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Research Article

VASORELAXANT MECHANISM(S) OF *CLERODENDRUM VOLUBILE* ETHANOL LEAF EXTRACT IN NORMAL AND DOXORUBICIN-TREATED ENDOTHELIUM INTACT AORTIC RINGS

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ABSTRACT

Objectives: Doxorubicin (DOX) is a highly effective antibiotics anthracycline cytotoxic agent with a broad spectrum of activity in the treatment of solid and hematological malignancies. However, DOX is notorious for inducing cardiotoxicity and vascular dysfunction as its common off-target side effects. This study evaluated the possible vasorelaxant activity and mechanism(s) of action of *Clerodendrum volubile* ethanol leaf extract (CVE) in normal and DOX-pretreated endothelium intact aortic rings in Physiological Salt Solution (PSS) *in vitro*.

Methods: The responses were recorded isometrically by an organ bath connected to Data Capsule Acquisition System. Effects of CVE on phenylephrine-precontracted endothelium intact rat aortic rings and the influence of the respective blockers for adrenergic, cholinergic, calcium channel, and prostacyclin receptors were investigated to unveil the possible underlying vasorelaxant mechanism(s) of CVE.

Results: Our findings showed that CVE significantly induced vasorelaxation in phenylephrine hydrochloride (PE) and KCl precontracted endothelium intact aortic rings in a concentration-dependent manner. Furthermore, the CVE-induced vasorelaxation in PE- and KCl-precontracted aortic rings were inhibited by pre-incubation with atropine and indomethacin indicating that the vasorelaxant effect of CVE was profoundly mediated through cholinergic and prostacyclin mechanisms.

Conclusion: Overall, results of this study report for the first time the vasorelaxant effect of CVE in isolated endothelium-intact doxorubicin-treated aortic rings of normotensive rats which was probably cholinergic and prostacyclin-mediated. Thus, results of this study provide further insight into the cardioprotective mechanism of CVE in doxorubicin-induced cardiotoxicity beyond the antioxidant and anti-apoptosis mechanisms that have been previously reported.

Keywords: Doxorubicin, Endothelium intact aortic rings, Vasorelaxant mechanisms, *Clerodendrum volubile*, Ethanol leaf extract.

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INTRODUCTION

Doxorubicin remains one of the most widely accepted and used anthracycline antibiotics cytotoxic drugs in clinical settings especially in the management of cancers such as lymphomas, Wilm's tumor, breast, lung, thyroid, ovarian, testicular, and gastric carcinomas as well as hematological malignancies since its first discovery in the 1960s [1-3]. However, despite its wide clinical use, doxorubicin is notorious for its cardiotoxicity profile [4], thereby, increasing the morbidity and mortality as well as reducing the quality of life of cancer patients on it [5,6]. The mechanisms of doxorubicin-induced cardiotoxicity is known to be multifactorial and includes mitochondrial reactive oxygen species, calcium overload through increased gene expression and activation of ubiquitin-ligase-proteasome system, as well as up-regulated cardiomyocyte apoptosis [7-9]. It is estimated that the level of oxidative stress generated by doxorubicin in the heart tissue is about 10 folds higher than that generated in the liver, kidney and spleen combined [5,9]. Similarly, accumulated evidence-based studies have shown that doxorubicin-mediated endothelial injury is a strong triggering factor in the etiology, development and course of doxorubicin-induced cardiomyopathy, mediated primarily through decreased biosynthesis, release as well as decreased activity of major endothelial regulating factors and upregulating endothelial apoptosis [10]. This has, thus, made the endothelium a novel target for the early detection, effective management and prevention of doxorubicin-mediated cardiomyopathy (Luu *et al.* 2018) [10]. However, there are cumulative facts indicating that antioxidants could play a leading role in effectively preventing doxorubicin-mediated cardiomyopathy and vasculopathy [11-13]. Thus, plant-derived antioxidants especially catechin- and flavonoids-rich medicinal plants and herbs have been reported to ameliorate doxorubicin-induced cardiac injury [11,13-15].

Clerodendrum volubile P. Beauv (commonly known as White butterfly and belonging to the *Labiatae* family) is a climbing vegetable that flourishes in the thick forests of West Africa [17]. In the Niger-Delta region of Nigeria where the plant is predominantly grown locally for consumption either as green leafy vegetable or as food condiment to improve soup taste, it is used for the local management of gouty arthritis, rheumatism, dropsy, swellings/edema, and ulcers [16,18]. *C. volubile* leaf extracts are reported to contain secondary metabolites such as alkaloids, flavonoids, saponins, anthraquinone, and cardiac glycoside [19]. *C. volubile*'s phenolic-rich leaf fractions have been reported to possess antihyperglycemic activity mediated via α-amylase and α-glucosidase inhibitory activities and improvement in glucose tolerance [16]. This phenolic-rich fraction is also reported to mediate antihypertensive activity through angiotensin I converting enzyme inhibition [17]. Similarly, *C. volubile*'s antioxidant, immunomodulatory, anti-inflammatory, and cytotoxic activities are well documented in the literature [19-22]. High flavonoids content have been implicated in the

reported potent antioxidant activities of *C. volubile*'s polyphenol-rich fraction [22-25].

Recently we reported antidotal activity of *C. volubile* ethanol leaf extract against doxorubicin-mediated cardiotoxicity [26] as well as its ameliorative effect in doxorubicin-mediated hepatorenal dysfunction [27] both in rats.

METHODS

Plant materials collection and extraction

Fresh mature *C. volubile* whole plant was obtained from same source reported in our earlier study [26]. The plant's botanical identification, authentication, and referencing were equally done as previously reported [26]. Similarly, processing and solvent extraction of dried pulverized *C. volubile* leaves as well as calculation of %yield was also done following earlier reported standard method [26].

Experimental animals and their care

Male Wistar Albino rats (age: 8–12 weeks old and body weight: 130–190 g) were obtained from the Animal House of the Lagos State University College of Medicine, Ikeja, Lagos State, Nigeria after ethical clearance for the study was granted. The rats were sham-handled in accordance with international principles guiding the Use and Handling of Laboratory Animals [28] and fed standard rat feed and potable water *ad libitum* under standard laboratory conditions.

Experimental treatment with doxorubicin

Before commencement of the experiment, rats were randomly allotted into two groups of five rats for each group. This was done such that the weight difference within and between groups does not exceed ±20% of the average weight of the sample population used for the study.

Groups I rats which served as untreated control were orally pretreated with 10 ml/kg/day of distilled water and equally treated with 1 ml/kg of 0.9% normal saline intraperitoneal while Group II rats were pretreated with 10 ml/kg/day of distilled water 3 h before intraperitoneally administering 2.5 mg/kg of doxorubicin hydrochloride dissolved in 0.9% normal saline administered on alternate days for 14 days [26,27]. On the last day of the experiment, treated rats were weighed and later fasted overnight but drinking water was made available *ad libitum*.

Drugs and reagents

Phenylephrine hydrochloride, acetylcholine chloride, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), potassium chloride (KCl), and sodium nitroprusside (SNP) were all sourced from Sigma-Aldrich Inc., St. Louis, USA, while nifedipine monochloride was from Bayer Plc, United Kingdom. Doxorubicin hydrochloride (®Celondoxily Injection 50) was purchased from CELON Laboratories Pvt. Ltd, Telangana State, India. All the drugs were dissolved and stock solutions were prepared in distilled water.

In vitro vasorelaxant effect of *Clerodendrum volubile* ethanol leaf extract (CVE) on endothelium-intact aortic rings of normal rats

Preparation and mounting of endothelium-intact aortic rings of normal and doxorubicin-treated rats

The rats were sacrificed humanely by cervical dislocation after light diethyl ether inhalation. The thoracic aorta was briskly identified, freed of adjoining connective tissue, dissected out *en bloc* and placed in a petri-dish containing physiological salt solution (PSS). The aortic lumen was gently flushed with PSS and sectioned into 2 mm ring segments. Each aortic ring was suspended in a 50 ml jacketed tissue bath containing PSS with the following composition: NaCl - 118.0 mmol/L, KCl - 4.7 mmol/L; KH_2PO_4 - 1.2 mmol/L; $MgSO_4$ - 1.2 mmol/L; $NAHCO_3$ - 15.0 mmol/L, $CaCl_2$ - 1.6 mmol/L, and glucose - 11.5. mmol/L. The tissue baths, maintained at 37°C and pH=7.4, were bubbled with a mixture of 95% O_2 and 5% CO_2. To record isometric tension, aortic rings were mounted on two stainless steel hooks, one fixed to the base of the inner chamber and the other to a force transducer (model 7004; Ugo Basile, Varese, Italy) connected to Data Capsule Acquisition System Model 17400 for recording of isometric contractions. The optimal tension, determined by preliminary experiments, was that which gave the greatest response to phenylephrine (10^{-7} M). Initially, a tension of 2 g (100%) was applied, and aortic rings were allowed to equilibrate for 90 min [29]. Thirty minutes after mounting the tissue, contractile responses were tested with 10^{-7} M phenylephrine. After exposure to 10^{-7} M phenylephrine or 10^{-7} M acetylcholine, tissues were rinsed 3 times with PSS to restore basal tension.

Experimental protocol

Concentration response of phenylephrine and KCl precontracted endothelium-intact aortic rings to CVE

Thirty minutes following the restoration of basal tension, 10^{-7} M phenylephrine was added to endothelium intact rat aortic rings, which elicited a steady contraction after 30 min. Thereafter, graded doses of CVE at 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.0 mg/ml, and 1.2 mg/ml were added cumulatively into bath solution. The effect of each concentration was allowed to reach a steady level before the addition of the next dose [29] and this was done at intervals of approximately 15 min. Tension was expressed as a percentage of phenylephrine-induced contraction (3.54 ± 0.25 g, 100%). Similar procedures described were also followed for precontraction of the endothelial intact rat aortic rings with 60 mM KCl.

Concentration response of acetylcholine pretreated endothelium-intact aortic rings to CVE

Thirty minutes following restoration of basal tension, 10^{-7} M acetylcholine, a positive control of vasorelaxation, was added to the endothelium intact rat aortic rings, following which graded doses of CVE at 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.0 mg/ml, and 1.2 mg/ml were added cumulatively into bath solution at intervals of approximately 10 min. Tension was expressed as a percentage of basal contraction (2.0 ± 0.16 g, 100%).

Eliciting the possible mechanism(s) of vasorelaxant action of CVE on aortic rings

Since CVE caused significant concentration-related vasorelaxation on phenylephrine-precontracted rat aortic segments, an attempt was made to elicit the vasorelaxant mechanism(s) involved. In an attempt to do this, 3 min after adding 10^{-7} M phenylephrine, aortic rings with endothelium were preincubated for 30 min with various antagonists to elicit the possible vasorelaxant mechanism(s) of CVE. In these experiments, the relaxation responses to sodium nitroprusside, indomethacin, and atropine were carried out. The endothelium intact aortic rings were incubated with CVE at a concentration of 0.4 mg/ml for 15 min. After the 15 min incubation period, the rings were pre-contracted with 10^{-7} M phenylephrine, and when the phenylephrine-induced contraction had reached stable plateau, cumulative doses of sodium nitroprusside (10^{-9}–10^{-5} M) were added. In other experiments, quiescent aortic rings with intact endothelium (not pre-contracted) were incubated with L-NAME (10 M to block the NO-mediated component of the response to CVE for 15 min. After the 15 min incubation period, the rings were pre-contracted with 10^{-7} M phenylephrine, after which cumulative doses of extract (0.4–1.2 mg/ml) were added to the organ bath [29]. This procedure was repeated for calcium channel blocker (nifedipine), nitric oxide synthase inhibitor (N[gamma]-nitro-L-arginine methyl ester, L-NAME), prostaglandin inhibitor (indomethacin), and anticholinergic (atropine) to explore possible vasorelaxant effect of CVE through calcium channel, nitric oxide, prostacyclin, and cholinergic mechanisms, respectively.

In vitro tissue studies of vasorelaxant effect of CVE on endothelium-intact aortic rings of doxorubicin-treated rat

Same procedures as described for endothelium-intact rat aortic rings of normal and untreated rats were repeated in the endothelium intact aortic rings of rats intoxicated with cumulative dose of 15 mg/kg doxorubicin over 2 weeks.

Data analysis

Data are expressed as mean±S.E.M of four observations. Statistical analysis was performed using a one-way repeated-measures analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test. Statistical significance was considered at $P < 0.05$. The statistical analysis was performed with Microsoft Excel (USA).

RESULTS

%Yield

The calculated yield was 8.39%. The resultant residue was a dark color, sticky and jelly-like, and sweet-smelling residue which was insoluble in water but methanol and ethanol.

Vasorelaxant effect of CVE on endothelium-intact aortic ring of normal rats

Effect of CVE on phenylephrine induced contraction in endothelium-intact aortic ring

After 10^{-7} M phenylephrine induced steady contraction, CVE induced a significant ($P < 0.05$) concentration-dependent relaxation in phenylephrine pre-contracted endothelium-intact aortic rings (Figs. 1a, b and 2a, b). The minimal and maximal relaxation responses in phenylephrine-contracted rings were 18.5 ± 0.65% and 49.6 ± 3.6%, respectively.

Effect of CVE on KCl-induced contraction in endothelium-intact aortic ring of normal rat

Similarly, CVE ethanol leaf extract also produced a significant ($P < 0.05$) concentration-dependent reduction of high K^+-induced contraction in the endothelium-intact aortic arteries. The percentage of relaxation response was 36.0 ± 1.5% ($P < 0.05$) (Fig. 3).

Relaxation response to 0.4-1.2 mg/ml of CVE on endothelium intact rat aortic ring of normal rat after 15 min incubation with acetylcholine

Fig. 4 depicts the effect of CVE on the maximum relaxation response of endothelium-intact aortic ring pre-contracted with 10^{-7}M phenylephrine in presence of 10^{-4} M acetylcholine. There was a significant ($P < 0.05$) concentration-dependent vasorelaxant effect induced by 0.4–1.2 mg/ml of CVE with the percentage maximum relaxation response recorded at the maximum concentration of the extract (1.2 mg/ml) which was comparable to that induced by acetylcholine (Fig. 4).

Relaxation effect of sodium nitroprusside on aortic rings of normal rat after 15 min incubation in presence of CVE

10^{-9}–10^{-5} M sodium nitroprusside was added cumulatively to aortic ring pre-contracted with 10^{-7}M phenylephrine after 15 min incubation in the presence of 0.4–1.2 mg/ml of CVE (Fig. 5). The relaxation caused by sodium nitroprusside was not significantly ($P > 0.05$) different from that of endothelium-intact aortic rings (Fig. 5).

Relaxation response to CVE after calcium channel inhibition by nifedipine on endothelium intact aortic ring of normal rat

Fig. 6 depicts the effect of CVE on the maximum relaxation response of endothelium-intact aortic ring pre-contracted with 10^{-7} M phenylephrine in presence of the calcium channel blocker (nifedipine). Comparison between the two groups shows that there was no significant change ($P > 0.05$) in the percentage maximum relaxation response between the endothelium-intact aortic rings pre-treated with nifedipine and endothelium-intact aortic rings treated with 0.4–1.2 mg/ml of CVE (Fig. 6).

Relaxation response to CVE after NOS inhibition by L-NAME in endothelium-intact aortic ring of normal rat

Fig. 7 depicts the effect of CVE on the maximum relaxation response of endothelium-intact aortic ring pre-contracted with 10^{-7} M phenylephrine in presence or absence of L-NAME. Comparison between the two groups

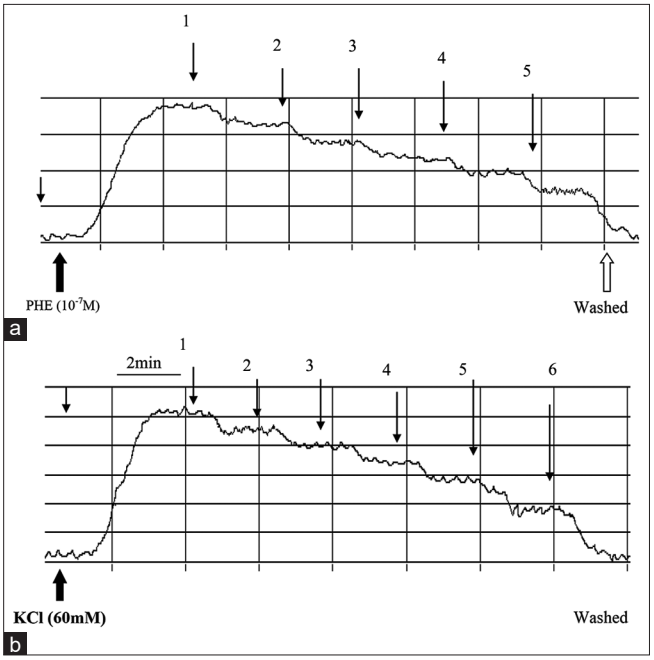

Fig. 1: (a and b) Typical tracings showing the vasorelaxant effects of graded concentration of *Clerodendrum volubile* ethanol leaf extract (CVE) on (a) Phenylephrine (PHE)- induced (b) KCl-induced contraction in the endothelium intact aortic ring preparation obtained from normotensive rat. Arrows 1-6 represent cumulatively administered CVE doses (0.4, 0.6, 0.8, 1.0, and 1.2 mg/ml, respectively). PHE, KCl, and CVE were washed out at the open upward arrow

shows that there was no significant change ($P > 0.05$) in the percentage maximum relaxation response between the endothelium-intact aortic rings pre-treated with L-NAME and endothelium-intact aortic rings treated with 0.4–1.2 mg/ml of CVE (Fig. 7).

Relaxation response to CVE after cholinergic inhibition with atropine in endothelium-intact aortic rings of normal rat

Fig. 8 depicts the effect of CVE on the maximum relaxation response of endothelium-intact aortic ring pre-contracted with 10^{-7} M phenylephrine in the presence of atropine. Comparison between the two groups shows that there was a significant ($P < 0.05$) concentration-dependent vasorelaxant effect induced by 0.4–1.2 mg/ml of CVE with the percentage maximum relaxation response recorded at the maximum concentration of the extract which was comparable to that induced by atropine (Fig. 8).

Relaxation response to CVE after prostacyclin inhibition with indomethacin in endothelium-intact aortic rings of normal rat

Fig. 9 depicts the effect of CVE on the maximum relaxation response of endothelium-intact aortic ring pre-contracted with 10^{-7} M phenylephrine in the presence of indomethacin. Comparison between the two groups shows that there was a significant ($P < 0.05$) concentration-dependent vasorelaxant effect induced by 0.4–1.2 mg/ml of CVE with the percentage maximum relaxation response recorded at the maximum concentration of the extract which was comparable to that induced by indomethacin (23.6 ± 1.7) (Fig. 9).

Vasorelaxant effect of CVE on endothelium-intact doxorubicin treated rat aortic rings

Effect of CVE on phenylephrine induced contraction in endothelium-intact aortic ring of doxorubicin treated rats

After 10^{-7} M phenylephrine induced steady contraction, CVE induced a significant ($P < 0.05$) concentration-dependent relaxation in phenylephrine pre-contracted endothelium-intact aortic rings (Fig. 10).

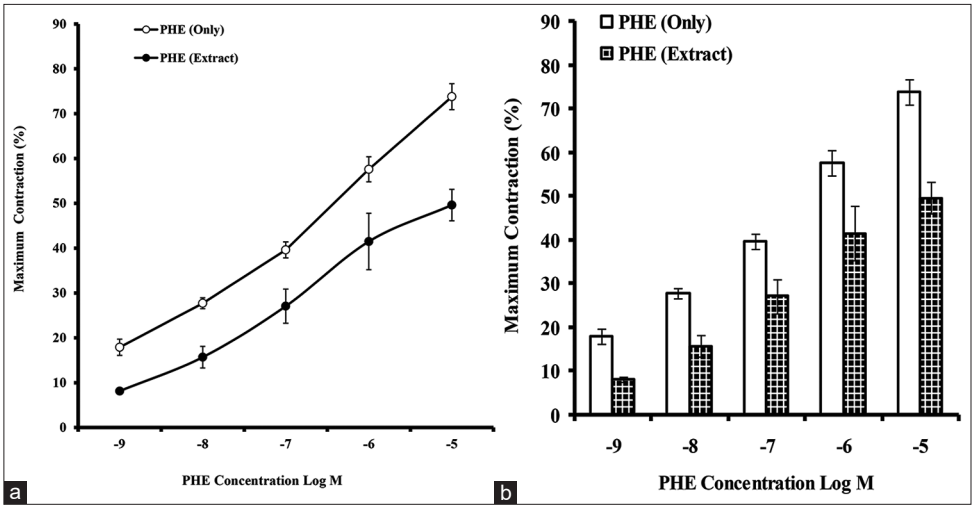

Fig. 2: (a) Log concentration-response of cumulatively administered *Clerodendrum volubile* ethanolic leaf extract on phenylephrine (PHE)-induced contractions in the endothelium-intact isolated aortic ring preparations obtained from normotensive rat.*P* < 0.05 at all concentrations. (b) Log concentration - Response of aortic rings to cumulative application of PHE in the absence (open bar and presence of extract (mg/ml, filled bar). Each point represents mean±SEM of 4 experiments. *P* < 0.05 at all concentration

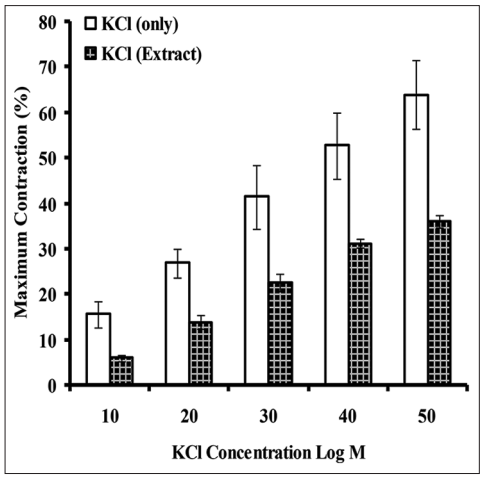

Fig. 3: Log concentration response of aortic rings to cumulative application of KCl in the absence (open bar) and presence of extract (mg/ml, filled bar). Each point represents mean±SEM of 4 experiments. *P* < 0.05 at all concentration

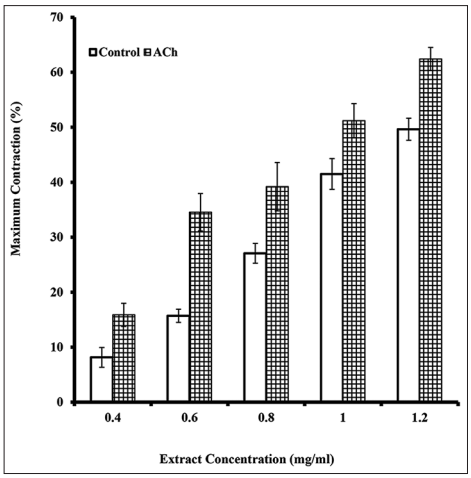

Fig. 4: Effect of 10^{-4} M acetylcholine on cumulative concentration response to 0.4–1.2 mg/ml of *Clerodendrum volubile* ethanolic leaf extract on aortic ring pre-contracted with 10^{-7} M of PHE. Each point represents mean±SEM of 4 experiments. *P* > 0.05 at all concentration

The minimal and maximal relaxation responses in phenylephrine-contracted rings were 06.1 ± 2.4% and 69.4 ± 7.5%, respectively.

Effect of CVE on KCl-induced contraction in endothelium-intact aortic ring of doxorubicin treated rats

Similarly, CVE also produced a significant ($P < 0.05$) concentration-dependent reduction of high K^+-induced contraction in the endothelium-intact arteries. The percentage of relaxation response was 77.5 ± 5.4% ($P < 0.05$) (Fig. 11).

Relaxation response of endothelium intact aortic rings of doxorubicin treated rats to CVE after calcium channel inhibition by nifedipine

Fig. 12 depicts the effect of CVE on the maximum relaxation response of endothelium-intact aortic ring pre-contracted with 10^{-7} M phenylephrine in presence of the calcium channel blocker (nifedipine). Comparison between the two groups shows that there was significant change ($P > 0.05$) in the percentage maximum relaxation response between the endothelium-intact aortic rings pre-treated with nifedipine and endothelium-intact aortic rings treated with 0.4–1.2 mg/ml of CVE (Fig. 12).

Relaxation response of endothelium intact aortic rings of doxorubicin treated rats to CVE after NOS inhibition by L-NAME

Fig. 13 depicts the effect of CVE on the maximum relaxation response of doxorubicin treated endothelium-intact aortic ring pre-contracted with 10^{-7} M phenylephrine in the presence or absence of L-NAME. Comparison between the two groups shows that there was no significant change ($P > 0.05$) in the percentage maximum relaxation response between the endothelium-intact aortic rings pre-treated with L-NAME and doxorubicin treated endothelium-intact aortic rings treated with 0.4–1.0 mg/ml except at the maximum dose (1.2 mg/ml) of CVE which is far greater than 35.0 ± 8.8 recorded for L-NAME only (Fig. 13).

Relaxation response of doxorubicin treated aortic ring to CVE after cholinergic inhibition with atropine

Fig. 14 depicts the effect of CVE on the maximum relaxation response of doxorubicin treated endothelium-intact aortic ring pre-contracted with 10^{-7} M phenylephrine in presence of atropine. Comparison between the two groups shows that there was a significant ($P < 0.05$) concentration-dependent vasorelaxant effect induced by 0.4–1.2 mg/ml of CVE

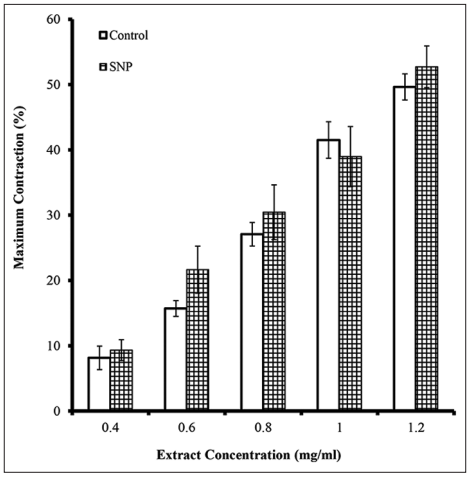

Fig. 5: Effect of 10^{-4} M sodium nitroprussideon cumulative concentration response to 0.4–1.2 mg/ml of *Clerodendrum volubile* ethanolic leaf extracton aortic ring pre-contracted with 10^{-7} M of PHE. Each point represents mean ± SEM of 4 experiments. *P* > 0.05 at all concentration

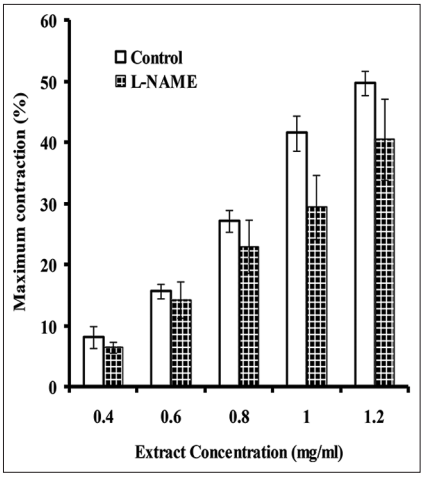

Fig. 7: Effect of 10^{-4} M L-NAME on cumulative concentration response to 0.4–1.2 mg/ml of *Clerodendrum volubile* ethanolic leaf extract on aortic ring pre-contracted with 10^{-7} M of PHE. Each point represents mean±SEM of 4 experiments. *P* > 0.05 at all concentration

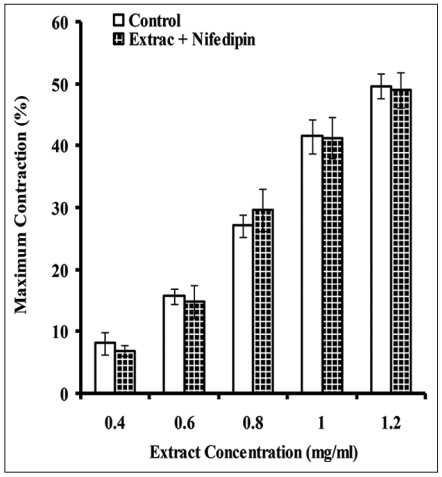

Fig. 6: Effect of 10^{-4} M of nifedipine on cumulative concentration response to 0.4–1.2 mg/ml of *Clerodendrum volubile* ethanolic leaf extract on endothelium-intact aortic ring pre-contracted with 10^{-7} M PHE. Each point represents mean±SEM of 4 experiments. *P* > 0.05 at all concentrations

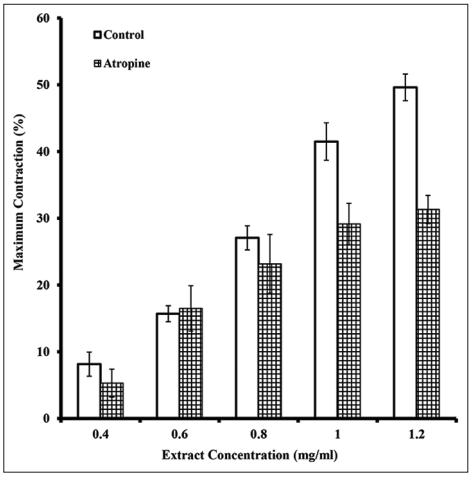

Fig. 8: Effect of 10^{-4} M atropine on cumulative concentration response to 0.4–1.2 mg/ml of *Clerodendrum volubile* ethanolic leaf extract on aortic ring pre-contracted with 10^{-7} M of PHE. Each point represents mean±SEM of 4 experiments. *P* < 0.05 at all concentration

with the percentage maximum relaxation response recorded at the maximum concentration (1.2 mg/ml) of the extract which was far greater than that induced by atropine (49.4 ± 2.7) (Fig. 14).

Relaxation response of doxorubicin treated aortic ring to CVE after prostacyclin inhibition with indomethacin

Fig. 15 depicts the effect of CVE on the maximum relaxation response of doxorubicin treated endothelium-intact aortic ring pre-contracted with 10^{-7} M phenylephrine in presence of indomethacin. Comparison between the two groups shows that there was a significant ($P < 0.05$) concentration-dependent vasorelaxant effect induced by 0.4–1.2 mg/ml of CVE with the percentage maximum relaxation response recorded at the maximum concentration of the extract which was comparable to that induced by indomethacin (30.7 ± 7.5) (Fig. 15).

DISCUSSION

The endothelium is known to play a crucial role in the development and course of doxorubicin-induced cardiomyopathy which over time culminates into heart failure [3,10]. The reported underlying etiopathological mechanisms of doxorubicin-mediated cardiomyopathy majorly involve increased vascular endothelial permeability [30], and disruptive effect of reactive oxygen species (ROS) on endothelial cell-derived endothelin(ET)-1, prostaglandin (PG) I_2, nitric oxide (NO), and neuregin (NRG)-1 [31-33] which promote arteriosclerosis development especially of the aorta [34]. As a result of this, it is imperative to take appropriate measures to protect the endothelium while still ensuring optimal doxorubicin efficacy [10]. In doing this, the effect of CVE at concentrations ranging from 0.4–1.2 mg/ml was investigated for its possible vasorelaxant effect and its underlying mechanism(s) in pre-contracted endothelium-intact aortic rings of normal and doxorubicin treated rats. Results of our study showed that CVE induced relaxation of rat aortic smooth muscle following pre-contraction with either phenylephrine or high K^+ in a concentration-dependent manner. Thus, this direct vasorelaxant effect induced by CVE on vascular smooth muscle lend credence to the earlier report on the vasorelaxant effect of CVE [16]. However, the molecular mechanism of arterial smooth muscle contraction produced by phenylephrine is uniquely different from that high K solution. Phenylephrine is a post-synaptic, selective α_1-adrenoceptor agonist which causes vasoconstriction by activating

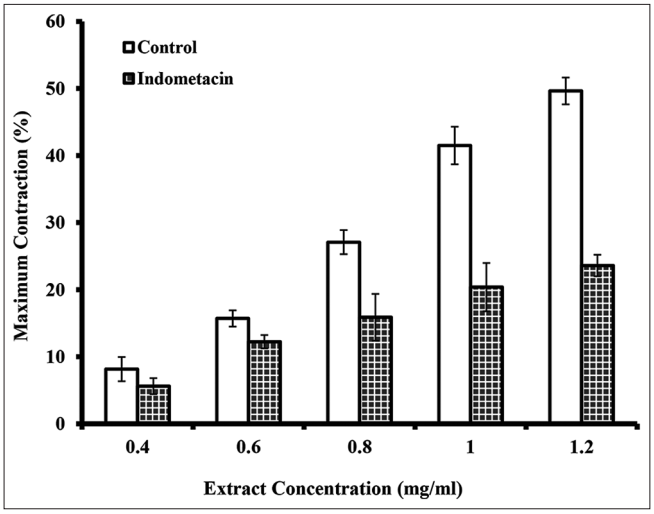

Fig. 9: Effect of 10^{-4} M Indomethacin on cumulative concentration response to 0.4–1.2 mg/ml of *Clerodendrum volubile* ethanolic leaf extract on aortic ring pre-contracted with 10^{-7} M of PHE. Each point represents mean±SEM of 4 experiments. *P* < 0.05 at all concentration

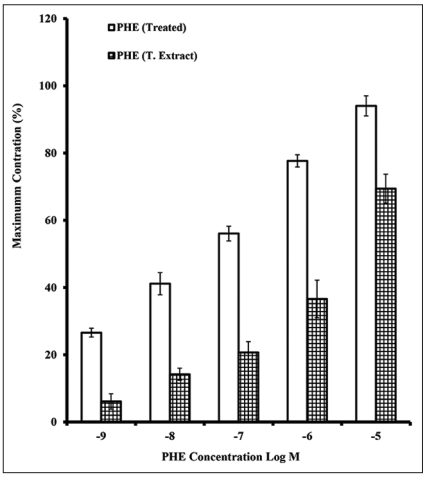

Fig. 10: Log concentration - Response of doxorubicin-treated aortic rings to cumulative application of PHE in the absence (open bar and presence of extract (mg/ml, filled bar). Each point represents mean±SEM of 4 experiments. *P* < 0.05 at all concentration

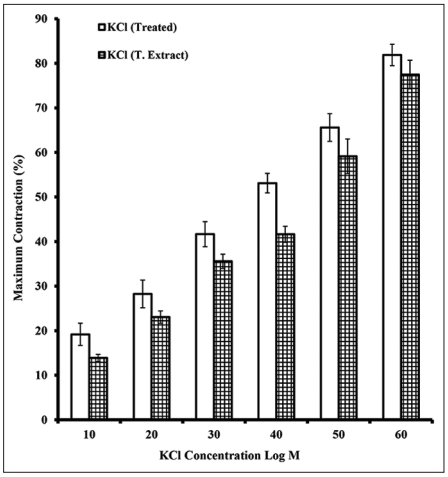

Fig. 11: Log concentration response of doxorubicin-treated aortic rings to cumulative application of KCl in the absence (open bar and presence of extract (mg/ml, filled bar). Each point represents mean±SEM of 4 experiments. *P* < 0.05 at all concentration

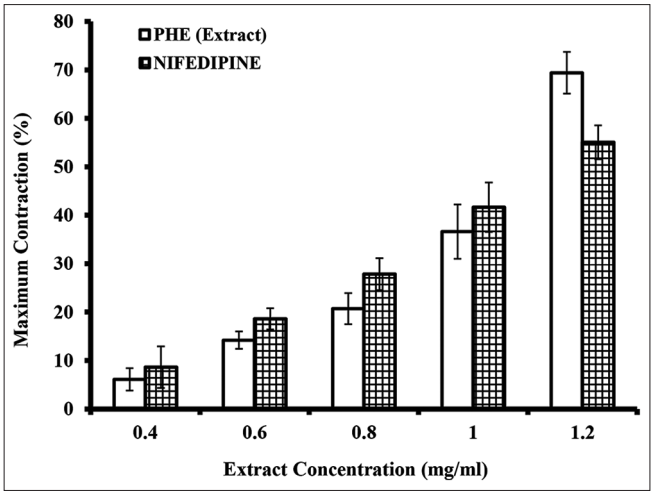

Fig. 12: Effect of 10^{-4} M of nifedipine on cumulative concentration response to 0.4–1.2 mg/ml of *Clerodendrum volubile* ethanolic leaf extract on endothelium-intact aortic ring pre-contracted with 10^{-7} M PHE. Each point represents mean±SEM of 4 experiments. *P* > 0.05 at all concentrations

phospholipase C which, in turn, primarily trigger Ca^{2+} release from the sarcoplasmic reticulum [35-38]. On the other hand, KCl is a non-specific agonist which produces contraction through activation of voltage-operated L-type Ca^{2+} channels that leads to increases in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$), Ca^{2+}-calmodulin-dependent myosin light chain (MLC) kinase activation, MLC phosphorylation and contraction [39,40]. Unfortunately, CVE did not inhibit the contraction of aortic smooth muscle produced by these two mechanisms, ruling out that blockade of voltage-gated Ca^{2+} as a possible vasorelaxant mechanism of CVE. This assertion was also corroborated by the non-relaxant effect produced by CVE following incubation with nifedipine. Nifedipine is an analogue of the dihydropyridine class of calcium channel blockers which targets the vascular L-type calcium channels [41]. Due to their high vascular selectivity, dihydropyridines are primarily used to reduce systemic vascular resistance and arterial pressure, and therefore are used to treat hypertension [41]. However, the pronounced vasorelaxant effect recorded for the extract in the high K^+ solution suggested that CVE could be mediating its direct vasorelaxant effect via this mechanism. This assertion is consistent with those previously reported for agonist stimulated and high extracellular K^+-depolarized rat aortic rings [29]. Similar profound vasorelaxant effects were observed with incubation with atropine and indomethacin, suggesting that CVE could be mediating its vasorelaxant effect through cholinergic and prostacyclin mechanisms.

The fact that application of sodium nitroprusside (SNP) and L-N^Gnitro arginine methyl ester (L-NAME) did not produce significant vascular relaxation of both the normal and doxorubicin-treated endothelium intact aortic rings suggested that the vasorelaxant effect of CVE is not nitric oxide mediated. It is well documented in literature that sodium nitroprusside (SNP) breaks down in circulation to release nitric oxide (NO) [42]. Similarly, NOS-inhibiting L-arginine analog, L-NAME, has been a mainstay tool in characterizing the role of NOS in the regulation of vascular tone [43]. The fact that CVE did not induce significant vasorelaxation in the face of the non-isoform-selective nitric oxide synthase (NOS) inhibitor, L-NAME, is equally suggestive of the fact the vasorelaxant effect of the extract is not mediated through nitric oxide–cGMP signaling mechanism/pathway.

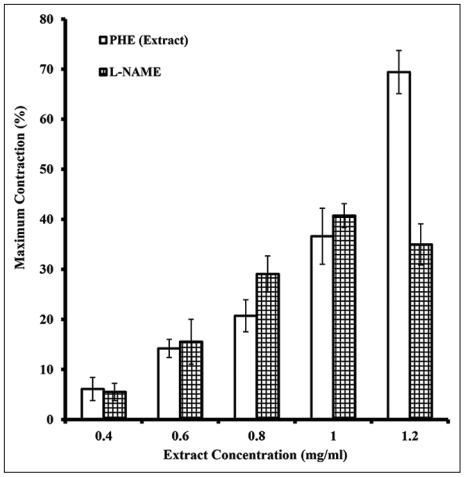

Fig. 13: Effect of 10^{-4} M L-NAME on cumulative concentration response to 0.4-1.2 mg/ml of *Clerodendrum volubile* ethanolic leaf extract (CVE) on doxorubicin-treated aortic ring pre-contracted with 10^{-7}M of PHE. Each point represents mean±SEM of 4 experiments. *P* < 0.05 at 1.2 mg/ml of *CVE*

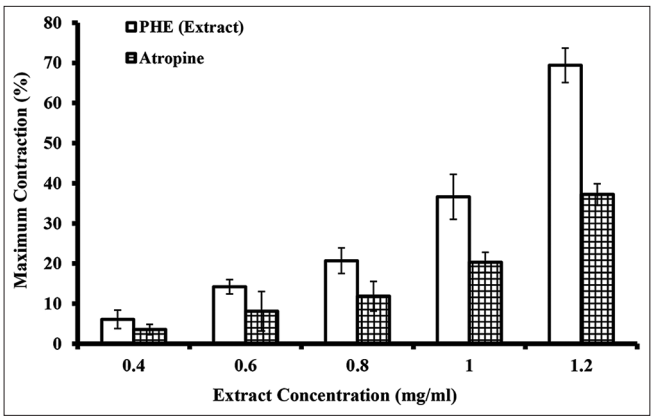

Fig. 14: Effect of 10^{-4} M atropine on cumulative concentration response to 0.4–1.2 mg/ml of *Clerodendrum volubile* ethanolic leaf extract (CVE) on doxorubicin-treated aortic ring pre-contracted with 10^{-7} M of PHE. Each point represents mean±SEM of 4 experiments. *P* < 0.05 at all concentration

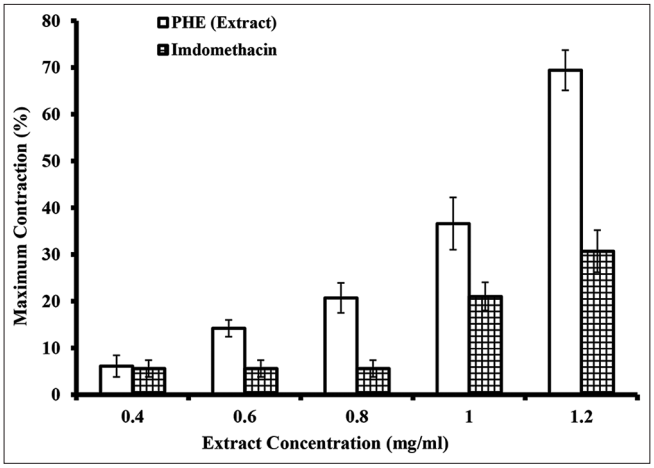

Fig. 15: Effect of 10^{-4} M indomethacin on cumulative concentration response to 0.4–1.2 mg/ml of *Clerodendrum volubile* ethanolic leaf extract on doxorubicin treated aortic ring pre-contracted with 10^{-7} M of PHE. Each point represents mean±SEM of 4 experiments. *P* < 0.05 at all concentration

L-NAME is known to be a precursor for NO which itself is known to activate guanylate cyclase in vascular smooth muscle and stimulates intracellular cyclic guanosine monophosphate (cGMP) synthesis which itself activates protein kinase G to inactivate myosin light chains that are involved in muscle contraction [44,45], thus, resulting in vascular smooth muscle relaxations [45]. Literature has shown that polyphenols (such as flavonoids, phenolic acids, stilbenes, flavones, and coumarins) are known naturally occurring phytochemicals that are useful in the control and management of cardiovascular diseases including hypertension and stroke [46-48]. Also natural compounds rich in flavonoid and terpenoid contents have also been reported to exert vasodilatory effect, at least in part, through K^+ channel activation [49,50]. Similarly, accumulated evidences have shown anthocyanins to elicit potent vasorelaxation in rat aorta in a concentration-dependent manner [51,52]. GC-MS analysis of CVE as previously reported [26] that it contained, ethyl-α-d-glucopyranoside and its esters (glycoside) which has been reported to be involved in the maintenance and improvement of skin and vascular homeostasis [53]; 7-octadecenoic acid methyl ester and 9-octadecenoic acid methyl ester (palmitic acidesters) (structurally related to n-hexadecanoic acid and its ethyl esters) which are not only known powerful antioxidants but have been reported to produce relaxations of rat aortas mediated through perivascular relaxing factors (PVRFs) and opening voltage-gated K^+ channels in vascular smooth muscle cells (VSMCs) [54]. Similar vasorelaxant and spasmolytic effects of palmitic acid esters have been reported in isolated rabbit aortic and jejunum preparations [55] as well as in isolated retinal preparations via retina-derived relaxing factor (RRF) [56]. Therefore, it can be suggested that these compounds that are contained in CVE may contribute to vasorelaxant effect through modulation of K^+ channel. Thus, the presence in high amount and single or multiple bioactivities of these phytochemicals in CVE could be responsible for the recorded vasorelaxant effect of the extract.

CONCLUSION

Overall, results of this study showed that CVE exhibited vasorelaxant effect in normal and doxorubicin treated endothelium intact aortic rings dose dependently which was mediated profoundly through cholinergic and prostacyclin mechanisms singly or by interactions of the secondary metabolites in CVE.

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AUTHORS' CONTRIBUTIONS

Akinyele Olubiyi Akinsola is an M.Sc. student in Olufunke Esan Olorundare's laboratory who performed the laboratory research; Hussein Moyosore Salahdeen was involved in the design of experimental protocol for the study and interpreted the tracings; Olufunke Esan Olorundare designed the experimental protocol for this study and read through the manuscript; Adejuwon Adewale Adeneye also contributed to the experimental design, coordinated the research, analyzed data and wrote the manuscript; Babatunde Adekunle Murtala provided technical guidance for the experiment; Hassan Mukhtar and Ralph M. Albrecht are our collaborator in the U.S.A. who are joint recipients of this research grant award read through the manuscript.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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