Inhibitory Effects of Glycosides from the Leaves of *Melaleuca quinquenervia* on Vascular Contraction of Rats

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Original Paper

Abstract

Two new glycosides, 3-hydroxy-5-methoxy-4-methylphenyl β-D-glucopyranoside (**1**) and 4-benzoyl-2-*C*-β-glucopyranosyl-3,5-dihydroxy-6-methylphenyl β-D-glucopyranoside (**2**), together with four known glycosides, 2-*endo*-β-D-glucopyranosyloxy-1,8-cineole (**3a**), 2-*exo*-β-D-glucopyranosyloxy-1,8-cineole (**3b**), roseoside (**4**), and citroside A (**5**), were isolated from the methanolic extract of leaves of *Melaleuca quinquenervia*. Their structures were elucidated on the basis of spectroscopic analysis. Compounds **1**, **2a** and **3** inhibited contractile response induced by phenylephrine in aortic rings from Sprague-Dawley rats. This inhibition was independent of the endothelium. Compounds **2** and **4** significantly relaxed precontracted aortic rings, in an endothelium-dependent manner. Pretreatment of N^{ω}-nitro-L-arginine (L-NNA), a nitric oxide synthase inhibitor, partially attenuated the vasorelaxation induced by both compounds, suggesting that nitric oxide was likely the responsible mediator. The rank-order potency (EC_{50} value) of vasorelaxing activities of these compounds is **4** > **2** > **2a** > **3** > **1**.

Key words
Melaleuca quinquenervia · Myrtaceae · glycosides · leaves · vasorelaxing activities

Introduction

Melaleuca quinquenervia (Myrtaceae), a large erect paper-bark tree, is widely cultivated in Taiwan. Its leaves containing bioactive agents have long been used in traditional folk medicine as sedative and pain-relieving agents [1]. Findings of chemical studies on leaves of the plant were reported, from which series of triterpenoids [2], [3], flavanones [4] and polyphenols [5] were identified. However, the glycosidic constituent of *M. quinquenervia* was not reported, a chemical investigation on the *n*-butanol soluble part of the methanolic extract of its leaves was thus undertaken that has led to the isolation and identification of two new phenolic glycosides as well as four known glycosides. In an attempt to assess their potential as antihypertensive agents, *in vitro* experiments were first conducted to verify the vasorelaxing properties. This study describes the isolation and structural elucidation of the new compounds as well as their biological activity toward blood vessels.

Materials and Methods

General experimental procedures

Optical rotations were measured using a JASCO DIP-180 digital spectropolarimeter. IR spectra were recorded on a Nicolet 510P FT-IR spectrometer. UV spectra were measured in MeOH or CH_2Cl_2 on a Hitachi U-2000 spectrophotometer. The NMR spectra were recorded in CD_3OD or $CDCl_3$ at room temperature on a Bruker DMX-500 SB spectrometer, and the solvent resonances were used as internal shift references. The 2D NMR spectra were recorded using standard pulse sequences. Positive ion FAB-MS and HR-FAB-MS data were obtained on a JOEL SX-102A mass

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spectrometer using *m*-nitrobenzyl alcohol (NBA) as the matrix. Sephadex LH-20 (Pharmacia Biotech) was used for open column chromatography. HPLC was performed using an ODS column (Hyperprep ODS, 10 mm i.d.×250 mm, Keystone Scientific Inc., Bellefonte, PA; detector, RI) and a silica column (Hyperprep HS silica, 10 mm i.d.×250 mm, ThermoQuest Hypersil, Runcorn, UK; detector, RI). TLC was performed using silica gel 60 F_{254} plates (200 μm, Merck, Germany).

Plant material

Leaves of *M. quinquenervia* (CAV.) S. T. Blake were collected at the Academia Sinica, Taipei on March 16, 2001 and were identified by Mr. Chii-Cheng Liao, Department of Botany, National Taiwan University. Voucher specimens (No. 20010316) have been deposited at the Institute of Botany, Academia Sinica, Nakang, Taipei, Taiwan.

Extraction and isolation

Fresh leaves of *M. quinquenervia* (650 g) were successively extracted three times with 3 L of MeOH at room temperature. The methanolic extract was adjusted to 85% in aqueous solution for an *n*-hexane partition, which generated two fractions soluble in aqueous methanol and *n*-hexane. Subsequently, the aqueous methanol-soluble fraction was then evaporated in vacuum to dryness (52 g) and further partitioned between chloroform (500 mL×2) and water (500 mL), and the remaining water solution was extracted three times with *n*-butanol (500 mL). The *n*-butanol layer was evaporated to a brown residue and redissolved in MeOH for chromatographic separation. The first separation step was carried out using gel filtration chromatography on a Sephadex LH-20 column (3×55 cm) and eluted by MeOH with a flow rate of 13 mL/min. Each fraction (15 mL) collected from the *n*-butanol layer was checked for their compositions by TLC using EtOAc/HCO_2H/H_2O (85:10:15) for development. Observation under UV 254 nm and dipping in vanillin-sulfuric acid (brown or orange spots) were used in the detection of glycosides. Subsequently, the glycoside fractions (#fr. 11–19) from above separation were combined to give subfraction 1 and 2. HPLC of subfraction 1 on a reversed-phase column with MeCN/H_2O (20:80) as eluent, 2 mL/min, afforded **3** (56 mg), **4** (45 mg), and **5** (6 mg), retention time: 16.2, 11.5 and 12.4 min, respectively. Subfraction 2 was purified by using the same column with MeCN/H_2O (15:85) as eluent to yield **1** (11 mg) and crude mixture of **2** (25 mg), retention time: 10.1 and 10.8 min, respectively. Crude mixture of **2** (10 mg) was dissolved in pyridine (3 mL) with Ac_2O (3 mL), and the mixture left overnight at room temperature. Then, ice water (30 mL) was added to the reaction mixture, and the resultant suspension was extracted with ethyl acetate (30 mL×2). The ethyl acetate layer was purified using a normal-phase column with *n*-Hex/$CHCl_3$/EtOAc (1:1:1) as eluent, 2 mL/min, to yield the pure peracetylated derivative **2a** (9.5 mg).

Acid hydrolysis: Compounds **1** and **2** (5 mg) were hydrolyzed by 2N HCl (2 mL) at room temperature overnight. The reaction mixture was then partitioned with EtOAc (2 mL×2). The lower layer was neutralized using Amberlite IRA-400 resin (Fluka, Switzerland), filtered with glass wool, and the filtrate was evaporated to give D-glucose: $[\alpha]_D^{25}$: +47.1° (*c* 0.075, H_2O).

3-Hydroxy-5-methoxy-4-methylphenyl β-D-glucopyranoside (**1**): amorphous white powder; m.p. 112–114 °C; $[\alpha]_D^{25}$: –65° (*c* 0.30, MeOH); UV (MeOH): λ_{max} (log ε) = 225 (4.1), 268 (3.4) nm; IR (KBr): ν_{max} = 3372, 1603, 1516, 1464, 1423, 1165, 1117, 1074 cm^{-1}; ^{1}H-NMR (CD_3OD, 500 MHz): δ = 6.27 (d, *J* = 2.0 Hz, H-6), 6.24 (d, *J* = 2.0 Hz, H-2), 4.79 (d, *J* = 7.3 Hz, H-1′), 3.90 (dd, *J* = 11.9, 1.2 Hz, H-6′a), 3.74 (s, H_3–7), 3.69 (dd, *J* = 11.9, 5.4 Hz, H-6′b), 3.2–3.45 (m, H-2′, 3′, 4′, 5′), 1.94 (s, H_3–8); ^{13}C-NMR (CD_3OD, 300 MHz): δ = 160.4 (C-5), 158.1 (C-1), 157.2 (C-3), 107.5 (C-4), 102.7 (C-1′), 97.6 (C-2), 93.4 (C-6), 78.2 (C-3′ or C-5′), 78.1 (C-5′ or C-3′), 74.9 (C-2′), 71.5 (C-4′), 62.6 (C-6′), 56.0 (C-7), 7.9 (C-8); FAB-MS (NBA): *m*/*z* = 339 $[M+Na]^+$ (34), 154 (100); HR-FAB-MS (NBA): *m*/*z* = 317.1250 $[M+H]^+$ calcd. for $C_{14}H_{20}O_8$ + H: 317.1236.

4-Benzoyl-2-C-β-glucopyranosyl-3,5-dihydroxy-6-methylphenyl β-D-glucopyranoside peracetate (**2a**): amorphous white powder; m.p. 125–126 °C; $[\alpha]_D^{25}$: –17.9° (*c* 0.48, CH_2Cl_2); UV (CH_2Cl_2): λ_{max} (log ε) = 256 (4.6), 230 (4.3) nm; IR (KBr): ν_{max} = 2942, 1755, 1674, 1599, 1435, 1369, 1223, 1039 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 7.77 (d, *J* = 7.5 Hz, H-3′, -7′), 7.56 (t, *J* = 7.5 Hz, H-5′), 7.44 (t, *J* = 7.5 Hz, H-4′, -6′), 5.47 (t, *J* = 9.5 Hz, H-2‴), 5.43 (t, *J* = 8.2 Hz, H-2″), 5.35 (t, *J* = 9.5 Hz, H-3‴), 5.26 (t, *J* = 8.2 Hz, H-3″), 5.25 (d, *J* = 9.5 Hz, H-1‴), 5.24 (t, *J* = 8.2 Hz, H-4″), 5.00 (t, *J* = 9.5 Hz, H-4‴), 4.84 (d, *J* = 8.2 Hz, H-1″), 4.48 (dd, *J* = 12.5, 3.2 Hz, H-6″a), 4.28 (dd, *J* = 12.6, 4.8 Hz, H-6‴a), 3.88 (br d, *J* = 12.5, H-6′b), 3.76 (br d, *J* = 12.6, H-6‴b), 3.72 (m, H-5‴), 3.65 (m, H-5″), 2.08, 2.07, 2.04, 2.01, 2.00, 1.98, 1.95, 1.94, 1.85, 1.78 (s, OAc×10), 2.06 (s, H_3–7); ^{13}C-NMR ($CDCl_3$, 300 MHz): 948; = 191.1 (C-1′), 170.3, 170.2×3, 169.5, 169.4, 169.2, 168.7, 167.8, 167.7 ($O\underline{C}OCH_3$×10), 154.1 (C-1), 148.7 (C-3), 146.3 (C-5), 136.8 (C-2′), 133.5 (C-5′), 129.8 (C-3′, -7′), 128.4 (C-4′, -6′), 126.0 (C-4), 122.9 (C-6), 122.5 (C-2), 101.7 (C-1″), 75.8 (C-5‴), 74.4 (C-3‴), 73.9 (C-1‴), 72.8 (C-3″), 72.2 (C-5″), 71.7 (C-2″), 70.4 (C-2‴), 68.6 (C-4‴), 67.2 (C-4″), 62.2 (C-6‴), 60.9 (C-6″), 20.7, 20.6×4, 20.5×2, 20.4, 20.2, 20.1 ($OCO\underline{C}H_3$×10), 10.1 (C-7); FAB-MS (NBA): *m*/*z* = 989 $[M+H]^+$ (12), 947 (7), 617 (8), 331 (70), 169 (100), 109 (37); HR-FAB-MS (NBA): *m*/*z* = 989.2935 $[M+H]^+$ calcd for $C_{46}H_{52}O_{24}$ + H: 989.2927.

Compound **3**, $[\alpha]_D^{25}$: –40.2° (*c* 0.64, MeOH), was characterized as a mixture (**3a**: **3b** = 4:1) of 2-*endo*-*β*-D-glucopyranosyloxy-1,8-cineole (**3a**) and 2-*exo*-*β*-D-glucopyranosyloxy-1,8-cineole (**3b**), obtained previously from *Eucalyptus perriniana* [6] and *Salvia bucharica* [7], respectively. Compound **4**, $[\alpha]_D^{25}$: +83.5° (*c* 0.50, MeOH), was identified as roseoside, having been isolated from aerial parts of *Epimedium grandiflorum* [8]. Compound **5**, $[\alpha]_D^{25}$: –85° (*c* 0.25, MeOH), was determined to be citroside A, and its spectral data were in good agreement with the published data [9].

Bioassays

In vitro *vascular tension study*: The details of the experimental procedures have been described previously [10]. Briefly, Sprague-Dawley rats were sacrificed by decapitation, and sections of the thoracic aorta between the aortic arch and the diaphragm were excised carefully. Isolated aortic rings 3 to 4 mm in length were fixed isometrically in organ chambers under passive tension of 1.8 g for 60 min. The changes of vascular tension were recorded with a polygraph (Gould, model 2400, Vally View, OH, USA) via a force displacement transducer (Grass FTO3, Quincy, MASS, USA) and simultaneously displayed on the monitor of an IBM-compatible computer after being digitized (PowerLab, ADInstruments Pty Ltd., Sydney, Australia). After equilibration,

near maximal contraction was induced by phenylephrine (0.3 μM). When the rings achieved a stable contractile tension, acetylcholine (1 μM) was added to the bath to assess endothelial integrity. In some preparations, the intima was gently frayed with a cotton swab to disrupt the endothelium. The absence of acetylcholine-induced relaxation indicated successful endothelial denudation.

Relaxation of phenylephrine-induced contraction: For the evaluation of relaxation, acetylcholine (1 μM, endothelium-dependent) and sodium nitroprusside (0.1 μM, endothelium-independent) were used as positive controls in this experiment. Compounds **1**, **2**, **2a**, **3**, **4** (0.01 – 100 μM) or vehicle (0.1%, DMSO) was added in a cumulative manner during the tonic phase of contraction induced by phenylephrine (0.3 μM) in both endothelium-intact and -denuded aortic rings. A 10-min time interval was required to obtain the maximal effect with each concentration of various compounds. The construction of concentration-response curves for compounds was based on the percentage of relaxation of the agonist-induced maximal contraction. The concentration evoking 50% relaxation (EC_{50} value) for each experiment was calcuated. To investigate the possible involvement of nitric oxide in the vasorelaxing effects of compounds, endothelium-intact aortic rings were preincubated with the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 100 μM) for 10 min. Cumulative concentrations of each of these compounds (0.01 – 100 μM) were then applied during the sustained phase of phenylephrine (0.3 μM)-induced contraction. The effects of the inhibitor were studied by comparing the degrees of vasorelaxation induced by compounds in the absence or presence of this inhibitor. The concentrations of the inhibitor used had been reported to be adequate to produce the necessary nitric oxide inhibition [11].

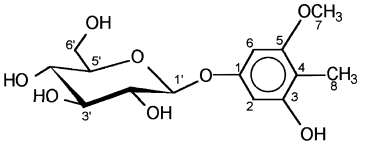
Statistical analyses

The data are presented as mean ± S.E. and *n* represents the number of experiments. In line graphs, S.E. values are indicated by error bars (in some cases the error bars were so small they were obliterated by the line symbols). Statistical analyses were carried out by Student's unpaired *t* test when applicable. P values of less than 0.05 were considered to be significant.

Results and Discussion

A methanolic extract of the fresh leaves of *M. quinquenervia* was fractionated by liquid-liquid partitioning into fractions soluble in *n*-hexane, chloroform, and *n*-butanol, successively. The *n*-butanol-soluble fraction was then subjected to Sephadex LH-20 column chromatography and HPLC to give six glycosides. Of these, compounds **1** and **2** were identified to be two novel glycosides based on their spectral analysis.

The molecular formula for **1**, $C_{14}H_{20}O_8$, was determined by ^{13}C-NMR and HR-FAB-MS data. The IR spectrum of **1** indicated the presence of hydroxy (3372 cm^{-1}) and aromatic (1603 and 1516 cm^{-1}) groups. Its UV spectrum exhibited maxima at 225 and 268 nm. The ^{1}H-NMR spectrum of **1** showed the signals for methylene protons at δ = 3.90 (1H, dd, J = 11.9, 1.2 Hz) and 3.69 (1H, dd, J = 11.9, 5.4 Hz), methyl protons at δ = 1.94 (3H, s), methoxyl protons at δ = 3.74 (3H, s), four methine protons at δ = 3.2–3.45 (1H×4, m), a glucopyranosyl anomeric proton at δ = 4.79 (1H, d, J = 7.3 Hz), and two meta-aromatic protons at δ = 6.27 (1H, d, J = 2.0 Hz) and 6.24 (1H, d, J = 2.0 Hz), suggesting the presence of a benzene ring and a β-D-glucopyranose. The β-D-glucopyranosyl, methoxy, methyl, and hydroxy groups were determined to be linked at C-1, C-5, C-4 and C-3, respectively, since correlations between glucopyranosyl H-1′ and C-1, H_3 – 7 and C-3, and H_3 – 8 and C-3, -4, -5 were observed in the HMBC spectrum. Three singlets at δ = 160.4, 158.1 and 157.2 in the ^{13}C-NMR spectrum of **1** further supported the oxygenation of the benzene ring at C-1, C-5 and C-3, respectively. Thus, the structure of **1** was determined as 3-hydroxy-5-methoxy-4-methylphenyl β-D-glucopyranoside.



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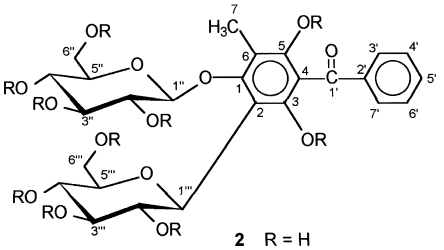

2 R = H
2a R = Ac

Compound **2** was purified as its peracetylated derivative **2a**. Compound **2a** was obtained as an amorphous powder, whose molecular formula was confirmed to be $C_{46}H_{52}O_{24}$ by the HR-FAB-MS and ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum of **2a** showed signals for fourteen sugar protons, and five aromatic protons in A_2X_2Y system, both being corroborated by a COSY spectrum, in addition to a signal for an aryl methyl group (δ = 2.06). Analysis of the signals of fourteen protons suggested two β-glucopyranosyl units with the anomeric protons at δ = 5.25 (d, J = 9.5 Hz, H-1‴) and 4.84 (d, J = 8.2 Hz, H-1″). The ^{1}H-NMR spectrum of **2a** also revealed ten acetyl methyl singlets, suggesting that there existed two aromatic hydroxy groups in addition to eight hydroxys on two glucosyl moieties. In the HMBC spectrum, the anomeric protons at δ = 5.25 and 4.84 showed interactions with aryl C-2 and C-1 at δ = 122.5 and 154.1, respectively, the glucose H-1‴ signal at δ = 5.25 correlated with the aryl C-1 and C-3 resonances at δ = 154.1 and 146.3, respectively, and the aryl methyl signal H_3 – 7 at δ = 2.06 correlated with the aryl C-1 and C-5 resonances at δ = 154.1 and 148.7, respectively, through a three-bond coupling. These evidences established the two glucosyl moieties to be attached at C-1 and C-2, the two hydroxys at C-3 and C-5, and the methyl group at C-6. The consecutive five phenyl protons with A_2X_2Y system were considered as a benzoyl moiety, which was discernible from their chemical shifts and coupling constants. Based on these findings, the structure of **2a** was concluded to be 4-benzoyl-2-*C*-β-glucopyranosyl-3,5-dihydroxy-6-methylphenyl β-D-glucopyranoside peracetate.

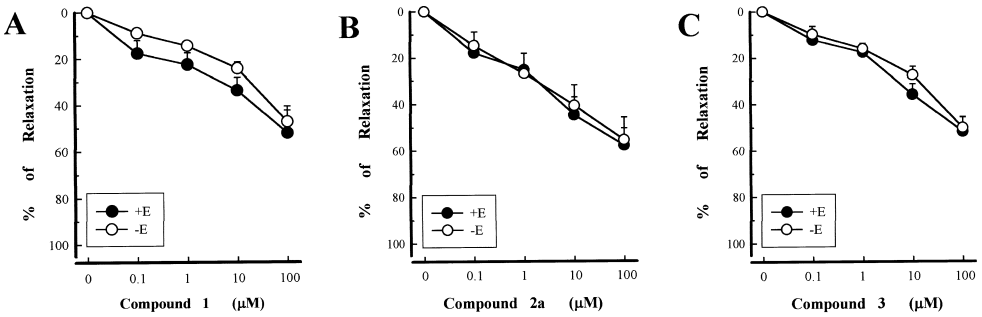

Fig. **1** Vasorelaxing effects of **1**, **2a,** and **3** on endothelium-intact (+E) and endothelium-denuded (–E) Sprague-Dawley rat thoracic aortic rings contracted with phenylephrine (0.3 μM). The tensions developed in the absence of compounds in intact and denuded rings were 1.50 ± 0.17 and 1.75 ± 0.15 g, respectively. Values are mean ± S.E.; n = 5 to 6 for each group.

Table **1** Vasorelaxing effects of compounds in endothelium-intact aortic rings isolated from Sprague-Dawley rats. Aortic rings were precontracted with phenylephrine (0.3 μM), relaxation was subsequently induced with cumulative concentrations of each of various compounds

Compound	n*	*EC_{50} (μM)*	*Max. relaxation (%)*
1	5	80.1 ± 7.2	52.2 ± 9.8
2	7	21.2 ± 6.5	68.1 ± 4.6
2a	6	31.0 ± 6.8	57.9 ± 7.4
3	5	71.4 ± 4.8	51.9 ± 6.2
4	5	16.3 ± 5.1	73.2 ± 7.9

*n denotes the number of experiments.

In a vasorelaxing bioassay each test compound given alone did not alter the baseline tension of the aortic rings (data not shown). In the rat thoracic aorta, phenylephrine caused an initial phasic and then a tonic contraction, which lasted for at least 30 min. During the tonic phase of contraction induced by phenylephrine, all the compounds produced concentration-dependent vasorelaxation compared with the vehicle-treated group (Fig. **2A**). The EC_{50} and the maximal relaxation of various compounds obtained by 100 μM are shown in Table **1**. The vasorelaxing effects of **1**, **2a** or **3** on precontracted aortic rings showed no significant difference in the presence or absence of endothelium, while the positive control acetylcholine produced a significant relaxation (95 ± 4%) in endothelium-intact rings. The results implied that these compounds acted directly on the arterial smooth muscle (Figs. **1A**, **1B**, **1C**). The rank-order potency (EC_{50} value) of the relaxation was **2a** > **3** > **1** (Table **1**). The vasorelaxing effect of sodium nitroprusside was used as a positive control in each test aortic rings without endothelium. The maximal relaxation was 93 ± 5%. As shown in Fig. **2**, the vasorelaxation induced by **2** or **4** was somewhat attenuated by removal of the endothelium, indicating that both **2** and **4** relaxed vascular smooth muscle via mechanisms which were partly endothelium-dependent. The magnitude of relaxation induced in endothelium-intact aortic rings had an order of **4** > **2** (Table **1**). To investigate whether the endothelial mediator nitric oxide is involved in the vasorelaxing effect of **2** or **4**, L-NNA, a nitric oxide synthase inhibitor, was preincubated in endothelium-intact aortic rings. The concentration-response curve of cumulative **2** or **4** before and after treatment with L-NNA are illustrated in Figs. **2B** and **2C**. The results showed that treatment with this inhibitor did not significantly affect the basal vascular tone (data no shown). In the presence of L-NNA, the vasorelaxing effect induced by both **2** and **4** were significantly reduced in endothelium-intact aortic rings, showing endothelium-derived nitric oxide was involved in the vasorelaxing effect of **2** and **4**. The quantity of **5** obtained was insufficient to perform this biological investigation.

Acknowledgements

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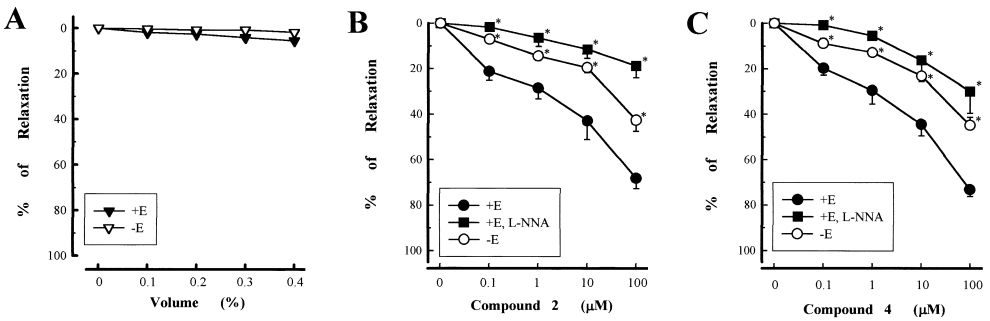

Fig. **2** Vasorelaxing effects of vehicle (0.1%, DMSO), **2,** and **4** on endothelium-intact (+E) and endothelium-denuded (-E) Sprague-Dawley rat thoracic aortic rings contracted with phenylephrine (0.3 μM). The tensions developed in the absence of compounds in intact and denuded rings were 1.50 ± 0.17 and 1.75 ± 0.15 g, respectively. Values are mean ± S.E.; n = 5 to 7 for each group. *, Statistically significant difference between test system with endothelium (+E) and test system without endothelium (–E) or with endothelium plus L-NNA (+E, L-LNNA).

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