The Vasorelaxant Activity of Marrubenol and Marrubiin from *Marrubium vulgare*

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Abstract

Crude extracts of the aerial parts of *Marrubium vulgare* show a potent *in vitro* inhibition of KCl-induced contraction of rat aorta. Bio-guided fractionations, spectroscopic analysis and chemical derivatization revealed the furanic labdane diterpenes marrubenol and marrubiin as the most active compounds.

Marrubium vulgare L. (Horehound, Lamiaceae) is a widespread Mediterranean plant used in folk medicine to cure a variety of diseases. Chemically, this genus is best known for its furanic labdane diterpene marrubiin, which has potent antinociceptive [1] and expectorant [2] effects. Other furanic labdane diterpenes including premarrubenol, marrubenol and premarrubiin have been repeatedly reported to occur in this plant [3], [4] but pharmacological information about these components is lacking. In a previous report, we have demonstrated that treatment with an aqueous extract of the aerial parts of *M. vulgare* significantly lowered the systolic blood pressure in spontaneously hypertensive rats (SHR), related to a vasodilatory effect exhibited by the extract *ex vivo* as well as *in vitro* [5]. The present study deals mainly with the isolation and structural elucidation of the compounds responsible for the vasorelaxant activity ascribed to this plant.

Preincubation of rat aorta with the cyclohexane fraction of *M. vulgare* water extract evoked a dose-dependent inhibition of KCl-induced contraction while the aqueous fraction showed no effect (Table **1**). Bio-guided fractionation of this cyclohexane extract by column chromatography gave several fractions, two of which showed a high activity. The first one was further purified using preparative TLC on silica gel (Si 60 F_{254} Merck) with hexane-diethyl ether (4 : 6) as mobile phase, yielding 10.9 mg of marrubiin {$[\alpha]_D^{22}$: + 35.6° (*c* 0.0025, $CHCl_3$)} which was identified by positive ES-mass spectra, comparison of the ^{1}H- and ^{13}C-NMR data with the literature data [6] and with an authentic sample. The second fraction contained a pure compound {9.9 mg, purity greater than 95 % by TLC analysis, $[\alpha]_D^{22}$: +15.4° (*c* 0.0013, $CHCl_3$)}, giving a molecular ion peak at *m/z* = 335 in negative ES-MS.

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Table **1** Effect of different fractions from *Marrubium vulgare* on contraction of aortic rings evoked by KCl (100 mM)

	Inhibition of KCl -induced contraction (%)			
Concentration (mg/mL)	***Crude water extract***	***Cyclohexane fraction***	***Aqueous fraction***	***Verapamil***
0.016	–	61.2	0.9	100
0.032	13.5	92.8	1.2	–
0.064	27.3	100	1.5	–
0.18	56.9	–	2.0	–
0.36	79.9	–	2.8	–
0.72	88.6	–	2.8	–

After recording of a first control contraction evoked by 100 mM KCl, aortic rings were pre-incubated in the presence of various concentrations of the tested fractions or the Ca^{2+} antagonist verapamil for 20 min before a second contraction was evoked. Contraction in the presence of the extract or verapamil was expressed as percentage of the control contraction.

Extensive NMR analysis (^{1}H, ^{13}C, COSY, HMQC, HMBC) allowed us to identify it as marrubenol, previously isolated from *M. vulgare* [7]. Table **2** gives, for the first time, the complete NMR assignments of marrubenol. Its structure was further confirmed by comparison with marrubenol obtained by reduction of marrubiin (Fig. **1**).

The effects of marrubiin and marrubenol on the contractions evoked by high-KCl depolarizing solution in aortic segments were compared to the effect of the calcium channel blocker verapamil, a well known antihypertensive drug which inhibits the contraction evoked by high-KCl solution in arteries [8]. Marrubenol and marrubiin inhibited the aortic contraction in a concentration-dependent manner (Fig. **2**). Marrubenol was slightly more

Table **2** ^{1}H-NMR and ^{13}C-NMR spectral data of marrubenol in $CDCl_3$

C	δ_C	δ_H	***HMBC correlations***
1	33.8	1.4 – 1.5 (m)	2, 3
2	18.5	1.38 – 1.49 (m)	
3	40.7	1.11 – 1.35 (m)	18, 2
4	38.9	–	5,18,19
5	49.3	1.56 (d: 1.2 HZ)	3,18,19,20
6	65.9	4.18 (d: 2.0 Hz)	5
7	38.9	1.47 (m) – 1.64 (dd: 13.4, 3.3 Hz)	17
8	31.1	2.25 (m)	11 A – B, 17
9	77	–	11 A – B , 20, 17
10	43.4	–	5, 11, 20
11	34.9	1.7 – 1.9 (m)	12
12	21.5	2.45 (m)	11 A-B
13	125.4	–	11, 12, 14, 15, 16
14	110.8	6.21 (br s.)	12, 15, 16
15	142.8	7.28 (br s.)	14, 16
16	138.5	7.17 (br s.)	12,14,15
17	16.2	0.9 (d: 6.8 Hz)	
18	27.8	0.99 s	19 A – B, 5, 3
19	69.1	3.14 (d: 11.5 Hz)-4.25 (d:11.5 Hz)	5, 18
20	19.6	1.25 (s)	5, 1

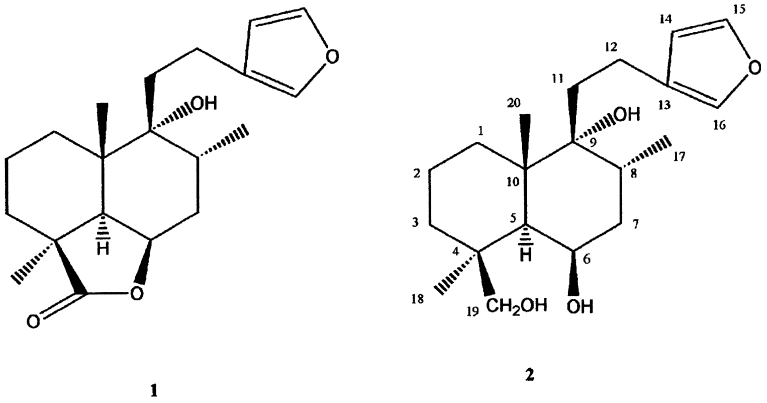

Fig. **1** Chemical structure of marrubiin (**1**) and marrubenol (**2**).

potent than marrubiin (IC_{50} values were 7.7 ± 1.9 μM and 24 ± 2.3 μM for marrubenol and marrubiin, respectively, $P < 0.05$). Both compounds were significantly less potent than verapamil: IC_{50} ratios of 199 and 64 were obtained for marrubiin and marrubenol, respectively, compared to verapamil. Further studies are being undertaken to determine the mode of action of these diterpenes. The presence of marrubiin and marrubenol may at least in part justify the vasorelaxant activity of aqueous extract of *M. vulgare*.

Materials and Methods

Material: Marrubium vulgare was collected in Sidi Sliman province (Morocco) in November 2000, identified and authenticated according to the French Pharmacopoeia. A voucher specimen was deposited at the National Scientific Institute in Rabbat, Morocco (reference number H35). Marrubiin was purchased from Extrasynthese (Natural products, France), verapamil and $LiAlH_4$ were obtained from Sigma.

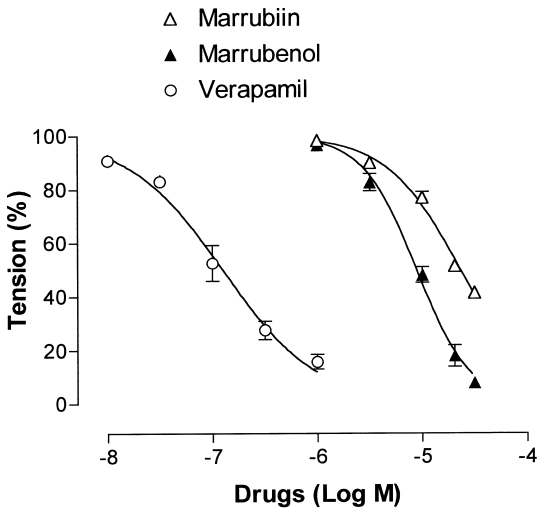

Fig. **2** Effect of marrubenol and marrubiin on the contraction of rat aortic rings exposed to 100 mM KCl depolarizing solution. Contractile response to 100 mM KCl in arteries incubated with marrubiin, marrubenol or the reference compound verapamil, is expressed as a percentage of the contraction measured in the same artery before the addition of the antagonists. Data are means ± S.E.M from 5 to 6 determinations.

Extraction: The aerial parts of the flowering plant were cleaned, and shade dried. The dried aerial parts (60 g) were extracted with 400 mL of distilled water at 90 °C for 15 min. The decoction was extracted three times with cyclohexane. The aqueous fraction was lyophilized and the cyclohexane fraction was evaporated under reduced pressure yielding a greenish oily residue (180 mg) which was chromatographed by MPLC on a silica gel Lichoprep Si60 (15 – 25 μm, 150 g) column (omnifit OM 6427 15 × 750 mm), eluted with CH_2Cl_2-MeOH (99 : 1), 1 mL/min. 8-mL fractions were collected, t_R of **1**: between 176 and 224 mL, **2:** 696 – 1024 mL. Fractionation was monitored by TLC (silica gel 60 F_{254}, Merck) using CH_2Cl_2-MeOH (95 : 5) as solvent system, detection: 1 % vanillin in 50 % phosphoric acid, R_f **1:** 0.78, R_f **2:** 0.35. Fractions with similar chromatographic profiles were combined and tested for their vasorelaxant activity.

Reduction of marrubiin to marrubenol: 5 mL of a 0.6 mg/mL solution of marrubiin in sodium-dried Et_2O was added to 5 mL of a 2 mg/mL ether solution of $LiAlH_4$ with vigorous stirring for 1 hour under nitrogen atmosphere. The excess of reagent was decomposed by the cautious addition of EtOAc (1 mL). The reaction mixture was poured gradually into excess of ice-cold dilute sulfuric acid (0.5 M). The ether layer was evaporated under reduced pressure and the aqueous solution was extracted with ether (three times) which was also evaporated. Purity of synthesized marrubenol was greater than 95 % by TLC analysis.

General experiment procedures: 1D and 2D spectra were recorded on a Bruker Avance DRX-400 spectrometer in $CDCl_3$ at 400.13 MHz (^1H) and 100.62 MHz (^{13}C), at 25 °C. Standard Bruker pulse sequences were used for homonuclear (COSY) and heteronuclear (HMQC-HMBC) correlation experiments. MS analyses were achieved using an LCQ mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization source. Optical rotations were measured with a Perkin-Elmer 241 spectropolarimeter in $CHCl_3$.

Measurement of aorta contraction: Contraction of rat isolated aorta was measured as described [9]. Briefly, aortic segments from Wistar rats were suspended in 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) bubbled with a gas mixture of 95 % O_2, 5 % CO_2 and maintained at 37 °C. Contraction was evoked by changing the physiological solution in the bath to a depolarizing 100 mM KCl solution (composition

(mM): NaCl, 27; KCl, 100; $NaHCO_3$, 15; $MgCl_2$, 1.25; $CaCl_2$, 1.25; glucose, 11). The amplitude of the contraction evoked in the presence of the tested compound was compared to the response measured in its absence. Verapamil was used as a reference compound [8]. Data analysis was performed by non-linear curve fitting (PRISM, GraphPad). Comparisons were made using Student's t test or by analysis of variance followed by a Bonferroni test (one-way ANOVA), when more than two groups were involved in the comparison. *P* values lower than 0.05 indicated significant differences.

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