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Spasmolytic Effects of Nonprenylated Rotenoid Constituents of *Boerhaavia diffusa* Roots

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Boerhaavia diffusa is an Ayurvedic remedy used traditionally for the treatment of a number of diseases, including those affecting the gastrointestinal tract. In the current investigation, a methanol extract obtained from roots of *B. diffusa* exhibited a significant spasmolytic activity in the guinea pig ileum, probably through a direct effect on the smooth muscle. A detailed phytochemical analysis of this methanol extract led to the isolation of one new (**12**) and six known (**6**−**11**) rotenoid derivatives. The structure of the new compound was determined through interpretation of its MS and NMR data. All the isolated rotenoids were evaluated for their effect on intestinal motility in vitro, and the results obtained showed unambiguously that they are active spasmolytic constituents. Preliminary structure−activity relationships for this class of compounds are suggested.

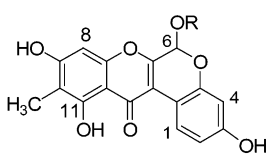
Several remedies of Ayurvedic medicine are used traditionally to relieve disorders of the gastrointestinal tract, and among them, *Boerhaavia diffusa* L. (Nyctaginaceae), a perennial herb that grows in tropical areas of India, Africa, and South America, plays an important role.[1] Despite the extensive use of *B. diffusa*, few phytochemical studies aimed at analyzing the effect of this plant on the digestive system have been performed. During a preliminary investigation, we found that a methanol extract obtained from roots of *B. diffusa* was able to inhibit the contractions induced by acetylcholine (ACh) in the isolated guinea pig ileum; in addition, through a bioassay-guided separation, five compounds belonging to the rotenoids class (**1**−**5**) were isolated.[2] The spasmolytic activities exhibited by these compounds (Table 1) clearly indicated a negative effect of *O*-methylation at C-6.[2]

Table 1. Inhibition of ACh-Induced Contractions by Rotenoids **1**−**12** at 30 μg/mL

rotenoid	E_{max}[a]
boeravinone D (**1**)	inactive[b]
boeravinone E (**2**)	100[b]
6-*O*-demethylberavinone H (**3**)	100[b]
boeravinone H (**4**)	inactive[c]
boeravinone G (**5**)	60.0 ± 6.9[b]
boeravinone C (**6**)	inactive[c]
10-demethylboeravinone C (**7**)	19.4 ± 1.3
coccineone E (**8**)	25.2 ± 2.0
2′-*O*-methylabronisoflavone (**9**)	36.8 ± 3.2
boeravinone F (**10**)	50.0 ± 8.2
coccineone B (**11**)	inactive[c]
9-*O*-methyl-10-hydroxycoccineone B (**12**)	36.1 ± 3.3
papaverine	72.3 ± 7.6

[a] E_{max} indicates the percentage of maximum inhibition. [b] Results obtained in a previous study.[2] [c] Inactive means with no effect on Ach-induced contractions.

To get further information on the smooth muscle relaxant activity of nonprenylated rotenoids contained in *B. diffusa* roots, we have carefully re-examined its methanol extract and isolated seven additional rotenoid derivatives (**6**−**12**), one which is a new compound (**12**). In this paper we detail the isolation procedure for **6**−**12** and the structure elucidation of the new compound **12**, and preliminary structure−spasmolytic activity relationships for the rotenoid class have been formulated, based on the relative potency of compounds **1**−**12**. The possible mechanism(s) for the spasmolytic activity exhibited by *B. diffusa* rotenoids are discussed.



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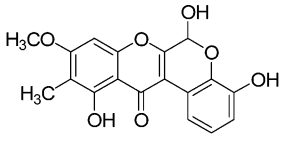
2 R = H



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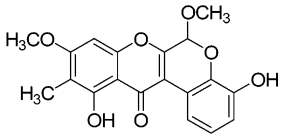

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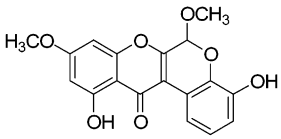

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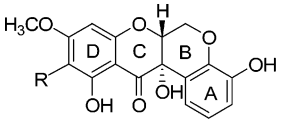

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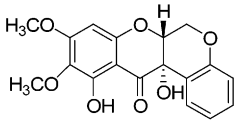
7 R = H



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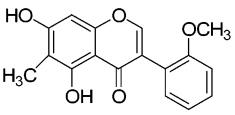

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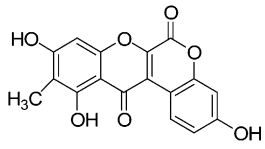

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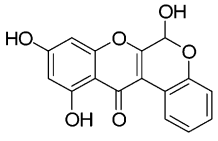

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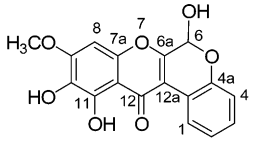

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Results and Discussion

The methanol extract obtained from *B. diffusa* roots was subjected to partitioning against different solvents according to a

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modified Kupchan procedure, obtaining four different fractions (*n*-hexane, CCl_4, $CHCl_3$, *n*-BuOH). Preliminary spectroscopic analysis showed that the CCl_4 and the $CHCl_3$ fractions contained rotenoids, and therefore, they were further separated. Purification of the CCl_4 fraction, carried out through column chromatography on silica gel followed by HPLC, led to the isolation of boeravinone C (**6**, 5.5 mg),[3] 10-demethylboeravinone C (**7**, 1.8 mg),[4] coccineone E (**8**, 7.9 mg),[5] 2′-*O*-methylabronisoflavone (**9**, 3.6 mg),[6] and the new compound **12** (1.5 mg) in the pure state. The $CHCl_3$ fraction was separated by column chromatography on silica gel, followed by HPLC, yielding boeravinone F (**10**, 2.2 mg)[7] and coccineone B (**11**, 2.1 mg).[8] The structures of the known compounds **6**−**11** were deduced by comparing their spectroscopic data with those reported in the literature.[3−8] It should be noted that compounds **7**−**9** and **11** have been isolated for the first time from *B. diffusa*. Of these, compounds **7**, **8**, and **11** have been obtained previously only from the Brazilian plant *B. coccinea*,[4,5,8] while compound **9** has been isolated as an antifungal principle of a plant cell culture of *Mirabilis jalapa*.[6]

Compound **12**, isolated as a pale yellow amorphous solid, showed a ESIMS pseudomolecular ion peak at *m*/*z* 327 $[M - H]^-$, while its HREIMS allowed the assignment of the molecular formula, $C_{17}H_{12}O_7$. The ^{1}H NMR spectrum of **12** (500 MHz, CD_3OD) showed only seven signals: six methine resonances (two singlets and four multiplets) in the region between δ_H 6.00 and 8.90 and a methoxy resonance at δ_H 3.89. The four multiplets of the ^{1}H NMR spectrum were assigned unambiguously to four aromatic methines arranged in sequence: the 2D COSY spectrum confirmed that the triplets at δ_H 7.08 and 7.29 (both $J = 7.8$ Hz) were mutually coupled and *ortho*-coupled with the doublets at δ_H 8.87 ($J = 7.8$ Hz) and 7.05 ($J = 7.8$ Hz), respectively. The ^{13}C NMR spectrum of **12** (125 MHz, CD_3OD) showed the resonances of a methoxy signal at δ_C 56.0 and of 16 carbons in the low-field region (with the typical pattern of rotenoid derivatives), of which 10 were nonprotonated. All the proton resonances were associated with those of the directly linked carbon atoms in the 2D HMQC NMR spectrum.

The $^{2,3}J$ correlations apparent in the 2D HMBC experiment proved to be useful in the assignment of the ^{1}H and ^{13}C NMR data of **12** and guided the assembly of the rotenoid skeleton of this molecule. In particular, the correlations of the hemiacetal proton at δ_H 6.06 (H-6) with C-4a (δ_C 147.7), C-6a (δ_C 153.6), and C-12a (δ_C 109.1), and those of the signal at δ_H 8.87 (H-1) with C-12a, C-4a, and C-1a (δ_C 116.6), corroborated by comparison with data reported for other boeravinones,[2] allowed us to elucidate the structures of rings A−C. Therefore, in accordance with the molecular formula, ring D must accommodate two hydroxy groups and a methoxy group on the four available positions. The bathochromic shift of band I, obtained in the UV spectrum with NaOAc−H_3BO_3 (diagnostic for an *ortho*-dihydroxy group) and with $AlCl_3$ and $AlCl_3$−HCl (diagnostic for an OH group at C-11), indicated the presence of hydroxy substitution at both C-10 and C-11.[9] Therefore, the protonated carbon of ring D could be either C-8 or C-9. It was identified as C-8 on the basis of the relatively large correlation (indicative of $^3J_{H-C}$, in planar systems) for H-8/C-11a (δ_C 101.3) and from the relatively small correlations (indicative of $^2J_{H-C}$ or $^4J_{H-C}$, in planar systems) of H-8/C-7a (δ_C 158.4) and H-8/C-12a, detected in a phase-sensitive HMBC experiment.[10] Consequently, C-9 must be linked to the methoxy group, thus completely defining the structure **12** as 9-*O*-methyl-10-hydroxycoccineone B. Compound **12** exhibited $[\alpha]_D = 0$, and thus, as in the previously isolated boeravinones,[2] it is racemic at the single chiral center, C-6.

The methanol extract of *B. diffusa* (0.001−1 mg/mL), significantly, and in a concentration-dependent manner, inhibited electrical field stimulation (EFS)-, acetylcholine-, histamine-, and barium chloride-induced contractions of isolated guinea pig ileum (Figure 1). The arithmetic mean IC_{50} values (95% C.L.) for EFS, acetylcholine, barium chloride, and histamine were 182 (138−240) μg/mL, 160 (121−212) μg/mL, 168 (132−213) μg/mL, and 158 (117−215) μg/mL, respectively. Inhibition produced by the methanol extract of *B. diffusa* was reversible, since at the end of the experiment (after washing the tissues) acetylcholine produced contractions with an amplitude similar to that archived at the beginning of the experiment (before the treatment with *B. diffusa* methanol extract).

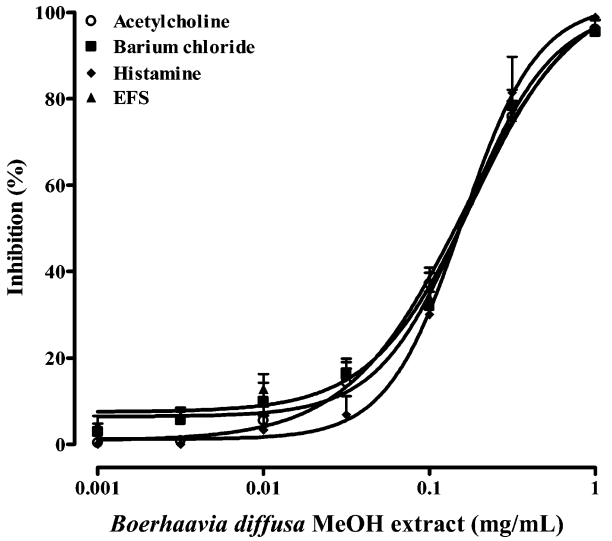

Figure 1. Effect of *Boerhaavia diffusa* root MeOH extract (0.001−1 mg/mL) on contractions induced by acetylcholine (10^{-6} M, ACh), histamine (10^{-6} M), barium chloride (10^{-4} M), and electrical field stimulation (EFS, 2.5 Hz for 2 s, 400 mA, 1 ms pulse duration) on guinea pig ileum. Each point represents the mean ± SEM of 6−8 experiments.

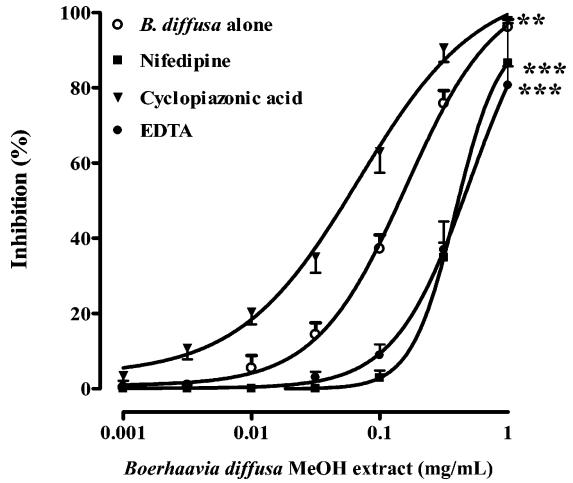

Figure 2. Acetylcholine-induced contractions in isolated guinea pig ileum: effect of *Boerhaavia diffusa* root MeOH extract (0.001−1 mg/mL) alone or in the presence of nifedipine (10^{-6} M), cyclopiazonic acid (10^{-5} M), and EDTA (10^{-3} M). Each point represents the mean ± SEM of 6−8 experiments. $^{**}p < 0.01$; $^{***}p < 0.001$ vs vehicle.

Since *B. diffusa* extract inhibited the contractions induced by three different spasmogens, we hypothesized a common site of action on smooth muscle, for example, the involvement of cytosolic Ca^{2+}, which is known to play an important role in contractile processes of smooth muscle.[11] Therefore, we evaluated the action of calcium antagonists/blockers on the spasmolytic effect of *B. diffusa* extract. The inhibitory response on ACh-induced contractions was significantly ($p < 0.05$) reduced by nifedipine (10^{-6} M) or EDTA (10^{-3} M) (Figure 2): IC_{50} values (95% C.L.) for *B. diffusa* in the presence of nifedipine or EDTA were 3.15×10^{-1} ($(2.04-4.88) \times 10^{-1}$) and 2.28×10^{-1} ($(1.79-2.9) \times 10^{-1}$) mg/mL,

respectively. In contrast, cyclopiazonic acid (10^{-5} M) significantly increased the inhibitory response induced by this extract [IC_{50} value (95% C.L.): 5.44×10^{-3} ($(3.95-7.49) \times 10^{-3}$) mg/mL (Figure 2). Thus, nifedipine, a blocker of L-type Ca^{2+} channels, and EDTA, a calcium chelator, reduced the inhibitory effect of *B. diffusa* methanol extract on ACh-induced contractions, suggesting an involvement of extracellular calcium and/or L-type calcium channels. Conversely, cyclopiazonic acid, a potent and specific inhibitor of the sarcoplasmatic reticulum Ca^{2+}-ATPase in smooth muscle, not only did not reduce the inhibitory effect of *B. diffusa* on ACh-induced contractions but instead produced a leftward shift of the inhibitory curve (Figure 2).

All the rotenoid analogues (**6**−**12**) isolated during the present study were evaluated for their spasmolytic effects on the isolated guinea pig ileum (Table 1). Compounds **7**−**10** and **12** reduced acetylcholine-induced contractions (at 30 μg/mL), while, in contrast, compounds **6** and **11** were completely inactive. None of these compounds completely inhibited acetylcholine-induced contractions: indeed, the maximal inhibition (E_{max}) achieved for each of compounds **7**−**10** and **12**, at the tested concentration, was about 20−50% (Table 1). Papaverine, used as a reference drug, reduced acetylcholine-induced contraction with a percentage of maximum inhibition of 72.3 ± 7.6 at a concentration of 30 μg/mL (Table 1).

In the present investigation, it has been shown that a methanol extract obtained from the roots of *B. diffusa* exhibited a concentration-dependent inhibition of both exogenous spasmogens (i.e., acetylcholine, histamine, and barium chloride) and electrical field stimulation-evoked contractions in the isolated guinea pig ileum. These stimulants induce ileum contraction by different mechanisms: acetylcholine and histamine through the activation of postjunctional receptors; EFS through the release of acetylcholine from enteric nerves; and barium chloride through a postjunctional nonreceptor-mediated mechanism. Since no significant difference was observed in the *B. diffusa* inhibition curves, the spasmolytic effect of *B. diffusa* is probably due to a direct effect on smooth muscle. Furthermore, results obtained in the presence of calcium antagonists/blockers demonstrate that the spasmolytic activity of *B. diffusa* might involve, at least in part, extracellular calcium, while intracellular calcium appears to negatively modulate the inhibitory effect of *B. diffusa* on intestinal motility.

We have described recently the activity of nonprenylated rotenoids from *B. diffusa* on intestinal motility, and through the pharmacological evaluation of compounds **1**−**5**, the negative effect of *O*-methylation at C-6 on this activity was observed (at 30 μg/mL, only **2** and **3** produced a complete inhibition of ACh-induced contractions).[2] Evaluation of the spasmolytic activity within the series of additional rotenoids isolated during the present study allows a more complete formulation of structure−activity relationships for this type of activity. In particular, the activities of compounds **6**−**10** clearly highlight the crucial role of ring B (see Table 1). Indeed, compounds **6**−**8**, showing hydration of the double bond, $\Delta^{6a(12a)}$, regardless of the presence of either a H, a CH_3, or an OCH_3 at C-10, showed reduced or no activity. By analogy, the isoflavone **9**, characterized by an opened ring B, possesses low activity. On the other hand, oxidation of the hemiacetal group at C-6 to a lactone group (as in compound **10**) appears to be better tolerated; however, the activity of boeravinone F (**10**) was markedly lower compared to that of its reduced analogue, boeravinone E (**2**), which appears to be the most potent rotenoid tested.[2] Finally, the low potencies of compounds **11** and **12** indicate that the presence of a hydroxylated pyran ring B is not sufficient for the exhibition of spasmolyitic activity. Most likely, a monohydroxylated ring A and/or a trisubstituted ring D are also needed.

Data presented herein strengthen the observation that nonprenylated rotenoids are responsible for the smooth muscle relaxant activity of the *B. diffusa* root extract investigated. It should be noted that an action on intestinal motility has not been reported previously for this class of molecule. On the other hand, the somewhat structurally comparable stilbenoids from *Nidema boothii* have been reported recently to show a spasmolytic action.[12] Interestingly, structure−activity relationships established for stilbenoids (hydroxylation of both phenyl rings is needed, while methylation of hydroxyl groups is detrimental) resemble those proposed herein for nonprenylated rotenoids.

Experimental Section

General Experimental Procedures. Optical rotations were measured in MeOH on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. UV spectra were taken with a Beckman spectrometer in CH_3OH solution. IR (KBr) spectra were recorded on a Bruker model IFS-48 spectrophotometer. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a Varian INOVA spectrometer; chemical shifts were referenced to the residual solvent signal (CD_3OD: $\delta_H = 3.32$, $\delta_C = 49.0$). The multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. One-bond heteronuclear $^1H-^{13}C$ connectivities were determined with the HMQC experiment. Two- and three-bond $^1H-^{13}C$ connectivities were determined by HMBC experiments optimized for a $^{2,3}J$ of 7 Hz. Heteronuclear coupling constants were qualitatively evaluated by using PS-HMBC. Low-resolution electrospray (negative ions) spectra were performed on a LCQ Finnigan MAT mass spectrometer; low- and high-resolution EIMS (70 eV, direct inlet) were performed on a VG Prospec Fisons mass spectrometer. Medium-pressure liquid chromatography (MPLC) was performed using a Büchi 861 apparatus with a silica gel (230−400 mesh) column. HPLC separations in isocratic mode were achieved on a Beckman apparatus equipped with a refractive index detector and with Phenomenex LUNA (250 × 4.6 mm) silica (5 μm) columns.

Drugs used in the present study (acetylcholine chloride, histamine, barium chloride, nifedipine, cyclopiazonic acid, tetrodotoxin, ethylenediaminetetraacetic acid disodium, and papaverine) were all from Sigma, Milan, Italy. Acetylcholine, histamine, barium chloride, TTX, and EDTA were dissolved in distilled water, while nifedipine and cyclopiazonic acid were dissolved in DMSO. In all experiments, vehicles (water or DMSO) did not modify per se acetylcholine (ACh)-, histamine-, barium chloride-, or EFS-induced contractions. *B. diffusa* root MeOH extract stock solution was suspended in DMSO 50%, and further dilutions were performed in water. All drugs were added in volumes less than 0.01% of the bath volume.

Plant Material. *Boerhaavia diffusa* roots were collected in Jammu, India, in June 2003, and the plant was identified by Dr. R. Longo (Carlo Sessa S.p.A., Milan). A voucher specimen (No. 22.02) is deposited in the Herbarium of Carlo Sessa S.p.A., a company established in Viale Gramsci 212, 20099 Sesto S. Giovanni, Milan, Italy.

Extraction and Isolation. Fresh roots of the plant (1.0 kg) were extracted (3 × 3 L) with methanol (Fluka, 99% pure) at room temperature for 1 h. Evaporation of the pooled extracts left a brown material (44.4 g). A portion of the crude methanolic extract (5.0 g) was subjected to a modified Kupchan partition scheme, as previously described,[2] obtaining CCl_4 (0.72 g), $CHCl_3$ (1.12 g), and *n*-BuOH (1.05 g) extracts. The CCl_4 extract was chromatographed by MPLC on a silica gel (230−400 mesh) column (750 × 25 mm), using a linear gradient system (400 mL for each solvent) from *n*-hexane to EtOAc to MeOH−EtOAc (1:1). All fractions obtained were subjected to preliminary spectroscopic investigation, and those apparently containing not previously isolated rotenoids were further separated by HPLC. The first fraction (*n*-hexane−EtOAc, 8:2) was purified by HPLC on an analytical column (250 × 4.6 mm) using *n*-hexane−EtOAc (85:15) as eluent, flow rate 1.0 mL/min, affording 2′-*O*-methylabronisoflavone (**9**, 3.6 mg, $t_R = 13.2$ min) and coccineone E (**8**, 7.9 mg, $t_R = 14.1$ min). A second fraction (*n*-hexane−EtOAc, 1:1) was purified by HPLC on an analytical column (250 × 4.6 mm) using *n*-hexane−EtOAc (55:45) as eluent, flow 1.0 mL/min, affording the known compound **7** (10-demethylboeravinone C, 1.8 mg, $t_R = 12.5$ min). A third fraction (*n*-hexane−EtOAc, 4:6) was purified by HPLC on an analytical column (250 × 4.6 mm) using *n*-hexane−EtOAc (4:6) as eluent, flow rate 0.8 mL/min, yielding boeravinone C (**6**, 5.5 mg, $t_R = 12.9$ min). Finally, a fourth fraction (*n*-hexane−EtOAc, 3:7) was purified by HPLC on an analytical column (250 × 4.6 mm) using *n*-hexane−EtOAc (35:65) as

eluent, flow rate 0.8 mL/min, affording the new compound **12** (1.5 mg, $t_R = 8.8$ min). The $CHCl_3$ extract was chromatographed by MPLC on a silica gel (230−400 mesh) column (750 × 25 mm), using a linear gradient system (400 mL for each solvent) from *n*-hexane−EtOAc (7:3) to EtOAc to EtOAc−MeOH (1:1). The fraction eluted with EtOAc−*n*-hexane (7:3) was further purified by HPLC (EtOAc−*n*-hexane, 6:4, flow rate 0.8 mL/min), yielding boeravinone F (**10**, 2.2 mg, $t_R = 7.9$ min) and coccineone B (**11**, 2.1 mg, $t_R = 9.0$ min) in the pure state.

9-*O*-Methyl-10-hydroxycoccineone B (12): pale yellow amorphous solid; purity 98% (by HPLC and NMR); $[\alpha]_D^{25}$ 0 (*c* 0.01, MeOH); UV (CH_3OH) λ_{max} (logϵ) 343 nm (3.70), 281 nm (4.50); UV (CH_3OH + $AlCl_3$) λ_{max} 378, 284 nm; UV (CH_3OH + $AlCl_3$/HCl) λ_{max} 381, 285 nm; UV (CH_3OH + NaOAc−H_3BO_3) λ_{max} 352, 285 nm; IR (KBr) ν_{max} 3270, 1650, 1616 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 8.87 (1H, d, $J = 7.8$ Hz, H-1), 7.29 (1H, t, $J = 7.8$ Hz, H-3), 7.08 (1H, t, $J = 7.8$ Hz, H-2), 7.05 (1H, d, $J = 7.8$ Hz, H-4), 6.30 (1H, s, H-8), 6.06 (1H, s, H-6), 3.89 (3H, s, OC*H*$_3$-9); ^{13}C NMR (125 MHz, CD_3OD) δ 179.2 (s, C-12), 158.4 (s, C-7a), 156.0 (s, C-9), 155.5 (s, C-11), 153.6 (s, C-6a), 147.7 (s, C-4a), 132.2 (s, C-10), 128.3 (d, C-3), 126.5 (d, C-1), 122.2 (d, C-2), 116.6 (s, C-1a), 116.2 (d, C-4), 109.1 (s, C-12a), 101.3 (s, C-11a), 97.3 (d, C-8), 94.2 (d, C-6), 56.0 (q, O*C*H$_3$-9); ESIMS (negative-ion) *m*/*z* 327 $[M - H]^-$; EIMS (70 eV) *m*/*z* 328 $[M]^+$ (30), 311 $[M - OH]^+$ (100); HREIMS found *m*/*z* 328.0576 (calcd for $C_{17}H_{12}O_7$, *m*/*z* 328.0583).

Spasmolytic Activity. Guinea pigs (New Zealand, weighing 250−400 g), supplied by Harlan Nossan Italy, Corezzana, MI, Italy, were used after 1 week of acclimation (temperature 23 ± 2 °C; humidity 60%). Animals were killed by asphyxiation with CO_2, and segments (1−1.5 cm) of ileum were removed, flushed of luminal contents, and placed in Krebs' solution (composition in mM: NaCl 119, KCl 4.75, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.5, $CaCl_2$ 2.5, and glucose 11). The segments were placed horizontally in a bath filled with warm (37 °C) and aerated (95% O_2−5% CO_2) Krebs's solution and set up as described previously.[13] The mechanical activity of the longitudinal muscle was recorded isotonically (load 0.5 g) with a transducer connected to a PowerLab data-acquisition system (Ugo Basile, Comerio, Italy). After a 1 h equilibration period, the ileum was stimulated with acetylcholine (10^{-3} M) in order to obtain a maximal contraction (100% contraction). Successively, single concentrations of acetylcholine (10^{-6} M), histamine (10^{-6} M), and barium chloride (10^{-4} M) were added to the bath and left in contact with the tissue for 30 s and then washed out. The concentrations of these spasmogens gave a contractile response that was about 50% of the contraction produced by acetylcholine (10^{-3} M). After at least three stable control contractions, the responses were repeated in the presence of increasing (noncumulative) concentrations of *B. diffusa* MeOH extract (0.001−1 mg/mL) added 20 min before the contacting stimulus (after washing the tissue). Preliminary experiments showed that a 20 min contact time was sufficient for the *B. diffusa* extract to achieve the maximal inhibitory effect.

Electrical field stimulation-induced contractions (EFS; 2.5 Hz for 2 s, 400 mA, 1 ms pulse duration) were performed on the guinea pig ileum via a pair of platinum electrodes placed around the intestine. The conditions of electrical stimulation were selected so that contractile responses were similar in amplitude to that of acetylcholine, histamine, and barium chloride. Stable and reproducible contractions for a time period of 4 h were obtained, with stimulations every 20 min. After stable control contractions evoked by EFS of the cholinergic nerves had been registered, the responses were observed in the presence of increased cumulative concentrations of *B. diffusa* methanol extract (0.001−1 mg/mL). The contact time for each concentration was 20 min.

In preliminary experiments, the effect of tetrodotoxin (TTX; 3×10^{-7} M, contact time: 20 min) was evaluated on both exogenous spasmogens- and electrical-induced contractions. Electrical stimulation of the guinea pig ileum gave a contractile response that was abolished by TTX (3×10^{-7} M) (data not shown). By contrast, the contractions induced by acetylcholine, histamine, and barium chloride were not modified by tetrodotoxin (3×10^{-7} M) (data not shown).

In some experiments, the effect of *B. diffusa* methanol extract on acetylcholine-induced contractions was observed in the presence of nifedipine (10^{-6} M), cyclopiazonic acid (10^{-5} M), and disodium edetate (EDTA = ethylenediaminetetraacetic acid) (10^{-3} M) (contact time: 20 min). The concentrations of nifedipine, cyclopiazonic acid, and EDTA were selected on the basis of previous studies.[13,14] When given alone (i.e., in the absence of *B. diffusa* methanol extract), nifedipine (10^{-6} M), EDTA (10^{-3} M), and cyclopiazonic acid (10^{-5} M) significantly ($p < 0.01$) reduced (% reduction: nifedipine: 47.3 ± 2.9, EDTA: 49.7 ± 5.2, cyclopiazonic acid: 52.3 ± 5.8, $n = 8$ for each drug) the contractions induced by acetylcholine.

Statistical Analysis. Results are expressed as the arithmetic mean ± SE mean [or 95% confidence limits (C.L.) of the IC_{50} values]. Comparisons between two sets of data were made by Student's *t*-test for paired data. When multiple comparisons against a single control were made, ANOVA was used, followed by the Bonferroni's multiple comparisons test. Analysis of variance (two-way) was used to compare different cumulative concentration−effect curves. The arithmetic mean IC_{50} values and 95% C.L. were calculated by analyzing the regression lines according to Tallarida and Murray.[15] E_{max} values were calculated using the Graph Pad Instat program version 4.01. A *p*-value of less than 0.05 was considered significant.

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