International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
www.florajournal.com
IJHM 2020; 8(3): 01-07
Received: 01-03-2020
Accepted: 03-04-2020

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Nephroprotective effects of methanolic extract of *Cucumis metuliferus* fruit in cockerels

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Abstract
Serum chemical and haematological analyses together with physical examination can provide important information on animal's health status; and are important in the diagnosis and treatment of patients and especially in avian species that somewhat show minimal overt clinical signs of disease, even when seriously ill. This study aimed at establishing the effect of crude methanolic extract of *Cucumis metuliferus* on serum biochemical indices associated with kidney functions in cockerels. The fruits of *C. metuliferus* were collected in Vom village, Jos South Local Government Area, Plateau State, Nigeria in Nov. 2012. The plant was identified and authenticated by a plant Taxonomist in the Department of Biological Sciences, University of Maiduguri, Maiduguri. The grounded fruit was serially extracted (cold maceration) using solvents of different polarities. This was kept in an air-tight container until used. The crude methanolic extract being the most active was used for sub-acute toxicity studies in cockerels. Serum biochemicals relevant to kidney function (urea, creatinine, sodium, potassium, chloride and bicarbonate) were determined weekly for three weeks (day 7, 14 and 21). After stoppage of the treatment, serum was also collected a week later (day 28), for the determination of same biochemicals in order to establish the withdrawal effect. Data obtained were analyzed using one way analysis of variance (ANOVA) and results expressed as mean ± standard deviation (S.D) where $p<0.05$ was considered significant. The result of the serum biochemicals and electrolytes of the treated groups when compared to their controls showed a significant ($p<0.05$) decrease in the level of urea, creatinine and sodium, with a significant ($p<0.05$) increase in potassium, chloride and bicarbonate. The result of 7 days post treatment, which is the withdrawal period (Day 28) when compared to its day 21 showed only creatinine to significantly ($p<0.05$) increase, whereas, sodium, potassium, chloride and bicarbonate significantly ($p<0.05$) decreased after treatment withdrawal. This result may mean that Cucumis metuliferus may have a nephroprotective effect and may be good in maintaining acid-base balance. In conclusion, the crude methanolic extract of the ripe fruit of Cucumis metuliferus has confirmed the folkloric use of the plant in the treatment of kidney disorders or as a diuretic.

Keywords: *Cucumis metuliferus*, electrolytes, kidney function indices, cockerels

1. Introduction
Serum chemical and haematological analyses together with physical examination can provide important information on animal's health status; it is the cornerstone of medical diagnosis of disease in any species and is useful for management purposes. Plasma or serum biochemistry is especially important in avian species, which frequently show minimal overt clinical signs of disease, even when seriously ill. Accurate and useful haematological and biochemical analyses are essential for successful diagnosis and treatment especially in terminal cases of kidney failure. The occurrence of kidney disease leading to kidney failure is of major concern to the clinician and the patient. Renal disorder is a major cause of death worldwide due to its association with diseases such as diabetes and hypertension [1-2]. In high blood pressure or vascular diseases, the vessels of the kidney tubules may be damaged and diabetes is almost always associated with nepropathy [3]. The kidneys' being the major organ for elimination of drugs and toxins is constantly exposed to damage by toxic chemical compounds. This damage could result to acute or chronic renal failure. In a bit to alleviate injury to the kidneys, natural products could be used as drugs or supplements. Plants have been the source of several synthetic drugs including, diuretics; theophyline and theobromine are obtained from *Theobroma cacao*. Plants such as *Silybum marianum*, *Picrorhiza kurroa*, *Astragalus membranaceus*, *Cordyceps sinensis*, *Salvia miltiorrhiza*, *Herniaria hirsute* and *panax notoginseng* are used as nephroprotective agents [4-6]. Horsetail, barberry, parsley, chamomile, licorice, are also used as medicinal plants to alleviate kidney pains and are nephroprotective [7]. Several Chinese herbs are used to ameliorate acute kidney injury induced by neprotoxicants like drugs, heavy metals, organic xenobiotics, and endotoxins [8].

Plants of the family Cucurbitaceae are used as diuretic or in the treatment of kidney disorders [9, 10]. Kidney damage is also caused by micro-organisms that invade the urinary tract system, therefore, isolated compounds from plant extracts of *Cucumis sativus* L., *Cucumis anguria* L. or plant-based tablet such as 'Sanji tablet' have shown activity against *Escherichia coli*; a microorganism that causes urinary tract infection [11-13]. Several other causes of kidney damage may be the use of unpurified or adulterated herbal preparations especially when consumed in large quantities [14]. Oxidative stress can enhance kidney damage due to production of oxidants. However, plants that have anti-oxidant activity may ameliorate the injury to the kidneys by acting against the Reactive Oxygen Species (ROS). Flavonoids are groups of phenolic compounds present in plants that pharmacologically possess antioxidant activity, anti-inflammatory and antimicrobial activities [15-17]. Troxerutin a natural flavonoid found in vegetables, fruits, tea, coffee, and cereals reduces kidney damage by reduction in ROS, decreased activity of cyclooxygenase-2, as well as reduces the urine albumin-creatinine ratio [18]. Ligustrazine obtained from the Chinese herb *Ligusticum wallichii* also exerted anti-oxidant, anti-inflammatory and anti-hypertensive effects [19]. Two antioxidant compounds have been identified in the melon seeds; γ-tocopherol and α- tocopherol. Both are organic types of Vitamin E with known anti-oxidant activity and are found in *Cucumis metuliferus* [20]. Nonetheless, taking a single anti-oxidant does not prevent against kidney damage because of the different mechanism of action of anti-oxidants [21]. Thus, several fruits and vegetables are necessary diets for daily consumption. The plant *Cucumis metuliferus* is a member of the cucumber family which is related to melon, zucchini and cucumber; it is renamed as kiwano. The health benefits of kiwano are in the nutrients it contains. These nutrients are made up of good levels of vitamin C, iron and potassium. It also has minerals such as phosphorus, magnesium, zinc, calcium, copper and sodium [20]. Linoleic and oleic acids found in melon seeds help with the lowering of blood pressure [22] consequently, may help in acute kidney failure. Alkaloids from the fruit of *C. metuliferus* reduced significantly the biomarkers of kidney function (urea, creatinine) in gentamicin-induced kidney injury of albino rats [23]. The plant has secondary metabolites such as alkaloids, carbohydrates, cardiac glycosides, flavonoids, saponins, Tannins, steroids, and terpenoids [24]. Flavonoids isolated from plants exert a nephroprotective effects in kidney damage caused by antibiotics such as gentamicin, cyclosporine an immunosuppressant and chemicals such as carbon tetrachloride [25]. Saponins from the leaves of *Panax quinquefolius* showed nephroprotective effects against kidneys damaged by anti-tumour drug cisplastin [26].

The purpose of this study was to determine the protective ability of *Cucumis metuiferus* on the kidneys in a sub-acute toxicity study. Considering the serum biochemicals related to kidney functions, this study will further add to the folkloric use of the plant for the treatment of kidney damage or as a diuretic.

2. Materials and Methods

Plant collection and identification, as well as preparation of extracts were described in our work [24].

2.1 Experimental Animals

Day old chickens were purchased from Ghamba Consultancy and Enterprises, Wulari, Maiduguri and kept at the Veterinary Physiology, Pharmacology and Biochemistry Laboratory, University of Maiduguri, Maiduguri, for Intensive Management. Throughout these periods, food and water were provided *ad libitum*. The feed given was pelletised Vital Feed, (Grand Cereals Ltd.), Zawan Roundabout, Jos, Plateau State. The biochemical research involving animals was approved by the Ethics Committee, Faculty of Veterinary Medicine, University of Maiduguri, Borno State, Nigeria and was carried out according to the principles of Council for International Organizations for Medical Science (CIOMS) and the International Council for Laboratory Animal Science (ICLAS) [27].

2.2 Sub-Acute Toxicity Study

Twenty, 7-week old cockerels were used for this study. They were randomly divided into four groups of 5 chicken each (groups A, B, C and D). Cockerels in group D served as the untreated control and were given only feed and distilled water daily for a period of 28 days. The cockerels in groups A, B and C were treated daily orally with graded doses of the most active of the fruit extracts, that is CME, (200, 400 and 600 mg/kg respectively). Effect of the prolonged administration of the extract was examined. Blood was obtained from the wing vein on weekly basis, centrifuged and the serum was used for the determination of blood urea nitrogen, creatinine and electrolyte ions (sodium, potassium, chloride and bicarbonates) for 3-weeks (Day 7, 14 and 21). Serum was also taken a week after the stoppage of the administration of the extract to monitor the above mentioned parameters (Day 28).

2.3 Statistical Analysis

Data were analyzed using the Computer Statistical Software Package, GraphPad Instat using one way analysis of variance (ANOVA) and results expressed as mean ± standard deviation (S.D) where $p<0.05$ was considered significant.

2.4 Determination of Serum Urea or Blood Urea Nitrogen (BUN) (mmol/L)

Urea was determined by the Urease-Berthelot method using the Randox test kit (Randox Lab. Ltd., Ardmore, UK).

2.5 Principle: For analysis of serum urea, diacetyl-monoxime colometric (Non-uv) method was employed (Berthelot's reaction). Urea is hydrolysed to ammonia in the presence of urease. The ammonia formed reacted with phenol and hypochlorite in alkaline medium to form indophenols. Nitroprusside is used to catalyse the reaction and the absorbance/optical density of the dissociated indophenol, a blue chromogene is measured at a wavelength of 560 nm [28, 29].

2.6 Procedure: Three test tubes individually labelled as; blank, standard and test were used. Hundred micro liters (100 µl) of reagent 1 (EDTA, sodium nitroprusside and urease) was added to each test tube. Ten microliters (10 µl) of distilled water was added to the blank test tube. Thereafter, 0.01 ml of the standard (100 mmol/L) and test solutions were placed in the tubes labelled standard and test respectively. This was mixed immediately and incubated at 37 ºC for 10 minutes. Thereafter, 2.5ml of reagent 2 (phenol concentrate) and reagent 3 (hypochlorite concentrate) were added to each test tubes, mixed with a vortex mixer and incubated at 37 ºC for 15 minutes. The absorbance/ optical density of the sample and standard were read against the blank using a spectrometer (Boeringer 4010, West Germany) at 546 nm. The concentration of the urea was calculated using the following formula

$$\text{Urea Concentration (mmol/L)} = \frac{\text{Sample Optical Density}}{\text{Standard Optical Density}} \times \text{Conc. of Standard}$$

2.7 Determination of Creatinine (μmol/L)

Creatinine was determined by the colorimetric method using commercial Randox test kits (Randox Lab. Ltd., Ardmore, UK). This is based on the principle that creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration. The absorbance of the complex was read at 492 nm wavelength [30].

2.8 Procedure

To the test tubes labeled standard and test were added 1ml working reagent 1a (picric acid) and 1b (sodium hydroxide). Thereafter 0.1ml of the standard solution and serum sample was added to the test tube labeled standard and test respectively. The contents were mixed and after 30 seconds the absorbance of the standard and test were taken (A_1). Exactly 2 minutes later the absorbance is still taken (A_2). All absorbance was read against the blank at 492 nm.

$A_2 - A_1 = \Delta A_{\text{sample}}$ or $\Delta A_{\text{standard}}$

The level of creatinine was calculated using the formula.

$$\frac{\Delta A \text{ sample}}{\Delta A \text{ standard}} \times \text{Standard concentration } (\mu mol/l)$$

2.9 Analysis of Electrolytes

2.9.1 Analysis of Sodium (mmol/L)

The amount of sodium in the serum samples of treated cockerels was determined by flame photometry [31]. The test is based on the principle that sodium solutions under carefully controlled conditions, when finely sprayed (aspirated) into a burner, the flame de- solvates the solution leaving solids (salts) which dissociate to give neutral ground state atom. Some of these atoms are excited in the flame, thus moving into a higher energy state. When these excited atoms fall back to the ground state, they emit light of characteristic wavelength (590 nm). This light then passes through a suitable filter onto a photosensitive element and the amount of current produced is measured and is directly proportional to the amount of sodium present in the sample. To 0.1ml of the serum in a universal bottle was added 9.9 ml of deionized distilled water, the bottle was capped and mixed by inversion. Distilled water was used as blank while 0.1ml of the standard working solution served as the standard.
The concentration of sodium in the serum sample was then obtained using the formula:

Sodium (mmol/L) = Absorbance reading × 2.

2.9.2 Analysis of Potassium (m mol/L)

The amount of potassium in the serum samples of treated cockerels were determined by flame photometry [31]. Briefly, 0.1ml of serum sample was taken in a universal bottle and 9.9 ml of deionized water was added to remove any ion present. The solution was mixed by inverting the bottle several times. Distilled water was used as the blank, while 0.1ml of the standard working solution was used as the standard. The potassium light filter was inserted into the galvanometer at an air pressure of 10 lb/sq.inch.

Potassium concentration in the serum sample was obtained using the formula:

Potassium (mmol/L) = Absorbance reading × 2.

2.9.3 Analysis of Chloride Ion (mg/dl)

Serum chloride levels were determined using the mercuric nitrate method [32]. About 0.2 ml of the test serum was added to 1.8 ml of distilled water and 2 drops of phenol red as an indicator solution were later added, mixed and titrated to a violet end point with mercuric nitrate. Similarly to 1.8 ml of the standard solution, 2 drops of the indicator solution was added, mixed and titrated to a violet end point with mercuric nitrate. The levels of the chloride ions in the serum were then calculated using the formula:

$$\text{Chloride ion (mg/dl)} = \frac{\text{Sample O.D}}{\text{Standard O.D}} \times \frac{100\ mg/dl}{1}$$

Where O.D = Optical Density

2.9.4 Analysis of Bicarbonate Ion (mmol/L)

Bicarbonate determination was done using back titration method[33]. About 3.0 ml of distilled water was added to 100 µl of serum and 1.0 ml of 0.01M sulphuric acid. Thereafter, 1 to 2 drops of the phenol red indicator solution was added to the solution and was titrated with 1.0ml of 0.01ml NaOH until the end point was reached (pink or yellow). The volume of excess NaOH solution left in the pipette of burette was then recorded. This volume multiplied by 100 gives the level of bicarbonate in mmol/L

Conc. of HCO_3^- (mmol /L) =

$$\text{Amount of acid x conc. of acid used} \times \frac{100}{\text{Volume of sample used}}$$

3. Results

3.1 The Effect of Crude Methanolic Extract of *Cucumis metuliferus* on Kidney Function Indices

There was a significant (p<0.05) decrease in the level of urea in all the treated groups from day 14 to 21, with no significant changes the result of 7 days post treatment (day 28) when compared to its day 21 as shown in Fig. 1.

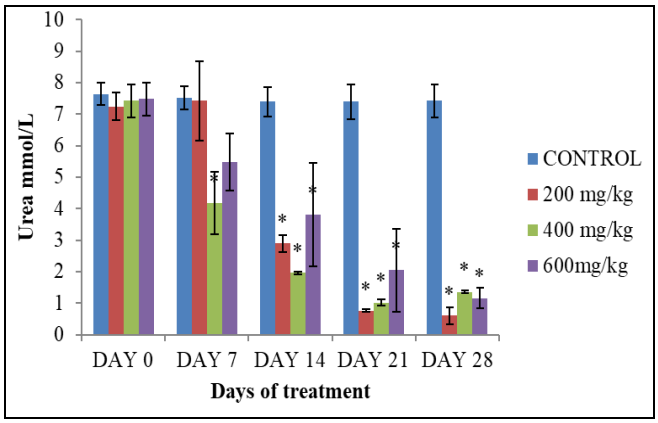

Fig 1: Effect of CME of *Cucumis metuliferus* on Urea

* (p<0.05) significant when compared to the day before dosing

[a] (p<0.05) significant when compared to values of day 21of treatment (withdrawal effect)

The level of creatinine significantly (p<0.05) decreased in all the treated groups from day 14 to 21 when compared to their controls. The result of 7 days post treatment showed a significant (p<0.05) increase when compared to its day 21 (Fig. 2).

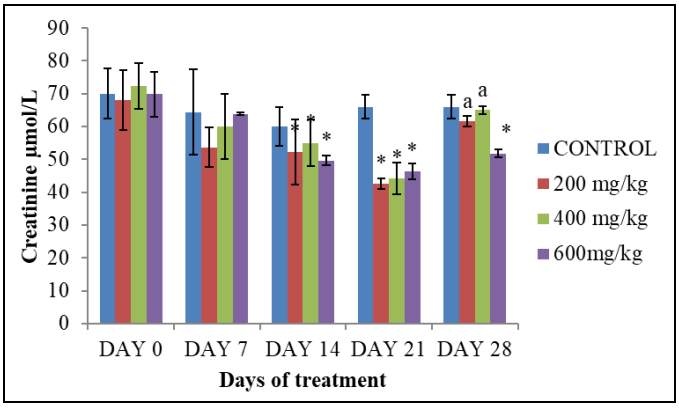

Fig. 2: Effect of CME of *Cucumis Metuliferus* on Creatinine

* (p<0.05) significant when compared to the day before dosing
[a] (p<0.05) significant when compared to values of day 21of treatment (withdrawal effect)

The result of serum sodium as shown in Fig. 3 showed a significant (p<0.05) decrease of those given 400 and 600 mg/kg, while the result of 7 days post treatment when compared to their day 21 showed a significant (p<0.05) decrease in all the treated groups (Fig. 3).

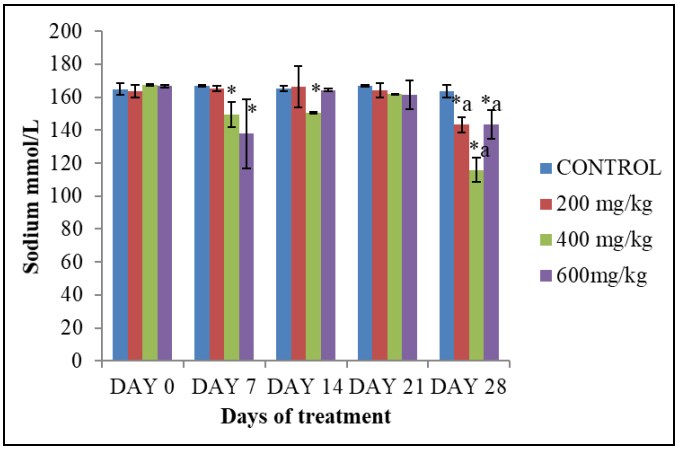

Fig 3: Effect of CME of *Cucumis metuliferus* on serum sodium

* (p<0.05) significant when compared to day before dosing
[a] (p<0.05) significant when compared to values of day 21of treatment (withdrawal effect)

The result of serum potassium showed a significant (p<0.05) increase while on day 7 post treatment only those that received 400 and 600 mg/kg showed a significant (p<0.05) decrease when compared to their day 21 (Fig. 4).

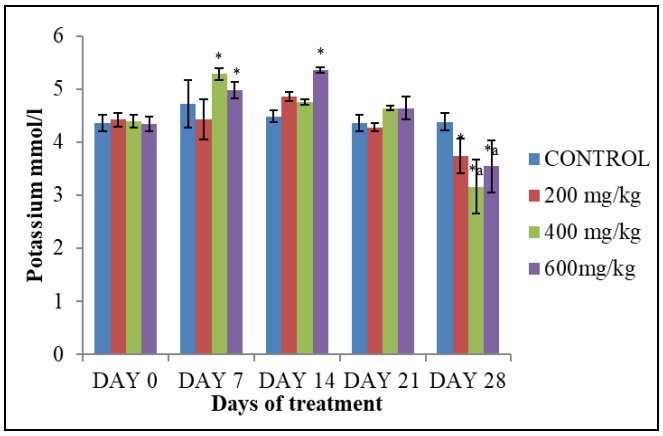

Fig 4: Effect of CME of *Cucumis metuliferus* on serum potassium

* (p<0.05) significant when compared to the day before dosing
[a] (p<0.05) significant when compared to values of day 21of treatment (withdrawal effect)

The result of serum chloride as shown in Fig. 5 showed that only the group given 400 mg/kg extract had a significant (p<0.05) increase on day 7 post treatment (day 28). The result of 7 days post treatment when compared to its day 21 showed that the groups given 200 and 400 mg/kg showed a significant (p<0.05) decrease (Fig. 5).

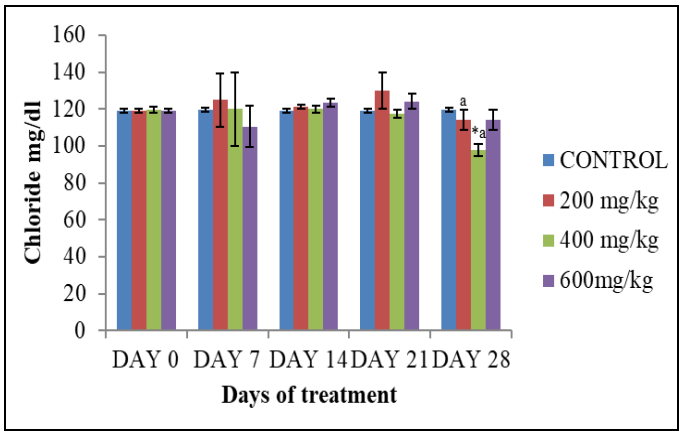

Fig 5: Effect of CME of Cucumis metuliferus on serum chloride

* (p<0.05) significant when compared to the day before dosing
[a] (p<0.05) significant when compared to values of day 21of treatment (withdrawal effect)

The result of serum bicarbonate of those treated with 600 mg/kg extract showed a significant (p<0.05) increase on day 7. The result of 7 days post treatment showed that the groups treated with 400 and 600 mg/kg had a significant (p<0.05) decrease when compared to their day 21 (Fig. 6).

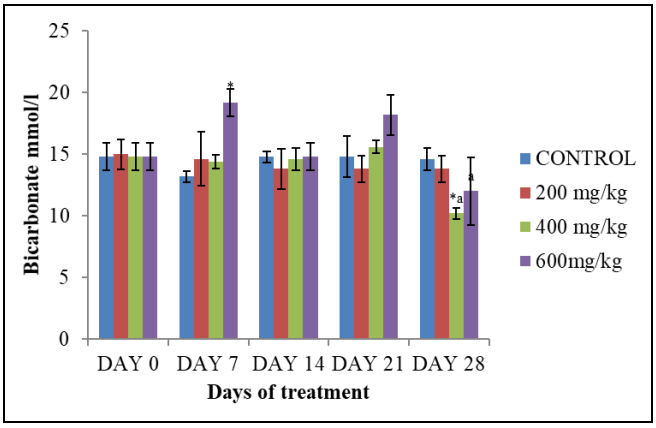

Fig. 6: Effect of CME of Cucumis metuliferus on serum bicarbonate

* (p<0.05) significant when compared to the day before dosing

a (p<0.05) significant when compared to values of day 21of treatment (withdrawal effect)

4. Discussion

Urea and creatinine are excellent indicators of protein metabolism and kidney function. In this study, there was a significant (p<0.05) decrease in the level of urea and creatinine throughout the treatment days. However, there was no significant (p<0.05) decrease in urea level after 7 days withdrawal when compared to day 21. This work is in contrast with that of Wannang *et al*. (2007) who reported a dose dependent significant (p<0.05) increase of blood urea nitrogen (BUN). Urea is the predominant end product of protein and amino acid catabolism and is made in the liver through the urea cycle. It is the main non protein nitrogen (NPN) constituent in the blood. Other NPN substances include amino acids, uric acid, creatinine and ammonia [34]. Azotemia results with increase in the level of urea in the blood. Urea increases due to excess dietary protein, poor quality dietary protein, carbohydrate deficiency, catabolic states, dehydration, congestive heart failure, renal failure, blocked urethra and ruptured bladder. It decreases due to low dietary protein, gross sepsis, anabolic hormonal effects, liver failure and inborn errors of urea cycle metabolism [35]. From the result of this experiment, it may probably be deduced that the fruit extract of this plant may be helpful in the treatment of azotemia and kidney dysfunction.

Creatinine, the biomarker of protein metabolism, derived from phosphocreatin in muscle is normally low in birds and its high level is associated with high level of activity [34]. Creatinine is an amino acid formed as a waste product of creatine metabolism, an important energy storage substance in muscle metabolism whose measurement provides an exceptional useful index of kidney function. Creatinine increases due to renal dysfunction, blocked urethra and ruptured bladder; it decreases due to sample deterioration [35]. Patients with a high muscle mass have high-normal creatinine concentrations, while patients with a low muscle mass have low-normal creatinine concentrations [35]. Up to 50% of the renal function must be lost before the serum creatinine level becomes abnormally elevated, thus making it not useful in early detection of renal dysfunction [30, 34]. Increased levels of creatinine is seen in shock, dehydration, congestive heart failure and diabetic patients, due to reduced renal blood flow, consequently leading to kidney damage [36]. The fruit extract of *C. metuliferus* was reported by [37,38] to have anti-diabetic property. The level of creatinine in this study is significantly (p<0.05) decreased, this may therefore mean that the fruit of *C. metuliferus* may aid in renal dysfunction or may probably be nephroprotective.

The balance of cations and anions maintains pH and regulates nervous, cardiac and muscular functions. Anions and cations also are essential cofactors in numerous enzymatic reactions [34]. The result of this present work on electrolyte ions shows a significant (p<0.05) decrease in the levels of sodium ions in the treated groups compared to the control. This work agrees with that reported by Jimam *et al*., 2011, that the aqueous fruit extract of *C. metuliferus* produced a significant (p<0.05) decrease in the level of sodium ions at 1000 mg/kg, although they reported an insignificant increased excretion of sodium and potassium ions at 500 mg/kg [39]. An increase in the concentrations of sodium in the bloodstream can be toxic. Hypernatremia causes various cells of the body, including those of the brain to shrink; this could result in acute mortality and mild to severe neurological signs, such as tremors, seizures and behavioural changes [40]. The fruit extract of *C. metuliferus* suppressed nervous signs of Newcastle Disease in chickens [41]. This may be associated with hyponatraemic effect of the plant as seen in this study.

The level of potassium in the fruit of *C. metuliferus* (286.59 mg/223 g) is more than the daily requirement recommended by the United State Department of Agriculture (USDA) [42]. The result of potassium ion in this present work showed a significant (p<0.05) increase; the fruit may probably be used in the treatment of hypokalaemia. This is further seen after 7 days treatment withdrawal where the level of potassium ion was significantly (p<0.05) decreased when compared to their control in all the treated groups and significantly (p<0.05) decreased at 400 and 600 mg/kg when compared to their day 21. This further showed that the fruit of *C. metuliferus* may increase the level of potassium ion. However, according to Odutola (1992), the serum potassium concentration is not a measure of total body potassium because the bulk of K^+ ion resides within the cells [43]. Thus a reduction in K^+ ion after treatment withdrawal in the present study will probably not affect the acid-base balance of the physiological system because the serum potassium only constitutes a small fraction of the total body potassium.

The measurement of bicarbonate, usually in conjunction with sodium, potassium and chloride, is used in the assessment of acid-base balance resulting from metabolic or respiratory disease. Disturbances of water homeostasis are seen in nervous, muscular or cardiac dysfunctions [35]. In their studies Kanda *et al*., 2013 reported that the level of serum bicarbonate is not associated with the progression of chronic kidney disease [44]. They reported that high serum bicarbonate level if maintained within the normal range could be associated with low risk of chronic kidney disease progression. The result of this study showed a significant (p<0.05) increase in serum bicarbonate level on day 7 of treatment of those given 600mg/kg. This may mean that the fruit could be of importance in treatment of conditions associated with loss of sodium bicarbonate such as diarrhoea and renal tubular acidosis. The withdrawal of this extract potentiates this fact by showing a significant (p<0.05) decrease in the serum bicarbonate (day 28) when compared to day 21 of those given 400 and 600 mg/kg extract (Fig. 6).

5. Conclusion

In conclusion, this research study showed that the fruit extract of *Cucumis metuliferus* may alleviate or reduce nephrotoxicity; and so, may be of pharmacological

importance as diuretics, nephroprotective agent and/or in the treatment of kidney dysfunction.

6. Acknowledgement
The authors sincerely appreciate the management of National Veterinary Research Institute, Vom, Plateau State, Nigeria, for giving the first author financial support to carry out this research.

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