Antispasmodic Effects and Structure−Activity Relationships of Labdane Diterpenoids from *Marrubium globosum* ssp. *libanoticum*

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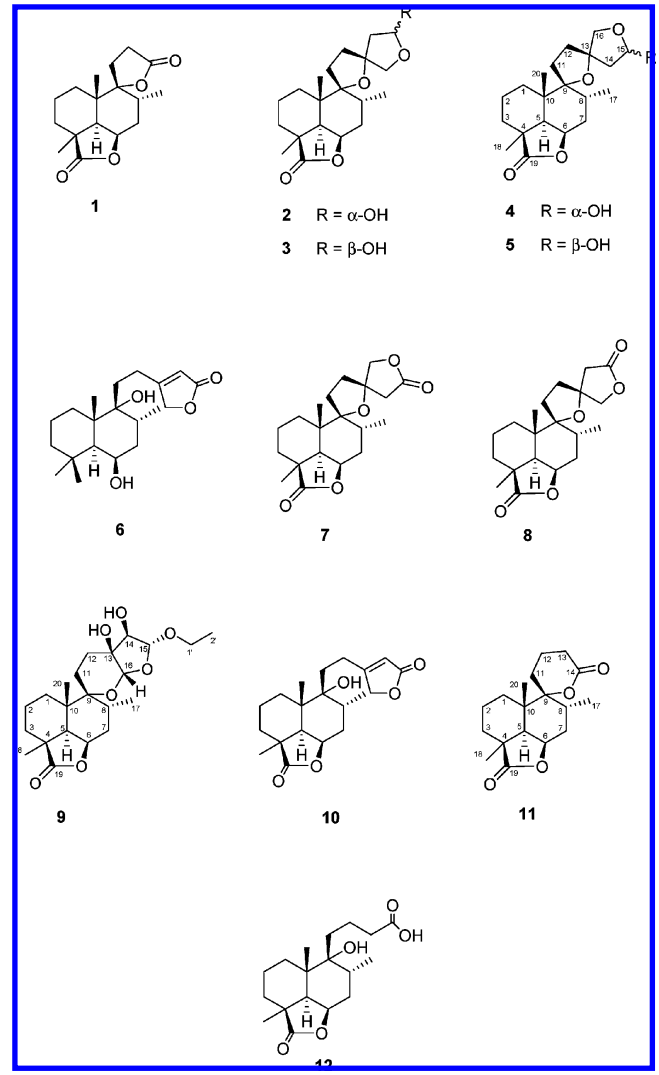
Marrubium globosum ssp. *libanoticum* is a medicinal plant used in Lebanon to reduce pain and smooth muscle spasms. A chloroform extract obtained from *M. globosum* aerial parts reduced acetylcholine-induced contractions in the isolated mouse ileum. The purification of this extract identified, among 12 isolated labdane diterpenoids, four new compounds, named 13-epicyllenin A (**4**), 13,15-diepicyllenin A (**5**), marrulibacetal (**9**), and marrulactone (**11**). Their structures were determined by spectroscopic methods. Compound **9**, which exerted antispasmodic activity, is likely the active ingredient of the extract. Preliminary structure−activity relationships for this class of compounds are suggested.

The genus *Marrubium* (Lamiaceae) includes about 40 species that grow mainly in the temperate regions of the Eurasian zone and in the area along the Mediterranean Sea.[1] Most of the species are annual or rhizomatous herbs. Extracts of flowering aerial parts are used in folk medicine to treat upper respiratory tract infections, lung congestion, and inflammatory and intestinal diseases.[2] In particular, it has been used to reduce pain and spasms from menstruation or intestinal conditions.[2] The genus is known to produce diterpenoids; many species of *Marrubium* were studied and several diterpenoids are described.[3,4]

M. globosum Montbr. et Auch. ex Benth. ssp. *libanoticum* Boiss. is a medicinal plant called "hashiashat el kelb" in Northern Lebanon. A decoction or infusion of the flowers and leaves is used in internal medicine as hypoglycemic, febrifuge, antispasmodic, and anti-inflammatory drugs and in external applications against snake bites and as a cicatrizant of wounds.[2] Recently, we have thoroughly studied this plant.[5−8] We showed that an acetone extract exerts anti-inflammatory effects in rat paw edema induced by carrageenin,[6] due to the labdane diterpene marrulibanoside.[8] No experimental or clinical studies have been performed to explain the traditional use of this herb in the treatment of intestinal spasms. The aim of our study was to investigate the effects of a chloroform extract, partially purified fractions, and pure isolated compounds from *M. globosum* aerial parts on intestinal motility. Bioassay-directed fractionation of the extract led to the isolation of four new labdane diterpenoids, **4**−**5**, **9**, and **11**, and eight known compounds, **1**,[4] **2**, **3**, **6**−**8**, **10**, and **12**.[6,8]



Results and Discussion

A chloroform extract obtained by sequential extraction of the aerial parts of *M. globosum* with petroleum ether and $CHCl_3$, significantly and in a concentration-dependent manner, inhibited the contractions evoked by acetylcholine (Figure 1). We can exclude that the antispasmodic effect of this extract is due to an antimuscarinic action, since it also inhibited, with a similar potency, the contractions induced by $BaCl_2$, which acts through a non-receptor-mediated mechanism (Figure 1). To provide more information on the *M. globosum* extract (MGE) mode of action, we evaluated the antispasmodic effect of the extract in the presence of calcium antagonists/blockers (nifedipine, EDTA, ω-conotoxin, and cyclopiazonic acid), because cytosolic calcium plays an important role in smooth muscle contractions.[9] The inhibitory response of MGE on acetylcholine-induced contraction was significantly reduced by

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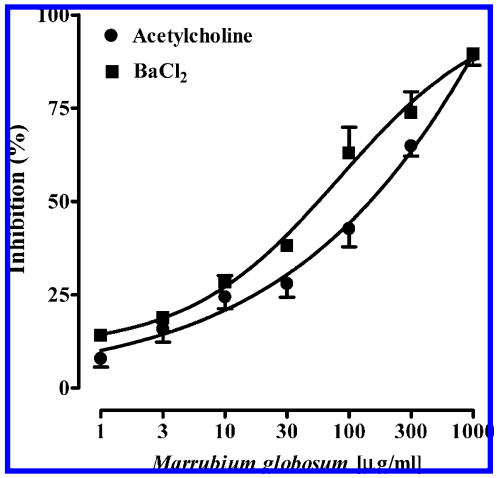

Figure 1. Effect of *M. globosum* extract (1−1000 μg/mL) on contractions induced by acetylcholine (10^{-6} M) and $BaCl_2$ (10^{-4} M) in the isolated mouse ileum. Each point represents the mean ± SEM of 6−8 experiments.

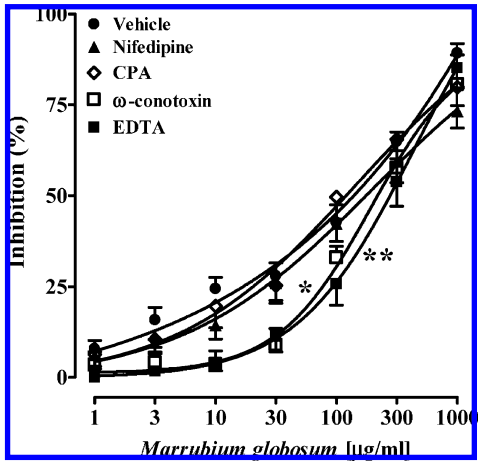

Figure 2. Acetylcholine-induced contractions in the isolated mouse ileum: effect of *M. globosum* extract (1−1000 μg/mL) alone (vehicle) or in the presence of nifedipine (10^{-6} M), cyclopiazonic acid (10^{-5} M, CPA), ω-conotoxin (3×10^{-8} M), and EDTA (10^{-3} M). Each point represents the mean ± SEM of 6−8 experiments. *$p < 0.05$; **$p < 0.01$ vs vehicle.

ω-conotoxin (an N-type calcium channel blocker, 3×10^{-8}) and EDTA (a calcium chelator, 10^{-3} M), but not by nifedipine (an L-type calcium channel blocker, 10^{-6} M) or cyclopiazonic acid (an inhibitor of the sarcoplasmatic reticulum Ca^{2+}-ATPase, 10^{-5} M), thus suggesting an involvement of the N-type calcium channel blocker in the MGE antispasmodic action (Figure 2). The antispasmodic effect of MGE observed *in vitro* resulted in *in vivo* inhibition of intestinal transit; indeed, MGE (0.1−3 mg/kg, IP) reduced, in a dose-dependent manner, intestinal transit (Figure 3).

MGE chromatographic analysis led to nine fractions (A−I). Four out of these fractions (B, D, E, and H) were the most active (data not shown).

Purification of the fractions by normal-phase HPLC led to the isolation of four new labdane diterpenoids, **4**, **5**, **9**, and **11**, and eight known compounds, **1**,[4] **2**, **3**, **6**−**8**, **10**, and **12**.[6,8]

Compounds **4** and **5**, apparently homogeneous, were isolated as an unresolvable mixture (7:3), as indicated by the duplicated signals observed in the ^{1}HNMR spectrum, and their ESIMS and elemental analysis were indicative of a $C_{20}H_{30}O_5$ formula. The ^{1}HNMR spectrum (see Experimental Section) was similar to those of the two (13*R*)-C-15 epimeric hemiacetals cyllenin A (**2**) and 15-epicyllenin A (**3**), co-occurring in this plant. The main differences were the chemical shifts of the signals of H-16a and H-16b with respect to cyllenin A and 15-epicyllenin A;[10] in fact in this case the major compound showed two doublets at δ_H 4.18 (d, $J = 8.8$) and 3.65 (d, $J = 8.8$), whereas the minor compound had a broad two-proton singlet at δ_H 3.86. This pattern clearly indicates the occurrence of a C-15 epimeric pair (δ_H 5.43, brs, H-15; δ_H 5.59, brs, H-15); moreover the chemical shifts of H_2-16 are in agreement with a 13*S* absolute configuration.[10]

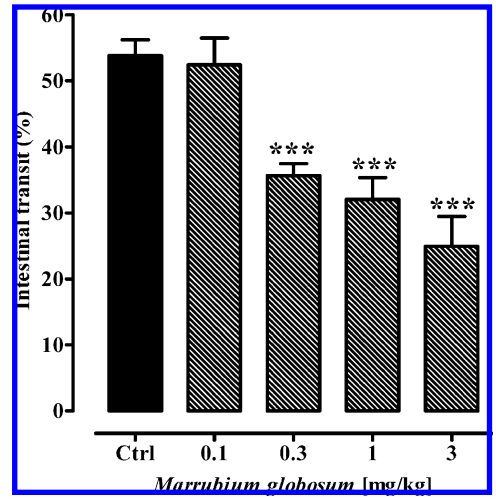

Figure 3. Effect of *M. globosum* extract (0.1−3 mg/mL) on gastrointestinal transit in mice. Results are mean ± SEM of 10−12 animals for each experimental group. ***$p < 0.00$ 1 vs control (Ctrl).

Consequently, these compounds were assigned the structures depicted in formulas **4** and **5** with trivial names of 13-epicyllenin A and 13,15-diepicyllenin A, respectively.

The ESIMS and elemental analysis as before of compound **9** were indicative of a $C_{22}H_{34}O_7$ formula. The signals of the ^{1}H and ^{13}C NMR spectra (Table 1) showed the presence of a saturated γ-lactone involving C-19 (δ_C 183.89) and C-6 (δ_H 4.77, 1H, brdd, H-6α; δ_C 76.47, C-6), two tertiary methyl groups (δ_H 1.26, 3H, s, Me-18; δ_C 23.20, C-18; δ_H 1.02, 3H, s, Me-20; δ_C 22.03, C-20), and a secondary methyl (δ_H 1.10, 3H, d, Me-17; δ_C 19.47, C-17), indicating an identical functionalization of the decalin moiety to other labdanes previously isolated from the same species[6,8] and other species of *Marrubium*.[10] The only difference was the value of the chemical shift of the quaternary oxygenated carbon C-9 (δ_C 80.41), downfield shifted with respect to deacetylvitexilactone (**6**) or marrulibanoside (**10**) (δ_C 75.9−75.6),[6,8] both carrying a free hydroxy group at C-9, and upfield shifted with respect to cyllenin A (**2**) (δ_C 92.1),[9] in which C-9 is part of a spiro five-membered (9,13)-epoxy ring. This fact suggested the presence of a tetrahydropyrano ring, in accordance with the C-9 chemical shift (δ_C 81.0) reported for semisynthetic labdanes with a similar structural moiety.[11]

The signals of the C-11/C-16 fragment indicated the presence of two acetals (δ_H 5.45, 1H, s, H-16; δ_C 105.39, C-16; δ_H 5.03, 1H, d, H-15; δ_C 108.70, C-15), two methylenes (δ_C 21.14, C-11; δ_C 29.61, C-12), a quaternary oxygenated carbon (δ_C 75.60), and an oxygenated methine (δ_H 3.94, 1H, brs, H-14; δ_C 78.55, C-14) coupling with the proton at δ_H 5.03 (H-15) and with the −OH at δ 2.73, as indicated by the correlations in the COSY spectrum. Furthermore, the correlations observed in the HMBC spectrum showed that an additional ethoxy group (δ_H 3.81, 1H, δ_H 3.50 1H, δ_H 1.20, 3H; δ_C 63.95, δ_C 15.05) was linked at C-15 and that C-9 and C-16 were linked at the same oxygen, forming a tetrahydropyrano ring. In order to assign the relative configuration of all the stereogenic centers, a NOESY experiment was carried out (Figure

Table 1. NMR Data of Compound **9** in $CDCl_3$ (*J* values in parentheses)

H	ppm	NOESY	C	ppm	HMBC
1a(α)	1.97	H-5, H-14	1	27.91	H-5, H-3a, H-2b, H-3b, Me-20
1b(β)	1.18				
2a	1.73	H-5	2	17.90	H-3a, H-3b, H-1b
2b	1.50				
3a(β)	2.10	Me-20	3	28.24	H-1a, Me-18
3b(α)	1.42	Me-18			
			4	43.92	H-5, H-3a, H-2b, H-3b, Me-18
5	2.35 d (4.5)	Me-18, H-1a	5	44.73	H-6, H7a, H-1b, Me-20
6α	4.77 bdd (5.3, 4.5)	Me-18	6	76.47	H-7a, Me-17 (J^4)
7a	2.17		7	32.34	H-6, H-8, Me-17
7b	1.87	Me-17			
8	2.08	Me-20	8	33.66	H-6, H-7a, H-7b, H-11a, H-11b, Me-17
			9	80.41	H-16, H-5, H7a, H-12a, H12b, H-11a, H-11b, Me-17, Me-20
			10	40.99	H-6, H-5, H-11a, H-11b, H-1b, Me-20
11a	1.80	H-14, Me-20	11	21.14	H-12a, H-12b
11b	1.75	H-14			
12a	2.08		12	29.61	H-14, H-11a, H-11b
12b	1.85	H-16			
			13	75.60	H-15, H-12a, H12b, H-11a, H-11b
14	3.94 bs	H-1a, H-12a, H-12b	14	78.55	H-15, H-12a, H-12b
15	5.03 d (1.7)	H-1′b,	15	108.70	H-16, H-14, H-1′a, H-1′b
16	5.45 s	Me17, H-12b	16	105.39	H-15, H-14, H12a, H-12b
Me-17	1.10 d (6.5)	H-16, H-7b	17	19.47	H-7a, H-8, H-7b
Me-18	1.26 s	H-6, H-5, H-3b	18	23.20	H-3a, H-3b
			19	183.89	H-5, H-3a, H-3b, Me-18
Me-20	1.02 s	H-8, H-11a, H-3a	20	22.03	H-5, H-1b
1′a	3.81 dq (14.2, 7.0)		1′	63.95	H-15
1′b	3.50 dq (14.2, 7.0)	H-15			
Me-2′	1.20 t (7.0)		2′	15.05	H1′a, H1′b
OH-13	2.80 bs				
OH-14	2.73 bs				

4). Diagnostic NOE cross-peaks were observed between Me-17 (δ_H 1.10) and H-16 (δ_H 5.45), indicating the β-orientation of the latter, and between both H-11 protons (δ_H 1.80 and 1.75) and H-14 (δ_H 3.94), showing a *cis* fusion of the two rings and consequently a β-orientation of the two hydroxy groups at C-13 and C-14. The α-orientation of H-14 was confirmed by its correlation with H-1α (δ_H 1.97). Finally, the configuration of C-15 was deduced by the small coupling constant (1.7 Hz) between H-14 and H-15 that indicated a *trans* relationship of the two protons.

On the basis of these results and by heteronuclear 2D $^1H-^{13}C$ correlations, one-bond HSQC, and long-range HMBC, we were able to assign unambiguously all the carbons and protons and to give the structure (16*S**)-(9α−16α),(15−16α)-diepoxy-13β,14β-dihydroxy-15α-ethoxylabdan-6β(19)-olide and the trivial name marrulibacetal to compound **9**.

The ESIMS and elemental analysis of compound **11** indicated a di-nor structure with a molecular formula of $C_{18}H_{26}O_4$. The signals of the ^1H and ^{13}C NMR spectra (see Experimental Section) showed an identical pattern of substitutions in the decalin moiety to compound **9** and the presence of a saturated δ-lactone involving C-14 (δ_C 172.07) and C-9 (δ_C 88.32). Further signals were the three methylene carbons at δ_C 34.47, 20.51, and 34.37, which were assigned to C-11, C-12, and C-13, respectively.

This information allowed us to assign the di-nor-labdane structure depicted in formula **11** to this compound, for which the trivial name marrulactone has been given.

The labdane diterpenoids (**1**−**12**) were evaluated for their potential antispasmodic effect on isolated mice ileum. Compound **9**, at a concentration of 0.001−10 μg/mL, significantly and in a concentration-dependent manner, reduced the acetylcholine-induced contraction (Figure 5). Compounds **4**, **5**, **7**, **8**, and **12** completely inhibited acetylcholine-induced contraction at a concentration of 100 μg/mL, while compounds **1** and **11** showed low activity (less

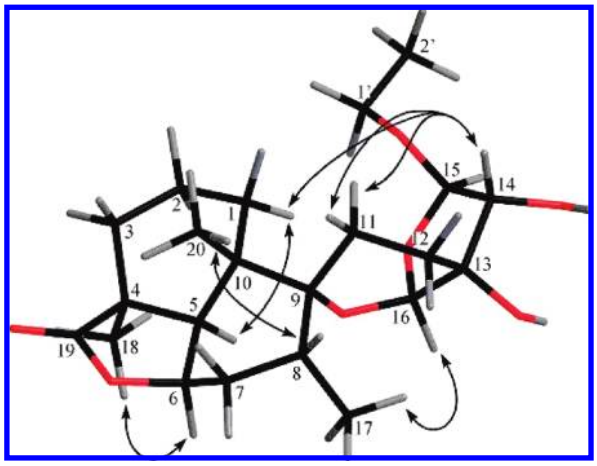

Figure 4. Selected NOE correlations for compound **9**.

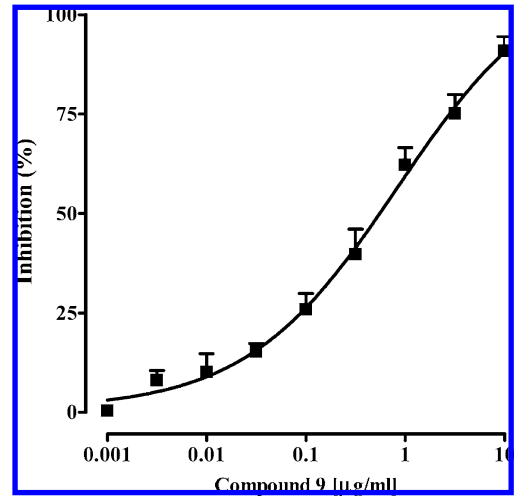

Figure 5. Effect of compound **9** (0.001−10 μg/mL) on contractions induced by acetylcholine (10^{-6} M) in the isolated mouse ileum. Each point represents the mean ± SEM of 6−8 experiments.

than 50% inhibition) to the highest concentration tested (100 μg/mL) (data not shown). Compounds **2**, **3**, **6**, and **10** were inactive up to 100 μg/mL concentration (data not shown).

Due to the small amount of the most active compound, **9**, we were unable to verify its possible inhibitory effect on intestinal transit *in vivo*.

Concerning the biological activity of these metabolites, the presence of a carbonyl group, also in the masked form (hemiacetal or acetal), at C-15 and the configuration of C-13 seem of pivotal importance. In fact, the spatial arrangement of the C-13 spiro atom induces no activity when its configuration is *R* (hemiacetals **2** and **3**), whereas the 13*S* configuration produces good activity (hemiacetals **4** and **5**). Thus, the activity of the mixture of lactones **7** and **8** can be ascribed to the former. The nature of the carbonyl group modulates the potency; in fact lactones or masked aldehydes show medium activity, free carboxylic acid (compound **12**) shows very good activity, whereas the α,β-unsaturated lactone (compounds **6** and **10**) is a structural feature that inhibits activity. All these considerations are compatible with a nucleophilic interaction of the carbonyl group with biological systems, and the high activity of compound **9** can be justified by the occurrence of two masked aldehyde groups at C-15 and C-16.

In conclusion, we have reported here that a chloroform extract of *M. globosum* exerted antispasmodic effects in isolated mice ileum, which possibly involves N-type calcium channels. *In vivo*, the extract inhibited intestinal transit. Bioassay-guided separation of the chloroform extract of aerial parts of *M. globosum* showed that compound **9** is the principal responsible for the antispasmodic activity.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Jasco P-1010 digital polarimeter. UV spectra were obtained on a Jasco 7800 UV−vis spectrophotometer. IR spectra were obtained on a Shimadzu FTIR-8300 spectrophotometer. Elemental analysis was carried out with a Perkin-Elmer 240 apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 400 MHz NMR spectrometer (^{1}H at 400.4 MHz, ^{13}C at 100.7 MHz), δ (ppm), *J* in Hz, using the residual solvent signal (δ 7.27 in ^{1}H and δ 77.0) as reference. ^{13}C NMR assignments were determined by DEPT and HSQC experiments. Standard pulse sequences were employed for COSY. HSQC and HMBC were optimized for $^1J_{C-H} = 135$ Hz and $^{2,3}J_{C-H} = 10$ Hz, respectively. ESIMS was obtained on an Applied Biosystem API-2000 mass spectrometer. Merck silica gel (70−230 mesh), deactivated with 15% H_2O, was used for column chromatography. Normal-phase HPLC was performed with a TSP SpectraSeries P100 instrument equipped with rheodyne injector and a refractive index detector, using a Hypersil silica column (Thermo, 250 × 4.6 mm, flow rate 1.5 mL/min). Thin-layer chromatography (TLC) was performed on plates coated with silica gel 60 F_{254} Merck, 0.25 mm.

Chemicals. Acetylcholine chloride, $BaCl_2$, nifedipine, cyclopiazonic acid, EDTA, and ω-conotoxin GVIA were purchased from Sigma (Milan, Italy). All solvents were obtained from Carlo Erba Reagenti (Milan, Italy). Acetylcholine, $BaCl_2$, EDTA, and ω-conotoxin were dissolved in distilled H_2O, while nifedipine and cyclopiazonic acid were dissolved in DMSO. The drug vehicles had no effect on the responses under study.

Plant Material. *M. globosum* aerial parts were collected from flowering plants in July 2004 on Col de Cèdres, Lebanon, at 2340 m above sea level. The identification was done by Prof. N. A. Arnold, University of Saint Esprit, Lebanon, and confirmed by Prof. T. Raus, Botanische Garten, Berlin. A voucher specimen (NAP #23) is deposited at the Herbarium Neapolitanum (NAP), Dipartimento di Biologia Vegetale, Università degli Studi di Napoli "Federico II", Italy. For *in vitro* experiments the $CHCl_3$ extract was dissolved in EtOH, while for *in vivo* experiments the extract was first dissolved in DMSO and then suspended in a lipophilic solution (Peceol/Gelucire 44/14, a gift from Indena, Milan, Italy; 8% DMSO, 92% lipophilic solution).

Extraction and Isolation. Dried and finely powdered aerial parts of *M. globosum* (285 g) were sequentially extracted by cold maceration with petroleum ether (40−60 °C, 3 × 2.5 L) and $CHCl_3$ (3 × 2.5 L). After filtration, the solvent was evaporated at low temperature (35 °C) to give a gum (8.2 g), which was dissolved in $CHCl_3$. Part of this gum was used for biological assays. The remainder of the extract (4.42 g) was dissolved in $CHCl_3$ and then submitted to column chromatography on Merck silica gel 60 (70−230 mesh, deactivated with 15% H_2O), eluting with *n*-hexane−EtOAc (from 100:0 to 0:100 gradient) and then MeOH, to afford 32 fractions of 250 mL each. The fractions, estimated by TLC (eluent system petrol−EtOAc (1:1 v/v), spray reagent $Ce(SO_4)_2$ in H_2SO_4), were collected, giving nine fractions (A−I) that were submitted for biological testing. Fractions B, D, E, and H, which were shown to be the most active, were further purified by normal-phase HPLC on a silica column to yield compounds **1**−**12**.

Fraction B (0.372 g), eluted with *n*-hexane−EtOAc (85:15), was subjected to HPLC (*n*-hexane−EtOAc, 1:1) to give 9.4 mg of **1**; t_R 4.4 min. Fraction D (0.292 g), eluted with *n*-hexane−EtOAc (75:25), was subjected to HPLC (*n*-hexane−EtOAc, 55:45) to afford 37.9 mg of a mixture of **2**−**5** (t_R 6.5 min) and **6** (t_R 7.2 min). A mixture of compounds **2**−**5** was further subjected to HPLC (*n*-hexane−EtOAc, 65:35) to afford 3.2 mg of **2/3** (t_R 9 min) and 33.2 mg of **4/5** (t_R 11 min). Fraction E (0.308 g), eluted with *n*-hexane−EtOAc (35:65), was subjected to HPLC (*n*-hexane−EtOAc, 35:65) to give 21.4 mg of compounds **7/8** (t_R 6 min), 14.2 mg of **9** (t_R 8.9 min), and 25.5 mg of **10** (t_R 11.5 min). Fraction H (0.697 g), eluted with EtOAc−MeOH (9:1), was subjected to HPLC (*n*-hexane−EtOAc, 30:70) to give 4.5 mg of **11** (t_R 2.9 min) and 19.2 mg of **12** (t_R 4.5 min).

Compounds 4 and 5: amorphous solid; ^{1}H NMR ($CDCl_3$) δ 5.59 (0.3H, brs, H-15), 5.43 (0.7H, brs, H-15), 4.69 (1H, dd, $J = 4.4, 3.6$, H-6), 4.18 (0.7H, d, $J = 8.8$, H-16a), 3.86 (0.6H, brs, 2H-16), 3.65 (0.7H, d, $J = 8.8$, H-16b), 2.44 (0.3H, m, H-14a), 2.28 (0.7H, brd, $J = 13.2$, H-14a), 1.25 (3H, s, Me-18), 1.04 (2.1H, s, Me-20), 1.02 (0.9H, s, Me-20), 0.94 (2.1H, d, $J = 5.2$, Me-17), 0.88 (0.9H, d, $J = 5.2$, Me-17); ESIMS (positive-mode) *m/z* 389 $[M + K]^+$, 373 $[M + Na]^+$, 351 $[M + H]^+$; *anal.* C 68.57%, H 8.61%, calcd for $C_{20}H_{30}O_5$, C 68.54%, H 8.63%.

Compound 9: amorphous solid; $[\alpha]^{25}_D$ −13.1 (*c* 0.29, $CHCl_3$); ^{1}H and ^{13}C NMR data see Table 1; ESIMS (positive mode) *m/z*: 449 $[M + K]^+$, 433 $[M + Na]^+$, 411 $[M + H]^+$; *anal.* C 64.33%, H 8.34%, calcd for $C_{22}H_{34}O_7$, C 64.37%, H 8.35%.

Compound 11: amorphous solid; $[\alpha]^{25}_D$ −23.80 (*c* 0.22, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 4.78 (1H, dd, $J = 5.4$, 4.4 Hz, H-6), 2.58 (1H, dt, $J = 17.4$, 3.9, H-13a), 2.32 (1H, d, $J = 4.4$, H-5), 1.31 (3H, s, Me-18), 1.11 (3H, s, Me-20), 0.98 (3H, d, $J = 6.3$, Me-17); ^{13}C NMR ($CDCl_3$) δ 183.41 (s, C-19), 172.07 (s, C-14), 88.32 (s, C-9), 75.71 (d, C-6), 45.02 (d, C-5), 44.09 (s, C-4), 39.90 (s, C-10), 34.48 (t, C-11), 34.37 (t, C-13), 32.45 (d, C-8), 31.75 (t, C-7), 28.79 (t, C-1), 28.54 (t, C-3), 23.13 (q, C-18), 22.56 (q, C-20), 20.51 (t, C-12), 18.41 (t, C-2), 16.80 (q, C-17); ESIMS (positive mode) *m/z* 635 $[2M + Na]^+$, 345 $[M + K]^+$, 329 $[M + Na]^+$, 307 $[M + H]^+$; *anal.* C 70.60%, H 8.53%, calcd for $C_{18}H_{26}O_4$, C 70.56%, H 8.55%.

Pharmacological Testing. Male ICR mice (20−22 g), purchased from Harlan Italy (S. Pietro al Natisone, UD, Italy), were used after 1 week of acclimation (temperature 24 ± 2 °C; humidity 60%). Animals had free access to water and food. All experiments complied with the Italian D.L. no. 116 of January 27, 1992, and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC).

For *in vitro* experiments, animals were killed by asphyxiation with CO_2 and segments (2−3 cm) of the distal ileum were quickly removed and flushed of luminal contents and placed in Krebs solution (mmol L): NaCl 119, KCl 4.75, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, $MgSO_4$ 1.5, and glucose 11). The segments were set up (in such a way so as to record contractions mainly from the longitudinal axis) in an organ bath containing Krebs (20 mL) equilibrated with 95% O_2, 5% CO_2 at 37 °C. The tissues were connected to an isotonic transducer (load 0.5 g) connected to a PowerLab system (Ugo Basile, Comerio, Italy). At the beginning of each experiment, the ileum was stimulated with acetylcholine (10^{-3} M) in order to obtain a maximum contraction (100% contraction). After a minimal 1 h equilibration period, the tissues were stimulated with spasmogens, namely, acetylcholine (10^{-6} M) or $BaCl_2$ (10^{-4} M). Acetylcholine and $BaCl_2$ were added to the bath and left in contact with the tissue for 30 s and then washed out. The interval between each stimulation was 20 min. After at least three stable control contractions, the contractile responses were repeated in the presence of increasing (noncumulative) concentrations of *M. globosum* $CHCl_3$ extract (MGE, 1−1000 μg/mL) added 20 min before the contacting

stimulus (i.e., after washing the tissue). Preliminary experiments showed that a 20 min contact time was sufficient for MGE to achieve the maximal inhibitory effect. In some experiments, the effect of MGE on acetylcholine-induced contractions was evaluated in the presence of nifedipine (an L-type calcium channel blocker, 10^{-6} M), EDTA (a Ca^{2+} chelator, 10^{-3} M), ω-conotoxin (an N-type calcium channel blocker, 3×10^{-8} M), or cyclopiazonic acid (an inhibitor of the sarcoplasmatic reticulum Ca^{2+}-ATPase, 10^{-5} M). The concentrations of nifedipine, EDTA, ω-conotoxin, and cyclopiazonic acid were selected on the basis of previous published work.[12−14] In another set of experiments, we evaluated the effect of labdane diterpenoids isolated from MGE on acetylcholine-induced contractions (contact time: 20 min for each drug).

Intestinal motility *in vivo* was measured as previously described.[12] Briefly, a black marker (0.1 mL 10 g/mouse; 10% charcoal suspension in 5% gum Arabic) was administered orally to mice. After 20 min the mice were killed by asphyxiation with CO_2 and the gastrointestinal tract was removed. The distance traveled by the marker was measured and expressed as a percentage of the total length of the small intestine from pylorus to cecum. MGE (0.1−3 mg/kg) or vehicle (0.4% DMSO, 96.6% lipophilic solution) was given intraperitoneally, 30 min before the administration of the fluorescent marker to the animals.

Statistical Analysis. Results are expressed as mean ± SEM. Nonlinear regression analysis for all concentration−response curves was performed (Graph Pad Instat program version 4.01; GraphPad Software, Inc., San Diego, CA). Data were analyzed by two-way ANOVA. A value of $P < 0.05$ was considered significant.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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