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Endothelium-dependent and independent vasorelaxant effects of aqueous extract of *Tridax procumbens* Lin. leaf in rat aortic rings

Hussein M Salahdeen[1]*, Gbolahan O Idowu[2] & Babatunde A Murtala[1]

[1]Department of Physiology, College of Medicine, Lagos State University, Ikeja, Lagos, Nigeria

[2]Department of Physiology, Olabisi Onabanjo University, Ikene, Ogun State, Nigeria

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Tridax procumbens leaf extract induced aortic relaxation in a concentration-dependent manner, for both phenylephrine (PE) and KCl- induced contractions in isolated rat aortic rings. The relaxation effect of the extract on PE-induced contraction was 57% greater than that on KCl- induced contraction. The extract caused dose-dependent relaxations in precontracted isolated rat aorta with phenylephrine; the relaxation was attenuated by the removal of endothelium. However, the relaxation responses to sodium nitroprusside were not significantly abolished by the removal of endothelium. The vasorelaxatory effect of the extract was completely abolished in presence of L-NAME. The results indicate that the vasorelaxant effect of *T. procumbens* extract is probably mediated by both endothelium-dependent and-independent mechanisms.

Keywords: Acetylcholine, Aortic ring, Hypertension, Smooth muscle, *Tridax procumbens*

Tridax procumbens Lin (Family Asteraceae) is a common weed found in the tropics that grows primarily during rainy season. The ethnopharmacology of the leaf and root of this plant have been studied[1]. The methanolic extract of *T. procumbens* has antimicrobial activity, wound healing property and immunomodulatory activity in experimental animal[2]. Its effect on liver antioxidant defense system during lipopolysaccharide-induced hepatitis in D-galactosamine sensitized rats was also studied[3,4]

In Nigeria, *Tridax procumbens* is traditionally used in the treatment of fever, typhoid fever, cough, asthma, epilepsy and diarrhea[5]. Previous studies indicate that intravenous injection of crude extract of *T. procumbens* caused bradycardia which was associated with a fall in arterial blood pressure in normotensive rats[6,7]. The precise mechanism of the hypotensive action of *T. procumbens* could not be established with these studies. Therefore, the aim of the present study is to investigate the vascular mechanisms involved in the antihypertensive effect of leaf aqueous extract of *Tridax procumbens* using rat aortic smooth muscle.

*Correspondent author
Telephone: +2348034094937, +2348187710087
E-mail: hmsalahdeen@yahoo.ca; hussein.salahdeen@lasucom.edu.ng

Materials and Methods

Plant material—Fresh leaves of *T. procumbens* were collected from open grassland on the Ikeja Campus of the College of Medicine Lagos State University, Lagos, Nigeria during September, 2011. Identification of the plant was carried out by the Taxonomist of the Lagos State University, Department of Botany. Following identification, a voucher specimen of the plant was deposited in the herbarium of the Botany Department of Lagos State University, Lagos State, Nigeria.

Preparation of T. procumbens leaf aqueous extract—Fresh *T. procumbens* leaves (1 kg) were air dried under shade at room temperature (26 ± 1 °C) for 2 weeks. The air dried leaves were milled into fine powder in a warring commercial blender. The powdered leaves were macerated in distilled water and extracted twice, on each occasion with 100 mL distilled water at room temperature (26 ± 1 °C) for 48 h (with occasional shaking). The combined aqueous extract was filtered and concentrated under reduced pressure in a rotary evaporator at 26 ± 1 °C. Freeze-drying and solvent elimination of the resulting aqueous extract yielded 23.6% of a light brown powdery crude *T. procumbens* leaf aqueous extract. Aliquot portions of the extract were weighed and dissolved in distilled water (at room temperature) for use at the time of experiments.

Animals—Healthy, young adult, male and female Wistar albino rats weighing 250-300 g were used. The animals were kept and maintained under conventional laboratory conditions of temperature, humidity and light, and allowed free access to standard pellet diet (Live Stock Feeds Nig. Ikeja, Nigeria) and water ad *libitum.* All the animals used were fasted for 18 h, but still allowed access to water before the commencement of the experiments.

Drugs—Acetylcholine chloride, L-phenylephrine chloride, L-NAME and sodium nitroprusside were obtained from E. Merck, Darmstadt, Germany. All chemicals and materials used were of analytical grade.

Experimental protocols and procedures used in this study were approved by the Animal Ethics Committee of the Lagos State University College of Medicine and conform to the 1985 guidelines for laboratory animal care of the National Institute of Health (NIH).

Preparation and mounting of aortic rings— The rats were sacrificed by cervical dislocation. The thoracic aorta was quickly removed, freed of connective tissue 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[15,16] (mmol/L): NaCl, 118.0; KCl, 4.7; KH_2PO_4 , 1.2; $MgSO_4$, 1.2; $NAHCO_3$, 15.0; $CaCl_2$, 1.6 and glucose, 11.5. The temperature of the bath was maintained at 37 ^oC and the solution bubbled with a 95% O_2 5% CO_2 gas mixture (*p*H 7.35-7.40). Each ring was mounted between a stainless steel hook connected to the base of the bath and a stainless steel rod anchored to a force transducer (model 7004; Ugo Basile Varese, Italy) connected to Data capsule acquisition system model 17400 for recording of isometric contractions. An initial tension of 2 g was applied to all arterial rings. This level of initial tension produces maximal active contractions in aortic ring stimulated with phenylephrine (PE) or a depolarizing solution[8]. An equilibration period of 60-90 min was allowed before the start of experiments, and during this time it was stimulated thrice with $10^{-7}M$ phenylephrine for 5 min at 30 min intervals[9]. In this experiment, the endothelium was removed by gently rubbing the inner lining of the aortic ring with a fine platinum wire. Successful removal of the functional endothelium was assessed by the presence or absence respectively of relaxant responses to acetylcholine ($10^{-7}M$)[8]. ACh-induced relaxation of 3-5% was taken as satisfactory removal of the functional endothelium.

Concentration response of aortic rings to T. procumbens extract—The effects of *T. procumbens* extract on base line tension was determined after which the ring was contracted with phenylephrine ($10^{-7}M$) or 60 m*M* KCI. After the contraction of the ring had stabilized, extract (0.3-1.05 mg/mL) was added cumulatively into bath solution. The effect of each concentration was allowed to reach a steady level before the addition of the next dose. In other experiments, relaxation response to *T. procumbens* was also assessed in aortic rings with and without endothelium pre-contracted with phenylephrine.

Influence of endothelium on vasorelaxant effect of T. procumbens extract against phenylephrine-induced contractions—In these experiments the relaxation responses to acetylcholine, histamine and sodium nitroprusside were carried out. Both denuded and endothelium intact aortic rings were incubated with *T. procumbens* extract (0.6 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 ($10^{-4}M$ to block the NO-mediated component of the response to *T. procumbens*) 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 (0.3-1.05 mg/mL) were added to the organ bath.

Statistical analysis—Data are expressed as means±SE, where *n* equals the number of animals from which blood vessels were isolated. The data were analyzed using two-way ANOVA. The Student-Newman-Keuls post hoc test was used to identify differences between individual means. The confidence interval was set at 95%, so that in all cases, results with a value of $P<0.05$ were considered to indicate statistical significance.

Results

Vasorelaxant effect of T. procumbens extract—After $10^{-7}M$ phenylephrine induced steady contraction, *T. procumbens* extract induced a significant ($P<0.05$) concentration-dependent relaxation in phenylephrine

pre-contracted aortic rings (Fig. 1a and b). The maximal relaxation responses in phenylephrine-contracted rings was 90.5±1.8 % and the IC_{50} was 0.6±0.01 mg/mL. Similarly, *T. procumbens* extract also produced a concentration-dependent reduction of high K^+-induced contraction in the endothelium-intact arteries. The percentage of relaxation response was 54.3 ± 5.2% ($P < 0.05$). The IC_{50} was 0.9 ± 0.01mg/mL (Fig. 2)

Effect of T. procumbens extract on phenylephrine induced contraction in endothelium-intact and-denuded aortic ring—T. procumbens extract caused a significant relaxation of endothelium-intact aortic rings. Functional removal of the endothelium significantly reduced the *T. procumbens* -induced relaxation of the rings (Fig. 3).

Relaxation effect of sodium nitroprusside on aortic rings after 15 min incubation in presence of 0.6 mg/mL T. procumbens extract—Sodium nitroprusside (10^{-9}-10^{-5} *M*) was added cumulatively to aortic ring contracted with 10^{-7}*M* phenylephrine after 15 min incubation in the presence of *T. procumbens* extract (0.6 mg/mL). The relaxation caused by sodium nitroprusside was not significantly different between endothelium- intact and denuded aortic rings (Fig. 4).

Relaxation response to T. procumbens extract after NOS inhibition by L-NAME—Figure 5 shows the effect of *T. procumbens* 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 shows that there was a significant decrease (P<0.05) in the percentage maximum relaxation response of the endothelium-intact aortic rings with L-NAME when compared with endothelium-intact aortic rings without L-NAME (Fig. 5).

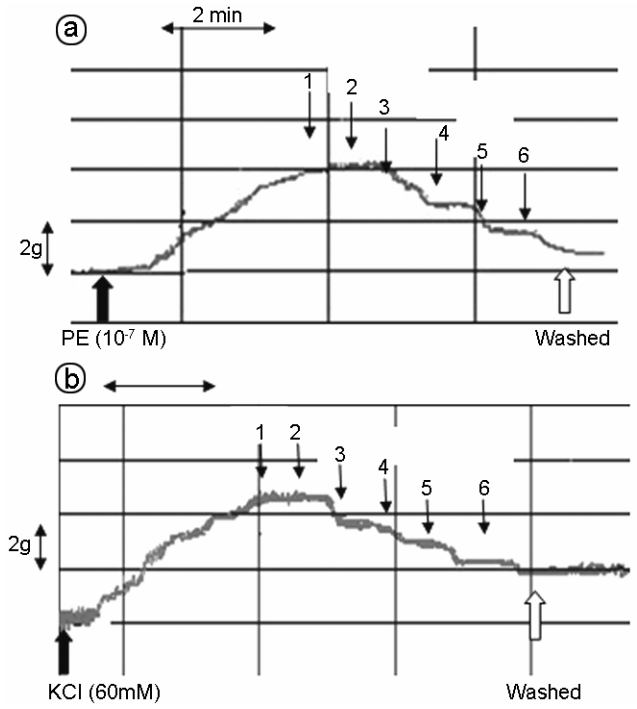

Fig. 1—Typical tracings showing the vasorelaxant effects of graded concentrations of *T. procumbens* aqueous leaf extract on (A) phenylephrine (PE)-induced and (B) KCl induced contractions in the endothelium-containing isolated aortic ring preparations obtaining from normotensive rat. Arrows 1-6 represent cumulatively administered *T. procumbens* extracts (0.3, 0.45, 0.6, 0.75, 0.9 and 1.05 mg/mL respectively). PE, KCl and *T. procumbens* were washed out at the open upward- arrow.

Discussion

Application of *T. procumbens* leaf aqueous extract to unstimulated rat aortic rings did not result in a change of baseline tension suggesting that *T. procumbens* extract has no contractile effect under resting condition. The lack of effect of *T. procumbens* on baseline tension is comparable to published reports that active tone is required to demonstrate that relaxant effect of a variety of vasoactive agents[10-12]. The results of the present study clearly show that

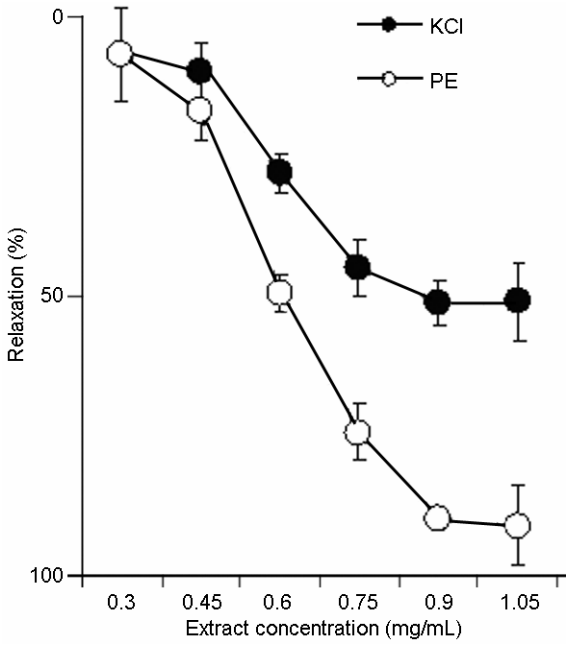

Fig. 2—Cumulative concentration response for *T. procumbens* extracts (0.3-1.05 mg/mL) on rat isolated aortic ring pre-contracted with PE (10^{-7}*M*) and (60 mM), KCl^-. Results are expressed as mean ± SE of 6 rings from each animal. Data were analyzed by two-way ANOVA followed by Newman-Keuls post hoc test. P<0.05.

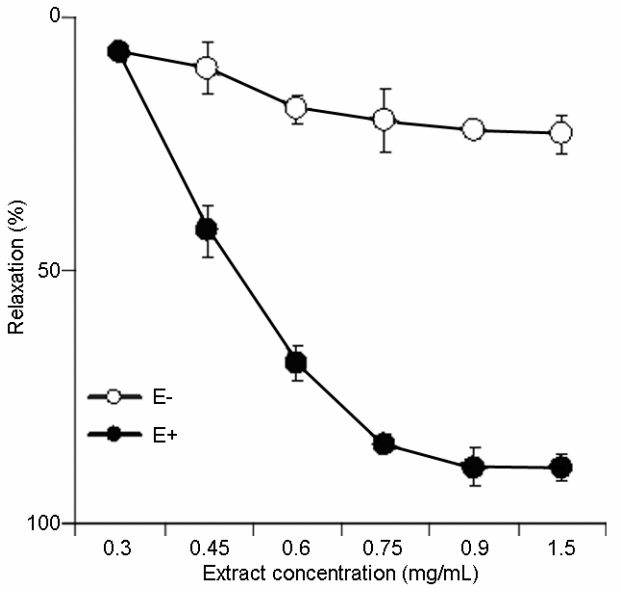

Fig. 3—Concentration-effect curve of *T. procumbens* aqueous leaf extract (0.3-1.05 mg/mL) on endothelium Intact (E+) and endothelium-denuded (E-) aortic ring pre-contracted with PE (10^{-7} *M*). Results are expressed as mean ± SE of 6 rings from each animal. Data were analyzed by two-way ANOVA followed by Newman-Keuls post hoc test. $P<0.05$.

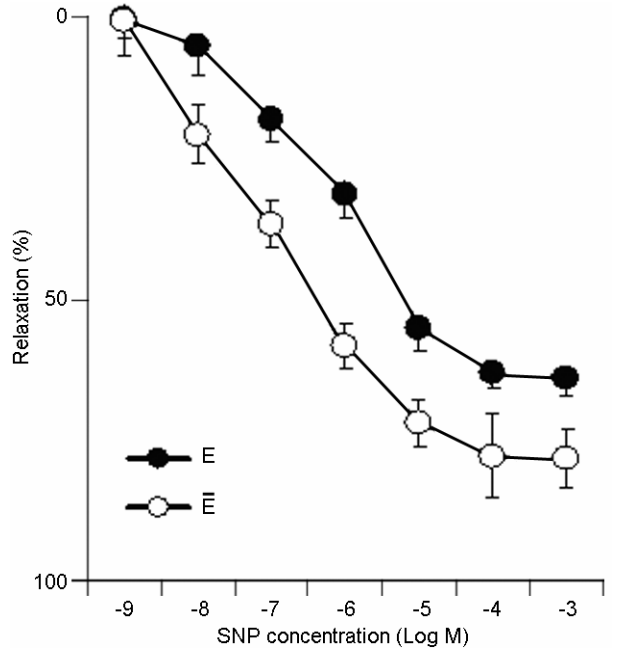

Fig. 4—Effect of *T. procumbe+ns* extract (0.6 mg/mL) on cumulative concentration response for sodium nitroprusside (SNP) (10^{-9}-10^{-3} *M*) in endothelium- intact (E+) and endothelium-denuded (E-) aortic ring pre-contracted with PE (10^{-7}*M*). Results are expressed as mean ± SE of 6 rings from each animal. Data were analyzed by two-way ANOVA followed by Newman Keuls post hoc test.

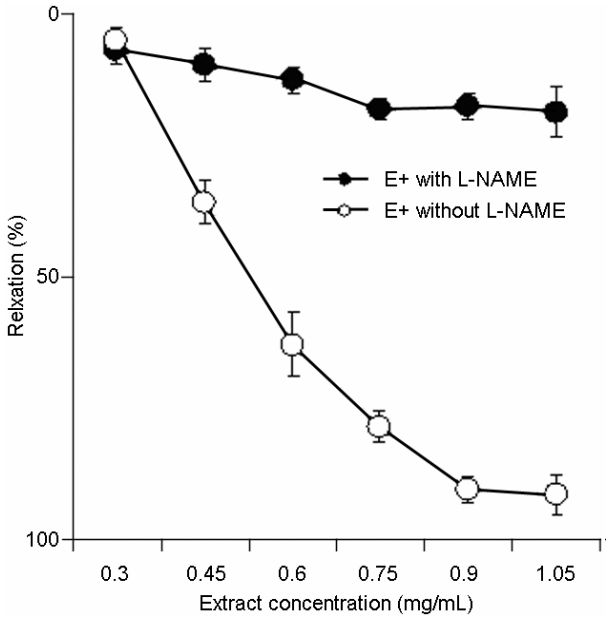

Fig. 5—Effect of L-NAME (10^{-4} *M*) on cumulative concentration response of *T. procumbens* extract (0.3-1.05 mg/mL) in endothelium- intact (E+) and endothelium denuded (E-) aortic ring pre-contracted with PE (10^{-7} *M*). Results are expressed as mean ± SE of 6 rings from each animal. Data were analyzed by two-way ANOVA followed by Newman Keuls post hoc test. $P<0.05$.

T. procumbens leaves extract induced relaxation of rat aortic smooth muscle following precontraction induced by phenylephrine or high-K^+ in a concentration-dependent manner. This observation of relaxation effect of *T. procumbens* is consistent with the previous report that *T. procumbens* extract possessed a direct vasodilatory effect on vascular smooth muscle[13].

The cellular mechanisms of contraction involved in response of arterial smooth muscle to phenylephrine and high-K solution is different. The former is a α_1 adrenoceptor agonist. It causes vasoconstriction by activating phospholipase C and primarily triggering Ca^{2+} release from the sarcoplasmic reticulum following by sustained Ca^{2+} entry[14], and the later induced contraction mainly by Ca^{2+} influx upon depolarization of the cell membrane, which activates voltage-dependent L-type Ca^{2+} channel[15]. *T. procumbens* extract inhibit the contraction of aortic smooth muscle produced by these two mechanisms to a similar extent, suggesting that the blockade of voltage-gated Ca^{2+} channels may not be an important factor in the relaxation induced by *T. procumbens* extract. That is, the inhibitory actions of *T. procumbens* extract may occur in a voltage –independent manner

in smooth muscle cells of the rat aorta. The present data showing that, *T. procumbens* extract significantly produce direct relaxation is consistent with those previously reported for agonist stimulated and high extracellular K –depolarized in rat aortic rings[13].

Endothelium cells lining blood vessels produced an endothelium-derived relaxing factor (EDRF) in response to many types of stimuli such as chemical agents and mechanical stimulation[16,17]. Nitric oxide (NO) may be chemical involved, and its diffusion to vascular smooth muscle cells appears to activate soluble gualylate cyclase to enhance the production of cyclic GMP[17]. The elevated cyclic GMP in vascular smooth muscle cells causes a relaxation of smooth muscle due to reduction of cytosolic Ca^{2+} through activation of Ca ATPase distributed in the membrane of the internal stores[14].

Tridax procumbens induced significant relaxation responses in aortic rings with endothelium-intact and was attenuated in endothelium-denuded aortic rings, suggesting that the relaxation response of *T. procumbens* is endothelium-dependent. One may therefore speculate that impairment of release of nitric oxide or other endothelium-dependent vasorelaxant substance may be held to account for the observed relaxant effect of *T. procumbens* in rat aortic smooth muscle. Endothelium-dependent involvement was also corroborated by the complete inhibition of *T. procumbens* -induced vasorelaxation by L-NAME[15]. In the present study, L-NAME, a nitric oxide synthase inhibitor, completely abolished the relaxation effect of *T. procumbens* extract in endothelium-intact aortic ring suggesting that the active components in *T. procumbens* act on the vascular endothelium via the NO synthase pathway. It has been recognized that L-NAME which inhibits of NO production in the endothelium had a greater effect on blood pressure demonstrating the role of NO as a potent vasodilator[16,17].

Application of sodium nitroprusside (SNP) induced significant relaxation of both endothelium-intact aortic rings suggesting that the relaxation of *T. procumbens* extract is endothelium-independent. Therefore, it is more likely that *T. procumbens* extract may lead to either an increase in endothelium production of NO or a premature activation of the produced NO in the aorta of these rats. It is well known that sodium nitroprusside (SNP) breaks down in circulation to release nitric oxide (NO). NO activates guanylate cyclase in vascular smooth muscle and increases intracellular production of cGMP. cGMP activates protein kinase G which activates phosphatases which inactivate myosin light chains. Myosin light chains are involved in muscle contraction[18]. The end result is vascular smooth muscle relaxations, which allow vessels to dilate[18]. Also in cardiac myocytes, NO has a prominent role in the regulation of Ca^{2+} handling. It modifies Ca^{2+} influx via L-type sarcolemmal Ca^{2+} channels, it regulates the sarcoplasmic reticulum (SR) Ca^{2+} release channel and it may inhibit Ca^{2+} -ATPase-mediated SR reuptake of Ca^{2+} (ref. 19).

The present experiments have shown that relaxation of aortic smooth muscle contracted with phenylephrine or high-K solution in response to *T. procumbens* extract may be directly related to the increased production of cyclic GMP or to hyperpolarization of the membrane[20].

Although, cGMP was not measured in the present study, the effect of phenylephrine in vascular smooth muscle is far more complicated[14]. It cannot be excluded that *T. procumbens* extract may inhibit non-voltage-sensitive Ca^{2+}channels and decrease Ca^{2+} -sensitive of the contractile proteins in the vascular smooth muscle. Additional experiment related to the effects of *T. procumbens* extract on contractile system such as the alteration of calcium sensitivity or regulatory proteins are required.

In summary, *T. procumbens* extract induced a concentration dependent relaxation in rat aortic rings preontracted by either phenylephrine or KCl. The primary mechanisms may include both endothelium-dependent and-independent relations in the vascular smooth muscle cells.

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