SUPPLEMENT MATERIAL

_Eucalyptus globulus_ possesses antihypertensive activity in L-NAME-induced hypertensive rats and relaxes isolated rat thoracic aorta through nitric oxide pathway

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Abstract

In the current study we determined the effect of the aqueous extract of leaves of _Eucalyptus globulus_ (AEEG) in anesthetized normal and L-NAME-induced hypertensive rats and on rings of isolated thoracic aorta from Wistar rats at a dose of 160 mg/kg. Our results show that AEEG extract reduced systolic, mean and diastolic blood pressure after repeated (7 days) oral administration of this extract in hypertensive rats. In addition, it was revealed from the present study that AEEG relaxed aortic rings in a dose-dependent (25 µg/ml–200 µg/ml) manner via the activation of nitric oxide production. Pre-treatment of aortic rings with indomethacin, glibenclamide, nifedipine or propranolol did not attenuate the AEEG-induced vasorelaxation. Our data elucidate the health-orientated virtues of using AEEG which may play an important role as an antihypertensive agent to reduce the burden of cardiovascular complications.

Key words: _Eucalyptus globulus_, blood pressure, antihypertensive, vasorelaxation, L-NAME, nitric oxide.
Experimental

**Plant material and preparation of the aqueous extract**

Fresh leaves of *Eucalyptus globulus* (*E. globulus*) were harvested in Tafilalet region (semi-arid area) of Morocco in April 2017, and air-dried at 40 °C. The plant was taxonomically identified and voucher specimen was deposited at the herbarium of the Faculty of Sciences and Techniques, Errachidia (EG150). Then, plant material was prepared according to the traditional method used in Morocco (decoction): 1 g of powdered leaves, mixed with 100 ml distilled water, was boiled for 10 min and then cooled for 15 min. Thereafter, the aqueous extract was filtered using a Millipore filter (Millipore 0.2 mm, St Quentin en Yvelines, France) to remove particulate matter. Finally, the filtration of the extract was lyophilized in a lyophilizator (LABCONCO, G.BOYER, materiel de laboratoire, Casablanca). The dose administered was 80 mg of lyophilized aqueous extract per kg of body weight.

**Experimental animals**

Healthy albino adult male rats (Wistar strain) weight about 175-280 g were obtained from a commercial vendor and were kept in individual polyethylene cages and maintained in standard condition (24 ± 1 °C, with 12 h/12 h light/dark cycle). The animals were fed *ad libitum* with normal laboratory chow standard pellet diet. Rats were allowed at least 3 week for a better acclimation from shipping stress.

**Chemical reagents and drugs**

Acetylcholine chloride, norepinephrine, phenylephrine hydrochloride, Nω-nitro- L-arginine methyl ester (L-NAME) and indomethacin were purchased from Sigma Chemical Co. (St. Louis, USA). All other reagents were of analytical grade from local sources. All cited drugs were dissolved in distilled water.

**Experimental protocol**

**Hypotensive activity**

Hypertension was induced in male albino Wistar rats by a repeated (once by day during a week) oral administration of L-NAME (80 mg/ kg body weight) dissolved in distillated water; normal control rats received distilled water as vehicle. The animals were
confirmed as hypertensive by the raised systolic blood pressure over 150 mmHg and were used for the study.

Normotensive and hypertensive rats were randomly assigned to three different groups consisting of six rats each. One control group received distilled water, a second treated group received the aqueous extract of aerial parts of *E. globulus* (AEEG) at a dose of 160 mg/kg body weight, and the third group received a reference drug (lasilix at a dose of 20 mg/kg). For repeated oral administration, rats were treated once daily for 7 days and the three last parameters were followed during this period. The rats (n=6 in each group) were treated orally. All experiments were performed after anesthetizing animals using ether, this in order to avoid immobilization stress induction. For estimation of blood pressure, rats were placed in the NIBP restrainers and appropriate cuff with sensor was then attached on its tail and warmed to about 35 °C for 15–20 min to allow reproducible blood pressure measurements (three measurements per animal per session) using a tail cuff and a computer-assisted monitoring device. The tail cuff was inflated to a pressure well above the expected systolic blood pressure (200 mmHg) and slowly released during which the pulses were recorded by using Power Lab data acquisition system and Lab chart 5.0 software. Systolic blood pressure (SBP), mean blood pressure (MBP) and heart rate (HR) were measured directly using pulse tracing while diastolic blood pressure (DBP) was calculated from SBP and MBP using formula: \( DBP = \frac{3 \times MBP - SBP}{2} \). All blood pressure measurements were carried out at the same time of day for the sub-chronic test.

**Vasorelaxant activity**

Male Wistar rats (260–300 g) were raised and housed in rooms with a controlled 12/12 h light/dark cycle at 25 °C with food and water *ad libitum*. Animals were sacrificed by stunning and exsanguination after anesthesia by pentobarbital sodium. Aortas were immediately excised and placed in cold buffer, then, quickly removed and carefully cleaned of adhering fat and connective tissue. The isolated arteries were spirally cut into rings 2–3 mm length and suspended at a resting force of 2 g in an tissue bath oxygenated (95% O\(_2\), 5% CO\(_2\)) and containing 40 ml Krebs-Henseleit solution (KH), maintained at 37 °C and pH 7.4 and containing (mM) NaCl (118), KCl (4.50), NaHCO\(_3\) (25), MgSO\(_4\) (1.2), CaCl\(_2\) (1.8), NaHPO\(_4\) (1.2) and glucose (11). Changes in length were recorded isotonically using a lever transducer (Erma, Tokyo). Aortic rings were mounted on two stainless steel hooks, one fixed to the bottom of the chamber and the other to a UFI force transducer (LCM Systems Ltd).
connected to a PowerLab/400 ADInstrument data acquisition system (HARVARD Apparatus) in order to record signal corresponding to the isometric tension.

**Functional and mechanistic approaches**

Optimal tension, selected from preliminary experiments, was that which gave the greatest response to $10^{-5}$ M norepinephrine. The rings were given 2 g (100%) of initial tension and allowed to equilibrate for 1 h. Thirty minutes after setting up the tissue bath; aortas rings were contracted with $10^{-5}$ M epinephrine (NE) or 80 mM potassium chloride (KCl) solution to test their contractile responses. After exposure to $10^{-5}$ M epinephrine, tissues were washed three times with Krebs solution to restore basal tension and this solution was renewed every 40 min.

In order to determine the cumulative dose-dependent curves for AEEG-induced relaxation and after equilibration, rings were contracted by NE (10 µM) and KCl (80 mM). After the plateau was reached, AEEG was added cumulatively (25, 50, 75, 100, 125, 150, 175 and 200 µg/ml), at each dose of AEEG, the curve was allowed to reach a plateau before the addition of subsequent dose. To determine the mechanistic route involved in the relaxant effect induced by AEEG on NE pre-contracted rat aortic rings, the following experiment was carried out: twenty minutes before adding NE, aortic rings were pre-incubated for 20 min with one of various standard drugs. Drugs used for pre-incubation were: 1) $10^{-4}$ M L-NAME, a direct inhibitor of NO synthase. 2) 10 µM glibenclamide, an ATP-sensitive K$^+$ channel blocker. 3) 10 µM indomethacin, a prostaglandin synthesis inhibitor. 4) 10 µM propranolol, a beta-blocker. 5) 10 µM nifedipine a calcium channel blocker. After the addition of NE ($10^{-5}$ M) and the plateau was attained, and then aortic relaxation was carried out by cumulative addition of AEEG (25 to 200 µg/mL), the vasorelaxant effect on the aortic rings was calculated as a percentage of contraction in response to NE. Before changing any of the compounds used in this experiment, the aorta ring was washed three times by cold buffer (aorta ring should be incubated 5 min intervals in each bath). The control was performed by cumulative addition of vehicle (distillated water) to aortic rings pre-contracted by NE ($10^{-5}$ M) and KCl (80 mM).

**Statistical analysis**

All the values were expressed as mean ± SEM. Statistical significance was tested between more than two groups using two-way ANOVA followed by the Bonferroni multiple comparisons test. Relaxations to AEEG were expressed as a percentage decrease of the NE and KCl contraction. Each cumulative dose-effect curve for AEEG-induced relaxation was
plotted, contingent on application of sigmoidal curve fitting and non-linear regression, using Prism version 7 (GraphPad Software Inc., San Diego, CA., U.S.A.) to generate the parameters \( R_{\text{max}} \) (maximal relaxant response) and \( \text{IC}_{50} \) (the concentration of AEEG dose required to give 50% of the maximum aortic-relaxant response). Differences were considered to be statistically significant when \( p<0.05 \).