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Rotenoids from *Boerhaavia diffusa* as Potential Anti-inflammatory Agents

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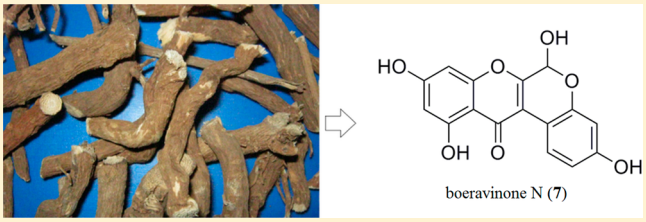
Supporting Information

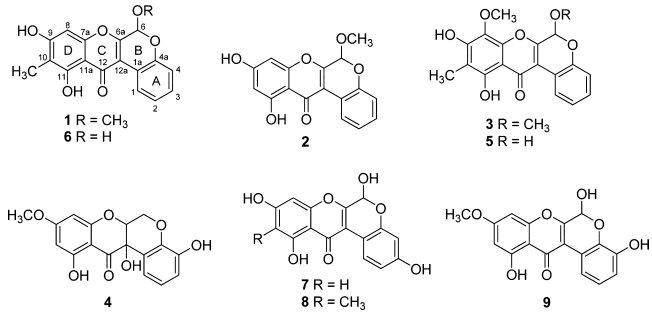
ABSTRACT: Five new (**2**, **3**, **5**, **7**, and **9**) and four known rotenoids (**1**, **4**, **6**, and **8**) were isolated from a methanol extract of *Boerhaavia diffusa* roots. The structures of the new rotenoids were elucidated by spectroscopic data interpretation. The 70% ethanol extract, a rotenoid-rich fraction, and all isolated rotenoids were evaluated for their COX-1 and COX-2 inhibitory activities. Among the rotenoids tested, compound **7** showed the most potent COX-1 and COX-2 inhibition, with IC_{50} values of 21.7 ± 0.5 and 25.5 ± 0.6 μM, respectively. Boeravinone B (**6**) exhibited significant anti-inflammatory activity (56.6% at 50 mg/kg) when evaluated in an in vivo carrageenan-induced rat paw model.



Boerhaavia diffusa L. (Nyctaginaceae) is a perennial herbaceous plant, found to occur in the tropical regions of India, South America, and Africa. In Ayurveda, *B. diffusa* roots are used widely for the treatment of various diseases such as jaundice, dyspepsia, nephrotic syndrome, convulsions, enlargement of spleen, abdominal pain, stress, and inflammation.[1] While a methanol extract of *B. diffusa* (whole plant) has been found to possess antiproliferative and antiestrogenic effects,[2] a corresponding ethanol extract was reported to exhibit hepatoprotective,[3] immunomodulatory,[4] and anti-inflammatory activities.[5] On the other hand, an aqueous extract of *B. diffusa* (leaves) has been reported to exhibit antidiabetic activity.[6] The earlier phytochemical investigation of *B. diffusa* revealed it to contain diverse classes of chemical constituents including phenolics, terpenoids, and organic acids.[7,8] In addition, boeravinones A–J, a group of rotenoids, have been reported from *B. diffusa* roots.[9−15] In view of the ethnopharmacological actions of *B. diffusa* indicated above, and as a part of our ongoing program in search of novel COX inhibitory compounds from Indian medicinal plants, the constituents of a methanol extract of the roots of this species were isolated. In this report the isolation and characterization of five new and four known rotenoids are described, with these compounds evaluated for their anti-inflammatory activity.

RESULTS AND DISCUSSION

Phytochemical investigation of the methanol extract of *B. diffusa* roots led to the isolation of five new rotenoids, boeravinones K (**2**), L (**3**), M (**5**), N (**7**), and O (**9**), and four known rotenoids, boeravinone A (**1**), 10-*O*-demethylboeravinone C (**4**), boeravinone B (**6**), and boeravinone E (**8**), using various chromatographic techniques.



Compound **2**, a pale yellow, amorphous powder, displayed a molecular formula of $C_{17}H_{12}O_6$ based on the HRESIMS, which gave a molecular ion peak at *m*/*z* 335.0531 $[M + Na]^+$ (calcd 335.0532). The ^1H NMR spectrum of **2** (Table 1) showed a total of seven methine proton signals and an oxygenated methyl group. Of these, four methine protons appeared in the region δ_H 7.16–8.69, with a characteristic splitting pattern (doublet, triplet, triplet, and doublet), indicating that they may be the neighboring protons. Further, these four methine protons showed $^1H-^1H$ COSY correlations that confirmed their locations at adjoining carbon atoms. Two methine protons at δ_H 6.42 and 6.26 appeared as doublets with J = 1.2 Hz, indicating meta coupling. In addition, a D_2O-exchangeable signal at δ_H 12.74 was also present, which was assigned to a C-11-linked hydrogen-bonded hydroxy group, similar to other rotenoids.[13−15] Association of the eight proton signals with directly linked carbons was determined from the ^{13}C NMR and HSQC spectra, which also confirmed the presence of an

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Table 1. ^{1}H NMR Spectroscopic Data for Compounds 2, 3, 5, 7, and 9 (400 MHz, δ_H, J in Hz)

H	2[a]	3[b]	5[c]	7[d]	9[a]
1	8.69, d (7.7)	8.82, dd (7.9, 1.6)	8.81, dd (7.8, 1.5)	9.12, d (8.6)	8.16, dd (7.8, 1.6)
2	7.16, t (7.6)	7.15, td (7.7, 1.3)	7.11, td (7.8, 1.2)	7.04, dd (8.7, 2.4)	6.93, t (7.9)
3	7.34, t (7.6)	7.30, td (7.8, 1.6)	7.28, td (7.8, 1.6)		6.86, dd (8.0, 1.6)
4	7.18, d (8.1)	7.12, dd (8.0, 1.2)	7.07, dd (8.1, 1.1)	7.17, s	
6	6.08, s	5.83, s	6.20, s	6.61, s	6.22, s
8	6.42, s			6.66, d (2.1)	6.71, d (2.2)
10	6.26, d (1.2)			6.72, d (2.1)	6.45, d (2.2)
OCH_3-6	3.52, s	3.61, s			
OCH_3-8		3.96, s	3.89, s		
OCH_3-9					3.87, s
CH_3-10		2.17, s	2.11, s		
OH-11	12.74, s	12.88, s		13.57, s	12.86, s

[a−d]Measured in DMSO-d_6, $CDCl_3$, methanol-d_4, and pyridine-d_5, respectively.

oxygenated carbon (δ_C 55.5) and nine quaternary carbons, including a carbonyl carbon (δ_C 179.4) in the structure. Detailed analysis of the HMBC spectrum of **2** (Figure 1) showed the correlation of a methoxy group singlet (δ_H 3.52) with only one carbon at δ_C 93.9, which was positioned at C-6. The methine proton singlet at δ_H 6.08 showed an HSQC correlation with C-6 and HMBC correlations with carbons at δ_C 108.0 (C-12a), 148.1 (C-4a), and 155.0 (C-6a), which confirmed its position at C-6. HMBC correlations were observed for the signal at region δ_H 7.18−8.69 as δ_H 8.69 with δ_C 129.0 (C-3), C-12a, and C-4a, of δ_H 7.16 with δ_C 116.6 (C-1a) and 117.2 (C-4), of δ_H 7.18 with δ_C 122.5 (C-1) and C-4a, and of δ_H 7.18 with C-2 and C4a, which revealed that these could be placed at C-1, C-2, C-3, and C-4, respectively. Thus, the structure of rings A−C in **2** was confirmed. Further, two meta-coupled methine protons showed HMBC correlations, of δ_H 6.42 with δ_C 99.8 (C-10), 104.2 (C-11a), and 156.4 (C-7a) and of δ_H 6.26 with δ_C 94.2 (C-8), C-11a, and 165.5 (C-9), which revealed the locations of these protons at C-8 and C-10 on ring D. In the case of ring D, the C-9 signal appeared in the downfield region (δ_C 165.5), which indicated that a hydroxy group is present at C-9. On the basis of the above inferences, the structure of compound **2** (boeravinone K) was deduced as shown.

Compound **3** was obtained as a pale yellow, amorphous powder. Its molecular formula was established as $C_{19}H_{16}O_7$ based on the $[M + Na]^+$ ion peak at m/z 379.0795 (calcd 379.0794) in the HRESIMS. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) of compound **3** were comparable in many respects to those of compound **2**, with the major difference being the absence of two meta-coupled methine proton signals of ring D in compound **3**. Instead of these proton signals, a methyl singlet at δ_H 2.17 and a methoxy singlet at δ_H 3.96 were present for **3**, which showed HSQC correlations for signals at δ_C 7.53 (C-10 methyl) and 62.1 (C-8 methoxy), respectively. Further, the resonance for C-8 (δ_C 126.1) in compound **3** appeared in a downfield region in comparison to the analogous signal for compound **2**, which appeared at δ_C 94.2. Detailed analysis of the HMBC spectrum of **3** (Figure 1) showed the correlations of one methoxy proton signal (δ_H 3.61) with C-6 at δ_C 95.0 and another methoxy proton signal (δ_H 3.96) with C-8 at δ_C 126.1, which confirmed their positions at C-6 and C-8, respectively. The methyl proton singlet at δ_H 2.17 showed HMBC correlations with carbons at δ_C 154.0 (C-9), 108.1 (C-10), and 156.0 (C-11), which revealed that the methyl group is present at the C-10 position. Thus, the substituents on all four rings (A−D) were confirmed, and compound **3** (boeravinone L) was characterized structurally as shown.

Compound **5**, a pale yellow, amorphous powder, was assigned the molecular formula $C_{18}H_{14}O_7$ based on the molecular ion peak at m/z 365.0628 $[M + Na]^+$ (calcd 365.0637), using HRESIMS. The ^{1}H NMR spectrum of **5** (Table 1) showed a total of five methine protons in the region δ_H 5.83−8.81. In this region, the 2D-NMR data of compound **5** were similar to those of compounds **2** and **3**, which confirmed that compound **5** has an unsubstituted ring A. An oxygenated methyl signal at δ_H 3.89 and a methyl proton signal at δ_H 2.11 showed HSQC correlations with carbons at δ_C 62.2 (C-8 methoxy) and 7.7 (C-10 methyl). The ^{13}C NMR data (Table 2) and HSQC spectrum of **5** showed the presence of a total of 18 carbon atoms, including a carbonyl at δ_C 181.8, a methyl at δ_C 7.7, a methoxy at δ_C 62.2, and five methine carbons. Detailed analysis of the HMBC spectrum of **5** (Figure 1) showed the correlations of the methoxy group signal at δ_H 3.89 with only one carbon at δ_C 128.5 and of the methyl protons at δ_H 2.11 with carbons at δ_C 109.5 (C-10), 156.4 (C-9), and 156.9 (C-11), which revealed methoxy and methyl groups to be present at C-8 and C-10, respectively. Further, the C-6 signal in **5**

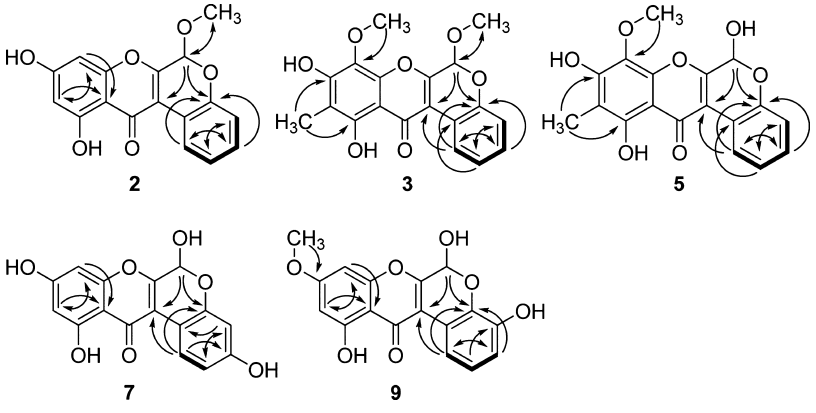

Figure 1. ^{1}H→^{1}H COSY (solid bond) and ^{1}H→^{13}C HMBC (arrow) correlations of new rotenoids (**2**, **3**, **5**, **7**, and **9**).

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Table 2. ^{13}C NMR Spectroscopic Data (100 MHz) for Compounds 2, 3, 5, 7, and 9

C	2[a] (δ_C)	3[b] (δ_C)	5[c] (δ_C)	7[d] (δ_C)	9[a] (δ_C)
1	126.3, CH	127.3, CH	128.0, CH	130.6, CH	117.1, CH
1a	116.6, C	116.8, C	118.1, C	111.1, C	117.6, C
2	122.5, CH	123.0, CH	123.2, CH	112.7, CH	122.3, CH
3	129.0, CH	129.1, CH	129.8, CH	162.1, C	116.4, CH
4	117.2, CH	117.1, CH	118.5, CH	107.7, CH	147.0, C
4a	148.1, C	148.3, C	150.3, C	153.6, C	137.3, C
6	93.9, CH	95.0, CH	89.9, CH	91.8, CH	88.1, CH
6a	155.0, C	153.7, C	157.9, C	158.2, C	158.2, C
7a	156.4, C	145.6, C	148.0, C	159.6, C	156.8, C
8	94.2, CH	126.1, C	128.5, C	96.7, CH	93.2, CH
9	165.5, C	154.0, C	156.4, C	165.6, C	165.9, C
10	99.8, CH	108.1, C	109.5, C	102.4, CH	99.0, CH
11	162.0, C	156.0, C	156.9, C	168.2, C	162.3, C
11a	104.2, C	105.1, C	105.5, C	107.6, C	105.8, C
12	179.4, C	180.3, C	181.8, C	182.8, C	180.4, C
12a	108.9, C	110.2, C	110.2, C	112.3, C	109.5, C
OCH_3-6	55.5, CH_3	56.1, CH_3			
OCH_3-8		62.1, CH_3	62.2, CH_3		
OCH_3-9					56.7, CH_3
CH_3-10		7.53, CH_3	7.7, CH_3		

[a–d]Measured in DMSO-d_6, $CDCl_3$, methanol-d_4, and pyridine-d_5, respectively.

occurred at a slightly upfield region (δ_C 89.9) in comparison to C-6 (δ_C 95.0) of compound **3**, which is generally the case when a free OH group is present at C-6. The remaining protons of **5** showed similar HMBC correlations to compound **3**. Thus, the substituents on all four rings (A–D) were confirmed, and the structure of compound **5** (boeravinone M) was deduced as shown.

Compound **7** was obtained as a pale yellow, amorphous powder with a molecular formula of $C_{16}H_{10}O_7$, which was determined by HRESIMS at m/z 337.0315 $[M + Na]^+$ (calcd 337.0324). The ^1H NMR spectrum of **7** (Table 1) showed a total of six methine proton signals. Of these, two methine protons at δ_H 9.12 and 7.04 showed ortho coupling, which revealed that these protons are situated at adjoining carbon atoms. Three methine proton signals at δ_H 7.17, 6.66, and 6.72 showed meta coupling, and a methine proton appeared as a singlet at δ_H 6.61. A D_2O-exchangeable signal at δ_H 13.57 was also present in the ^1H NMR spectrum of **7**. The association of six proton signals with directly linked carbons and the presence of 10 quaternary carbons, including one carbonyl carbon at δ_C 182.8, were confirmed from the $^1H-^{13}C$ HSQC spectrum. The methine proton singlet at δ_H 6.61 showed an HSQC correlation with a carbon signal at δ_C 91.8 (C-6) and HMBC correlations with carbons at δ_C 112.3 (C-12a), 153.6 (C-4a), and 158.2 (C-6a) (Figure 1), which confirmed its position at C-6. An ortho-coupled methine proton at δ_H 9.12 showed HMBC correlations with signals at δ_C 162.1 (C-3), 112.3 (C-12a), and 153.6 (C-4a), and another ortho-coupled methine proton at δ_H 7.04 showed HMBC correlations with signals at δ_C 111.1 (C-1a) and 107.7 (C-4), which confirmed their positions at C-1 (δ_C 130.6) and C-2 (δ_C 112.7), respectively. The positions of the remaining three meta-coupled methine protons were confirmed by correlations in the HSQC and HMBC spectra as follows: the δ_H 7.17 signal showed an HSQC correlation with C-4 and HMBC correlations with C-2 and C-1a; the δ_H 6.66 signal showed an HSQC correlation with C-8 and HMBC correlations with C-10 and C-11a; and the δ_H 6.72 signal showed an HSQC correlation with C-10 and HMBC correlations with C-8 and C-11a, which revealed these protons to be situated at C-4, C-8, and C-10, respectively. Thus, the substituents on all the four rings (A–D) were confirmed, and the structure of compound **7** (boeravinone N) was determined as shown.

Compound **9** was obtained as a pale yellow, amorphous powder. Its molecular formula was established as $C_{17}H_{12}O_7$ by HRESIMS, m/z 351.0481 $[M + Na]^+$ (calcd 351.0481). The ^1H NMR spectrum of **9** (Table 1) showed the occurrence of six methine proton signals. Of these, three aromatic methine protons at δ_H 8.16, 6.93, and 6.86 showed correlations in the COSY spectrum, which revealed that these are situated at adjoining carbon atoms. Two aromatic methine protons at δ_H 6.71 and 6.45 showed meta coupling to one another, in an analogous manner to compound **7**, and a methine proton at δ_H 6.22 appeared as a singlet. An oxygenated methyl proton singlet at δ_H 3.87 and a D_2O-exchangeable signal at δ_H 12.86 were also present in the ^1H NMR spectrum of **9**. The association of all the proton signals with directly linked carbons and the presence of 10 quaternary carbons, including a carbonyl carbon at δ_C 180.4 and an oxygenated methyl at δ_C 56.7, were evident from the HSQC spectrum. A detailed analysis of the HMBC spectrum of **9** (Figure 1) showed correlations of a methine proton singlet at δ_H 6.22 with a signal at δ_C 109.5 (C-12a), 137.3 (C-4a), and 158.2 (C-6a) and an HSQC correlation with a signal at δ_C 88.1 (C-6), which confirmed its position to be at C-6. Three methine protons in the region δ_H 6.86–8.16 showed correlations in the HMBC spectrum as follows: δ_H 8.16 with δ_C 122.3 (C-2), C-12a, and C-4a; δ_H 6.93 with δ_C 117.6 (C-1a) and 147.0 (C-4); and δ_H 6.86 with C-1 and C-4a, which indicated these to be situated at C-1, C-2, and C-3, respectively. The methoxy proton signal at δ_H 3.87 showed an HMBC correlation with only one carbon at δ_C 165.9 (C-9), which confirmed its position at C-9. The positions of the remaining two meta-coupled methine protons were confirmed by correlations in the HSQC and HMBC spectra. Thus, the proton resonating at δ_H 6.71 showed an HSQC correlation with C-8 and HMBC correlations with C-10 and C-11a, and the

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Table 3. In Vitro COX-1 and COX-2 Inhibitory and in Vivo Anti-inflammatory Activity

		COX % inhibition[a]			% inhibition of edema at[b]	
extract/compound	concentration	COX-1	COX-2	dose, mg/kg	3 h	5 h
1	40[c]	32.9 ± 1.1[d]	48.5 ± 2.1			
2	40[c]	59.4 ± 2.8	44.6 ± 0.8			
3	40[c]	33.1 ± 1.2[d]	31.4 ± 0.8[d]			
4	40[c]	47.2 ± 2.9	41.3 ± 0.8			
5	40[c]	25.9 ± 0.3[d]	55.7 ± 0.9			
6	40[c]	25.0 ± 1.2[d]	54.6 ± 3.1	50	48.3 ± 1.7[g]	56.7 ± 1.6[f]
7	40[c]	21.7 ± 0.5[d]	25.5 ± 0.6[d]			
8	40[c]	23.9 ± 0.1[d]	61.5 ± 0.7			
9	40[c]	59.9 ± 0.5	61.2 ± 0.7			
indomethacin[e]	30[c]	98.2 ± 0.3	53.4 ± 1.0			
celecoxib[e]	30[c]	13.1 ± 0.9	95.6 ± 0.5			
ibuprofen[e]				50	35.8 ± 1.6[g]	43.5 ± 2.4[g]
				100	42.7 ± 2.7[g]	50.4 ± 2.2[g]

[a]Data are expressed as means ± SEM (n = 2−3). [b]Data are expressed as means ± SEM (n = 6). [c]In μM. [d]IC_{50} μM. [e]Positive control. [f]$p < 0.01$. [g]$p < 0.001$ in comparison to control group.

signal at δ_H 6.45 showed an HSQC correlation with C-10 and HMBC correlations with C-8 and C-11a, which revealed these protons to be situated at C-8 and C-10, respectively. Accordingly, the substituents on all four rings (A−D) were confirmed, and the structure of compound **9** (boeravinone O) was deduced as shown.

Compounds **2**, **3**, **5**, **7**, and **9** were found to be optically inactive, as these exhibited $[\alpha]^{25}_D = 0$, which revealed that they are racemic at the single chiral center, C-6, in agreement with literature reports for previously isolated boeravinones.[13−15] The structures of the known compounds were elucidated by comparing the observed spectroscopic data with values reported in the literature and were identified as boeravinone A (**1**),[11] 10-*O*-demethylboeravinone C (**4**),[14,16] boeravinone B (**6**),[11] and boeravinone E (**8**).[12] An analysis of the 70% ethanol extract of *B. diffusa* roots by HPLC revealed the presence of three major rotenoids, **6**, **8**, and **9**. The concentration levels of these three rotenoids were determined by an analytical HPLC method and were found to be 119.69, 18.32, and 27.95 μg/g, respectively, in dried *B. diffusa* roots.

All the isolated rotenoids were evaluated for COX-1 and -2 inhibitory activity at 40 μM concentration. Compound **7** exhibited the most potent COX-1 inhibitory effect (IC_{50}, 21.1 μM), followed by **8**, **5**, **6**, **1**, and **3** among all compounds tested. Compound **7** also showed the most potent COX-2 inhibition (IC_{50}, 26.7 μM), followed by **3** among all the tested compounds (Table 3). Compound **3** exhibited the best COX-2 selectivity index (COX-1/COX-2 ratio) of 1.09, whereas compound **7** showed a COX-2 selectivity index of 0.79. The results revealed that rotenoids act as nonselective inhibitors of the COX-1 and -2 enzymes, thus accounting at least in part for their anti-inflammatory activity. The 70% ethanol extract and a rotenoid-rich fraction were also evaluated for their COX-1 and COX-2 inhibitory activities at 100 and 50 μg/mL concentration, respectively. The results showed that the COX-1 and COX-2 inhibitory effects of these were weak in comparison to that of rotenoids isolated.

Finally, boeravinone B (**6**), a major rotenoid isolated from *B. diffusa* roots, was evaluated for its in vivo anti-inflammatory activity, by a carrageenan-induced rat paw edema assay. It showed significant anti-inflammatory activity (56.6% at 50 mg/kg), better than the positive control, ibuprofen (43.52% and 50.40% at 50 and 100 mg/kg po, respectively) (Table 3). The anti-inflammatory activity of the 70% ethanol extract and the rotenoid-rich fraction was also studied, by the same method as described above. The rotenoid-rich fraction exhibited a more potent anti-inflammatory (48.5% at 100 mg/kg po) effect as compared to the 70% ethanol extract (34.3% at 100 mg/kg po).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Autopol IV automatic polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). UV spectra were recorded on a Shimadzu 2450 UV−vis spectrophotometer (Chiyoda-ku, Tokyo, Japan). IR spectra were recorded on a Nicolet Impact 410 FT-IR spectrometer (Thermo Scientific, Chiyoda-ku, Tokyo, Japan). The mass spectra were recorded on a Bruker maXis UHR-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). ^{1}H and ^{13}C NMR spectra were recorded in deuterated solvents on a Bruker 400 Ultra Shield NMR spectrometer (Bruker Biospin AG, Faellanden, Switzerland) with TMS as an internal standard. Analytical HPLC was performed on a Waters 600 HPLC system equipped with a Delta 600 quaternary solvent pump, an in-line degasser, a 717 Plus autosampler, a temperature control module, a 2996 photodiode array (PDA) detector, and Empower 2 software (Waters, Milford, MA, USA). The separation was carried out on a C_{18} Xbridge column (4.6 × 250 mm, 5.0 μm) (Waters, Milford, MA, USA). Column chromatography was performed with silica gel (60−120 and 100−200 mesh) (Qualigens Fine Chemicals, Mumbai, Maharashtra, India). Gel permeation chromatography was performed on Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO, USA). TLC was performed on silica gel 60 F_{254} plates (0.20 mm thickness, Merck, Darmstadt, Germany). The rat paw volume was measured using a plethysmometer (model 7140, Ugo Basile, Comerio, Varese, Italy). The COX (ovine) inhibitory screening assay kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Plant Material. The roots of *B. diffusa* were purchased from a local herbal market in Chandigarh, India, in May 2012, and were authenticated by Dr. A. S. Sandhu, botanist, Department of Natural Products, National Institute of Pharmaceutical Education and Research (NIPER). A voucher specimen (NIP-H-175) is preserved in the herbarium, Department of Natural Products, NIPER, S.A.S. Nagar, India.

Extraction and Isolation. Extracts of *B. diffusa* roots were prepared by two different methods. In the first of these, the dried powdered root material (1 kg) of *B. diffusa* was macerated with 3 × 2 L of 70% ethanol, at room temperature, each for 24 h. The extracts were collected, filtered, and then concentrated on a rotary evaporator under reduced pressure to 180 mL volume and were finally freeze-dried to give 82 g of a dried extract (8.20% w/w). Another lot of dried

powdered root material (8 kg) of *B. diffusa* was first defatted with hexanes and then extracted with MeOH using a Soxhlet apparatus. The MeOH extract was filtered and then concentrated on a rotary evaporator under reduced pressure to give 692 g of a dried methanol extract (8.65% w/w). The dried 70% ethanol extract (20 g) of *B. diffusa* was subjected to column chromatography over Diaion HP-20 resin using gradient elution with H_2O−MeOH. The fractions collected on elution with 90% and 100% MeOH were concentrated on a rotary evaporator under reduced pressure to give 1.1 g of the rotenoid-rich fraction. The alcohol extracts (70% ethanol extract and methanol extract) and the rotenoid-rich fraction were analyzed by HPLC. While the alcohol extracts exhibited a similar HPLC profile, the rotenoid-rich fraction as described above showed the presence of enriched concentration levels of rotenoids.

The methanol extract (650 g) was suspended in water (3 L) and partitioned with EtOAc (4 × 2 L). The combined EtOAc phase was concentrated on a rotary evaporator under reduced pressure to give 55 g of a dried EtOAc fraction. This EtOAc fraction (50 g) was subjected to column chromatography over silica gel (100−200 mesh) and was eluted gradient-wise using hexanes−EtOAc (100:1 to 30:70). The fraction (3 L) from each eluent (2.5%, 5%, 10%, 15%, 20%, 30%, 40%, and 50%) was collected, and a final fraction of 70% EtOAc in hexanes (5 L) was also collected. Fractions were combined based on TLC to give six pooled fractions (BDM-F1−BDM-F6). Fraction BDM-F3 (0.84 g) was subjected to CC using Sephadex LH-20, eluting with MeOH, to give four subfractions (F3-I−F3-IV). Subfraction F3-IV was further purified by preparative HPLC using isocratic elution with CH_3CN−H_2O (70:30; flow rate 7 mL/min; detection at 273 nm) to afford compounds **1** (t_R 18.3 min, 103 mg) and **2** (t_R 13.2 min, 45 mg). Fraction BDM-F4 (0.95 g) was subjected to CC using Sephadex LH-20, eluting with MeOH, to afford compound **3** (36 mg). Fraction BDM-F5 (5 g) was subjected to CC using silica gel (230−400 mesh) and eluted gradient-wise, with hexanes−EtOAc as eluent, to give four subfractions (F5-I−F5-IV). Subfraction F5-III (660 mg) was subjected to CC over Sephadex LH-20, eluting with MeOH, to afford compounds **4** (6 mg), **5** (9 mg), and **6** (118 mg). Subfraction F5-IV (250 mg) was subjected to preparative HPLC using isocratic elution by CH_3CN−H_2O (70:30; flow rate 10 mL/min; detection at 273 nm) to afford compound **7** (t_R 7.2 min; 9 mg). The remaining amount of subfraction F5-IV (250 mg) was subjected to preparative HPLC, using isocratic elution by CH_3CN−H_2O (65:35; flow rate 5 mL/min; detection at 273 nm), to give compounds **8** (t_R 13.2 min; 10 mg) and **9** (t_R 15.9 min; 10 mg).

The three major rotenoids (**6**, **8**, and **9**) were quantitatively determined in the 70% ethanol extract by HPLC, on a RP-18 column (250 × 4.6 mm, 5 μm), using the mobile phase 0.1% acetic acid in water (A) and MeOH (B) in a gradient manner (0−5 min 40% B; 5−12 min 60% B; 12−25 min 70% B; 25−30 min 80% B; 30−35 min 90% B; and 35−45 min 40% B). Detection was performed at 273 nm using a PDA detector (Figure S1, Supporting Information).

Boeravinone K (2): pale yellow, amorphous powder; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 216 (4.13), 268 (4.10) nm; IR (KBr) ν_{max} 3334, 1651, 1589 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) data, see Table 1; ^{13}C NMR (DMSO-d_6, 100 MHz) data, see Table 2; HRESIMS *m*/*z* 335.0531 $[M + Na]^+$ (calcd for $C_{17}H_{12}O_6Na$, 335.0532).

Boeravinone L (3): pale yellow, amorphous powder; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 216 (4.05), 279 (4.01) nm; IR (KBr) ν_{max} 3334, 1652, 1589 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) data, see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 2; HRESIMS *m*/*z* 379.0795 $[M + Na]^+$ (calcd for $C_{19}H_{16}O_7Na$, 379.0794).

Boeravinone M (5): pale yellow, amorphous powder; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 216 (4.28), 278 (4.26) nm; IR (KBr) ν_{max} 3265, 1653, 1593 cm^{-1}; ^1H NMR (methanol-d_4, 400 MHz) data, see Table 1; ^{13}C NMR (methanol-d_4, 100 MHz) data, see Table 2; HRESIMS *m*/*z* 365.0628 $[M + Na]^+$ (calcd for $C_{18}H_{14}O_7Na$, 365.0637).

Boeravinone N (7): pale yellow, amorphous powder; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 212 (3.95), 272 (3.98) nm; IR (KBr) ν_{max} 3381, 1651, 1621 cm^{-1}; ^1H NMR (pyridine-d_5, 400 MHz) data, see Table 1; ^{13}C NMR (pyridine-d_5, 100 MHz) data, see Table 2; HRESIMS *m*/*z* 337.0315 $[M + Na]^+$ (calcd for $C_{16}H_{10}O_7Na$, 337.0324).

Boeravinone O (9): pale yellow, amorphous powder; $[\alpha]^{25}_D$ 0 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 216 (4.21), 269 (4.20) nm; IR (KBr) ν_{max} 3398, 1654, 1622 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) data, see Table 1; ^{13}C NMR (DMSO-d_6, 100 MHz) data, see Table 2; HRESIMS *m*/*z* 351.0481 $[M + Na]^+$ (calcd for $C_{17}H_{12}O_7Na$, 351.0481).

In Vitro COX-1 and COX-2 Inhibitory Activity. The COX-1 and COX-2 enzymes are well-established targets for test compound anti-inflammatory evaluation. Hence, the *B. diffusa* 70% ethanol extract, a rotenoid-rich fraction, and the compounds isolated were tested for COX-1 and COX-2 inhibitory activity using the COX (ovine) inhibitor screening kit, as per the manufacturer's instructions. The COX-1 and COX-2 inhibitory assays have been used earlier in our laboratory.[17] The COX-1 and COX-2 enzymes catalyze the biosynthesis of PGH_2 from arachidonic acid. In this assay, the $PGF_{2\alpha}$ produced by the reduction of PGH_2 with stannous chloride ($SnCl_2$) was measured in this enzyme immunoassay. The stock solutions were prepared in DMSO, and the final concentration was set as 40 μM for the isolated rotenoids, 50 μg/mL for the rotenoid-rich fraction, and 100 μg/mL for the 70% ethanol extract of *B. diffusa*. The percentage inhibition was calculated by comparison with control incubations. The IC_{50} values of the most active rotenoids were calculated from concentration−inhibition response curves. Indomethacin and celecoxib (Sigma, St. Louis, MO, USA) were used as reference standards.

In Vivo Anti-inflammatory Activity. The anti-inflammatory activity of the 70% ethanol extract, a rotenoid-rich fraction, and boeravinone B (**6**) of *B. diffusa* was evaluated using the carrageenan-induced paw edema method, described by Winter et al.[18] In brief, rats were fasted overnight before the start of each experiment. The animals were divided into seven groups having six animals each (n = 6). The standard and test samples were prepared by suspension in freshly prepared 0.5% hydroxypropyl methyl cellulose solution. The control group was treated with vehicle (normal saline) only, and the positive-control groups were given the reference standard drug ibuprofen at a dose level of 100 and 50 mg/kg po. The remaining three groups were treated with the 70% ethanol extract at a dose of 100 mg/kg po, the rotenoid-rich fraction at a dose of 100 mg/kg po, and boeravinone B (**6**) at a dose of 50 mg/kg po. Edema was induced by subcutaneous injection of 100 μL of 1% carrageenan in saline solution into the left hind paw of the rats, 1 h after test compound administration. The paw volume was measured immediately before (basal) and after (3 and 5 h) the carrageenan injection using a plethysmometer. Edema was calculated by subtracting the initial paw volume from the paw volume measured at the 3 and 5 h time points.

Animals. Female Sprague−Dawley rats (160−180 g) were used in the in vivo study. The Institute Animal Ethics Committee (IAEC) of NIPER approved the experimental protocol (IAEC/12/37), and experiments were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. Animals were housed in plastic cages and maintained under standard laboratory conditions with a natural 12 h light and dark cycle. The animals were fed with standard pellet diet and water ad libitum.

Statistical Analysis. In vivo activity data were determined as mean % ± standard error of the mean (SEM). Statistical differences between the treatment and the control groups were evaluated by one-way ANOVA followed by Dunnett's tests, $^{**}p < 0.01$ significant from control; $^{***}p < 0.001$ extremely significant from control; ns, not significant.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{13}C NMR, HSQC, and HMBC spectra of compounds **2**, **3**, **5**, **7**, and **9** are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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