Endothelium-dependent vasorelaxant effects of the methanol stem bark extract of *Vepris heterophylla* (Engl.) R. Let. (Rutaceae) on rat aorta

**ABSTRACT**

*Vepris heterophylla* is a medicinal plant used empirically in northern part of Cameroon by traditional healers for the treatment of various illnesses including arterial hypertension. Experiments were carried out to investigate mechanisms of relaxant activities of the methanol extract from *V. heterophylla* in rat isolated thoracic aortic strips. The relaxant effects of *V. heterophylla* on vascular preparation from rat aorta pre-contracted with KCl or norepinephrine was concentration dependent and endothelium-dependent. This relaxing effect was significantly reduced with KCl-induced contraction following mechanical damage to the aortic endothelium. Relaxation elicited by *V. heterophylla* was not significantly affected by tetraethylammonium (10^{-6} M), a non selective K^+ channel blocker. However, the present results using norepinephrine contraction indicate that, the relaxation induced by *V. heterophylla* in aorta pre-contracted with norepinephrine was also significantly affected by endothelial removal. The relaxation action of the plant extract was significantly reduced in endothelium intact aorta pre-contracted with norepinephrine in the presence of indomethacin (10^{-5} M), a cyclo-oxygenase inhibitor. These findings show that the vasorelaxation effect of the methanol stem bark extract of *V. heterophylla* may be mediated at least in part by prostacyclin. In the presence of 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) (10 μM), a sCG inhibitor, the relaxation induced by *V. heterophylla* (0.5-3 mg/ml) was almost completely abolished, suggesting a strong participation of the NO–cGMP pathway in the vasorelaxant response induced by *V. heterophylla*. The inhibitory effects induced by *V. heterophylla* in the presence of Nω-nitro-L-arginine methyl esther (L-NAME, 10^{-4} M) were completely reversed by the addition of the biological precursor of NO synthesis L-arginine (10^{-4} M). The results from this study show that the vasorelaxation of the methanol stem bark extract of *V. heterophylla* was endothelium-dependent, likely via the NO–cGMP pathway; or independent associated mediators such as prostacyclin, NO, and guanylyl cyclase. *V. heterophylla* is able to provoke a concentration-dependent vasorelaxation of the rat aortic strips.

**Key words:** *Vepris heterophylla*, aorta, endothelium, NO–cGMP pathway, vasodilation

**INTRODUCTION**

*Vepris heterophylla* (Engl.) R. Let. is a medicinal plant used empirically in the mountainous massifs in the northern part of Cameroon for the treatment of various illnesses such as malaria, cardio-vascular disorders, arterial hypertension...
etc (Letouzey 1968; Ngamo et al. 2001). The vernacular names of this species are Kounikoutchoum (Guizia, Mofou), Hohoum (Zoulgo), Gouguvetche (Mafa), Kotokolhi (fulfulde) which testifies its importance in this region (Hamawa et al., 2010). The medicinal virtues of this Rutaceae on high blood pressure and malaria have already been pointed out by several authors: Letouzey (1968), Moulis et al. (1994) and Keita and Ouattara (1995). Research on Cameroonian medicinal plants reputed to have cardiovascular effects will help us to scientifically validate their traditional use and to rationalize local use of the plant extracts for the management of these diseases. In an attempt to provide a pharmacological rationale (or otherwise) for the use of *V. heterophylla* stem bark in the management of hypertension, the present study was undertaken to investigate the effects of the methanol stem bark extract of *V. heterophylla* in isolated rat vascular smooth muscles.

**MATERIALS AND METHODS**

**Animals**

Wistar rats (250 – 350 g) were used for all experiments. Animals were housed under conditions of controlled temperature (20-24°C) and humidity (55% ± 10%). In addition, they had free access to food (Harlan Teklad, Global diets, Pavia, Italia) and tap water *ad libitum*. The animal handling was under the control of the veterinary surgeon of the University of Pavia. Experimental protocols and procedures were approved by the institutional Animals Care and Use Committee and the research was approved by the Ethical Committee of the University of Pavia.

**Plant material**

The stem bark of *V. heterophylla* was collected in February in the Mokolo region, far North of Cameroon (10° 39'214'N, 14° 24'145'E, 375 m altitude). This savannah region has annual average humidity of 73% and an average temperature of 29°C. The average annual rainfall reaches 1002 mm (IRAD, 2007). This plant was selected on the basis of ethno-pharmacological properties and a sample was identified and deposited (N°: 1UCN) EN A1c, B1+2C at the National Herbarium in Yaoundé, Cameroon. The stem bark was cut, air-dried and crushed. 2.5 kg powder of the plant material was introduced into the extraction solvent, at room temperature. The extraction lasted for 48 h. After decantation and filtration, the macerate was collected in a volumetric flask. The operation was repeated two times with the solvent. Each extract was concentrated to dryness under reduced pressure using a rotary evaporator (BUCHI). From this procedure, 175 g of methanol extract were obtained and kept at -0.5°C until use, with 7% of the extraction. Percolation and maceration were used as extraction techniques in this study. The powder obtained after grinding was placed in a glass column fitted with a sintered glass at its end. A thin layer of Celite (Diatomaceous Earth) was used to cover and protect the fritted glass disk; the pores might be clogged by fine particles. The plant powder was then placed in the column surrounded with a sheet of aluminium foil to protect the product from light. After addition of 2.5 liters of solvent into the column and maceration of 4 h, the extract was collected in a volumetric flask. The operation was repeated twice with two liters of solvent. The extracts were combined in the flask and evaporated to dryness under reduced pressure using a rotary evaporator. The volumetric flask was weighed and stored in the fridge until use (Duez and Stévigny, 2007). After this operation, 160 g plant extract were obtained.

**Drug administration**

The following drugs were used: methanol stem bark extract of *V. heterophylla*, dimethylsulfoxide (DMSO), Norepinephrine, acetylcholine chloride, No-nitro-L-arginine methyl ester (L-NAME), indomethacin, Verapamil, tetraethylammonium, L-arginine, 1H-[1,2,4]oxadiazolo-[4,3-a]quinolin-1-one (ODQ), all from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions were prepared in distilled water and kept at −4°C. Methanol barks extract of *V. heterophylla* was solubilized in DMSO and diluted to the desired concentrations with distilled water just before use. ODQ was dissolved in DMSO. The other compounds were dissolved in distilled water. The final concentration of DMSO in the bath never exceeded 0.1%, and has no effect when tested in control preparations (data not shown).

**Tissue preparation and experimental procedure**

The Wistar rats were sacrificed by stunning and bleeding. The thoracic aorta was dissected out and perfused with physiological solution (Tyrode) after which the aortic artery was removed and cleaned from connective tissue and fat. Aortic strips were obtained and placed in oxygenated physiological salt solution. The composition of the Tyrode’s solution used was 158.3 mM NaCl, 4.0 mM KCl, 2.0 mM CaCl2, 1.05 mM MgCl2, 0.42 mM NaH2PO4, 10.0 mM NaHCO3, and 5.6 mM glucose. The thoracic aorta was cleaned of all adhering tissue and then cut into helical strips (1.0 mm × 10 mm). The endothelium was kept intact in aorta strips, but when appropriate, the endothelium was removed by gently rubbing the luminal surface with cotton thread. Each tissue was mounted in a 4 ml organ bath at an initial tension of 1 g. The baths contained Tyrode solution kept at 37°C and bubbled with 95% O2 and 5% CO2. Isometric contractions were evoked by stimulation with 0.5 s trains of three pulses of 110% maximal voltage (CED
Micro 1401 mkII, train frequency 500 kHz; pulse duration 16-bit ADC) through a platinum electrode attached to the upper end and a stainless steel electrode attached to the lower end of each bath. Stimuli were generated by a Grass S48 stimulator (which is a general purpose stimulator intended for nerve and muscle stimulation procedures with applications extending from single cell to entire muscle stimulation). It can be set to deliver single, repetitive, twin pulses, trains of pulses and trains of twin pulses from its single output, then amplified (Med-Lab 4 channel attenuator) and divided to yield separate outputs to four organ baths (Med-Lab StimuSplitter II). Contractions were monitored by computer program Spike2 (Macintosh LCIII and Performa 475) using a data recording and analysis system (MacLab) that was linked via pre amplifiers (Mac bridge) to Dynamometer force transducers (Dubam 47C). The organ bath was maintained at 37°C, pH 7.4, and bubbled continuously with O₂. Each tissue was subjected to several 5 min periods of stimulation, the first of these beginning after the tissue had equilibrated but before drug administration. Then, endothelium integrity was functionally assessed by evaluating the ability of acetylcholine (ACH, 10⁻⁴M) to produce relaxation of preparations pre-contrasted with norepinephrine (NE 10⁻⁴M). Preparations were considered to contain a viable endothelium when Acetylcholine (ACH) evoked relaxations exceeding 64% of pre-contraction, and were considered to be endothelium denuded when ACH failed to cause relaxation (Furchgott and Zawadski 1980). After ACH testing, the aorta strips were washed with physiological salt solution three times during the next hour, prior to the next sequence (Dimo et al. 2005). Following the equilibration period, concentration response of strips with or without endothelium were studied by pre-contracting each aortic strip with KCl (60 mM) or 10⁻⁴M norepinephrine for 30 min and then allowing them to relax in the presence of V. heterophylla extract. When the contractile response to each agonist was stable, aortic strip was challenged with respective doses of V. heterophylla. All concentrations are expressed as final bath concentrations. In the second group of experiments, tissues containing an intact endothelium were incubated for 30 min with indomethacin (10⁻⁶M), a cyclo-oxygenase inhibitor and the relaxant effect of the plant extract was tested. The preparations were exposed to L-NAME (10⁻⁴M), a nitric oxide synthase (NOS) inhibitor (Moncada et al., 1993); L-NAME (10⁻⁴M) plus L-arginine (10⁻⁴M M), the endogenous substrate of NOS (Toda and Okamura, 2003); ODQ (10⁻⁴M), a soluble guanylyl cyclase (sGC) inhibitor (Garthwaite et al., 1995); Verapamil (10⁻⁴M) a α₁- adrenoreceptor antagonist and L-type calcium channel blocker. Tetraethylammonium (10⁻⁴M) a non specific K-ATP-dependent inhibitor, or indomethacin (10⁻⁵M), a cyclooxygenase (COX) inhibitor (Moncada et al., 1991; Shiraki et al., 2001), were used separately. These inhibitors were added 30 min before the application of KCl or norepinephrine. In the tonic phase of the second contraction, V. heterophylla extract (0.5-3 mg/ml) was cumulatively added to the preparations. Inhibition was calculated by comparing the response of V. heterophylla extract before and after the addition of the inhibitor or antagonist.

**Statistical analysis**

Data are expressed as mean ± SEM, n representing the number of rats used in each experiment. Changes in aortic tension were expressed in percentage of either norepinephrine or KCl-induced tension. Statistical significance (p <0.05) was evaluated by the Student t test and one-way ANOVA, using Origin Graph, (Microcal Origin 6.0) software version 6.0. The pEC₅₀ value was calculated by non-linear regression and the maximal relaxation corresponds to the maximal effect (Emax) of the highest concentration used. Values of P<0.05 were considered statistically significant.

**RESULTS**

**Phytochemical screening**

The phytochemical screening of the crude extracts was made with methanol. The results revealed the presence of several primary and secondary metabolites. Phenolic compounds, triterpenes, volatile oils, sterols, alkaloids, fatty acids, flavonoids, athraquinones, coumarines, catecho tannins, are present in the extract of the bark. The presence of alkaloids and flavonoids is remarkable.

**Effects of V. heterophylla extract on contraction**

The vasorelaxant effects of the methanol stem bark extract of V. heterophylla was assessed in rat vascular smooth muscle following a method previously described (Dimo et al. 2005). All tests showed concentration-dependent relaxations with the maximal response >80%. DMSO (0.1% in distilled water), used as vehicle had no effect on the vascular tone. The maximal relaxation effects to the stem bark extract of V. heterophylla generated in the absence or presence of the endothelium are compared in Table 1.

**KCl-induced contractions**

To determine the effect of V. heterophylla on contraction caused by the release of intracellular Ca²⁺, many concentration of V. heterophylla (0.5-3 mg/ml) was used to induce vasorelaxation in the presence or absence of endothelium. The relaxant effects of V. heterophylla on vascular smooth muscle from rat aorta pre-contracted with
Table 1. Relaxation induced by \textit{V. heterophylla} in the different experimental conditions

<table>
<thead>
<tr>
<th>Experimental condition with KCL (60 mM)</th>
<th>% relaxation</th>
</tr>
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<tbody>
<tr>
<td>-Intact endothelium (E+)</td>
<td>85.69 ± 1.53</td>
</tr>
<tr>
<td>-Endothelium denuded</td>
<td>11.93 ± 3.11***</td>
</tr>
<tr>
<td>-E+ plus Tetraethylammonium ((10^{-6}\text{M}))</td>
<td>80.75 ± 1.94</td>
</tr>
<tr>
<td>-E+ plus Verapamil ((10^{-4}\text{M}))</td>
<td>10.77 ± 1.51</td>
</tr>
</tbody>
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<thead>
<tr>
<th>Experimental condition with NE (10^{-4}\text{M})</th>
<th>% relaxation</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Intact endothelium (E+)</td>
<td>87.63 ± 2.11</td>
</tr>
<tr>
<td>-Endothelium denuded</td>
<td>14.21 ± 3.12***</td>
</tr>
<tr>
<td>-E+ indomethacin ((10^{-5}\text{M}))</td>
<td>19.05 ± 4.13***</td>
</tr>
<tr>
<td>-E+ plus L-NAME ((10^{-4}\text{M})) plus L-arginine ((10^{-5}\text{M}))</td>
<td>79.92 ± 1.49</td>
</tr>
<tr>
<td>-E+ plus L-NAME ((10^{-4}\text{M}))</td>
<td>12.31 ± 1.84***</td>
</tr>
<tr>
<td>-Endothelium denuded plus L-NAME ((10^{-4}\text{M}))</td>
<td>16.75 ± 2.94***</td>
</tr>
<tr>
<td>-E+ plus ODQ ((10^{-4}\text{M}))</td>
<td>11.21 ± 2.50***</td>
</tr>
</tbody>
</table>

\(n=5\), number of experiments. *** \(P<0.001\); significant difference compared to the intact aorta pre-contracted with KCl (60 mM) or norepinephrine \((10^{-4}\text{M})\).

Figure 1. Vasorelaxant activity of methanol extract stem bark of \textit{Vepris heterophylla} on the rat aortic strips pre-contracted with KCl (60 mM). Each value represents the percentage of relaxation ± SEM. \(n = 5\). *** \(P<0.001\) vs. endothelium-intact ring.

KCl (60 mM) are shown in Figure 1. \textit{V. heterophylla} extract induced relaxation of the pre-contracting strips in a concentration-dependent manner. The potent vasorelaxation on endothelium-intact arteries at the highest concentration \((3 \text{ mg/ml})\) of the plant extract when aortic muscle was pre-contracted with KCl were pEC\(_{50}\) = 10.21 ± 1.33. In endothelium-denuded aortic strips, the relaxant response induced by \textit{V. heterophylla} was almost completely abolished \((\text{pEC}_{50} = 3.08 ± 1.47)\), demonstrating the important participation of the vascular endothelium in the relaxation induced by \textit{V. heterophylla}. There was a significant difference (\(P<0.001\)) between effects on intact and denuded aortic strips in all concentrations tested. It was observed that pre-treatment of intact aortic strips with
Figure 2. Relaxation responses induced by the methanol extract stem bark of *Vepris heterophylla* (0.5-3 mg/ml) on endothelium-intact or -denuded in rat aorta rings pre-contracted with norepinephrine (10⁻⁴M). Values are means ± S.E.M., n = 5; *** P<0.001 vs. endothelium-intact ring.

Norepinephrine-induced contractions

In rat aortic strips with intact endothelium pre-contracted with norepinephrine (10⁻⁴M), the addition of the *V. heterophylla* (0.5-3 mg/ml) induced potent vasorelaxation (pEC₅₀ = 11.26 ± 0.06) in a concentration-dependent manner. The effect of the plant extract on norepinephrine-induced contractions was altered in the absence of the functional endothelium when compared to endothelium-intact aortic strips (Figure 2, Table 1). In the presence of ODQ (10⁻⁴M), a sCG inhibitor, the relaxation induced by *V. heterophylla* (0.5-3 mg/ml) was almost completely abolished (Figure 3, Table 1). There was no difference observed between the relaxation induced by *V. heterophylla* in endothelium-denuded rings (Figure 4, Table 1) and the NOS inhibitor, L-NAME (10⁻⁴M) with or without endothelium. The inhibitory effects induced by *V. heterophylla* in the presence of L-NAME (10⁻⁴M) were completely reversed by the addition of the biological precursor of NO synthesis L-arginine (10⁻⁵M) (pEC₅₀ = 8.76 ± 0.06) (Figure 4, Table 1). The contribution of the relaxant arachidonic acid derivatives pathway was also investigated in this study. Incubation of intact aortic strips with indomethacin (10⁻⁴M) significantly shifted the concentration effect curves of *V. heterophylla* to the right (pEC₅₀ = 3.12 ± 0.18) (Figure 3, Table 1), suggesting that arachidonic acid derivatives are involved in the *V. heterophylla* vasodilator effect. The relaxation provoked by the plant extract (3 mg/ml) on intact aorta muscle was higher (87.63%) than that obtained (19.05%) in the presence of indomethacin.

DISCUSSION

This study provides new insights into the functional interaction between endothelium-derived NO and endothelium-dependent vasorelaxation. Our data demonstrated that aorta rings with intact endothelium pre-exposed to *V. heterophylla*, inhibited the contractile response in vascular reactivity induced by the administration of KCl or norepinephrine. The vascular endothelium is a central target for intervention given its multiple roles in the physiology (in health) and pathophysiology (in disease) and its direct accessibility to circulating ligands (Eglen and Whiting, 1985). The regulation of the vasodilation by endothel-
Figure 3. Effects of the methanol extract stem bark of *Vepris heterophylla* (0.5-3 mg/ml) on endothelium-intact rat aortic strips pre-contracted with norepinephrine (10^-4M); after pre-treatment of the rings with ODQ (10^-4M) and with indomethacin (10^-5M). Values are means ± S.E.M., n = 5 *** <0.001; significant difference compared to the intact aorta pre-contracted with norepinephrine (10^-4M).

Figure 4. Relaxation responses induced by the methanol extract stem bark of *Vepris heterophylla* (0.5-3 mg/ml) on endothelium-intact or -denuded in rat aorta rings, in the presence of L-NAME (10^-4M) or using endothelium-intact rings after pre-treatment with L-NAME (10^-4M) plus L-arginine (10^-5M). Values are means ± S.E.M., n = 5, *** <0.001 vs. significant difference compared to the intact aorta pre-contracted with norepinephrine (10^-4M).

Endothelial function is determined by three main mediators: NO (Palmer et al., 1998), prostacyclin (PGI2) (Gryglewski et al., 1986), and endothelium-derived hyperpolarizing factor (EDHF) (Nakane et al., 1991). The relaxation action of the plant extract was significantly reduced in endothelium intact aorta rings pre-contracted with norepinephrine in the presence...
of indomethacin, a cyclo-oxygenase inhibitor. This suggests that vasorelaxation caused by *V. heterophylla* was mediated at least in part by prostacyclin. One of the interesting results reported in this present study is the non response observed in vessels treated with the NO synthase inhibitor L-NAME in aortic strips with or without endothelium. Indeed, these results imply that in vessels with endothelial dysfunction in which a significant decrease in NO production is observed, it is possible to maintain the vascular tone at normal levels. Moreover, our results showed that treatment with L-NAME (10^{-4}M) plus L-arginine (10^{-5}M) did not alter the relaxations induced by *V. heterophylla*. To determine the involvement of guanylyl cyclase in the relaxant effect induced by *V. heterophylla*, the effect of ODQ was studied. Our results also indicated that the inhibitor of cGMP production in smooth muscle, ODQ, enhanced norepinephrine contraction in control rings with intact endothelium. The relaxation effects induced by *V. heterophylla* was almost completely abolished by ODQ. This hypo-reactivity and the associated relaxation mechanisms involved the NO/cGMP pathway. These results confirm previous studies that soluble guanylyl cyclase mediates vasorelaxant activity associated with endogenous NO production or exogenous sources such as nitric oxide donors (Olson et al., 1997; Muller et al., 1998; Terluk et al., 2000). The possible mechanism of action of *V. heterophylla* extract may involve the production of NO in the endothelium, which results in the formation of cGMP, causing the relaxation of the preparation contracted by KCl. It was observed that pre-treatment of intact aortic strips with the α_{1}-adrenoreceptor antagonist, L-type calcium channel blocker, Verapamil reduced significantly the contraction induce by KCl. This finding suggest that the contractile responses induced by high KCl (60 mM) are due to the influx of extracellular Ca^{2+} through L-type voltage-sensitive channels (Godfraind et al., 1986; Carmeliet, 1986; Gilani et al., 1994) and have been used to provide a simple means of studying drug with possible Ca^{2+} entry blocking properties (Duarte et al. 1995). The plant extract (3 mg/ml) inhibited these contractile responses (85.69%), which suggested that it inhibited Ca^{2+} through voltage-sensitive channels. It appears that the relaxation response to *V. heterophylla* extract may be due to the blockade of the influx of extracellular Ca^{2+}. Tetraethylammonium (a non specific K-ATP-dependent inhibitor, Duarte et al. 1995) had no effect on *V. heterophylla* -induced relaxation, thereby excluding opening of K-ATP-dependent channels to the vasorelaxation of rat aortic rings of the plant extract.

**Conclusion**

The results from this study show that the vasorelaxation of the methanol stem bark extract of *V. heterophylla* was endothelium-dependent, via the NO–cGMP pathway. *V. heterophylla* is able to provoke a concentration-dependent vasorelaxation of the rat aortic strips. The vascular relaxa-

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