Preventive Beneficial Effect of an Aqueous Extract of Phyllanthus amarus Schum. and Thonn. (Euphorbiaceae) on DOCA-Salt–Induced Hypertension, Cardiac Hypertrophy and Dysfunction, and Endothelial Dysfunction in Rats

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Preventive beneficial effect of an aqueous extract of *Phyllanthus amarus* Schum. & Thonn. (Euphorbiaceae) on DOCA-salt-induced hypertension, cardiac hypertrophy and dysfunction, and endothelial dysfunction in rats

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Conflicts of interests

The authors declare no conflicts of interest.

Abstract

This study investigated the preventive effect of an aqueous extract of the whole plant of *Phyllanthus amarus* (AEPA) on blood pressure, cardiac and endothelial function in the deoxycorticosterone acetate (DOCA)-salt-induced hypertensive rat model. Male Wistar rats were assigned into five groups receiving either vehicle (control and DOCA-salt), DOCA-salt combined with AEPA at 100 or 300 mg/kg, or AEPA (100 mg/kg) alone for 5 weeks. In addition, DOCA-salt-treated rats were allowed free access to water containing 1% NaCl. Systolic blood pressure (SBP), left ventricle parameters, vascular reactivity of primary mesenteric artery rings, the vascular level of oxidative stress and the level of target proteins were determined, using respectively tail-cuff sphygmomanometry, echocardiography, organ chambers, dihydroethidium staining, and immunofluorescence methods.

After 5 weeks, AEPA treatments (100 or 300 mg/kg/day) significantly prevented the increase in SBP in DOCA-salt rats, respectively by about 24 and 21 mmHg, improved cardiac diastolic function, and reduced significantly the increased posterior and septum diastolic wall thickness and the left ventricle mass in hypertensive rats. Moreover, the DOCA-salt-induced endothelial dysfunction, and the blunted nitric oxide- and endothelium-dependent hyperpolarization-mediated relaxations in primary mesenteric artery were improved after the AEPA treatments.

AEPA also reduced the level of vascular oxidative stress and the expression level of target proteins (eNOS, COX-2, NADPH oxidase subunit $p22^{phox}$) in DOCA-salt rats.

Altogether, AEPA prevented hypertension, improved cardiac structure and function, and endothelial function in DOCA-salt rats. Such beneficial effects appear to be related, at least in part, to normalization of the vascular level of oxidative stress.

Keywords: *Phyllanthus amarus*, DOCA-salt, hypertension, cardiac hypertrophy, endothelial dysfunction

INTRODUCTION

Hypertension is an important public health problem,[1] which can lead to major cardiovascular complications such as atherosclerosis, coronary artery diseases, congestive heart failure and stroke, in chronic situation.[2-4] Previous studies have indicated that the spontaneously hypertensive rat (SHR), the angiotensin II (Ang II)-induced hypertensive rat, the Dahl salt-sensitive hypertensive rat or the DOCA-salt rat are used as experimental models of human essential hypertension to better understand the underlying pathophysiology.[5,6] The DOCA-salt model of hypertension is obtained following administration of a synthetic mineralocorticoid deoxycorticosterone acetate (DOCA) for several weeks to rats allowed free access to water containing high NaCl, promoting volume overload induced hypertension with sodium retention [5, 7], which is associated with a low level of renin and hypokalemia.[5,8] Endothelial dysfunction, cardiac hypertrophy, renal damage, and inflammation, common deleterious effects associated with hypertension in Humans, are also observed in experimental models of hypertension.[9,10] In case of hypertension-related endothelial dysfunction, the blunted endothelium-derived relaxing factors-mediated relaxations is often observed affecting both the nitric oxide (NO) and the endothelium-dependent hyperpolarization (EDH)

components.[11,12] Moreover, they are also often related to an excessive formation of reactive oxygen species (ROS), predominantly by vascular NADPH oxidase.[13]

Antihypertensive therapy in first line uses classic drugs, including diuretics, angiotensin-converting enzyme inhibitors, angiotensin II type 1 receptor (AT1R) blockers, calcium channel blockers and beta-blockers. However, during the last few decades, the modification of lifestyle and the use of natural products derived from plants have gained much attention in the prevention and the treatment of cardiovascular pathologies, such as high blood pressure.[10, 14]

Traditional medicine is managing hypertensive disease through the use of plants from the Pharmacopoeia. However, traditional healers have limited resources for the diagnosis of hypertension. Therefore, beyond suggestive signs such as recurrent vertigo, they refer to their patient's medical examinations, confirming high blood pressure prior to treatment by plants extract.

In Ivorian folk medicine, many traditional herbal preparations are offered to the population for the treatment of high blood pressure. For example, an ethnopharmacological survey conducted on markets in the district of Abidjan (Côte d'Ivoire) identified 27 antihypertensive plants commonly used by hypertensive patients, including *Phyllanthus amarus* (Euphorbiaceae), with a frequency of use of about 42 %.[15]

Moreover, previous data have also reported that *Phyllanthus amarus* extract is used by most of the Ivorian local population, especially by Abbey and Krobou of Agboville (Côte d'Ivoire) as decoction of leaves or whole plant to treat hypertension and other pathologies such as diabetes, fever, malaria and stomach ulcer.[15-17] Numerous studies have reported that *Phyllanthus amarus* extracts can induce several kinds of pharmacological activities such as anti-inflammatory, [18] antioxidant,[19] vasodilatation, hypotensive and antihypertensive.[20-22] Preliminary pharmacological investigations have suggested the involvement of muscarinic receptors, an increased formation of endothelium-derived NO, and blockade of calcium

channels in the mechanism of the hypotensive activity of aqueous extracts of leaves and whole plant of *Phyllanthus amarus* in normotensive rabbits, and the induction of diuresis in rats.[21,23,24] Moreover, the ethanolic fraction of *Phyllanthus amarus* promoted urinary excretion of water and Na^+, which involved prostaglandins in rats.[25] Furthermore, according to a recent study using high-fructose (HF) fed Wistar rats, the aqueous extracts of *Phyllanthus amarus* prevented cardiac and vascular damage by improving the increased level of cardiac and aortic lipids and stress markers, and also the decreased level of antioxidants defense markers such as vitamin C, superoxide dismutase and catalase.[26] Although, *Phyllanthus amarus* is known as a potential remedy against high blood pressure, to our knowledge cardioprotective and antihypertensive effects of *Phyllanthus amarus* in chronic hypertension has not been assessed. Therefore, the aim of the present study was to evaluate the preventive effect of an aqueous extract of *Phyllanthus amarus* (AEPA) obtained by decoction of the whole plant, on hypertension, cardiac hypertrophy and endothelial dysfunction induced by DOCA-salt in rats.

METHODS

Plant material and extraction procedure

The whole plant of *Phyllanthus amarus* (Euphorbiaceae) was previously identified by Professor Aké-Assi Laurent (expert in botany), at the National de Floristic Center of Félix Houphouët-Boigny University (Abidjan, Côte d'Ivoire) where several samples of *Phyllanthus amarus* are registered, respectively under voucher specimen No. 3, 141 and 248. The website http://www.theplantlist.org has been used to check the plant name. The plant used to prepare the extract was harvested in the district of Cocody (Abidjan, Côte d'Ivoire) in April 2014, in accordance with the international convention concerning the biodiversity right.[27,28] In Ivorian traditional medicine, *Phyllanthus amarus* is known under some vernacular name, such as

sougnassi (*Baoulé*) or *dè-la-kôla* (*Malinké*). Moreover, its common name in English is *carry me seed.*

Phyllanthus amarus aqueous extract was prepared according to a method previously described.[23] Briefly, after the harvest, the whole plants collected were washed. Next, 500 g of plant were put in 1 liter of distilled water and boiled for 30 min. Thereafter, the decoction was filtered and lyophilized to obtain the powder of the aqueous extract of *Phyllanthus amarus* (AEPA). The extraction procedure provided 11.72 g of AEPA from 1 kg of plant. The extract was stored at -20 °C for subsequent pharmacological studies.

Animals and experimental groups

Experiments were performed according to the US guidelines for laboratory animal use and care, previously published (publication No. 85-23, revised 1996) by the National Institutes of Health (NIH).[29] Male Wistar rats (Janvier, Le Genest-Saint-Isle, France) weighing between 180 and 200 g were maintained under standard laboratory conditions (21-22 °C) with dark and light cycle (12/12 h) and received on free access a standard dry pellet diet (A04, SAFE, France) and water *ad libitum*. Non-uniephrectomized rats were randomly assigned 7-8 rats per group, into the control group, the AEPA group, the DOCA-salt group, and the DOCA-salt + AEPA groups. The AEPA group received by gavage AEPA (100 mg/kg/day) solubilized in H_2O for 6 weeks. The DOCA-salt group received subcutaneously injection of DOCA (50 mg/kg) in corn oil once weekly for 5 weeks. In addition, water containing 1% NaCl was available to the DOCA-salt-treated rats for free access. The DOCA-salt + AEPA groups were pretreated by gavage with AEPA (100 or 300 mg/kg/day) for one week before the injection of DOCA, and, thereafter, the AEPA treatments were continued as appropriate for 5 weeks.

Blood pressure measurements

The blood pressure analysis system (BP-2000 Serie II, Visitech Systems) was used to determine systolic blood pressure (SBP), by tail-cuff sphygmomanometry, twice weekly during 5 weeks. Prior to start of blood pressure monitoring, rats were trained daily for one week to get used to the system. Blood pressure was always monitored at 9 a.m and included at least 12 measurements.

Echocardiographic studies

After 5 weeks of treatment, prior to start echocardiographic studies, rats were anaesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Cardiac structure and function were subsequently obtained using the transthoracic echocardiography (Phillips Sonos 5500 equipped with a 12-MHz phased-array transducer). Two-dimensional short axis views of the left ventricle and M-mode tracings were recorded through anterior and posterior left ventricle (LV) walls at the papillary muscle level. LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), posterior and septum diastolic wall thickness (PWT and SWT, respectively) were determined for characterization of the cardiac left ventricle. In addition, the LV ejection fraction (% LVEF), left ventricular mass (LVM) and cardiac output (CO) were determined.[9] In addition, heart rate (HR) was determined from the cardiac cycles recorded on the M-mode tracing, from the echocardiography measurements. The pulsed Doppler was used to assess the isovolumetric relaxation time (IVRT), measured as the interval between aortic closure and the start of the mitral flow.

Morphological analyses of the heart and kidney

At the end of the study, rats were weighed, and euthanized by an intraperitoneal injection of pentobarbital (120 mg/kg) and blood was collected from the left cardiac ventricle. The heart and the left kidney were carefully excised, cleaned, and weighed. The heart was also dissected into septum, left ventricle and right ventricle, which were weighted separately. Cardiac and renal weight ratios to the body weight were also determined.

Biochemical analysis

Heparinized tubes were used to collect blood. Thereafter, the blood was centrifuged at 1500 *g* for 15 min. Plasma aliquots were prepared and kept at -80 °C for subsequent analysis of electrolyte, urea and uric acid (Advia 20400, Siemens Healthineers).

Vascular reactivity studies

Vascular reactivity studies were performed using primary mesenteric artery rings according to the method previously described.[30] Rings were contracted with 1 µM of phenylephrine (PE) before the addition of increasing concentrations of acetylcholine (ACh) ranging from 0.1 nM to 10 µM to obtain concentration-response curves. In some experiments, rings were incubated with an inhibitor for 30 min before contraction with PE. In order to assess the NO-mediated component of relaxation, indomethacin (10 µM), and TRAM-34 (1 µM) plus apamin (100 nM) were respectively used to inhibit cyclooxygenases and EDH-mediated relaxation, respectively whereas the EDH-mediated component of the relaxation was evaluated in the presence of indomethacin (10 µM) and N^{ω}-nitro-L-arginine (L-NA, 300 µM) to inhibit the formation of prostanoids and NO, respectively. In endothelium intact rings, increasing concentrations of PE (0.1 nM-10 µM) were used to construct a concentration-contraction curve.

Immunofluorescence studies

OCT compound (Tissue-Tek®, Sakura Finetek, Villeneuve d'Ascq, France) was used as embedding medium to snap-froze primary mesenteric artery rings in liquid nitrogen. Frozen artery rings were cryosectioned at 14 µm. Sections were air-dried for 15 min and kept in the freezer at -80 °C until use. The slides were fixed with paraformaldehyde (4%), washed and finally treated with 10% milk containing 0.1% Triton X-100 for 1 h at room temperature in order to block non-specific binding. After an overnight at 4 °C, different primary main mesentery artery sections were respectively incubated with an antibody directed against either eNOS (1:50), NADPH oxidase subunit $p22^{phox}$ (1:50), cyclooxygenase-1 (COX-1, 1:250), cyclooxygenase-2 (COX-2, 1:200), or connexin 37 (Cx, 1:100). Sections were then washed with PBS, incubated with the secondary antibody (1/400, 633-conjugated goat anti-rabbit or anti mouse IgG) for 2 h at room temperature in the dark before being washed with PBS and mounted in Dako fluorescence mounting medium (Dako, Carinteria, USA) and cover-slipped. The primary antibody was omitted for negative control. Before immunofluorescence level examination using a confocal laser-scanning microscope (Leica TSC SPE-Mannheim, Germany), samples were protected from lights. Image J software (NIH, Bethesda, Maryland, USA) was used to perform analysis of immunofluorescence signals.

Determination of vascular reactive oxygen species formation

In primary mesenteric artery rings, *in situ* formation of reactive oxygen species (ROS) was determined using the redox-sensitive fluorescent dye dihydroethidium (DHE). Primary mesenteric artery rings (3-4 mm length) were subsequently incorporated in OCT compound as embedding medium and snap-frozen in liquid nitrogen. Frozen arteries were cryosectioned at 25 µm. Sections were air-dried for 15 min and stored at -80 °C until use. Dihydroethidium (2.5 µM, Sigma) was applied onto unfixed cryosections of mesenteric arteries for 30 min at

37 °C in a light-protected humidified chamber, before being mounted in Dako fluorescent mounting medium and cover-slipped. Samples were kept in the dark until the fluorescence level was determined using a confocal laser-scanning microscope and analyzed using Image J.

Drugs and antibodies

DOCA was obtained from Sigma (France) and antibodies as indicated: mouse anti-eNOS (61029, BD Transduction Laboratories, East Rutherford, New Jersey, USA), COX-1 monoclonal antibody (ab109025, Abcam, Paris France), COX-2 polyclonal antibody (ab15191, Abcam, France), rabbit anti-$p22^{phox}$ (sc11712, Santa Cruz Biotechnology, Santa Cruz, CA, USA), connexin 37 polyclonal antibody (CX37B12-A, Alpha diagnostic international, San Antonio, Texas, USA), Alexa 633-conjugated goat anti-rabbit and anti-mouse IgG, (A-21070 and A-21050, respectively, Life technologies, USA).

Statistical analysis

Data are expressed as means ± standard error of mean (SEM) of n experiments. Mean values were compared by ANOVA followed by the Bonferroni post-hoc test to identify significant differences between treatments, using GraphPad Prism (version 5 for Microsoft windows, GraphPad software, Inc, San Diego, CA, USA). The difference was considered to be significant when $P < 0.05$.

RESULTS

Effect of AEPA treatment on DOCA-salt-induced changes of plasma electrolytes, uric acid and urea levels

The DOCA-salt administration significantly increased the plasma uric acid level and decreased the plasma level of potassium in hypertensive rats ($P < 0.05$; Table 1). Both AEPA

treatments (100 and 300 mg/kg/d) normalized respectively plasma levels of uric acid and potassium in the DOCA-salt group ($P < 0.05$; Table 1). In addition, the plasma sodium level in the DOCA-salt group increased slightly, but not significantly compared to that of the control group (Table 1). The plasma chloride and urea levels were similar in all groups (Table 1).

Effect of AEPA treatment on DOCA-salt-induced changes on heart and kidney morphological parameters

Morphometric analysis of organs showed that except the right ventricle, the DOCA-salt treatment increased significantly the cardiac, the left ventricular, and the left kidney weight index, compared to those of the control group ($P < 0.05$; Table 2). The cardiac weight index was reduced in the DOCA-salt + AEPA groups (100 or 300 mg/kg/day), and also that of the kidney in the DOCA-salt + AEPA (300 mg/kg/day) compared to the DOCA-salt group ($P < 0.05$; Table 2).

Intake of AEPA prevents the DOCA-salt-induced hypertension

Following administration of DOCA-salt, SBP increased progressively and reached significantly increased levels starting at 1 week in the DOCA-salt group compared to the control group ($P < 0.05$; Figure 1). Daily oral administration of AEPA (100 or 300 mg/kg/day) significantly prevented the increase of SBP observed in DOCA-salt rats by 24 and 21 mmHg, respectively, after 5 weeks (Figure 1). The SBP of rats receiving only AEPA (100 mg/kg/day) remained similar compared to that of the control group ($P > 0.05$; Figure 1).

Intake of AEPA prevents the DOCA-salt-induced alteration of cardiac structure and function

After 5 weeks, DOCA-salt hypertensive rats presented a left ventricular hypertrophy indicated by changes of the cardiac structure, revealed after transthoracic echocardiography, compared to those of the control group (Figure 2). A significant increase of PWT (1.58 ± 0.02 mm versus 2.24 ± 0.05 mm; $P < 0.05$; Figure 2A) and SWT (1.54 ± 0.03 mm versus 1.77 ± 0.05 mm; $P < 0.05$; Figure 2B) was observed in the DOCA-salt group compared to the control group. The LVM was also significantly greater in the DOCA-salt group than the control group (1103.61 ± 40.13 mg versus 755.29 ± 24.66 mg; $P < 0.05$; Figure 2C) whereas LVEDD was not affected ($P > 0.05$; Figure 2D). The AEPA treatments (100 and 300 mg/kg/day) reduced significantly the increase of PWT in the DOCA-salt groups by 0.36 mm and 0.30 mm, respectively, SWT by 0.18 mm and 0.17 mm, respectively and LVM by 268.55 mg and 251.61 mg, respectively ($P < 0.05$; Figures 2A-C). Parameters for the AEPA group (100 mg/kg/day) were similar to those of the control group ($P > 0.05$; Figure 2).

Analyses of the cardiac function indicated that, IVRT was significantly increased in the DOCA-salt group compared to that of the control group (45.71 ± 1.96 ms versus 35.5 ± 2.83 ms respectively; $P < 0.05$; Figure 2E). The AEPA treatments (100 and 300 mg/kg/day) improved IVRT in DOCA-salt treated rats with values close to normal, 36.67 ± 3.47 and 36.88 ± 2.37 ms, respectively ($P < 0.05$; Figure 2E). In contrast, the AEPA (100 mg/kg/day) alone did not affect IVRT (Figure 2E). The LVEF was slightly decreased in DOCA-salt rats compared to that of the control group, but this effect did not reach statistical significance ($P > 0.05$; Figure 2F). In addition, CO and HR were slightly and not significantly decreased in the DOCA-salt group compared with the control group, and not affected by the AEPA treatments (100 and 300 mg/kg/day; $P > 0.05$; Figures 2G,H).

Intake of AEPA improves the DOCA-salt-induced endothelial dysfunction in the primary mesenteric artery: role of NO and EDH

The DOCA-salt treatment increased significantly phenylephrine-induced vasoconstriction in primary mesenteric artery rings with endothelium compared to the control group ($P < 0.05$; Figure 3A). Intake of AEPA (300 mg/kg/day) normalized the phenylephrine induced-contractile response in the DOCA-salt group, whereas 100 mg/kg/day was without effect (Figure 3A).

The DOCA-salt group presented an endothelial dysfunction as indicated by a significantly reduced acetylcholine induced endothelium-dependent relaxation compared to that of the control group ($P < 0.05$; Figure 3B). Treatment with AEPA (100 or 300 mg/kg/day) improved the DOCA-salt-induced endothelial dysfunction to acetylcholine ($P < 0.05$; Figure 3B).

In primary mesenteric artery rings with endothelium, both the NO-mediated (assessed in the presence of indomethacin and Tram-34 plus apamin) and the endothelium-dependent hyperpolarization (EDH)-mediated (assessed in the presence of indomethacin and N^{ω}-nitro-L-arginine) relaxations to acetylcholine were significantly reduced in the DOCA-salt group compared to those of the control group ($P < 0.05$; Figures 4A,B). Treatment with AEPA (100 or 300 mg/kg/day) improved the DOCA-salt-induced blunted NO- and EDH-mediated relaxations in the primary mesenteric artery (Figures 4A,B).

AEPA prevents the DOCA-salt-induced increased primary mesenteric artery oxidative stress and up-regulation of NADPH oxidase subunit $p22^{phox}$

Immunofluorescence studies showed that the DOCA-salt treatment was associated with oxidative stress throughout the primary mesenteric artery wall as shown by an increased DHE fluorescence signal, which amounted to 124.17 ± 4.89 % ($P < 0.05$; Figure 5A). Moreover, the increased level of oxidative stress in primary mesenteric artery induced by DOCA-salt

was associated with a significant increased expression of the NADPH oxidase subunits $p22^{phox}$ in the arterial wall, amounted to about 198.82 ± 17.17 % in the DOCA-salt group ($P < 0.05$; Figure 5B). Both AEPA treatments prevented the DOCA-salt-induced increased vascular oxidative stress and up-regulation of NADPH oxidase ($P < 0.05$; Figures 5A,B).

AEPA prevents the DOCA-salt-induced increased expression of eNOS and decreased expression of connexin 37 in the primary mesenteric artery

Immunofluorescence staining indicated a significant increased expression of eNOS (188.22 ± 12.91 %; $P < 0.05$; Figure 5C) and a decreased expression of connexin Cx37 (32.39 ± 3.23 %; $P < 0.05$; Figure 5D) in the primary mesenteric artery of the DOCA-salt group compared to those of the control group. Both AEPA treatments prevented the deleterious effect of the DOCA-salt treatment on the expression of eNOS, and also the AEPA (100 mg/kg/day) treatment on the expression of Cx37 ($P < 0.05$; Figures 5C,D).

AEPA prevents the DOCA-salt-induced increased expression of COX-2, but not COX-1 in the primary mesenteric artery

The immunofluorescence COX-2 signal was significantly increased in the primary mesenteric artery of the DOCA-salt group, which amounted to 153.72 ± 12.44 %; ($P < 0.05$; Figure 5F). After AEPA treatment, a significant reduction of the COX-2 immunofluorescence level was observed in the DOCA + AEPA (100 and 300 mg/kg/day) groups ($P < 0.05$; Figure 5F). Also, the expression level of COX-2 was slightly reduced in AEPA (100 mg/kg/day) group compared to that of the control group, however this effect did not reach statistical significance ($P > 0.05$; Figure 5F). In contrast to the COX-2 signal, the expression level of COX-1 was similar in the different groups ($P > 0.05$; Figure 5E).

DISCUSSION

The major findings indicate that daily oral intake of an aqueous extract of *Phyllantus amarus* for 5 weeks prevented effectively the hypertensive response observed after the subcutaneously injection of deoxycorticosterone acetate (DOCA) and intake of 1% NaCl in rats. The preventive beneficial effect of the AEPA is related to an improved cardiac structure and function, and also with an improved endothelial dysfunction affecting both the NO and EDH components.

Consistent with previous studies, DOCA-salt induced a progressive hypertensive response in rats, which reached a plateau phase after about 4 weeks, and is associated with the development of cardiac hypertrophy, especially in the left ventricle.[5,9,7] The transthoracic echocardiography analysis of DOCA-salt rats has indicated an increase of both PWT and SWT associated with an increase of LVM without dilation of the left ventricular chamber, indicating a concentric type of hypertrophy.[31] In addition, analysis of the cardiac function showed an impairment of diastolic function in DOCA-salt rats, as confirmed by an increase of IVRT. The ejection fraction (LVEF) was slightly reduced in the DOCA-salt group, but the value remained within the normal range i.e. greater than 50 %.[32]. Moreover, abnormal heart function observed in DOCA-salt rats is associated with minor changes of cardiac output and heart rate. Altogether, these findings are in good agreement with previous ones indicating that at early stages of hypertension, the cardiac diastolic function is impaired before the systolic function.[33] The present findings reveal that daily oral intake of AEPA was able to significantly prevent the hypertensive response to DOCA-salt, with a cardioprotective action including an antihypertrophic effect with improved PWT, SWT and LVM values, and an improved diastolic cardiac function with normal IVRT. Thus, these findings provide experimental evidence of the antihypertensive potential of *Phyllantus amarus* as well as its ability to prevent consequences of hypertension at the heart function and structure in chronic

situation. A previous study has also reported that oral intake of an extract of *Moringa oleifera* (Moringaceae) seeds decreased the LVM and improved the diastolic function in the SHR, associated with an up-regulation of peroxisome proliferator-activated receptor (PPAR)-α and δ signaling, which play a crucial role in fatty acid catabolism in the heart.[34] Moreover, *Euterpe oleracea* (Arecaceae) prevented cardiac disorders such as hypertrophy and fibrosis in rats after myocardial infarction.[14]

Besides the heart, chronic hypertension is known to cause damage to several end organs and, in particular, the kidney affecting its function and structure.[35,36] The present findings indicate that DOCA-salt hypertension is associated with an increased left kidney weight and plasma uric acid level, and a reduced plasma potassium level. Moreover since chronic hypokalemia has been involved in cardiac hypertrophy when hypertension is associated with primary aldosteronism,[8,37,38] the ability of the AEPA treatment to normalize the plasma potassium level might contribute to explain, at least in part, the improved kidney function and structure in the AEPA-treated DOCA-salt group. An improved kidney function and structure has also been observed with sesame oil and quercetin in the DOCA-salt hypertensive rat.[5,8]

Endothelial dysfunction is an early common arterial deleterious effect appearing before structural changes of the arterial wall in experimental models of hypertension, and also in hypertensive humans.[10, 39] In the DOCA-salt hypertensive group, the endothelial dysfunction of the main mesenteric artery is characterized by blunted endothelium-dependent relaxations to acetylcholine, and also the appearance of an exaggerated contractile response to phenylephrine. Moreover, the characterization of the endothelial dysfunction to acetylcholine indicated that both the NO and EDH components were significantly reduced in the primary mesenteric artery of the DOCA-salt group. Interestingly, the AEPA treatment at both 100 and 300 mg/kg/day prevented the DOCA-salt-induced endothelial dysfunction as indicated by normal NO and EDH components in response to acetylcholine, and also at 300 mg/kg/day a

normal contractile response to phenylephrine was observed. In addition, the *Phyllanthus amarus* aqueous extract induced pronounced concentration-dependent relaxations in coronary artery rings solely in the presence of a functional endothelium (unpublished data).

The AEPA treatments at 100 and 300 mg/kg/day protected the cardiovascular system of DOCA-salt hypertensive rats to a similar extent. Such effect, is possibly due to the fact that the antihypertensive effect of AEPA reached already a maximal response at a dose of 100 mg/kg/day. In addition, according to a previous study, the observed AEPA-induced hypotensive effects in rabbits was characterized by an ED_{50} of 25.77 mg/kg.[23].

Besides *Phyllantus amarus*, several other types of natural products such as a red wine polyphenols extract, an ethanolic extract of *Lindera obtusiloba* or the sesame lignan sesamin have been shown to prevent endothelial dysfunction in several experimental models of hypertension including the DOCA-salt model, in part, by preventing vascular oxidative stress.[30,40-44] Indeed, an increased level of oxidative stress is observed throughout the arterial wall of hypertensive arteries subsequent to the up-regulation of the expression of NADPH oxidase [45,46] and it has been shown to promote uncoupling of endothelial NO synthase most likely triggered by oxidation of the essential eNOS cofactor tetrahydrobiopterin,[30,47] alteration of calcium-dependent K^+ channels and connexins such as Cx37 involved in EDH,[12,48,49] and an increased expression of cyclooxygenases involved in endothelium-dependent contractile responses.[50,51] The present findings indicate that the AEPA treatment was able to effectively prevent the DOCA-salt-induced oxidative stress and the up-regulation of the NADPH oxidase subunit $p22^{phox}$ in the primary mesenteric artery wall. They further indicate that the AEPA treatment prevented the DOCA-salt-induced up-regulation of eNOS expression most likely part of a compensatory mechanism subsequent of an enhanced degradation of NO by superoxide anions, and also partially the down-regulation of Cx37. Moreover, the AEPA treatment prevented the up-regulation of COX-2, a redox-sensitive NF-kB-controlled pro-

inflammatory enzyme, in the primary mesenteric artery of the DOCA-salt group. Such an effect is in good agreement with previous *in vivo* and *in vitro* findings indicating that Phyllanthus species such as *Phyllanthus amarus* and *Phyllanthus acidus* reduced inflammatory responses by preventing COX-2 overexpression in RAW 264.7 cells,[18,51] and the high-fructose diet-induced cardiac oxidative stress in rats.[53] Moreover, *Phyllanthus amarus* hexane and hydroalcoholic extracts induced a potent anti-inflammatory effect in RAW 264.7 macrophages by preventing the expression of iNOS, COX-2, and pro-inflammatory cytokines such as IL-1β and IFN-γ, in response to activation of NF-kB.[18]

Moreover, lignans, hydrolysable tannins, flavonoids, alkaloids, triterpenes, sterols and volatile oils are known as main secondary metabolites present in *Phyllanthus amarus*.[54, 55] According to our previous study, the phytochemical analysis of the aqueous extract of whole plant of *Phyllanthus amarus* has revealed the presence of alkaloids, polyphenols, terpenes and sterols.[23] Although the contribution of these different classes of natural products to the beneficial effect of *Phyllanthus amarus* on DOCA-salt-induced hypertension, cardiac hypertrophy and dysfunction, and endothelial dysfunction in rats remains to be determined, it is possibly related to the combined action of these secondary metabolites, acting in a synergistic manner.

Altogether, the findings indicate that AEPA has antihypertensive properties, and prevents endothelial dysfunction, and cardiac hypertrophy and dysfunction in DOCA-salt hypertensive rats. Furthermore, the protective vascular effect of the AEPA treatment is associated with an improvement of both the NO and EDH components and also of the expression of major target proteins involved in these pathways including eNOS and Cx37, in part, by reducing the level of vascular oxidative stress.

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Figure legends

Figure 1: Effect of AEPA treatment on systolic blood pressure (SBP) measured by tail-cuff sphygmomanometry. Results are shown as mean ± SEM of 7 to 8 different rats. $^{*}P <$ 0.05 versus the control group; $^{\#}P < 0.05$ versus the DOCA-salt group.

Figure 2: Effect of AEPA treatment on left ventricle cardiac hypertrophy and function. (A) PWT: posterior diastolic wall thickness, (B) SWT: septum diastolic wall thickness, (C) LVM: left ventricular mass, (D) LVEDD: left ventricle end-diastolic diameter, (E) IVRT: isovolumetric relaxation time, (F) LVEF: left ventricle ejection fraction, (G) CO: cardiac output, and (H) HR: heart rate. Results are shown as mean ± SEM of 7 to 8 different rats. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the DOCA-salt group.

Figure 3: AEPA treatment prevents endothelial dysfunction induced by DOCA-salt in rats. (A) Arterial rings from the primary mesenteric artery with endothelium were subjected to increasing concentrations of phenylephrine to construct concentration-contraction curves. (B) Arterial rings from the primary mesenteric artery with endothelium were precontracted with 10^{-6} M of phenylephrine before the addition of increasing concentrations of acetylcholine to construct concentration-relaxation curves. Results are shown as mean ± SEM of 4-5 rats per group. $^{*}P < 0.05$ versus the control group; $^{\#}P <0.05$ versus the DOCA-salt group.

Figure 4: Improvement of both the NO and EDH components of the endothelium-dependent relaxation to acetylcholine by the AEPA treatment in DOCA-salt hypertensive rats. Arterial rings from the primary mesenteric artery with endothelium were precontracted

with 10^{-6} M of phenylephrine before the addition of increasing concentrations of acetylcholine to construct concentration-relaxation curves. (A) Nitric oxide (NO)-mediated relaxations were obtained in presence of indomethacin (10 μM, to prevent the formation of vasoactive prostanoids), and charybdotoxin (100 nM) plus apamin (100 nM; two inhibitors of EDH-mediated relaxations) for 30 min before construction of the concentration-response curve. (B) Endothelium-dependent hyperpolarization (EDH)-mediated relaxations were obtained in the presence of indomethacin (10 μM) to prevent the formation of vasoactive prostanoids, and N^G-nitro-L-arginine (100 μM, an inhibitor of endothelial NO synthase) for 30 min before construction of the concentration-response curve. Results are shown as mean ± SEM of 4-5 rats per group. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the DOCA-salt group.

Figure 5: Effect of AEPA treatment on the DOCA-salt-induced changes in vascular oxidative stress, and markers of NO, EDH and cyclooxygenase pathways. (A) The level of oxidative stress (*in situ* ROS formation) was determined in primary mesenteric artery sections by fluorescence histochemistry using the redox-sensitive probe dihydroethidium. The expression level of (B) NADPH oxidase subunit $p22^{phox}$, (C) eNOS, (D) connexin 37, (E) COX-1 and (F) COX-2 was assessed in mesenteric artery sections by immunofluorescence. Sections were observed using a confocal laser-scanning microscope. Values are shown as mean ± SEM of 4-5 rats per group. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the DOCA-salt group.

Table 1: Effect of AEPA treatment on biochemical parameters in the control and DOCA-salt hypertensive group of rats.

Groups	Control	AEPA (100 mg/kg/d)	DOCA	DOCA + AEPA (100 mg/kg/d)	DOCA + AEPA (300 mg/kg/d)
Urea (g/l)	0.29 ± 0.01	0.28 ± 0.02	0.30 ± 0.04	0.31 ± 0.01	0.32 ± 0.02
Uric acid (mg/l)	7.00 ± 1.00	6.66 ± 0.68	11.25 ± 0.48*	6.67 ± 0.52$^{\#}$	8.00 ± 0.50$^{\#}$
Sodium (mmol/l)	143.20 ± 1.11	144.4 ± 1.03	147.50 ± 0.65	147.8 ± 0.66	146.75 ± 0.75
Potassium (mmol/l)	5.54 ± 0.2	5.07 ± 0.10	4.18 ± 0.08*	5.26 ± 0.34$^{\#}$	5.14 ± 0.39$^{\#}$
Chloride (mmol/l)	99.00 ± 1.08	101.20 ± 1.52	100.00 ± 3.63	99.00 ± 2.35	102.5 ± 0.29

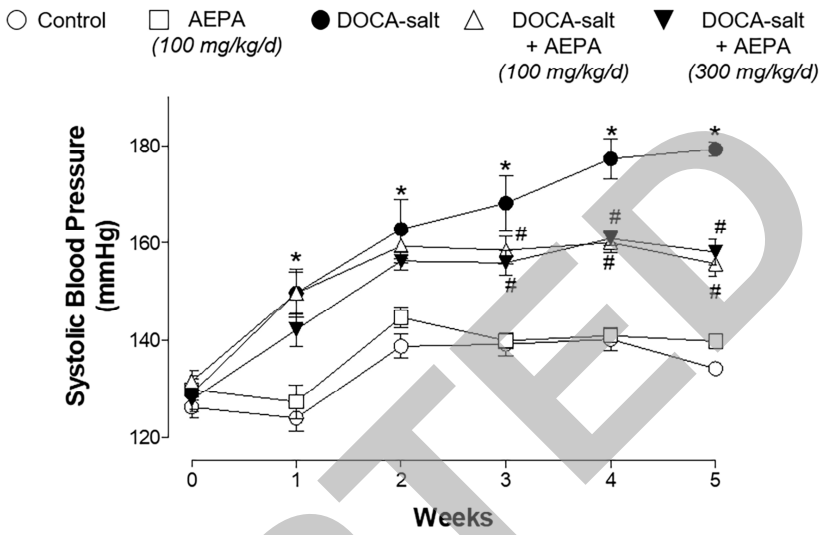
Results are shown as mean ± SEM of 4 to 5 different rats. $^{*}P < 0.05$ versus control group and $^{\#}P < 0.05$ versus DOCA-salt group.

Table 2: Effect of AEPA treatment on physiological parameters in the control and DOCA-salt hypertensive group of rats.

Groups	Control	AEPA (100 mg/kg/d)	DOCA	DOCA + AEPA (100 mg/kg/d)	DOCA + AEPA (300 mg/kg/d)
BW (g)	401.43 ± 11.55	407.83 ± 8.38	397.14 ± 10.32	402.50 ± 19.11	413.42 ± 7.11
HW/BW (mg/g)	2.73 ± 0.08	2.60 ± 0.05	3.40 ± 0.23*	2.75 ± 0.01#	2.73 ± 0.01#
LVW/ BW (mg/g)	1.04 ± 0.05	0.98 ± 0.03	1.34 ± 0.11*	1.15 ± 0.04	1.08 ± 0.05#
RVW/BW (mg/g)	0.46 ± 0.02	0.45 ± 0.03	0.58 ± 0.04	0.53 ± 0.03	0.47 ± 0.02
LKW/BW (mg/g)	3.18 ± 0.12	2.81 ± 0.06	3.71 ± 0.18*	3.21 ± 0.12	3.09 ± 0.08#

BW, body weight; HW/BW, cardiac weight index, LVW/ BW, left ventricle weight index; RVW/BW, right ventricle weight index; LKW/BW, left kidney weight index. Results are shown as mean ± SEM of 7 to 8 different rats. $^{*}P < 0.05$ versus control group and $^{\#}P < 0.05$ versus DOCA-salt group.

Figure 1



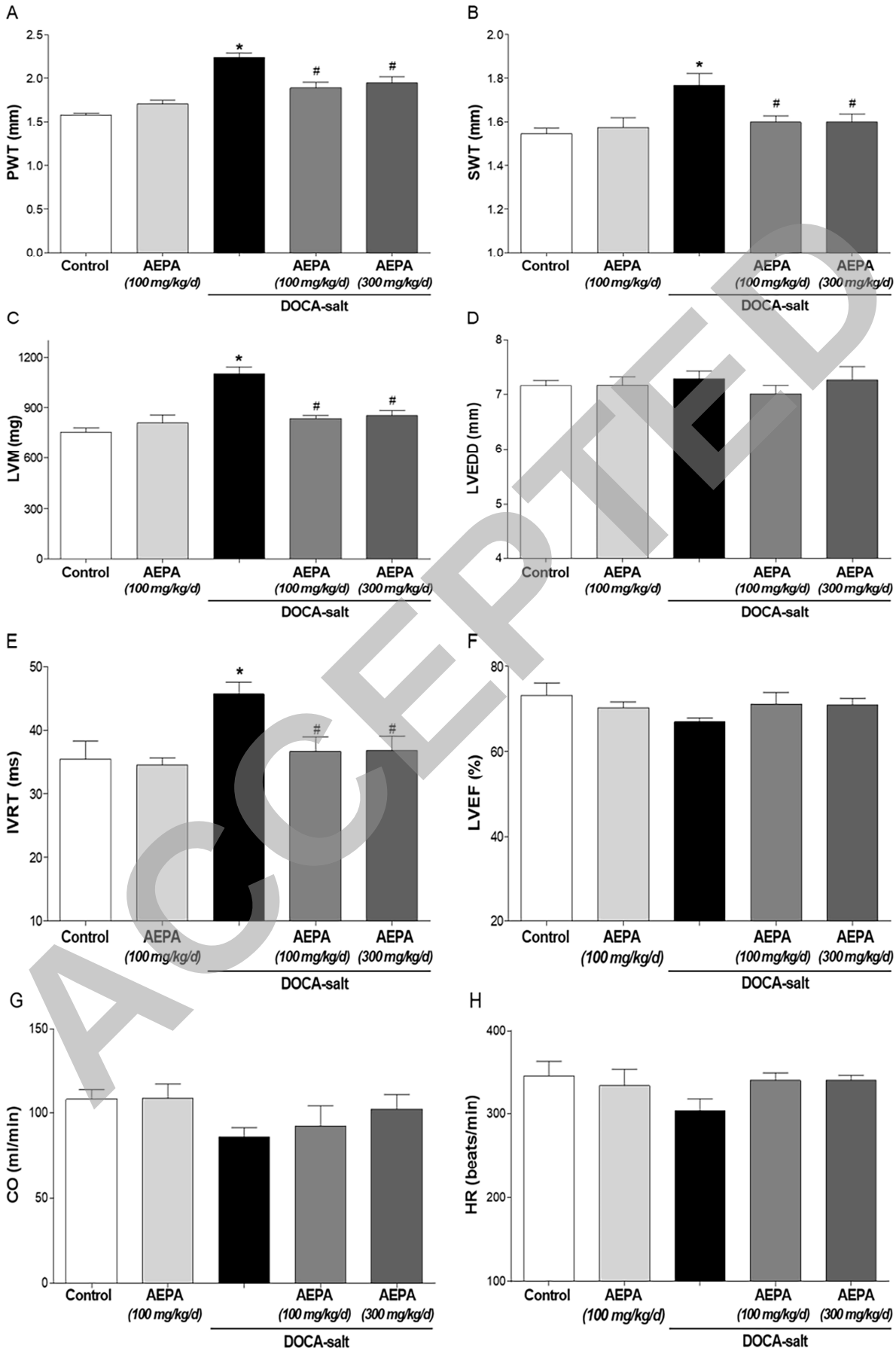


Figure 3

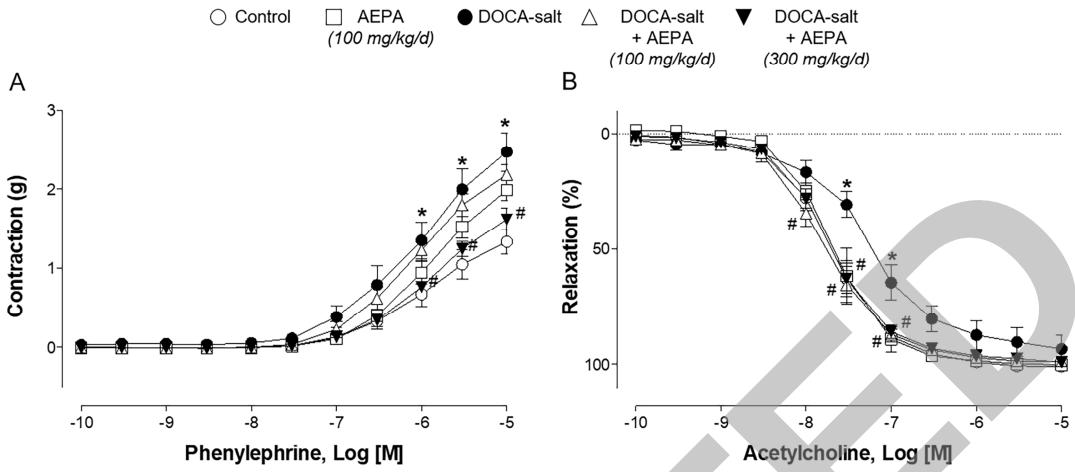


Figure 4

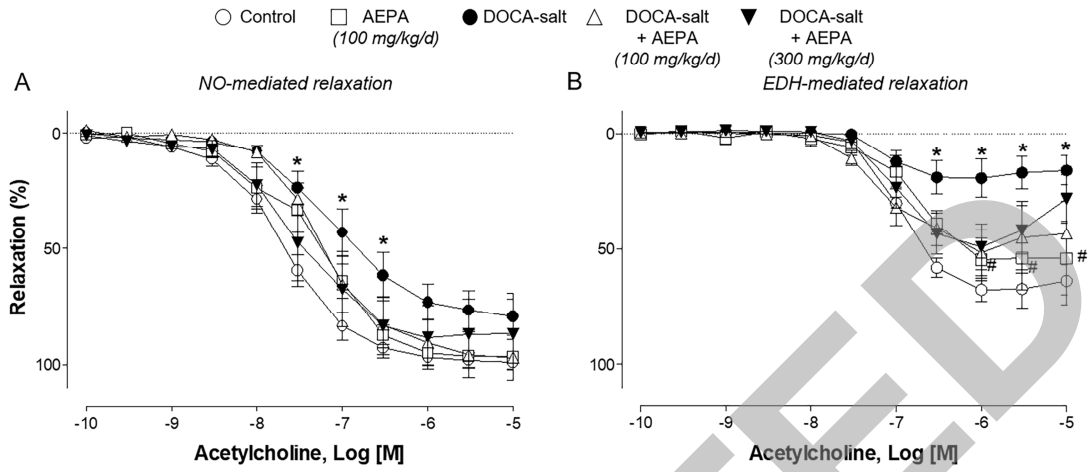


Figure 5