Vasorelaxant Activity of Essential Oils from *Croton zambesicus* and Some of Their Constituents

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Abstract
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In this study, we determined the vasorelaxant activity of essential oils of different samples of *Croton zambesicus* collected in the same area in Benin at different periods and analysed their compositions by GC-FID and GC-MS. 68 compounds were identified among which 20 have not been described previously in this plant's essential oils. We observed quantitative differences among essential oils but all possess significant vasorelaxant activity on intact rat aortae contracted by KCl (IC$_{50}$ 5.6–11.8 µg/mL). This activity may, at least in part, be explained by the presence of vasorelaxant diterpenes such as *ent*-18-hydroxy-trachyloban-3-one, isopimara-7,15-dien-3β-ol, and *ent*-18-hydroxy-isopimar-7,15-dien-3β-ol, previously isolated from the dichloromethane extract of the leaves, but also to linalool (IC$_{50}$ 43.4 µg/mL) and particularly to caryophyllene oxide (IC$_{50}$ 2.5 µg/mL).

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction
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Croton zambesicus Muell. Arg. (syn. *C. amabilis* Muell. Arg., Euphorbiaceae) is a decorative shrub or tree cultivated around villages in West Africa. The leaves, characterised by a pleasant aromatic odour, are used as home deodorant [1].
They are also traditionally used in West and Central Africa as a household remedy to treat urinary infection, fever associated with malaria or convulsions, and dysentery [2]. Furthermore, aqueous decoctions of *C. zambesicus* leaves are used in Benin to treat hypertension, and baths with these leaves are employed for the same treatment.
The compositions of the essential oils from the flowering tops, stem barks, roots, and leaves are described in the literature. The presence of monoterpenes and sesquiterpenes, as well as some diterpenes was reported in samples from Sudan, Tchad, Cameroon, and Benin [3–6].
In previous studies, we showed the vasorelaxant activity of some diterpenes contained in the dichloromethane extract of *C. zambesicus* leaves [7, 8]. In this work we investigated the vasorelaxant activity of essential oils of different samples of leaves on intact rat aortae contracted with KCl and analysed their compositions to search for the presence of vasorelaxant diterpenes. We also analysed the vasorelaxant properties of some of their major constituents.

Materials and Methods
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Plant material
Leaves of *C. zambesicus* were collected in the same area 20 km from Cotonou (Benin) in December 2001, 2003, 2005, as well as in May and December 2006. The samples were identified by Prof. V. Adjakidje from the Abomey-Calavi University in Benin. A voucher specimen was deposited at the herbarium of the Belgian Botanical Garden at Meise (BR S.P. 848.148).
Leaves were dried in the dark at room temperature, then blended to obtain a homogeneous powder and stored at room temperature in a dry and dark place.

Oil isolation
30 g of each leaves sample were hydrodistilled for 4 hours in a Clevenger type apparatus as described in the European Pharmacopoeia (6th edition) [9]. The volume of essential oil was measured by using a Hamilton syringe. The obtained

essential oils were stored at +4 °C until they were tested and analysed by GC-FID and GC-MS.

Gas chromatography

GC-FID analyses were performed on a ThermoFinnigan Focus GC instrument with a FID detector using a DB-XLB capillary column (column length 15 m × 0.25 mm with 0.25 µm film thickness; J&W Scientific, Agilent Technologies). Samples (1 µL of 10-fold dilutions in n-hexane) were injected in the split mode (split ratio 1/10) under the following conditions: injector temperature, 250 °C; detector temperature, 250 °C; oven temperature: from 60 °C to 240 °C at 3 °C/min. Helium was used as carrier gas with a 1 mL/min flow rate.

GC-EIMS analyses were performed on a TRACE GC 2000 series ThermoQuest instrument equipped with an autosampler AS2000 ThermoQuest and interfaced to a TRACE MS mass spectrometer. Separations were performed on the same column and under the same conditions as above.

Compounds identification

The identification of the constituents was based on the NIST/EPA/NIH mass spectral library (1998, version 1.6) of the GC-MS instrument integrated with a home-made library of mass spectra of some commercial references and pure compounds isolated from plants analysed in our laboratory. The linear retention indices (LRI) were determined by comparing them to a series of C_5-C_{28} FAME (fatty acid methyl esters).

Cembrene derivatives were identified in the NIST library on the basis of their molecular weight (typical C20H32 ion at m/z 272) and by comparing fragmentation with literature data [10].

Pure substances (>99% – GC-FID) previously isolated from *C. zambesicus* [6,7,11,12] dissolved in dichloromethane (10 mg/mL) were used as reference compounds. (±)Linalool (GC, 97%), β-caryophyllene (GC, ≥98.5%), caryophyllene oxide (GC, 99%), α-copaene (GC, 95%), and longifolene (GC, ≥99%) were obtained from Fluka®; marrubenol was isolated from *Marrubium vulgare* (TLC, >95%) as described previously [13].

The percentages of the essential oil constituents were calculated by electronic integration of GC-FID peak areas using the normalisation method. Values are calculated as means of 3 different injections and the variation coefficients were lower than 2%.

Measurement of aorta contraction

Endothelium-intact aortic rings (2 mm length) of male Wistar rats weighing 200 to 300 g were suspended under a resting tension of 20 mN in 12.5 mL organ baths filled with a physiological solution [composition (mM): NaCl, 122; KCl, 5.9; NaHCO$_3$, 15; MgCl$_2$, 1.25; CaCl$_2$, 1.25; glucose, 11] maintained at 37 °C and bubbled with a gas mixture of 95% O$_2$ and 5% CO$_2$. Contractile responses were measured as described previously [7,8]. When required, endothelium was gently rubbed out, and N-nitro-L-arginine (Sigma-Aldrich) was added to the solutions to inhibit any NO synthase activity. Endothelium integrity was tested by adding acetylcholine (1 µM) to the bath solution when KCl contraction had reached a plateau. In endothelium-intact preparations, acetylcholine relaxed KCl contraction by at least 30%, while in endothelium-denuded preparations no response to acetylcholine was observed. Contraction was evoked by changing the physiological solution in the bath to a depolarising solution [composition (mM): NaCl, 27; KCl, 100; NaHCO$_3$, 15; MgCl$_2$, 1.25; CaCl$_2$, 1.25; glucose, 11]. Samples were added cumulatively on the plateau of contraction to obtain a final concentration in the bath from 1 to 30 µg/mL for tested oils or from 0.3 to 100 µg/mL for reference compounds. Oils were dissolved in a physiological solution with 2% Tween 80. Stock solutions at 10 mg/mL of caryophyllene oxide, β-caryophyllene, α-copaene, and linalool were prepared in DMSO while longifolene and marrubenol were dissolved in EtOH. Further dilutions were made in a physiological solution. The amplitude of contraction evoked in the presence of the tested samples was compared to the response obtained before addition of the compound in the bathing solution. Blanks were performed with the solvent alone (the highest concentration was 0.1%), and inhibition of the contraction measured in the presence of the samples was normalised to time-matched controls receiving the same volume of the solvent. Maximum relaxation measured in the solvent-treated aortic ring was 22% and was similar with ethanol, Tween 80, or DMSO. Experiments were repeated at least on 3 different aortas.

Data analysis and statistics

Concentration-response curves were analysed to give the concentration producing 50% inhibition of the contractile response (IC50) by sigmoidal curve-fitting analysis. All results are expressed as means ± the standard error of the mean (SEM). IC$_{50}$ values are expressed as mean values with their 95% confidence intervals. Differences in the concentration-response curves were analysed by two-way analysis of variance, followed by Bonferroni's test with a criterion set for statistical significance at p < 0.05.

Supporting information

Concentration-response curves for essential oils are available as Supporting Information.

Results and Discussion

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The essential oils prepared from different samples of the leaves of *Croton zambesicus* displayed vasorelaxant activity in KCl-contracted rat aorta with similar potency. ◗ **Table 1** gives the IC$_{50}$ values of the vasorelaxant activity of the essential oils prepared from different samples of *C. zambesicus* leaves collected in the same area in December 2001, 2003, 2005, and 2006 as well as in May 2006. Oil samples produced concentration-dependent relaxations (cfr. **Fig. 1S** Supporting Information). This effect was significant (p < 0.001) at 1 µg/mL except for the essential oils of December 2001 and 2003 that became significant at 3 µg/mL.

To explain this activity, we analysed the compositions of these essential oils. The results obtained from GC-FID and GC-MS allowed us to identify 68 compounds (◗ **Table 2**); all unidentified peaks were small.

Table 1 *In vitro* vasorelaxant activity of the essential oils from the leaves of *Croton zambesicus*.

Sample	IC$_{50}$ (µg/mL)
Dec 01	11.8 (6.3 to 21.9)
Dec 03	8.1 (5.9 to 11.1)
Dec 05	7.7 (6.3 to 9.2)
Dec 06	5.6 (4.1 to 7.4)
May 06	8.5 (6.2 to 11.3)

Each value represents the mean of the IC$_{50}$ values (with the 95% confidence interval) obtained by nonlinear curve fitting of the concentration-effect curve in 3 different aortas

**Table 2** Chemical composition of the essential oils of *Croton zambesicus*.

Compounds	LRI*	% in oil				
		Dec 01	Dec 03	Dec 05	Dec 06	May 06
α-Thujene (M, B)	686	tr	0.1	tr	tr	0.1
α-Pinene (M, B,C)	693	tr	0.5	0.1	tr	0.3
Camphene (M, B)	709	0.1	0.4	0.1	tr	0.1
Sabinene (M, B)	729	tr	tr	0.2	–	tr
β-Pinene (M, B)	735	0.1	1.0	1.0	tr	2.4
p-Cymene (M, B)	747	0.2	0.3	0.8	0.3	1
Limonene (M, B,C)	760	tr	tr	tr	tr	0.1
Cineole (M, B, C)	795	0.2	0.4	0.8	tr	0.4
Linalool (M, B, C)	838	tr	0.1	0.3	tr	0.2
Octanol (B)	846	tr	tr	tr	1.1	tr
Nonanol (B)	852	tr	tr	tr	tr	0.1
trans-Pinocarveol (M, B)	868	0.1	1	tr	0.4	2.6
Sabinol (M, B)	910	tr	tr	0.5	tr	0.5
Camphor (M, B)	921	0.4	1.6	1.7	tr	0.1
Borneol (M, B)	938	tr	tr	0.7	tr	0.3
4-Terpineol (M, B)	956	tr	0.1	0.3	tr	0.3
δ-Elemene (S, B)	1090	–	–	–	2.0	–
Myrtenol (M, B)	1100	–	–	–	1.5	–
Azulene (M, B)	1110	tr	0.1	0.1	0.1	tr
Cyclosativene (S, B)	1127	0.3	0.2	0.2	tr	0.2
α-Copaene (S, B, C)	1139	0.8	1.5	3.0	2.0	1.3
β-Bourbonene (S, B)	1148	1.3	1.9	5.1	1.6	1.4
Cyperene (S, B)	1153	tr	0.1	0.1	tr	tr
Isolongifolene (S, B)	1158	0.1	0.3	0.4	0.3	0.4
β-Caryophyllene (S, B)	1169	1.3	tr	tr	tr	tr
β-Cedrene (S, B)	1173	0.2	0.1	0.1	1.1	tr
α-Humulene (P, B)	1180	0.3	1.1	1.9	0.2	1.7
Germacrene-D (S, B)	1195	0.1	0.3	0.7	0.4	0.2
Longipinene (S, B)	1207	–	–	–	4.8	–
y-Muurolene (S, B)	1212	0.6	0.1	0.1	3.3	0.4
t-Gurjunene (S, B)	1228	0.2	0.7	1.2	0.2	0.1
Longifolene (S, B)	1242	0.4	0.6	0.8	13.2	26.4
t-Cadinene (S, B)	1253	–	–	–	1.0	–
β-Chamigrene (S, B)	1261	–	–	–	1.2	–
β-Eudesmene (S, B)	1270	–	–	–	1.2	–
β-Cadinene (S, B)	1286	8.8	0.4	0.9	1.5	1.4
Ledene oxide (II) (S, B)	1293	1.4	0.9	1	0.7	0.7
Bisabolene oxide (S, B)	1352	–	–	–	2.1	–
Caryophyllene oxide (S, B, C)	1364	8.6	17.8	25.9	2.9	17.2
Aromadendrene oxide (S, B)	1377	0.3	0.5	1.0	0.4	0.2
Isoaromadendrene epoxide (S, B)	1391	0.7	1.9	0.3	0.4	1.9
Longipinocarveol (S, B)	1395	tr	tr	3.3	0.7	0.3
ent-Spathulenol (S, B)	1410	–	–	–	1.2	–
β-Eudesmol (S, B)	1417	–	–	–	1.4	–
Globulol (S, B)	1437	–	–	–	1.5	–
Unknown compound	1443	–	–	–	1.2	–
Longiverbenone (S, B)	1463	1.6	2.6	2.9	0.3	2.2
Alloaromadendrene oxide (S, B)	1473	0.8	2.0	1.7	0.9	0.8
trans-Phytol (D, A, B, C)	1593	2.6	0.1	0.1	0.1	–
Cembrene derivative 1 (D, B)	1681	0.7	tr	–	tr	0.6
Cembrene derivative 2 (D, B)	1686	–	–	–	1.3	–
Cembrene derivative 3 (D, B)	1722	tr	tr	–	0.2	–
Cembrene derivative 4 (D, B)	1737	–	–	–	0.4	0.3
Trachylobane derivative 1 (D, B)	1750	0.5	0.2	–	0.8	0.2
Trachylobane derivative 2 (D, B)	1766		1.9	0.4	0.6	tr
Retinol (D, B)	1817	0.3	tr	tr	tr	4.6
Labdandiol (D, B)	1827	–	–	–	10.1	–
Pimarane derivative 1 (D, B)	1879	1.5	2.8	1.0	3.8	2.3
Pimarane derivative 2 (D, B)	1886	–	–	–	2.3	1.6
Labdanol (D, B)	1915	–	–	–	3.6	–
Kaurene derivative (D,B)	1978	0.3	0.2	0.2	tr	tr
ent-18-Hydroxy-trachyloban-3-one (**7**) (D, A)	1991	6.0	3.4	2.0	0.3	tr
						(continued)

Table 2   *Continued*

Compounds	LRI*	% in oil				
		Dec 01	Dec 03	Dec 05	Dec 06	May 06
Pimarane derivative (D, B)	1998	1.5	0.2	0.3	0.1	0.2
Unknown diterpene (D, B)	2042	0.6	0.1	0.3	tr	0.1
ent-Trachyloban-3-one (**6**) (D, A)	2076	28.0	22.7	11.0	1.5	1.4
ent-Trachyloban-3β-ol (**3**) (D, A)	2217	6.4	6.2	2.5	0.3	0.2
Isopimara-7,15-dien-3β-ol (**4**) (D, A)	2239	4.4	1.5	1.6	0.3	0.2
ent-18-Hydroxy-trachyloban-3β-ol (**1**) (D, A)	2401	0.7	1.6	0.2	0.1	tr
ent-18-Hydroxy-isopimara-7,15-dien-3β-ol (**2**) (D, A)	2404	0.6	0.5	0.3	tr	–
Total monoterpenes		1.2	5.5	6.4	2.1	8.5
Total sesquiterpenes		27.9	32.9	50.6	49.3	56.8
Total diterpenes		54.0	41.5	19.8	25.9	11.7
Total identified		83.1	79.9	76.9	77.4	77.0
E. O. yield (% v/w)		0.1	0.1	0.1	0.2	0.2

* LRI indices relatives to C_5 to C_{30} FAME on a DB-XLB column; tr = trace (< 0.05%); M = monoterpene, S = sesquiterpene, D = diterpene, P = phloroglucinol derivative; A = identified by comparison with pure compound isolated from *Croton zambesicus* [8]; B = identified by comparison of MS spectra with NIST library; C = identified by comparison of MS spectra with spectra obtained from pure commercially available compound

Fig. 1   Structure of tested compounds.
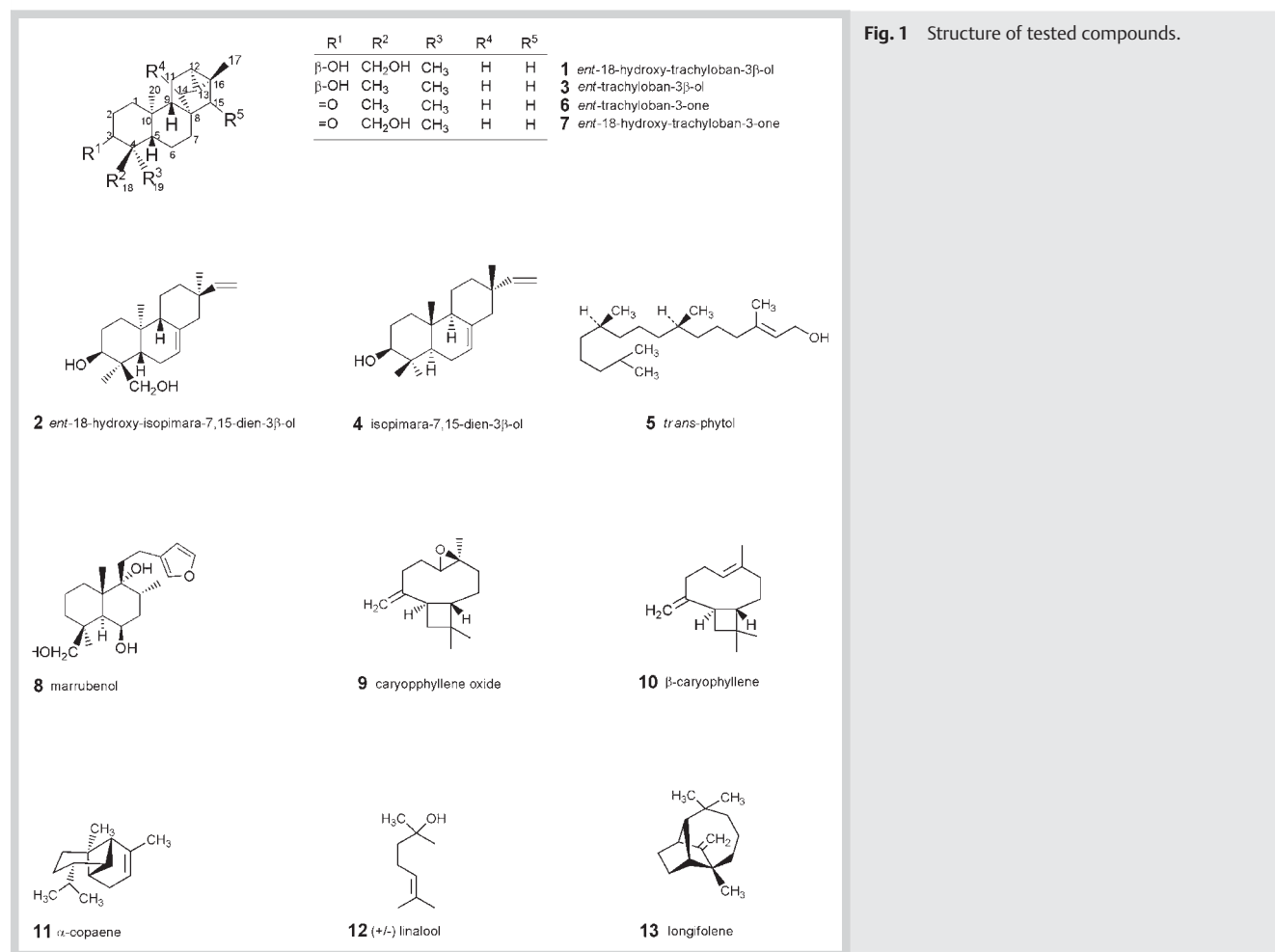
The analysed essential oils contained a high amount (from 28 to 57%) of sesquiterpenes (especially caryophyllene oxide and/or longifolene) and of diterpenes (from 12 to 54%), especially *ent*-trachyloban-3-one, *ent*-18-hydroxy-trachyloban-3-one, and *ent*-trachyloban-3β-ol, as well as a smaller amount of monoterpenes (from 1 to 9%). We noted that the diterpene content was more important in the oldest essential oils (except the sample of December 2001 which was prepared in 2002 and conserved at +6°C; the other essential oils were prepared at the same time in 2007, so dried leaves were conserved at room temperature from the collected date to 2007). This difference could be due, at least in part, to the evaporation of the most volatile compounds during storage of the leaves.

Among the 68 identified compounds, 20 were found for the first time in *C. zambesicus*: sabinol, δ-elemene, azulene, isolongifolene, longipinene, τ-gurjunene, longifolene, β-chamigrene, β-eudesmene, ledene oxide (II), bisabolene oxide, aromadendrene oxide, isoaromadendrene epoxide, longipinocarveol, β-eudesmol, globulol, longiverbenone, labdanol, retinol, and labdandiol. The presence of diterpenes previously isolated by our team from the leaves of *C. zambesicus* was observed in all oil samples especially those from the leaves collected in December 2001 and December 2003. Among them, *ent*-trachyloban-3-one was the most abundant (28% and 23%, respectively), but we observed in most samples a relatively high amount of *ent*-trachyloban-3β-ol, isopimara-7,15-dien-3β-ol and *ent*-18-hydroxy-trachyloban-3-one, the last one possessing a marked vasorelaxant activity as described previously [8–14]. We also detected the presence of *ent*-18-hydroxy-trachyloban-3β-ol and *ent*-isopimara-7,15-dien-3β-ol, another vasorelaxant diterpene previously isolated from *C. zambesicus* [8]. In the essential oils from the leaves collected in December 2006 and in May 2006, which seem to be quite different from the others, we detected smaller amounts of trachylobanes and pimaranes and for the first time significant amounts of cembrene derivatives and of other unknown diterpenes, although in relatively small amounts. The presence of high amounts of labdanol (3.6%) and labdandiol (10.1%) was only detected in the essential oil from the December 2006 sample.

All the essential oils except those of the December 2006 sample were particularly rich in caryophyllene oxide. Both samples of 2006 were relatively rich in longifolene (between 13 and 26%). Concentration-response curves of the different essential oils (see Supporting Information) did not reveal any significant difference in activity although we observed a marked decrease of the vasorelaxant diterpenes in the oils from the December 2006 and May 2006 samples. As the activities of essential oils were in the same range as those of the pure diterpenes [8], we decided to evaluate the effect of other major constituents of the essential oils that we obtained and those described in the literature [6]: caryophyllene oxide (**9**), β-caryophyllene (**10**), α-copaene (**11**), linalool (**12**), and longifolene (**13**) and compared them with those of the diterpenes that we isolated.

Marrubenol (**8**), a diterpene with known vasorelaxant activity associated with Ca^{2+}-channel antagonist properties, was used as reference compound [13]. The structures of the tested compounds are represented in ❍ **Fig. 1**.

Caryophyllene oxide, linalool, and α-copaene relaxed K⁺-induced contractions but neither β-caryophyllene nor longifolene showed any effect. The concentration-response curves of compounds **9** and **12** are represented in ❍ **Fig. 2** while their IC_{50} as well as those of the vasorelaxant diterpenes are reported in ❍ **Table 3**. Diterpenes **1, 3, 5,** and **6** did not display vasorelaxant activities ($IC_{50} > 80 \mu M$). Unfortunately, it was not possible to calculate the
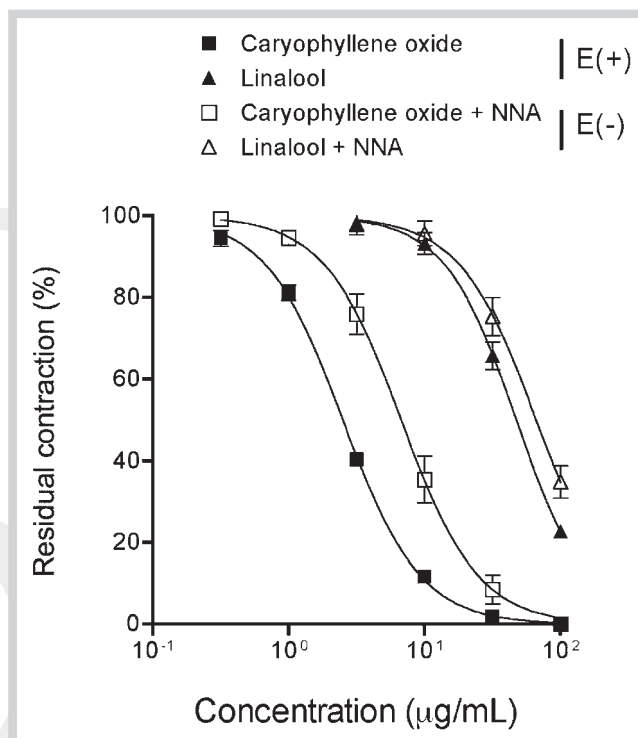

Fig. 2 Concentration-response curves of caryophyllene oxide and linalool on KCl-evoked contraction of rat aorta with or without functional endothelium. Aortas without endothelium were incubated in the presence of nitro-L-arginine (NNA). Each point represents the mean ± SEM from 3 determinations performed in different aortas.

IC_{50} of α-copaene because of solubility problems starting at 10 μg/mL, which inhibited contraction by about 50%.

KCl-evoked contraction is associated with the activation of voltage-operated calcium channels. The vasorelaxing effect of caryophyllene oxide in KCl-depolarised aorta might be related to the reported inhibitory effect of this compound on calcium channel current in the guinea-pig heart [15]. The relaxing effect of caryophyllene oxide was significantly depressed in the absence of functional endothelium, while the effect of linalool was less affected by the endothelium, but concentration-effect curves with and without endothelium were significantly different (Anova; ❍ **Fig. 2**). A similar influence of a functional endothelium is observed with most calcium channel blockers and has been attributed to a synergy between endothelium-released NO and calcium channel blockers [16].

Caryophyllene oxide showed a good inhibitory activity on KCl-induced contraction in a concentration range similar to the most vasorelaxant diterpenes suggesting that it may also be responsible for at least a part of the effect observed with some essential

Compound	IC_{50} (μM)	IC_{50} (μg/mL)
ent-18-Hydroxy-isopimara-7,15-dien-3β-ol (**2**)*	4.5 (4.1 to 4.9)	1.4 (1.3 to 1.5)
Isopimara-7,15-dien-3β-ol (**4**)*	46.2 (42.5 to 50.1)	13.3 (12.3 to 14.5)
ent-18-Hydroxy-trachyloban-3-one (**7**)*	6.3 (5.9 to 6.4)	1.9 (1.8 to 1.9)
Marrubenol (**8**)*	10.0 (7.5 to 10.5)	3.4 (2.5 to 3.6)
Caryophyllene oxide (**9**)	11.4 (10.8 to 12.0)	2.5 (2.4 to 2.6)
Linalool (**12**)	281.5 (260.8 to 304.2)	43.4 (40.2 to 46.9)

Table 3 Vasorelaxant activity (IC_{50} values in μM and μg/mL) of diterpenes isolated from *Croton zambesicus* and some constituents of *C. zambesicus* essential oils on KCl-induced contraction (100 mM).

Each value represents the mean IC_{50} (with the 95% confidence interval) of 3 determinations on 3 different aortas; * according to ref. [8]

oils. As concentrations vary from 1- to 10-fold, depending on the oil batch, its contribution to the observed effect also varies. Synergy or additivity of activities with other constituents should also be considered, as essential oils, which are complex mixtures, have a vasorelaxant activity at about the same range of concentration and their effect is close to the one of isolated active compounds. Furthermore, the essential oil of the sample of December 2006 does not have a lower activity than the others even though it contains much less identified vasorelaxing compounds. This could perhaps be explained by the presence of pimarane unidentified derivatives or other unidentified compounds.

Relaxant effect of essential oils from several plants has been reported in tracheal and ileal smooth muscles [17]. In this study, we showed for the first time the vasorelaxant properties of different samples of essential oils of *C. zambesicus* as well as of caryophyllene oxide and identified vasorelaxant diterpenes in oils. Linalool is already known to have moderate antispasmodic activity [18], and caryophyllene oxide has been reported to have a negative inotropic effect in the heart [15], but it is also the first time that it is shown to have a vasorelaxant effect on KCl-induced contraction. Although the concentrations of these compounds do not allow to totally explain the activity of the essential oils, they contribute, at least in part, to the vasorelaxant properties of the leaves of *C. zambesicus*.

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