Ameliorative Potential of *Clerodendrum volubile* Ethanol Leaf Extract on Doxorubicin-Induced Hepatorenal Toxicities in Rats

Adejuwon A. Adeneye^{1*}, Olufunke E. Olorundare², Akinyele O. Akinsola², Daniel A. Sanni³, Ikechukwu I. Okoye⁴, James M. Ntambi⁵, Hasan Mukhtar⁶

¹Department of Pharmacology, Therapeutics and Toxicology, Faculty of Basic Clinical Sciences, Lagos State University College of Medicine, 1-5 Oba Akinjobi Way, G.R.A., Ikeja, Lagos State, Nigeria. ²Department of Pharmacology and Therapeutics, Faculty of Clinical Sciences, College of Health Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria. ^{3,4}Department of Pathology and Forensic Medicine, Faculty of Basic Clinical Sciences, Lagos State University College of Medicine, 1-5 Oba Akinjobi Way, G.R.A., Ikeja, Lagos State, Nigeria. ⁵Department of Biochemistry, University of Wisconsin, Madison, 433 Babcock Drive, Madison WI, 53706, USA. ⁶Department of Dermatology, University of Wisconsin, Madison, Medical Science Center, 1300 University Avenue, Madison, WI 53706, U.S.A

*Corresponding Author Email: adejuwon.adeneye@lasucom.edu.ng

Phone: +2348035835589

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ABSTRACT

Background and Purpose: Hepatorenal toxicity is a side effect of the anthracycline cytotoxic antibiotics, doxorubicin that is used in cancer treatment. The study investigated the ameliorative potential of *Clerodendrum volubile* ethanol leaf extract (CVE) on doxorubicin (DOX)-induced hepatorenal toxicities.

Methods: Male Wistar rats were pretreated with *Clerodendrum volubile* ethanol leaf extract (50 - 400 mg/kg/day, p.o) followed by intraperitoneal injection of 2.5 mg/kg of DOX on alternate days for 14 days. Hepatorenal toxicity was assessed using renal function parameters (serum electrolytes, blood urea and creatinine), hepatic function endpoints [aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), total protein (TP), albumin (ALB) and total bilirubin (TB)]. In addition, the antioxidant activity in the kidney and liver tissues were assayed and histological studies of these tissues were also conducted.

Results: Oral pretreatment with 50 mg/kg/day, 100 mg/kg/day, 200 mg/kg/day and 400 mg/kg/day of CVE remarkably ameliorated DOX-induced liver and kidney injury by lowering the serum ALT, AST, ALP, Cr and BUN levels. CVE pretreatment remarkably ameliorated DOX-induced increases in the CAT, SOD and GPx activities and MDA levels compared to the DOX-treated rats. The biochemical changes were corroborated by improvements in the DOX-induced histological lesions seen in the hepatic and renal tissues examined.

Conclusions: Overall, these findings suggest that *Clerodendrum volubile* ethanol leaf extract elicits protective effect against DOX-induced hepatorenal toxicities mediated primarily via oxidative stress suppression and improvement in the free radicals scavenging activities of CVE.

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INTRODUCTION

Doxorubicin (DOX) (also known as Adriamycin) is one of the widely used broad spectrum anthracycline antibiotics and one of the most potent cytotoxic drugs use in the clinical management of solid and hematological malignancies (Injac and Strukelj, 2008; Chen *et al.*, 2016). These malignancies include but not limited to cancers of the breast, lungs, thyroid, urinary bladder and ovaries, Kaposi's sarcoma, Hodgkin's lymphoma and acute lymphocytic leukemia for which it is often combined with other cytotoxic agents (Tacar *et al.*, 2013). Its mechanisms of cytotoxic action involve DNA intercalation and inhibition of topoisomerase II, an enzyme which relaxes supercoils in DNA for transcription. Thus, DOX stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby halting replication (Pommier *et al.*, 2010). It is often combined with other cytotoxic agents such as cyclophosphamide, bleomycin, vinblastine, vincristine, prednisolone, 5-fluoruracil, in cancer treatment "cocktails" (Rivankar, 2014). These "cocktails" include but not limited to combinations involving doxorubicin /cyclophosphamide/ vincristine (for non-oat cell lung carcinoma), doxorubicin/vincristine/prednisolone /etoposide/ cyclophosphamide/bleomycin (for non-Hodgkin's lymphoma), doxorubicin/docetaxel/ cyclophosphamide (for primary breast cancer), doxorubicin/cyclophosphamide (metastatic breast cancer) (Injac and Strukelj, 2008; Jones *et al.*, 2009).

However, despite its wide clinical application in cancer treatment, its use is limited by its off-target common side effects, one of which is hepatorenal toxicity (Guo *et al.*, 2016; Kuzu *et al.*, 2019). There are reports that DOX treatment has been associated with injuries to the heart, and other body organs such as the liver, kidney, brain and testes (Kalender *et al.*, 2005; Bardi *et al.*, 2007; Mohan *et al.*, 2011; Badkoobeh *et al.*, 2013). Studies have also reported that between 30% and 40% of cancer patients on doxorubicin treatment suffered hepatotoxicity (Yang *et al.*, 2002; Damodar *et al.*, 2014). Unfortunately, the exact mechanisms involved in DOX-induced hepatic and renal toxicities are complex and remain largely unclear although excessive reactive oxygen species (ROS) generation, oxidative damage and the activation of nuclear factor- κ B have been implicated (Kalender *et al.*, 2005; Lamas *et al.*, 2015). However, in managing DOX-related organ toxicities, pharmacological agents such as antioxidants, hematopoietic cytokines and iron-chelating agents have been studied and proven to have beneficial effects in reducing oxidative stress but associated with little therapeutic benefits in DOX-associated hepatorenal toxicities (Wang *et al.*, 2019). Therefore, the need to

identify an effective therapeutic agent against DOX-induced hepatorenal complications becomes challenging (Granados-Principal *et al.*, 2015). In view of this, *Clerodendrum volubile* ethanol leaf extract is investigated for its possible chemopreventive potential in doxorubicin-induced hepatorenal toxicity.

Clerodendrum volubile P. Beauv (belonging to the **Labiatae** family) is a climbing vegetable of deciduous West African forest and ranging secondary jungles of Senegal to Fernando Po (Burkill, 1985). Its local names include "koŋwoli" (Susu tribe, Sierra Leone), "nebu" or "sussuru" (Kru tribe, Ivory Coast), "ayadebi" or "nombi" (Kyama tribe, Ivory Coast), "kumamuno" (Akan-Brong tribe, Ghana), "ēbenote", "nruchi ntà" (Edo and Igbo tribe, respectively, Nigeria) and "ebenetete" among the Itsekiri tribe and "eweta" among the Ilaje people of Nigeria (Erukainure *et al.*, 2014; Happy, 2016). The leaves are generally consumed as vegetable and as soup condiments. Decoctions of *Clerodendrum volubile* leaves have been used traditionally as pain-killers, sedatives and anti-abortionifacient (Burkill, 1985) and in the local management of gouty arthritis, rheumatism, dropsy and body swellings/edema (Erukainure *et al.*, 2016). *Clerodendrum volubile* is reported to contain significant amount of secondary metabolites such as ajugoside, pectolinarigenin, protocatechuic acid, biochanin and 5,7,4'-trimethoxykaempferol and other secondary metabolites that have been reported to elicit a very wide range of biological activities including antihyperglycemic, antihyperlipidemic, antihypertensive, anti-inflammatory, antioxidant, hepatoprotective, anticancer, antimicrobial activities (Erukainure *et al.*, 2014; Adefegha and Oboh, 2016; Erukainure *et al.*, 2016, Fred-Jaiyesimi and Adekoya, 2012; Erukainure *et al.*, 2017; Ajao *et al.*, 2018; Erukainure *et al.*, 2018; Afolabi *et al.*, 2019) and in improving cognitive neurodegenerative diseases such as Alzheimer's disease (Oboh *et al.*, 2017). Recently, we reported the protective effect of 50-400 mg/kg/day of *Clerodendrum volubile* ethanol leaf extract in DOX-intoxicated cardiotoxicity in Wistar rats (Olorundare *et al.*, 2020).

In continuation of our investigation into the possible therapeutic potentials of *Clerodendrum volubile* ethanol leaf extract (CVE) against doxorubicin-induced organ toxicities, this study investigated the possible potential of CVE against DOX-induced hepatorenal toxicities. In addition, the antioxidative profile of CVE in hepatic and renal tissues was evaluated following DOX-induced toxicity.

MATERIALS AND METHODS

Plant Materials

Fresh mature whole plants of *Clerodendrum volubile* were purchased from herbal vendors in Isikan Market in Akure, Ondo State, Nigeria in the month of March 2019. Samples of the plant were subjected to botanical identification and referencing at the University of Ilorin (UNILORIN) Herbarium, with voucher specimen number: UILH/01/019/1254 allotted.

Extraction Process

Fresh leaves of *Clerodendrum volubile* were de-stalked from the whole plant, gently rinsed under running tap water and then air-dried at the room temperature (28-33°C) until the weight of the leaves was constant. The dried leaves were then pulverized using a milling machine and kept in a water- and air-tight container.

A portion (1.50 kg) of the pulverized leaves was macerated in 8 liters of absolute ethanol at room temperature for 5 days but intermittently shaken to ensure complete extraction. Thereafter, it was first filtered with cotton wool and then 110 mm Whatmann filter paper. The resultant filtrate was concentrated *in vacuo* using a rotary evaporator (BÜCHI Rotavapor® Model R-215, Switzerland) with Vacuum Module V-801 (EasyVac®, Switzerland) set at 70 rpm and 36°C. Drying was completed over a water bath preset at 40°C. The dark-colored, sticky and jelly-like, sweet-smelling residue obtained was weighed, stored in air- and water-proof container in a refrigerator. The yield (% w/w) was calculated using the formula:

$$\frac{\text{Weight of crude extract obtained (g)} \times 100}{\text{Weight of pulverized dried leaf extracted}}$$

Experimental Animals

Young adult male Wistar rats (aged 8-12 weeks old and weighing 130-190 g) used in this study were obtained from the Animal House of Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria, after an ethical approval for the study (Protocol Identification code: UERC/BMS/134 and UERC Approval number: UERC/ASN/2019/1703) was obtained from the University of Ilorin Ethical Review Committee for Postgraduate Research. The rats were handled in accordance with international principles guiding the Use and Handling of Experimental Animals (US National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). The rats were fed *ad libitum* on standard rat feed (Ladokun Feeds, Ibadan, Oyo State, Nigeria) and potable water. They were maintained at

an ambient temperature between 28-30°C, humidity of 55 ± 5%, and 12 hours light and dark cycles.

Table 1: Treatment groups for the study

Groups	Treatments
Group I	10 mL/kg of distilled water <i>p.o.</i> for 14 days + 2.5 mg/kg of doxorubicin hydrochloride in normal saline given <i>i.p.</i> on alternate days for 14 days
Group II	200 mg/kg/day of CVE in 5% DMSO-distilled water <i>p.o.</i> for 14 days + 1 mL/kg of normal saline given <i>i.p.</i> on alternate days for 14 days
Group III	50 mg/kg/day of CVE 5% DMSO-distilled water <i>p.o.</i> for 14 days + 2.5 mg/kg of doxorubicin hydrochloride in normal saline given <i>i.p.</i> on alternate days for 14 days
Group IV	100 mg/kg/day of CVE in 5% DMSO-distilled water <i>p.o.</i> for 14 days + 2.5 mg/kg of doxorubicin hydrochloride in normal saline given <i>i.p.</i> on alternate days for 14 days
Group V	200 mg/kg/day of CVE in 5% DMSO-distilled water <i>p.o.</i> for 14 days + 2.5 mg/kg of doxorubicin hydrochloride in normal saline given <i>i.p.</i> on alternate days for 14 days
Group VI	400 mg/kg/day of CVE in 5% DMSO-distilled water <i>p.o.</i> for 14 days + 2.5 mg/kg of doxorubicin hydrochloride in normal saline given <i>i.p.</i> on alternate days for 14 days
Group VII	20 mg/kg/day of Vitamin C in distilled water <i>p.o.</i> for 14 days + 2.5 mg/kg of doxorubicin hydrochloride in normal saline given <i>i.p.</i> on alternate days for 14 days
Group VIII	10 mL/kg/day of distilled water <i>p.o.</i> for 14 days + 1 mL/kg of 0.9% normal saline given <i>i.p.</i>

Induction of Hepatorenal Toxicity and Treatment

The rats were randomly allotted into 8 groups of 7 rats per group. Doxorubicin-induced hepatorenal toxicity and subsequent oral treatments with 50-400 mg/kg/day CVE for 14 days were done as previously reported by Olorundare *et al.* (2020). These treatments are as indicated in Table 1. Treated rats were sacrificed and whole blood samples collected with 21G needle and 5 mL syringe directly from the heart under inhaled diethyl ether anesthesia. The liver and kidneys were excised and weighed.

Biochemical Assays

The blood samples were allowed to clot and then centrifuged at 5000 rpm to obtain separate clear sera for serum renal function parameters (Na⁺, K⁺, HCO₃⁻, Cl⁻, BUN and creatinine) and were assayed for according to the method described by Imafidon *et al.* (2019). Hepatic function parameters (AST, ALT, ALP, lipids, total protein, albumin, total bilirubin and conjugated bilirubin) were assayed using methods described by Afsar *et al.* (2019).

Measurement of Whole Body and Organ Weights

The rats were weighed on days 1 and 14 of the experiment using a digital rodent weighing scale ([®]Virgo Electronic Compact Scale, New Delhi, India). Excised organs were rinsed with normal saline, blotted with filter paper and weighed with electronic balance (Virgo Electronic Compact Weighing Scale, New Delhi, India). The weight of each organ relative to the 100 g of body weight of the rats from which the organ was excised was calculated as:

$$\frac{\text{Weight of organ (g)} \times 100}{\text{Weight of rat}}$$

Evaluation of Antioxidant Activities in Hepatic and Renal Tissues

The right lobe of the liver and right kidney was dissected out and immediately rinsed in ice cold 1.15% KCl solution in order to preserve the activities of oxidative enzymes. Thereafter, the organs were placed in clean sample bottles in an ice-packed cooler. Superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione-S-transferase (GST) activities and malonaldehyde (MDA) levels were measured using methods described by Weydert and Cullen (2010).

Histology of Liver and Kidney

Macroscopic examination was performed on the liver and kidney excised from randomly selected rats from each experimental group. After rinsing in normal saline, the organs were preserved in 10% formo-saline and processed for histological evaluation as previously described by (Slaoui and Fiette, 2011). Each organ sample was embedded in routine paraffin blocks from which 4-5 µm sections were prepared and stained with hematoxylin and eosin. The slides were examined for associated histopathological lesions using procedure earlier described by Slaoui and Fiette (2011).

Chemicals and Reagents

Doxorubicin hydrochloride (Celondoxily[®] Injection 50, CELON Laboratories PVT. Limited, Gajularamaram, Ranga Reddy District-500 090, Telangana State, India), test detection kits for the liver and renal function parameters were all procured from Sigma Chemicals Company (St. Louis, MO, USA). All other chemical and reagents used in the experiment were of analytical grade.

Statistical Analysis

Data are presented as mean ± S.E.M. of seven observations for the *in vivo* studies. Statistical analysis was done using one-way analysis of variance followed by post-hoc test, Student-Newman-Keuls test on GraphPad Prism

Version 5. Statistical significance was considered at $P < 0.05$.

RESULTS

Percentage Yield

The extraction of the pulverized dried leaves of *Clerodendrum volubile* in absolute ethanol was calculated to be 8.39% w/w.

Effect of CVE on Body Weight Changes of Doxorubicin-treated Rats

Table 2 shows the effect of repeated intraperitoneal doxorubicin injections and oral pretreatments with 50, 100, 200 and 400 mg/kg/day of CVE on the average body weight on day 1 and day 14 as well as percentage weight change (%Δwt.) of rats. Doxorubicin treatment caused significant and ($P < 0.0001$) reduction in the weight in the rats given doxorubicin only (Group I) when compared to untreated control (normal) rats (Group VIII). Treatment with 50, 100 and 200 mg/kg of CVE, significantly ($P < 0.05$, $P < 0.0001$) caused a dose related increase in the weight of rats (Table 2). Vitamin C also had similar effect on the weight gain pattern in the doxorubicin-treated rats (Table 2).

Table 2: Effect of repeated oral treatment with 50-400 mg/kg/day of CVE on the body weight of doxorubicin-treated rats

Treatment Groups	Day 1 Body Weight (g)	Day 14 Body Weight (g)	Percentage Weight Changes (% Δwt.)
I	159.9 ± 15.1	143.1 ± 19.0	-10.7 ± 04.7 ^{c-}
II	178.3 ± 18.0	191.1 ± 20.6	07.1 ± 04.1 ^{c+}
III	144.2 ± 15.8	141.5 ± 25.2	-02.4 ± 10.5 ^{a+}
IV	163.9 ± 08.0	153.0 ± 11.0	-06.6 ± 04.8 ^{a+}
V	154.0 ± 19.6	151.6 ± 23.1	-09.0 ± 05.5 ^{c+}
VI	165.2 ± 12.4	147.9 ± 12.8	-10.6 ± 05.8
VII	151.3 ± 12.9	146.0 ± 15.4	-03.5 ± 04.5 ^{a+}
VIII	163.8 ± 16.8	187.4 ± 20.2	14.5 ± 06.2

^{c-} represents a significant decrease at $P < 0.0001$ when compared to untreated (normal) negative control (Group VIII); ^{a+} and ^{c+} represent significant increases at $P < 0.05$ and $P < 0.0001$, respectively, when compared to untreated (doxorubicin-treated) negative control (Group I) values.

Effect of CVE on Relative Liver and Kidneys Weight in Doxorubicin-treated Rats

Treatment with doxorubicin only and co-treatments with 50-400 mg/kg/day of CVE induced significant ($P < 0.001$, $P < 0.0001$) increases in the relative liver weight when compared to the untreated control (Group VIII) values (Table 3). Similarly, treatment with 20 mg/kg/day of

Vitamin C caused significant ($P<0.0001$) increase in the relative organ weight in the treated rat weight (Table 3). However, the different doses of *CVE* did not reverse increase in the relative liver weight induced by doxorubicin treatment (Table 3). There were no significant alterations in the relative kidney weight with *CVE* pretreatment when compared to Group I values (Table 3).

Table 3: Effect of repeated oral treatment with 50-400 mg/kg/day of *CVE* on the relative hepatic and renal organ weight of doxorubicin-treated rats

Treatment Groups	Relative liver weight	Relative kidney weight
I	$3.59 \pm 0.24^{c+}$	0.76 ± 0.10
II	2.76 ± 0.45	0.68 ± 0.06
III	$3.41 \pm 0.41^{c+}$	0.76 ± 0.09
IV	$3.40 \pm 0.41^{c+}$	0.71 ± 0.05
V	$3.21 \pm 0.27^{b+}$	0.74 ± 0.06
VI	$3.54 \pm 0.55^{c+}$	0.73 ± 0.10
VII	3.01 ± 0.24	0.69 ± 0.09
VIII	2.79 ± 0.18	0.66 ± 0.05

b^+ and c^+ represent significant increases at $P<0.001$ and $P<0.0001$, respectively, when compared to untreated negative control (Group VIII) values and untreated positive control (doxorubicin-treated) (Group I) values

Effect of *CVE* on Serum Lipid Levels of Doxorubicin-treated Rats

Administration of doxorubicin resulted in significant ($P<0.0001$) increase in the serum TG and VLDL-c concentrations while there were no significant alterations in the serum HDL-c, LDL-c concentrations in DOX-treated rats when compared to those of untreated rats (Table 4). However, in rats pretreated with 50-400 mg/kg/day of *CVE* orally, there were significant ($P<0.05$ and $P<0.0001$) dose unrelated decreases in the serum concentration of TG and VLDL-c without significant alterations in the serum concentration of HDL-c and LDL-c when compared to doxorubicin only treated rats (Table 4). Vitamin C had similar pattern of effects on the measured serum lipids parameters (Table 4).

Effect of *CVE* on Hepatic Enzymes, Serum Proteins, and Bilirubin in Doxorubicin-treated Rats

Table 5 shows the effect of pretreatment with 50-400 mg/kg/day of *CVE* on serum hepatic enzymes (ALT, AST and ALP), and the levels of total protein (TP), albumin (ALB) and total bilirubin (TB) in doxorubicin-treated rats. Intraperitoneal injection with doxorubicin induced significant ($P<0.01$ and $P<0.0001$) increases in the serum ALT, AST and ALP levels when compared to untreated normal (Group VIII) values while causing significant ($P<0.05$) decreases in the serum TP and ALB levels when compared to untreated normal (Group VIII) values (Table

5). However, with oral pretreatment with 50-400 mg/kg/day of *CVE*, there were significant ($P<0.05$ and $P<0.0001$) reversals in the doxorubicin-related alterations of the serum hepatic enzymes, TP and ALB levels bringing these values to values recorded for the untreated control (Group I) rats (Table 5).

Effect of *CVE* on Renal Function in Doxorubicin-treated Rats

Table 6 shows the effect of pretreatment with 50-400 mg/kg/day of *CVE* on the serum electrolytes (sodium, potassium, chloride and bicarbonates), urea and creatinine in doxorubicin-treated rats. Intraperitoneal injection with doxorubicin induced significant ($P<0.001$ and $P<0.0001$) decreases in the serum Na^+ and K^+ levels when compared to untreated normal (Group VIII) values while causing significant ($P<0.05$ and $P<0.001$) increases in the serum BUN and creatinine (Cr) levels when compared to untreated normal (Group VIII) values (Table 6). However, doxorubicin treatment did not significantly alter serum HCO_3^- and Cl^- levels when compared to the untreated normal (Group VIII) values (Table 6). With oral pretreatments with 50-400 mg/kg/day of *CVE*, there were significant ($P<0.05$, $P<0.001$ and $P<0.0001$) reversals in the doxorubicin-related alterations of the serum Na^+ , K^+ , BUN and Cr bringing these values to nearly normal values (Table 6).

Effect of *CVE* on Hepatic Tissue Oxidative Stress Markers in Doxorubicin-treated Rats

Doxorubicin injection induced significant ($P<0.05$, $P<0.0001$) decreases in the GST, GPx and CAT activities and GSH levels while significantly ($P<0.05$) increasing MDA levels in the liver tissues when compared to the untreated control (Group VIII) (Table 7). However, oral pretreatments with *CVE* significantly ($P<0.05$, $P<0.001$ and $P<0.0001$) improved the hepatic GST, GPx and CAT activities while it significantly ($P<0.05$ and $P<0.001$) reduced MDA levels (Table 7).

Effect of *CVE* on Renal Tissue Oxidative Stress Markers in Doxorubicin-treated Rats

Doxorubicin injection induced significant ($P<0.05$, $P<0.001$, $P<0.0001$) decreases in the GPx and CAT activities and GSH levels in the liver tissues of doxorubicin-treated rats when compared to the untreated control (Group VIII) (Table 8). However, oral pretreatments with *CVE* significantly ($P<0.05$, $P<0.001$ and $P<0.0001$) improved the hepatic GPx and CAT activities but it significantly ($P<0.0001$) reduced MDA levels (Table 8).

Table 4: Effect of 50-400 mg/kg/day of *CVE* on serum lipid profile of doxorubicin-treated rats

Treatment Groups	TG (mmol/L)	TC (mmol/L)	HDL-c (mmol/L)	LDL-c (mmol/L)	VLDL-c (mmol/L)
I	1.3 ± 0.1 ^{c+}	2.3 ± 0.1 ^{a+}	0.6 ± 0.0	1.2 ± 0.1	1.3 ± 0.1 ^{c+}
II	0.8 ± 0.2 ^{d-}	1.8 ± 0.2 ^{d-}	0.6 ± 0.0	0.9 ± 0.1 ^{d-}	0.4 ± 0.1 ^{f-}
III	0.8 ± 0.1 ^{d-}	2.3 ± 0.1	0.7 ± 0.0	1.3 ± 0.1	0.5 ± 0.1 ^{f-}
IV	1.1 ± 0.1	2.3 ± 0.1	0.6 ± 0.0	1.2 ± 0.1	0.5 ± 0.1 ^{f-}
V	0.8 ± 0.1 ^{d-}	2.3 ± 0.1	0.6 ± 0.0	1.3 ± 0.1	0.4 ± 0.0 ^{f-}
VI	1.0 ± 0.1 ^{d-}	2.3 ± 0.2	0.6 ± 0.0	1.2 ± 0.1	0.5 ± 0.1 ^{f-}
VII	0.9 ± 0.1 ^{d-}	2.1 ± 0.1	0.6 ± 0.0	1.1 ± 0.1	0.4 ± 0.1 ^{f-}
VIII	0.6 ± 0.1	2.0 ± 0.1	0.6 ± 0.0	1.1 ± 0.0	0.3 ± 0.0 ^{f-}

^{a+} and ^{c+} represent significant increases at $P < 0.05$ and $P < 0.0001$, respectively, when compared to untreated negative control (Group VIII) value while ^{d-} and ^{f-} represent significant decreases at $P < 0.05$ and $P < 0.0001$ when compared to untreated positive (doxorubicin treated only) control (Group I) values, respectively. TG = serum triglyceride; TC = serum total cholesterol; HDL-c = serum high density lipoprotein-cholesterol; LDL-c = low density lipoprotein-cholesterol; VLDL-c = very low-density lipoprotein-cholesterol. n=7.

Table 5: Effect of 50-400 mg/kg/day of *CVE* on serum hepatic enzymes, proteins and total bilirubin of doxorubicin-treated rats

Treatment Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	TP (g/L)	ALB (g/L)	TB (mg/dL)
I	154.7 ± 6.3 ^{c+}	590.9 ± 92.0 ^{c+}	184.1 ± 47.4 ^{a+}	49.7 ± 1.7 ^{a-}	23.6 ± 0.7 ^{a-}	0.2 ± 0.0
II	104.9 ± 15.6 ^{d-}	595.4 ± 84.1 ^{d-}	141.1 ± 12.5	66.3 ± 4.1 ^{d+}	28.3 ± 1.4 ^{d+}	0.2 ± 0.0
III	71.9 ± 2.3 ^{d-}	454.7 ± 60.1	106.4 ± 13.2 ^{d-}	66.6 ± 2.7 ^{d+}	29.3 ± 0.9 ^{f-}	0.2 ± 0.0
IV	47.4 ± 4.3 ^{f-}	241.3 ± 34.3 ^{f-}	111.4 ± 17.3 ^{d-}	54.3 ± 5.5	23.1 ± 1.7 ^{a-}	0.2 ± 0.0
V	74.4 ± 6.8 ^{f-}	422.1 ± 43.8 ^{f-}	107.9 ± 22.0 ^{d-}	60.0 ± 1.3 ^{d+}	26.3 ± 0.3 ^{d+}	0.1 ± 0.0
VI	51.4 ± 3.0 ^{f-}	351.3 ± 3.0 ^{f-}	88.4 ± 3.9 ^{e-}	54.4 ± 1.2	25.6 ± 1.2	0.2 ± 0.1
VII	59.9 ± 11.9 ^{f-}	272.0 ± 41.2 ^{e-}	132.6 ± 16.9	63.6 ± 7.8 ^{d+}	27.1 ± 1.1 ^{d+}	0.2 ± 0.1
VIII	57.6 ± 5.8	225.4 ± 20.7	136.7 ± 12.3	63.4 ± 1.7	28.7 ± 0.9	0.1 ± 0.0

^{a+} and ^{c+} represent significant increases at $P < 0.05$ and $P < 0.0001$, respectively, when compared to untreated negative control (Group VIII) values while ^{d-}, ^{e-} and ^{f-} represent significant decreases at $P < 0.05$, $P < 0.001$ and $P < 0.0001$, respectively, when compared to untreated positive (doxorubicin treated only) control (Group I) values; ^{d+} represents a significant increase at $P < 0.05$ when compared to untreated positive (doxorubicin treated only) control (Group I) values. ALT = serum alanine aminotransferase; AST = serum aspartate aminotransferase; ALP = serum alkaline phosphatase; TP = serum total protein; serum albumin; serum total bilirubin. n=7.

Table 6: Effect of 50-400 mg/kg/day of *CVE* on serum electrolytes, urea and creatinine (Cr) of doxorubicin-treated rats

Group s	Na ⁺ (mmol/L)	K ⁺ (mmol/L)	Cl ⁻ (mmol/L)	HCO ₃ ⁻ (mmol/L)	Urea (mmol/L)	Creatinine (mmol/L)
I	154.7±6.3 ^{c+}	590.9±92.0 ^{c+}	184.1±47.4 ^{a+}	49.7±1.7 ^{a-}	23.6±0.7 ^{a-}	0.2±0.0
II	104.9±15.6 ^{d-}	595.4±84.1 ^{d-}	141.1±12.5	66.3±4.1 ^{d+}	28.3±1.4 ^{d+}	0.2±0.0
III	71.9±2.3 ^{d-}	454.7±60.1	106.4±13.2 ^{d-}	66.6±2.7 ^{d+}	29.3±0.9 ^{f-}	0.2±0.0
IV	47.4±4.3 ^{f-}	241.3±34.3 ^{f-}	111.4±17.3 ^{d-}	54.3±5.5	23.1±1.7 ^{a-}	0.2±0.0
V	74.4±6.8 ^{f-}	422.1±43.8 ^{f-}	107.9±22.0 ^{d-}	60.0±1.3 ^{d+}	26.3±0.3 ^{d+}	0.1±0.0
VI	51.4±3.0 ^{f-}	351.3±3.0 ^{f-}	88.4±3.9 ^{e-}	54.4±1.2	25.6±1.2	0.2±0.1
VII	59.9±11.9 ^{f-}	272.0±41.2 ^{e-}	132.6±16.9	63.6±7.8 ^{d+}	27.1±1.1 ^{d+}	0.2±0.1
VIII	57.6±5.8	225.4±20.7	136.7±12.3	63.4±1.7	28.7±0.9	0.1±0.0

^{a+} and ^{c+} represent significant increases at $P < 0.5$ and $P < 0.0001$, respectively, when compared to untreated negative control (Group VIII) value while ^{d-}, ^{e-} and ^{f-} represent significant decreases at $P < 0.05$, $P < 0.001$ and $P < 0.0001$ when compared to untreated positive (doxorubicin treated only) control values, respectively; ^{d+} represents a significant increase at $P < 0.05$ when compared to untreated positive (doxorubicin treated only) control (Group I) values. n=7.

Effect of *CVE* on the Histology of Liver tissues of Doxorubicin-treated Rats

Intraperitoneal injection with doxorubicin induced portal vascular congestion and hepatocyte vacuolation (Figure 1)

when compared to normal rat hepatocyte (Figure 2). However, there were significant improvements in the DOX-induced hepatic distortion with oral *CVE* pretreatments especially at 400 mg/kg/day of *CVE* which was marked by mildly dilated hepatic sinusoids with bilirubin plugs (Figure 3). Similarly, oral pretreatment

with vitamin C also improved DOX-induced hepatic histological distortion which was marked by mild portal

vascular congestion and scattered pyknotic nuclei in the hepatocytes (Figure 4).

Table 7: Antioxidant activities of 50-400 mg/kg/day of *CVE* in doxorubicin-treated rat hepatic tissue

Groups	GSH (U/mg protein)	GST (U/mg protein)	GPx (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (U/mg protein)
I	85.7±5.2 ^{a-}	31.1±0.1 ^{c-}	79.5±4.8 ^{c-}	1.7±0.1	7.7±1.2 ^{a-}	3.4±0.4 ^{a+}
II	53.5±3.3 ^{f-}	30.6±1.4	52.8±3.2 ^{e-}	1.5±0.1	5.5±0.3 ^{b-}	3.9±0.3 ^{a-}
III	78.5±5.7 ^{d+}	31.6±0.4	77.4±5.6 ^{d+}	1.2±0.0 ^{f-}	6.6±0.2 ^{a-}	3.3±0.2 ^{d-}
IV	84.8±8.2 ^{e+}	29.7±0.7	82.4±8.3 ^{d+}	1.4±0.1 ^{e-}	7.5±0.5	2.7±0.5 ^{e-}
V	50.6±3.3 ^{f-}	35.3±0.8 ^{d+}	53.9±4.0 ^{e-}	1.2±0.0 ^{f-}	7.2±0.3	3.0±0.2 ^{d-}
VI	65.5±3.4 ^{d-}	32.9±1.0	68.0±3.0 ^{d+}	1.6±0.0	9.1±0.3 ^{d+}	6.6±0.8
VII	122.7±1.8 ^{f+}	43.3±0.7 ^{f+}	134.0±2.0 ^{f+}	1.8±0.0 ^{d+}	10.9±0.2 ^{e+}	3.4±0.3 ^{d-}
VIII	105.4±5.9	41.9±1.6	115.1±6.4	1.8±0.0 ^{d+}	9.0±0.3 ^{d+}	3.0±0.2 ^{d-}

^{a-} and ^{c-} represent significant decreases at $P<0.05$ and $P<0.001$, respectively, when compared to untreated negative control (Group VIII) values. ^{d+}, ^{e+} and ^{f+} represent significant increases at $P<0.05$, $P<0.001$ and $P<0.0001$, respectively while ^{d-}, ^{e-} and ^{f-} represent significant decreases at $P<0.05$, $P<0.001$ and $P<0.0001$, respectively when compared to untreated positive (doxorubicin treated only) control (Group I) values respectively. GSH = reduced glutathione; GST = glutathione transferase; GPx = glutathione peroxidase; SOD = superoxidase dismutase; CAT = catalase; MDA = malondialdehyde dehydrogenase n=7.

Table 8: Antioxidant activities of 50-400 mg/kg/day of *CVE* in doxorubicin-treated rat renal tissue

Treatment Groups	GSH (U/mg protein)	GST (U/mg protein)	GPx (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (U/mg protein)
I	90.1±4.3 ^{a-}	28.7±0.1	83.6±4.0 ^{b-}	2.0±0.3	8.1±0.5 ^{c-}	3.3±0.5
II	57.3±2.0 ^{f-}	19.2±1.4 ^{f-}	56.9±1.9 ^{f-}	1.2±0.1 ^{d-}	6.8±0.3	3.0±0.3
III	106.5±3.1 ^{d+}	20.0±0.6 ^{f-}	105.0±3.0 ^{f+}	1.1±0.0 ^{d-}	7.3±0.5	3.6±0.3
IV	83.6±2.9	30.1±0.4	81.1±3.0	2.2±0.3	10.2±0.5	3.6±0.3
V	57.7±1.7 ^{f-}	33.2±0.8 ^{e+}	57.0±1.7 ^{f-}	2.0±0.2	10.8±1.0	7.6±0.4 ^{f+}
VI	58.2±7.1 ^{f-}	35.2±1.2 ^{f+}	56.1±6.2 ^{f-}	3.0±0.2	13.4±1.2 ^{a+}	6.8±1.0 ^{f+}
VII	103.1±3.9	29.2±1.3	101.6±3.9 ^{e+}	3.4±0.1	21.7±0.7 ^{f+}	3.2±0.2
VIII	104.1±3.8	25.9±0.3	101.7±3.7	2.4±0.2	18.5±1.6	5.3±0.3

^{c+} represent a significant increase $P<0.0001$ when compared to untreated negative (normal) control value while ^{b-} represents significant decreases at $P<0.001$ and $P<0.0001$ when compared to untreated negative (normal) control value control values. ^{d+}, ^{e+} and ^{f+} represent significant increases at $P<0.05$, $P<0.001$ and $P<0.0001$, respectively when compared to untreated positive (doxorubicin treated only) control values respectively while ^{d-}, ^{e-} and ^{f-} represent significant increases at $P<0.05$, $P<0.001$ and $P<0.0001$, respectively when compared to untreated positive (doxorubicin treated only) control values respectively. GSH = reduced glutathione; GST = glutathione transferase; GPx = glutathione peroxidase; SOD = superoxidase dismutase; CAT = catalase; MDA = malondialdehyde dehydrogenase. n=7.

Effect of *CVE* on the Histology of Renal Tissues of Doxorubicin-treated Rats

Intraperitoneal DOX injection resulted in glomerular atrophy, tubular degeneration with lymphocytic infiltration (Figure 5) compared to normal rat renal architecture (Figure 6). However, oral *CVE* pretreatment, caused remarkable improvements in the DOX-induced renal histological distortion especially at 400 mg/kg/day of *CVE* which was marked by occasional glomerular atrophy and increased Bowman space with normal adjacent proximal convoluted tubules (Figure 7). Similarly, oral pretreatment with vitamin C also improved DOX-induced renal histological distortion and this was marked by occasional glomerular cellularity (Figure 8).

Figure 1: A cross sectional representative of DOX only-treated rat liver showing moderate portal vascular congestion (indicated in blue thick arrow) and hepatocyte cytoplasmic ballooning/vacuolation (in black thin arrows) indicative of hepatic fatty degeneration (x400 magnification, Hematoxylin-Eosin stain).

Figure 2: A cross sectional representative of the normal rat liver showing normal hepatic histo-architecture (x100 magnification, Hematoxylin-Eosin stain).

Figure 3: A cross sectional representative of 400 mg/kg/day of *CVE* pretreated, DOX-treated liver showing mildly dilated hepatic sinusoids (indicated in blue thick arrows) with bilirubin plugs (indicated in red thin arrows) (x400 magnification, Hematoxylin-Eosin stain).

Figure 4: A cross sectional representative of 20 mg/kg/day of ascorbic acid pretreated, DOX-treated rat liver showing by mild portal vascular congestion (indicated in blue thick arrow) and scattered pyknotic nuclei of hepatocytes (indicated in black thick arrows) suggestive of hepatic recovery from doxorubicin toxicity (x400 magnification, Hematoxylin-Eosin stain).

Figure 5: A cross sectional representative of DOX only-treated rat kidney showing glomerular atrophy (indicated in yellow thick arrows), tubular degeneration with global lymphocytic infiltration (indicated in yellow thin arrows) (x100 magnification, Hematoxylin-Eosin stain).

Figure 6: A photomicrograph of cross sectional representative of a normal rat kidney showing normal glomeruli and tubules (x400 magnification, Hematoxylin-Eosin stain).

Figure 7: A photomicrograph of cross-sectional representative of 400 mg/kg/day *CVE* pretreated, DOX-treated rat kidney showing occasional glomerular atrophy (indicated in yellow thick arrows) and increased bowman space with normal adjacent proximal convoluted tubules (indicated in green thick arrows) (x100 magnification, Hematoxylin-Eosin stain).

Figure 8: A photomicrograph of cross-sectional representative of 20 mg/kg/day of vitamin C pretreated, DOX-treated rat kidney showing occasional glomerular cellularity (indicated in orange thick arrow) and global tubular congestions (indicated in orange thin arrows) (x400 magnification, Hematoxylin-Eosin stain).

DISCUSSION

Hepatorenal toxicities remain one of the most fatal dose-limiting toxicities often associated with DOX treatment (Guo *et al.*, 2016; Kamble and Patil, 2018; Kuzu *et al.*, 2019; Al-Shabanah *et al.*, 2019). In the study, DOX-induced hepatorenal toxicity was established after repeated intraperitoneal DOX injections for 14 days. The hepatic and renal toxicities were measured by the effect of DOX and concomitant *CVE* treatments on the hepatic and renal function parameters, respectively. The hepatic function endpoints included serum liver enzymes (ALT, AST, ALP and GGT), total proteins, albumin, total bilirubin and lipid profile while the renal function endpoints included serum electrolytes, blood urea nitrogen and creatinine (Ayla *et al.*, 2011). Classically, elevated hepatic transaminase activities are considered reliable index end-points of hepatotoxicity linked to oxidative stress, thus, indicating the source of the hepatic injury (pre-hepatic, intra-hepatic or post-hepatic origin) (Hall and Cash, 2012; Contreras-Zentella and Hernández-Muñoz, 2016). Of the hepatic transaminases, ALT which is mainly and abundantly produced and confined to hepatocytes remains the most specific marker of hepatocellular injury (Giannini *et al.*, 2005). Although, AST is present in hepatocytes, its cytosolic isoenzyme is abundantly present in skeletal muscle, heart muscle and kidney tissue also making it less reliable; while ALP which is synthesized in the membranes of cells lining bile ducts and canaliculi is released in response to the accumulation of bile salts or cholestasis (Hall and Cash, 2012). Similarly, gamma-glutamyl transferase (GGT) is abundantly present in the liver, kidney, pancreas and intestine. It is found in the microsomes of hepatocytes and biliary epithelial cells.

Thus, serum elevation of GGT in association with a rise in ALP is highly suggestive of a biliary tract obstruction

(Hall and Cash, 2012). Intraperitoneal DOX injections resulted in DOX-induced hepatorenal toxicities which were marked by profound increases in the serum transaminases (ALT and AST), ALP, and profound decreases in the serum TP and ALB without any significant alterations in TB levels which strongly suggest that DOX injury in this study was hepatobiliary in nature. Thus, the results of this study are consistent with those previously reported (Damodar *et al.*, 2014; Chauhan *et al.*, 2016; Anber, 2018). However, the fact that oral pretreatments with *CVE* profoundly prevented elevations in the serum hepatotoxicity parameters showed that *CVE* has mitigating effect against DOX-induced hepatotoxicity. These biochemical ameliorations were corroborated by improvement in the histological lesions previously marked by centrilobular necrosis and hepatocyte fatty degeneration. In the same vein, repeated intraperitoneal DOX injection was associated with nephrotoxicity which was marked by significant alterations in serum electrolytes, serum creatinine and serum blood urea nitrogen (Ayla *et al.*, 2011; Rashid *et al.*, 2013; Nagai *et al.*, 2018). Again, these renal function alterations were effectively attenuated by oral pretreatments with *CVE*, and these biochemical attenuations were similar to those also reported by Molehin (2020) with oral pretreatment of methanol leaf extract of *Clerodendrum volubile* against DOX-induced nephrotoxicity in rats. Improvements in these measured renal function parameters were also corroborated by improvements in the DOX-induced renal histological lesions. However, DOX-induced renal histological lesions could include glomerular vacuolation, sclerosis and atrophy as well as tubular dilatation and necrosis (de Jonge and Verweij, 2006; Lee and Harris, 2011; Kamble and Patil, 2018).

Other notable findings of this study are the effects of DOX and *CVE* pretreatments on the oxidative stress markers in the hepatic and renal tissues. Doxorubicin, as well as others anthracyclines, are known to also undergo one- and two-electron reduction, being members of the quinone family, and as such generate highly reactive free radicals that can damage macromolecules and lipid membranes through lipid peroxidation mechanism (Injac and Strukelj, 2008). The generation of highly reactive free radicals as metabolites of DOX metabolism is considered to be the primary mechanism of DOX-induced organ toxicities (Quiles *et al.*, 2002; Yilmaz *et al.*, 2005). The mechanisms responsible for DOX-induced hepatorenal toxicities are complex. In recent studies, oxidative stress has been considered as one major mechanism of oxidation-induced hepatorenal toxicities. It is reported that DOX induces oxidative stress that is characterized by ROS accumulation and the decrease of antioxidant defense on oxygen imbalance, culminating in attacking and oxidizing DNA and then inducing liver cell apoptosis (Wang *et al.*, 2019;

Song *et al.*, 2019). In the present study, DOX-induced hepatorenal toxicities resulted in oxidative stress as evidenced by the reduced GSH levels, reduced GST, GPx and CAT activities; and a high MDA level in the hepatic and renal tissues examined. However, these were effectively ameliorated by *CVE* and these findings are also similar to those earlier reported by Molehin (2020) in *Clerodendrum volubile* methanol leaf extract-pretreated DOX-intoxicated renal tissues.

Previous studies have reported *CVE* to contain abundant secondary metabolites such as saponins, flavonoids and phenols amongst others phytochemicals (Ogunwa *et al.*, 2016; Senjobi *et al.*, 2017; Afolabi *et al.*, 2019). Currently, much attention has been paid to the use of plant secondary metabolites otherwise known as phytochemicals as a protective strategy against DOX-induced organ toxicities including hepatorenal toxicities. Flavonoids are a group of polyphenol compounds that exhibit numerous pharmacological properties that are beneficial for human health partly due to their radical-scavenging, antioxidative, and iron-chelating properties (Kaiserová *et al.*, 2007; Du *et al.*, 2010). Similarly, proanthocyanidins have been reported to ameliorate doxorubicin-induced immunosuppression and hepatorenal toxicity (Abdel-Sattar *et al.*, 2019). Thus, these phytochemicals are considered potential protectors against DOX-induced hepatorenal toxicities and as such could significantly improve the therapeutic index of DOX (Du *et al.*, 2010; Vaclavikova *et al.*, 2008). The abundant presence of these phytochemicals in *CVE* could be responsible for the ameliorative effects of *CVE* in DOX-induced hepatorenal toxicities.

CONCLUSION

It can safely be concluded that *Clerodendrum volubile* extract offers protection against DOX-induced hepatorenal toxicities which is mediated via suppression of oxidative stress, free radical scavenging activity and/or antioxidant mechanisms.

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