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Combined leaf extracts of *Vernonia amygdalina* and *Occimum gratissimum* ameliorates cadmium-induced nephro-toxicity in Wistar rats

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Abstract

Medicinal plants have been shown to ameliorate cadmium (Cd) toxicity, which is prevalent due to its ubiquitous nature. Thus, this study examined the effects of combining *Vernonia amygdalina* and *Occimum gratissimum* leaf extracts on cadmium-induced nephrotoxicity in Wistar rats. Sixteen adult female Wistar rats (120-140g) were separated into four groups and subjected to the following treatment: group 1: control - given normal saline; group 2: cadmium group - received 20 mg/kg body wt. $CdCl_2$ daily for two weeks; group 3: extract group - received 200 mg/kg body wt. of the extract daily for two weeks and group 4: cadmium and extract group - received Cd and extract as in group 2 and 3. Administration of normal saline, Cd and the extracts were done orally using a gastric tube daily for 4 weeks. Cd toxicity in the kidney was manifested via depletion of catalase (CAT), superoxide dismutase (SOD), glutathione (GSH) with elevation in lipid peroxidation and serum levels of urea and creatinine. These results were confirmed by the histopathological assessment of the kidney which revealed that Cd-exposure stimulated hepatocellular injuries. The combined leaf extract of *V. amygdalina* and *O. gratissimum,* on the other hand, significantly restored CAT and SOD activities and normalized Urea and creatinine Levels in Cd-exposed rats. The results demonstrate that the administration of combined leaf extracts of *V. amygdalina* and *O. gratissimum* to Wistar rats orally might give considerable protection against cadmium-induced toxicity.

Keywords: *Vernonia amygdalina, Occimum gratissimum,* Kidney, Toxicity, Wistar rats, Leaf extracts

1. Introduction

Cadmium (Cd) is a toxic heavy metal that occurs naturally in the earth usually as a complex with other elements [1]. Its wide application in several industrial processes ranging from welding, electroplating, paints and pigment manufacture and the making of nickel-cadmium battering, has increased its availability in the environment and exposure to humans and animals [2]. Cadmium's long half-life of over 25 years, which contributes to its ability to bio-accumulate, makes it a metal of serious concern [3,4]. It induces oxidative stress and peroxidation of the membrane in the kidneys, liver and other organs which are extremely sensitive to cadmium's toxic effects [5]. Cd-induced oxidative stress is noted as the chief mechanism responsible for many diseases in the liver and kidneys [6,7]. According to Yuan et al [8] Cd's toxic effects on kidneys are also shown via damage to its structural architecture.

The search for antidotes to Cd toxicity is an interesting area of scientific research and in this regard fruits, leaves, pigments and many plant products have been shown to have antioxidant effects against Cd toxicity [9,10]. These have been accepted as very useful in mitigating the effects of toxic metals due to the presence of phytochemicals [11]. *Vernonia amygdalina* and *Occimum gratissinum* are common plants in Nigeria whose leaves are used as vegetables in cooking and in the preparation of herbs used for the treatment of diverse disease conditions [12]. *V. amygdalina* has a different local name in different languages and regions of the world. It is known as Mululuza and Omubirizi in Uganda, Ewuro, Onugbu, Oriwo, Etidot and Ityuna in Nigeria, Ebichaa in

Ethiopia, Awonwono in Ghana and South African leaf in Malaysia. *O. gratissimum* leaf, often known as clove basil or lemon basil, is a highly branching, aromatic shrub that grows to be about 0.5 to 3 meters tall and belongs to the Lamiaceae family. [12] *V. amygdalina* leaf has been demonstrated to have antioxidant qualities as well as the capacity to treat renal diseases [12,13]. The antioxidant and renal protective effects of *O. gratissimum* leaf extracts have also been reported [14,15]. Both leaves have been shown to contain several bioactive compounds such as bioactive compounds; flavonoids, saponins, triterpenes, methyl cinnamate, alkaloids, citral, eugenol, anthraquinone, linalool, tannins, and steroids [14]. Although the combination of *V. amygdalina and O. gratissimum* leaf extract has been reported to possess antidiabetic [16], cardioprotective [17], kidney restorative [18] as well as antioxidant properties [19], no study has reported the effects of their combined administration on Cd-induced nephro-toxicity. Udeh and Mene [20] reported that both bitter leaf and scent leaf extracts showed ameliorative results on diabetes mellitus disease, but they worked better when combined. In addition, Okunlola et at [21] stated that the plants have great nutritional value when consumed together.

Therefore, this study investigated the ameliorative potentials of combined leaf extracts of *V. amygdalina* and *O. gratissimum* on cadmium-induced nephro-toxicity in Wistar rats.

2. Materials and methods

2.1 Chemicals

Standard analytical grade chemicals used were products of Lobal Chemic Laboratory Regents and Fine Chemicals, Mumbai - India (Cadmium Chloride, ethanol), BDH Chemical Company, Poole, England (Thiobarbituric acid, Dichromate, and acetic acid), and Randox Laboratories, England (Urea and Creatinine assay kits).

2.2 Plant materials

Fresh leaves of *Vernomia amydalina* and *Occitum gratissum* were procured from a garden in Yenagoa, Bayelsa State, Nigeria and were identified and authenticated by a botanist in the Department of Biological Sciences, Edwin Clark University, Kiagbodo, with voucher number ECU/BIO/2021/02. The leaves were de-stalked, rinsed with clean water to remove the dust and dirt and air-dried under shade until a constant weight was obtained. Thereafter, they were blended using an electric blender and stored in an airtight can until further use.

2.3 Preparation of plant extracts

This was accomplished in accordance with the procedure outlined by Alara et al [22] with some modifications. In a Soxhlet apparatus, equal weights (100 g each) of powdered *V. amydalina* and *O. gratissum* leaves were inserted, and extraction was carried out with 2000 mL of ethanol (60% v/v). The extract was then concentrated to dryness at 400°C using a rotary evaporator and refrigerated until needed.

2.4 Experimental animals and experimental design

For the investigation, sixteen (16) mature female Wistar rats (120-140 g) were employed. The rats were collected from the Animal House of Delta State University's Faculty of Basic Medical Sciences in Abraka. Prior to experiments, the rats were acclimated in a well-ventilated room maintained at 25 ± 2°C with a 12 h light/dark cycle for one week and were allowed access to clean water and feed *ad libitum*. They were divided into four groups and treated as follows:

Group 1: Control. Rats here were given normal saline.
Group 2: Cadmium Group. Received 20 mg/kg body wt. $CdCl_2$ daily for two weeks.
Group 3: Extract Group: Received 200 mg/kg body wt. of the extract daily for two weeks.
Group 4: Cadmium and Extract Group: Received Cd and Extracts as in group 2 and 3 above.

For four weeks, normal saline, Cd, and the extract were administered orally through a stomach tube. At the completion of the experiment, the animals were sacrificed via cervical dislocation. Blood samples were taken through cardiac puncture and centrifuged for 10 min at 3000xg in heparinized vials. Kidneys were excised carefully, and 1 g was homogenized in ice-cold phosphate buffer (pH 7.0) and centrifuged at 3000g for 10 min. The collected sera and supernatants were kept refrigerated until they were analyzed. A section of the kidneys was also taken and fixed in 10% formalin solution before being processed for histological investigation.

2.5 Determination of oxidative stress parameters in the kidney

2.5.1 Catalase (CAT) activity determination

The procedure of Singha et al [23] was employed for the determination of CAT activity of samples. The experiment was carried out by filling tiny test tubes with varying amounts of H_2O_2 (10 to 100 moles) and adding 2 mL of dichromate/acetic acid to each. As a result, an unstable blue perchromic acid precipitate was generated. The solution combination was then boiled in boiling water for 10 min. As a result of the synthesis of chromic acetate, the color of the solution changes to a stable green. The volume of the reaction mixture was decreased to 3 mL after cooling at room temperature. A spectrophotometer was then used to measure the optical density at 570 nm. By graphing the standard concentrations against absorbance, a standard Catalase curve was generated.

2.5.2 Superoxide dismutase (SOD) activity determination

The approach was used to determine SOD activity in samples via superoxide dismutase preventing the autoxidation of adrenaline at pH 10.2. To begin, a 1:10 dilution of the sample was created by diluting 1 mL of sample in 9 mL of distilled water [24]. After that, the spectrophotometer was calibrated with an aliquot (0.2 mL) of the diluted sample in 2.5 mL of 0.05 M carbonate buffer pH 10.2. The reaction was then started by adding 0.3ml of freshly made 0.3 mM adrenaline to the liquid, which was quickly mixed by inversion. A blank curette was also produced using 2.5 mL buffer, 0.3 mL substrate (adrenaline), and 0.2 mL distilled water. The absorbance increase at 480 nm was calculated by measuring it every 30 sec for 150 sec and calculating it as follows:

$$\text{Increase in absorbance per min} = \frac{A_3 - A_0}{2.5} \quad (1)$$

$$\%\text{Inhibition} = 100 - \frac{\text{increase in absorbance for substance}}{\text{Increase absorbance for blank}} \times 100 \quad (2)$$

One unit of SOD activity was defined as the quantity of SOD required to limit the oxidation of adrenaline to adrenochrome by 50% for one min.

$$\text{SOD (unit/g tissue)} = \frac{\%\ \text{inhibition} \times 1 \times G \times D}{X^1 \times 50} \quad (3)$$

X^1 = mg of tissue in the reaction mixture, D = dilution factor, and 50 = 50% inhibition

One unit of SOD activity is the amount of SOD necessary to restrict adrenaline oxidation to adrenochrome by 50% for one min.

2.5.3 Glutathione level (GSH) estimation

The technique was used to determine the amount of reduced GSH [25]. After mixing 0.2 mL of the sample with 1.8 mL of distilled water, 3 mL of the precipitating solution was added. The mixture was then let to stand for five (5) min before being filtered. After five min, 1 mL of the filtrate was added to 4 mL of 0.1 M phosphate buffer. After that, the Ellman's reagent was added in 0.5 mL increments. 4 mL of 0.1 M phosphate buffer, 1 mL of diluted precipitating solution (3 parts distilled water to 2 parts distilled water), and 0.5 mL of Ellman's reagent were used to make a blank solution. The optical density was measured at 412 nm, and GSH was discovered to be proportional to absorbance, as calculated by the GSH standard curve.

2.5.4 Tissue lipid peroxidation estimation

The approach was used to detect the quantity of Thiobarbituric acid reactive substances (TBARS), which is an indicator of lipid peroxidation [26]. A 0.4 mL aliquot of the sample was mixed with 1.6 mL of Tris-KCL buffer and 0.5 mL of 30% trichloroacetic acid (TCA). The mixture was then heated in a water bath at 800°C for 45 min with 0.5 mL of 0.75% thiobarbituric acid (TBA). This was then refrigerated with ice before being centrifuged at 3000 g for 10 min. The absorbance of the clear supernatant was measured at 532 nm in comparison to a reference blank of distilled water. To compute liquid peroxidation in units/mg protein or gram tissue, the molar extinction coefficient of 1.56 x 10^5 M-1 CM-1 was utilized. TBAS levels are expressed as mole malondialdehyde (MDA)/g tissue in terms of MDA. The molar extinction value of 1.56x105 m/cm was used to determine the quantity of MDA in the samples.

2.5.5 Urea concentration estimation

This was done according to Randox assay kit instruction following the method of Weatherburn et al [27]. The test operates on the concept that in the presence of urease, urea in serum is hydrolyzed to ammonia. The ammonia is then photometrically measured using Bethelot's reaction. Exactly 10 µL each of distilled water, sample, and standard (Cal) were pipetted into 'blank', 'Test', and 'Standard' test tubes respectively. Following that, 100 µL of Reagent 1 was added to each of the three test tubes, stirred, and incubated at 370°C for 10 min. The three test tubes were then filled with 250 µl of Reagent 2 and 3, mixed immediately, and incubated at 37°C for 15 min. Absorbance of the sample (A_{Sample}) and standard ($A_{Standard}$) were read against the blank at 546 nm and calculated as follows:

$$\text{Urea concentration (mmol/L)} = \frac{A_{Sample} \text{ x } 13.3}{A_{Standard}} \quad (4)$$

2.5.6 Determination of serum creatinine level

This was done in accordance with the procedure of Bartels et al [28], as indicated in the Randox assay kit, it is based on the concept that creatinine in an alkaline solution combines with picric acid to form a colorful complex. The amount of complex generated is related to the creatinine concentration. 1.0 mL of the working reagent was placed in two test tubes labelled 'standard' and 'sample.' The standard solution (0.1 mL) was then put to the 'standard' test tube, and the sample (0.1 mL) was placed to the 'sample' test tube. They were combined and after 30 sec the absorbance A1 of the standard and sample were read. The absorbance A2 of the reference and sample was measured at 492 nm after exactly 2 min and calculated as follows:

$$\frac{A_{Sample} \text{ x } 2.06 \text{ mg/dl}}{A_{Standard}} \quad (5)$$

2.6 Histopathological examination of the kidney

Tissue samples were promptly fixed in formalin and processed for light microscopic inspection. The tissue blocks were then sliced into serial slices, which were then deparaffinized and stained with hematoxylin. The kidney microscopic architecture of experimental rats was histologically studied on hematoxylin stained slides. Images of stained tissues were obtained using a digital microscopic eyepiece, Brunel light microscope, 20 megapixels (Brunel SP35 Digital Trinocular) linked to a computer's USB connection.

2.7 Data analysis

The study's findings are reported as Mean $\pm$ SD. The Statistical Package for Social Sciences (SPSS) software (IBM SPSS Statistics for Windows, version 21 - IBM Corp., Armonk, N.Y., USA) was used for statistical analysis. The one-way analysis of variance (ANOVA) was used to compare the level of significance of various parameters assessed, and the difference between means was judged significant at $p < 0.05$.

3. Results

3.1 Effect of combined leaf extracts of V. amygdalina and O. gratissimumon cadmium-induced changes in oxidative stress parameters in the kidney of Wistar rats

The effect of combined leaf extracts of *V. amygdalina* and *O. gratissimum*on Cadmium-induced changes in oxidative stress parameters in the kidney of Wistar rats are shown in Table 1.

CAT activity in the kidneys of rats exposed to Cadmium alone (Group 2) was significantly ($p<0.05$) lower ($p>0.05$) than in the control group. There was no significant change in CAT activity in the kidneys of rats administered the extract alone (Group 3) relative to the control group. The treatment of Cd-exposed rats with a combination of *V. amygdalina* and *O. gratissimum* leaf extracts (Group 4) significantly boosted CAT activity when compared to untreated animals (Group 2). A similar pattern was seen in SOD activity. GSH levels in the kidneys of rats exposed to Cd alone (Group 2) were also significantly ($p<0.05$) lower than in the control group. When rats were exposed to Cadmium and leaf extracts (Group 4), GSH levels showed no ($p>0.05$) significant effect relative to rats exposed to Cd only (Group 2).

In contrast, MDA levels increased ($p<0.05$) significantly in group 2 administered Cd only in comparison to the control (group 1) and extract (group 2) groups. A substantial decrease in lipid peroxidation levels was seen

in Cd-exposed rats treated with a combination of *V. amygdalina* and *O. gratissimum* leaf extract (Group 4) compared to animals that were exposed to Cd alone (Group 2).

Cd reduced endogenous antioxidant enzymes and increased lipid peroxidation in the kidneys of Wistar rats, however treatment with a combination of *V. amygdalina* and *O. gratissimum* leaf extract (Group 4) reversed this trend with the exception of GSH.

Table 1 Effect of combined leaf extracts of *V. amygdalina* and *O. gratissimum* on cadmium-induced changes in oxidative stress parameters in the kidney of Wistar rats.

GROUPS	Oxidative stress parameters			
	CAT mmoles H_2O_2 consumed min/ mg protein	SOD μmole/mg protein	GSH μmole/mg protein	MDA μmole/mg protein
Group 1	7.08 ±0.54[a]	5.35±0.35[a]	32.77±0.90[a]	10.69±0.22[a]
Group 2	1.6 ± 0.19[b]	2.88±0.43[b]	21.08±0.87[b]	16.01±1.67[b]
Group 3	7.61±0.25[a]	6.07±0.88[a]	35.33±0.50[a]	9.43±0.25[a]
Group 4	3.74±0.16[c]	4.10±0.07[c]	24.34±0.48[b]	11.86±0.55[c]

Values are expressed as Mean ± SD. n=4.
Mean values with various superscript alphabets in the same column varied substantially at $p<0.05$: Group 1 (control); Group 2 (cadmium); Group 3 (extract); Group 4 (cadmium and extract).

3.2 Effect of combined leaf extracts of V. amygdalina and O. gratissimumon cadmium-induced changes in kidney function parameters of Wistar rats

The impact of combined leaf extracts of *V. amygdalina* and *O. gratissimum* on Cadmium-induced alterations in renal function parameters in Wistar rats are shown in Table 2. The administration of cadmium to rats (Group 2) significantly ($p<0.05$) increased the levels of urea and creatinine compared to the control (Group 1). However, when Cd-exposed rats were given the extract (Group 4), their blood levels of urea and creatinine were reduced considerably ($p<0.05$) as compared to Cd-exposed rats that were not given the extract (Group 2). The levels of urea and creatinine were not significantly different in rats maintained on the extract alone (Group 3) compared to the control (Group 1). The results indicate Cd caused a significant elevation in the assayed kidney function parameters, which was normalized by the administration of the extract.

Table 2 Effect of combined leaf extracts of *V. amygdalina* and *O. gratissimum* on cadmium-induced changes on selected kidney function parameters of Wistar rats.

Groups	Kidney function parameters	
	Urea (mg/dl)	Creatinine (mg/dl)
Group 1	109.64±1.82[a]	0.66±0.03[a]
Group 2	213.12±12.01[b]	1.57±0.02[b]
Group 3	106.45±1.42[a]	0.62±0.09[a]
Group 4	150.18±2.19[c]	1.15±0.02[c]

Values are expressed as Mean ± SD. n=4.
Mean values with various superscript alphabets in the same column varied substantially at $p<0.05$: Group 1 (control); Group 2 (cadmium); Group 3 (extract); Group 4 (cadmium and extract).

3.3 Effect of combined leaf extracts of V. amygdalina and O. gratissimum on the histology of the kidney of Cd-exposed Wistar rats

Figures 1 show the effect of combined leaf extracts of *V. amygdalina* and *O. gratissimum* on Cadmium-induced changes in the histology of the kidney of Wistar rats.

Histological examination of kidney of rats in the control group showed a visible normal renal corpuscle (long arrow) interstitial and tubules (short arrow) (Figure 1A), but the exposure of rats to Cd resulted in a visible distortion of atrophied renal corpuscle (long arrow) interstitial and tubular necrosis (short arrow) (Figure 1B). Administration of only the extract (Group 3) did not cause distortion of the tissues (Figure 1C), however the administration of the extract to Cd-exposed rats normalized the renal corpuscle (long arrow) interstitial and tubules (short arrow) (Figure 1D).

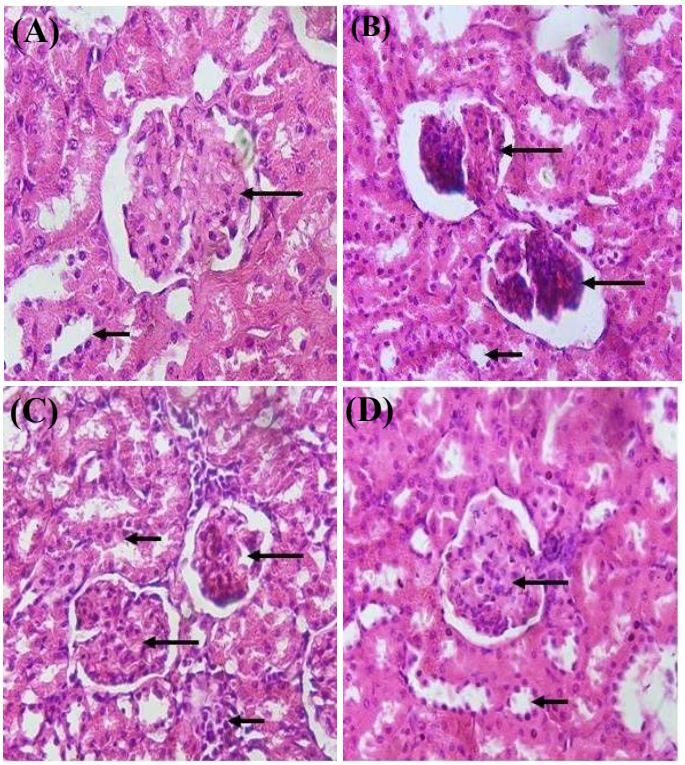

Figure 1 Kidney reveals visible: (A) Group 1 - control, (B) Group 2: cadmium, (C) Group 3: extract, and (D) Group 4: cadmium and extract.

4. Discussion

The kidneys, being essential excretory organs of the body are frequently exposed to toxins, of which Cd is a typical example [29]. It has been shown that plants possess phytochemicals that are nephro-protective [14].

In this study, administration of Cd depleted endogenous antioxidant enzymes (CAT, SOD and GSH) and caused increased lipid peroxidation in the kidney of Wistar rats, but treatment with the combined leaf extracts of *V. amygdalina* and *O. gratissimum* (Group 4) reversed this trend significantly. This result is in line with reported nephro-toxic effects of Cd [10]. Cd has been shown to cause a significant reduction in the levels and activities of endogenous antioxidant defense systems, which has been attributed to the increased use of GSH, SOD and CAT and to arrest oxidative stress induced by Cd [2,30,31]. The three fundamental components of the endogenous defense mechanism that protects cells from the damaging effects of reactive oxygen species are GSH, SOD, and CAT [32]. Increased lipid peroxidation rate as a result of oxidative stress owing to increased formation of reactive oxygen species is a typical symptom of Cd-induced toxicity seen in this study [33].

The ability of the extract to quench oxidative stress induced by Cd, restore the activities of SOD and CAT and reduce lipid peroxidation levels, as witnessed in this study points to the fact that the extracts contain potent antioxidant. Imafidon et al [2] had previously shown that extract of *V. amygdalina* attenuated Cd-induced alteration in lipid peroxidation levels in the plasma of rats and Abdulazeez et al [34] demonstrated the antioxidant properties of a combination of *V. amygdalina and O. gratissimum* leaf extract.

As indicated in Table 2, Cd significantly increased the amounts of Urea and creatinine in the blood, but was normalized by the administration of the extract. According to Atta et al [35], the levels of urea and creatinine in the blood are significant estimators of kidney function. The result confirms the reported ability of Cd to distort the effective excretion of urea and creatinine by cells [36]. Thus, Cd may have tempered with the normal functioning of the kidneys. This is confirmed by an increase in lipid peroxidation (Table 1) and the histological examination which showed visible distortion of atrophied renal corpuscle interstitial and tubular necrosis in rats exposed to Cd alone (plate 2).

The ability of the combined *V. amygdalina* and *O. gratissimum* leaf extracts to normalize the levels of urea and creatinine in Cd-exposed rats in this study, again shows that the extract possesses antioxidant properties against Cd-induced nephro-toxicity. Although the antioxidant prowess of *V. amygdalina and O. gratissimum* have been shown separately [13,14], this is the first study reporting the ability of their combination to offer protection against Cd-induced nephro-toxicity.

5. Conclusion

Cd toxicity in the kidney is manifested via depletion of CAT, SOD, GSH with elevation in lipid peroxidation and serum levels of urea and creatinine as confirmed by the results obtained from the histopathological assessment of the kidney. However, treatment of Cd-exposed rats with the combined *V. amygdalina and O. gratissimum* leaf extracts significantly restored kidney activities. Thus, oral administration of combined *V. amygdalina* and *O. gratissimum* leaf extracts to Wistar rats could provide significant protection against cadmium-induced toxicity in Wistar rats.

6. Ethical approval

Study approval was obtained from the Research Ethics Committee, of the Faculty of Science, Edwin Clark University, Kiagbodo, Delta State, Nigeria with approval number ECU/FOS/2021/04. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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