.5 M NaOH. The loosely stoppered tubes were immersed in boiling water bath for 1 h. The tubes were then cooled under running tap water and absorption measured at 532 nm (JENWAY Spectrophotometer, Barloworld Scientific U. K.) against MDA reactive [16].

SOD activity

To assay the SOD activity, 0.2 mL of homogenate was added to 2.5 mL of sodium carbonate 0.05 M pH10.2. The reaction began when 0.3 mL adrenaline was added. The reaction mixture was stirred vigorously and absorption measured at 480 nm against the blank (Sodium carbonate + adrenaline + distilled water). The specific activity of SOD was expressed as number of units/mg protein. A unit is the quantity of SOD that inhibits 50 % adrenaline oxidation per min [17].

Histology study

After dissection, the kidney sections were fixed in 10 % buffered formalin solution. The processed tissues were then embedded in paraffin, 5 μm thickness sections were stained with haematoxylin and eosin for microscopic assessment.

Statistical analysis

Data were expressed as mean ± SEM. The parameters were analysed statistically using one way ANOVA with Graph Pad Instat software 3.6, followed by Dunnett's multiple-comparison test. P values less than 0.05 were considered as statistically significant.

RESULTS

The phytochemical analysis revealed the presence of different classes of compounds such as tannins, phenols, sterol, anthraquinones, triterpens, phobotanins and polyphenols in the methanol extract of *Kalanchoe crenata* (MEKC).

In acute toxicity test on the MEKC, the doses 4, 6, 8 and 10 g/kg reduced the sensitiveness to noise and touch, jerkiness, lethargy, and caused soft faeces and 66 % mice death within 30 min of administration. The dose 10 g/kg caused 100 % mice death. The lethal dose 50 (LD_{50}) was 4.4 g/kg. There were no gross behavioural changes. Macroscopically, the organs (liver, kidney, heart) did not show any discoloration.

During the experiment, 6 weeks after the treatment of the rats, the adriamycin induced significant increase ($P<0.01$) of creatinemia, urinary volume, proteinuria, when the creatinuria/creatinemia ratio and creatinuria significantly ($P<0.01$) decreased (Table 1).

The serum level of sodium and the urinary level of potassium also significantly ($P< 0.01$) decreased while blood potassium, urinary sodium significantly ($P< 0.01$) increased (Fig. 1).

The MDA concentration in renal tissue was significantly raised (fig. 2A) when the SOD (fig. 3B) decreased in a significant way ($P<0.01$). The pathological assessment exhibited a significant change and damage of kidney in nephropathic control rat. In most glomeruli the expansion of the mesangium was observed, with disappearance of urinary space. Glomerular pathology was accompanied by prominent tubule interstitial changes, including tubular inflammation (fig. 3B).

Table 1: Blood and urinary biochemical parameters of adriamycin-induced kidney dysfunction after 6 weeks daily treatment with *Kalanchoe crenata* methanolic fraction or losartan

Treatment	Proteinuria(g/L)	Creatinuria (µmol/L)	Creatinemia (µmol/L)	Creatinuria/creatinemia	Urinary volume (mL/24 h)
NC	1.86 ± 0.09	11.3 ± 0.81	56.99 ± 1.23	0.19 ± 0.65	13.05± 0.13
NeC	5.02 ± 0.24**	3.28 ± 0.02**	91.82 ± 1.28**	0.03±0.02**	15.50± 0.43**
NeK50	1.82 ± 0.14^{b}	8.31 ± 0.60^{b*}	58.37 ± 1.50^{b}	0.99± 0.38^{b}	8.44± 0.51^{b*}
NeK68	1.76 ± 0.01^{b}	8.42 ± 1.04^{b*}	60.95 ± 1.8^{b*}	0.95 ±0.57^{b}	8.70± 0.40^{b*}
NeL10	1.23 ± 0.16^{b}	9.75 ± 0.35^{b}	63.68 ± 1.45^{b}	1.25 ± 0.24^{b}	9.45± 0.25^{b*}

Normal (NC) and nephropathy (NeC) control rats. Nephropathy rats treated with 50 mg/kg bw (NeK50), 68 mg/kg bw (NeK68) and with losartan 10 mg/kg bw (NeL10). Data are means ± SEM, n = 5. Significant difference: *P< 0.05 and **P< 0.01 compared with NC values; ^{a}P< 0.05 and ^{b}P< 0.01 compared with NeC values.

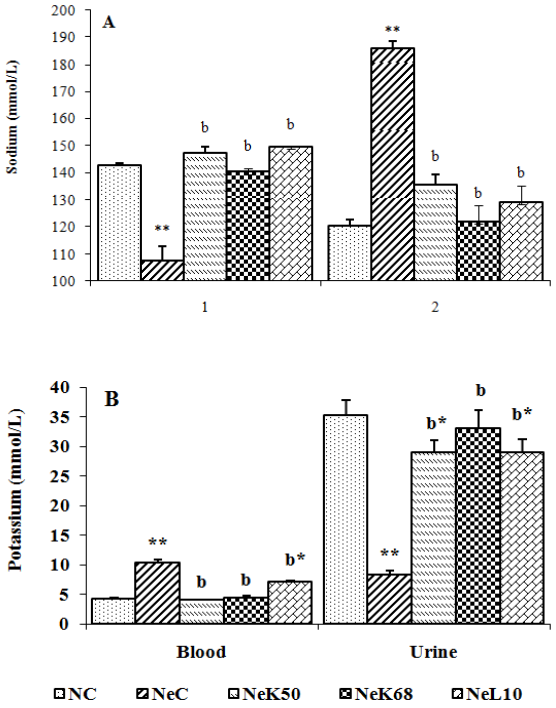


Fig. 1: Blood and urine sodium (A) and potassium (B) of adriamycin-induced nephropathic rats after 6 week daily treatment with *K crenata* methanolic extract 50 mg/kg bw (NeK50), 68 mg/kg bw (NeK68) or losartan 10 mg/kg bw (NeL10). Normal (NC) and nephropathy (NeC) control rats treated with 5 mL/kg bw distilled water. Data are means ± SEM, n = 5. Significance difference: *P< 0.05 and **P< 0.01 compared with NC values; ^{a}P< 0.05 and ^{b}P< 0.01 compared with NeC values

The *Kalanchoe crenata* methanolic extract (MEKC) 50 and 68 mg/kg bw, and the 10 mg/kg bw losartan, after 6 weeks treatment, significantly (P< 0.01) reduced the blood creatinine and the urinary volume and proteinuria in nephropathy rats. The Creatinuria/creatinemia ratio and the creatinuria were enhanced (P< 0.01) by these different drugs (table 1). The MEKC and the losartan have markedly (P< 0.01) raised the blood sodium, the urinary potassium and decreased the blood potassium and the urinary sodium levels in nephropathic rats (fig. 1).

K. crenata extract and losartan, after 6 weeks daily administration, markedly (P< 0.01) reduced the renal MDA level and significantly (P <0.01) raised the SOD activities. The extract and losartan improved the morphology of kidney in nephropathy rats (fig. 3). The treatment reduced the tubular inflammation, the expansion of the mesangium and improved the urinary space in nephropathic rats.

DISCUSSION

The main aim of this work was to assess the effect of *Kalanchoe crenata* methanol extract (MEKC), an anti-inflammatory and antihyperglycemic [7] plant on the renal function through the hydroelectrolytic balance (sodium, potassium), the proteinuria, creatinemia, creatinuria and on the renal morphology through the activities of MDA, SOD and the histology in Adriamycin-induced nephropathy in rats.

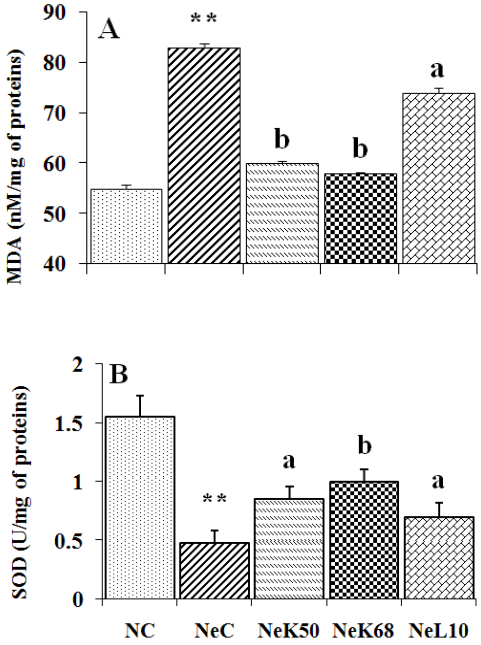
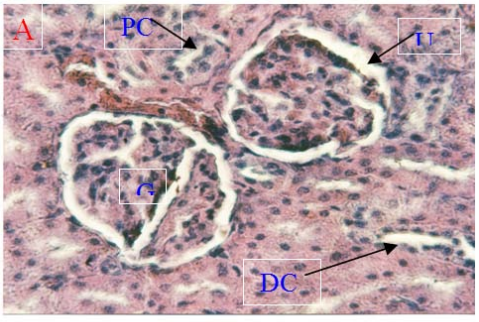
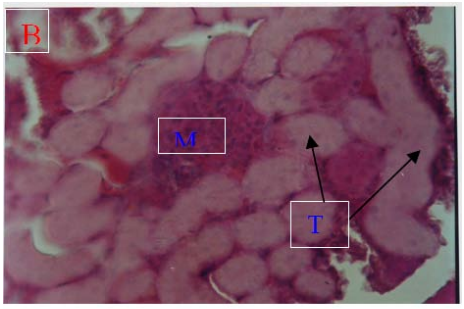


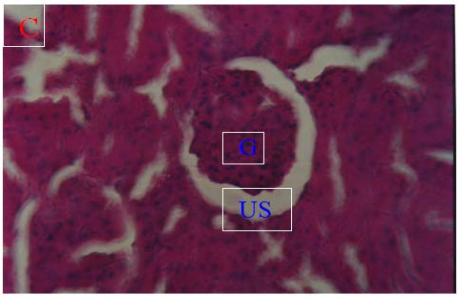
Fig. 2: Lipid peroxidation (MDA) level (A) and superoxide dismutase (SOD) activity (B) in the kidney homogenate of adriamycin-induced nephropathic rats after 6 weeks daily treatment with *K crenata* methanolic extract 50 mg/kg bw (NeK50), 68 mg/kg bw (NeK68) or losartan 10 mg/kg bw (NeL10). Normal (NC) and nephropathy (NeC) control rats treated with 5 mL/kg bw distilled water. Data are means ± SEM, n = 5. Significance difference: *P< 0.05 and **P< 0.01 compared with NC values; ^{a}P< 0.05 and ^{b}P< 0.01 compared with NeC values



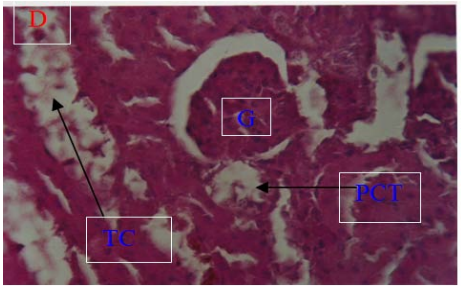
A- NC



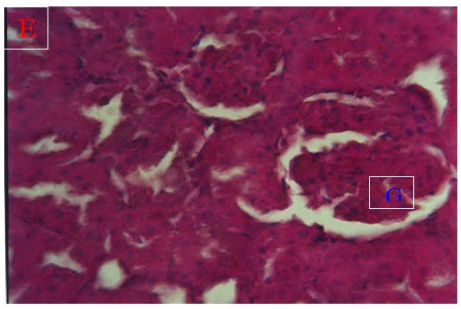
B- NeC



C- NeK50



D- NeK68



E- NeL10

Fig. 3: Morphology of adriamycin-induced nephropathic rats after 6 week daily treatment with *K crenata* methanolic extract 50 mg/kg b.w. (NeK50) (c), 68 mg/kg b.w. (NeK68) (d) or losartan 10 mg/kg b.w. (e) (NeL10). Normal (NC) (a) and nephropathy (NeC) (b) control rats treated with 5 mL/kg b.w. distilled water. G: Glomerulus, US: urinary space PCT: proximal convoluted tubule, DCT: distal convoluted tubule, ME: mesanguim expansion, TO: tubular oedema, TC: tubular clarification. (HE – x400).

In the acute toxicity study, single oral dose of the MEKC up to 2 g/kg was not lethal to both male and female mice. The apparent cause of death of mice in the current study at the higher doses might be due to respiratory depression or/and to methanol (solvent) poisoning; it is noteworthy that the aqueous-ethanol extract of *K. crenata* did not show lethalithy in a previous acute toxicity [8]. The results suggest that MEKC possesses low toxicity since its LD_{50}, (inferior to 5 g/kg b. w.) is 65 and 88 times respectively the assay doses [18].

Adriamycin-induced nephropathy resulted in hyperproteinuria. Parallel to the increase of proteinuria, there was hypercreatinemia and hypocreatinuria, along with the decrease of creatinuria/creatinemia ratio. Normally, the kidneys excrete creatinine and only a slight amount of low molecular weight protein passes through the glomerular [19]. Usually hypercreatinemia and hypocreatinuria observed in nephropathic states are characteristic of glomerular hyperfiltration[20]. The alteration of the glomerular filtration may explain the hyperproteinuria and the decrease of creatinuria/creatinemia ratio [21].

The MEKC showed significant dose-dependent effect on protein excretion in nephropathic rat, similar to that of the losartan. When compared with losartan effect, the decrease of urinary protein level could be due to a potential capability of the plant extract to restore the altered glomerular capillary function in nephropathic rats, and by the inhibition of enzyme-converting angiotensin (ECA) or the blockage of angiotensin II receptors, that reduces capillary vessel contraction and then decreases the retention of water and salt [19,22-24]. This could explain the decreased level of urine volume and urinary sodium in treated nephropathic rats.

Normally, the tubular reabsorbs water and Na^+, to form the hypotonic urine. In nephropathic rats, Adriamycin caused an electrolyte disorder due to the failure of tubular reabsorption causing sodium leak and potassium retention. Thus, the raise of urinary Na^+ excretion, which may be due to the decrease of proximal reabsorption, also causes an increased urinary volume [25]. In the treated rat, the decrease of urinary Na^+, urinary volume, and the increase of blood potassium, could then result from an improvement of the proximal tubular reabsorption function by *K. crenata*. The plant by normalizing the levels of sodium and potassium in the media also normalizes urinary volume.

In the nephropathic rats, the adriamycin provoked the lipid peroxidation products (MDA) increase and the SOD activities decrease in the kidneys. SOD is the major scavenging enzyme that removes toxic free radicals *in vivo* and protects tissues against oxygen free radicals by catalyzing the removal of superoxide radical (O_2^-) which damages the cell membrane and biological structure in kidney tissues. Reduced activities of SOD associated with adriamycin nephropathy in rats, may lead to a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide [26]. Increased in lipid peroxidation (MDA) in adriamycin nephropathy may cause peroxidative tissue damage and inflammation [2,10]. Otherwise, the proteinuria increase could lead to the accumulation of the protein in the proximal tubular cells cytoplasm, which with associated interstitial inflammatory reaction, may cause glomerular and kidney tubule damage [24].

In addition, glomerular damage, mesangium expansion and the tubular inflammation in nephropathic rats could result from the oxidant stress markers (SOD and MDA) increase by inflammatory mechanisms in the nephropathy progression [27,28]. In the treated rats, plant extract and losartan exhibited antioxidative properties by normalizing SOD activity and MDA level, and also improved the renal morphology. Restoration to the normal level SOD activity, MDA level in tissues, and kidney morphology could be attributed to the presence of polyphenols, triterpens and tannins in the extract, as they are known to possess antioxidant properties[29]. This indicates that *K. crenata* could prevent the alteration of the renal cells structure and/or function that could be induced by adriamycin.

CONCLUSION

Methanolic fraction of *K. crenata* could improve glomerular and renal tubular, and the expansion of the mesangium, could lower the urinary protein level, prevent the increase of creatinemia, MDA level and the decrease of SOD activities in nephropathy rats. Thus, this extract shows a potential to produce an alternative medicine for the treatment or management of the kidney disease.

CONFLICTS OF INTEREST

The authors have none to declare.

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