Vasorelaxing Activity of Two Coumarins from *Murraya paniculata* Leaves

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In the search for novel chemical scaffolds leading to potential antihypertensive agents, the methanol extract of *Murraya paniculata* leaves was assessed for its effects on isolated rat aorta rings. The vasorelaxing effect of the chloroform fraction of the methanol plant extract was the most potent for its vasorelaxing activity on rat aorta rings contracted by 60 mM K⁺ (K60). Two coumarins were isolated from the chloroform fraction: the novel kimcuongin (1) and the known murracarpin (2). Their structures were determined from spectroscopic evidences including ¹H- and ¹³C-NMR, correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC), and high resolution mass spectrometry (HR-MS). Kimcuongin and, to a lesser extent, murracarpin, showed vasorelaxing activity with IC₅₀ values of 37.7 μM and 139.3 μM, respectively. The coumarins kimcuongin and murracarpin may thus represent a novel class of vasodilators of natural source.

Key words *Murraya paniculata*; vasoactive agent; kimcuongin; coumarin

The Rutaceae family in Vietnam comprises about 30 genera. Species of *Clausena*, *Glycosmis*, *Micromelum*, *Zanthoxylum*, and *Murraya* genera are widely distributed throughout the country.¹⁾ *Murraya paniculata* (L.) JACK, local name “Nguyet que,” is mostly grown as ornament in Vietnamese gardens and houses for its glossy green foliage and clusters of white fragrant flowers. In traditional medicine, this plant is used as anti-dysenteric, analgesic, digestion stimulant, anti-swelling, anti-ecchymotic, expectorant, tonic, and toothache remedy.²⁾ Phytochemical studies of *Murraya* species have led to the isolation and characterization of indole alkaloids,³⁾ polymethoxylated flavonoids,⁴⁾ and a number of coumarins^{5–8)} including osthole, a vascular Ca_v1.2 channel antagonist potentially useful for the treatment of systemic hypertension.⁹⁾

As part of our search for novel vasoactive agents from selected Vietnamese medicinal plants, we report here the findings of an investigational, screening procedure of *Murraya paniculata*. The chloroform fraction of the methanol plant extract showed a remarkable vasorelaxing effect on rat aorta rings contracted by K60. The chemical analysis of this fraction gave rise to two coumarins, the novel kimcuongin (1) and the known murracarpin (2). These coumarins showed vasodilatory effects worth to be further investigated in detail.

MATERIALS AND METHODS

General Experimental Procedures ¹H-NMR (500 MHz), ¹³C-NMR (125 MHz) with tetramethylsilane (TMS) as an internal standard were performed on a Bruker Avance 500 MHz spectrometer; melting point was measured on an Electro-thermal Series 9200 (Denmark) with capillary 100×2 mm outer diameter (OD). The high resolution mass spectrometry (HR-MS) were obtained with a Bruker Daltonics, 255748 MicroQ-TOF III mass spectrometer, electrospray ionization (ESI)

(+) mode; column chromatography was carried out on silica gel (230–400 mesh, Merck). UV and IR spectra were obtained on a JASCO V-630 and an Impact 410 Nicolet FT-IR spectrometer, respectively.

All solvents were redistilled before use. Precoated plates of silica gel 60F₂₅₄ were used for TLC analysis. Compounds were visualized under UV radiation (254, 365 nm) and by spraying plates with 10% H₂SO₄ followed by heating with a heat gun.

Plant Material The leaves of *Murraya paniculata* (L.) JACK., Rutaceae, were collected in Cuc Phuong National Park, Hoa Binh Province, North Vietnam. The plants were identified by the botanist Dr. Tran The Bach, Institute of Ecology and Biological Resources, VAST. A voucher specimen (C-425) is deposited in the herbarium of the Institute of Natural Products Chemistry, VAST, Hanoi, Vietnam.

Extraction and Isolation Dried powdered leaves of *Murraya paniculata* (3.2 kg) were extracted with MeOH over the period of 5 d at room temperature and concentrated under reduced pressure to yield a black, crude MeOH extract (120 g). The MeOH extract was subsequently partitioned with *n*-hexane, chloroform, ethylacetate, *n*-butanol, and water. The chloroform fraction, after evaporation to dryness under reduced pressure (7.0 g) was subjected to CC on flash silica gel column (400–630 mesh) with gradient solvents *n*-hexane/EtOAc: 1/0–0/1; EtOAc/MeOH: 1/0–0/1 to afford 9 fractions (F-2A–F-2I). The sub-fraction F-2B (3.1 g) was further chromatographed on a silica gel column (230–400 mesh) and eluted with gradient of *n*-hexane in EtOAc (1/0–0/1) to obtain 29 fractions (F-2B-1–F-2B-29). Fraction F-2B-8 (55 mg), after concentration to brown crude oil, was dissolved in a mixture of *n*-hexane–EtOAc (2:1, v/v). A white compound was crystallized and cleaned-up 3 times with *n*-hexane (1 mL) to yield the pure compound kimcuongin (1, 42 mg). Fraction F-2B-11 was dried at room temperature. A white compound was thus obtained and cleaned 3 times with cold MeOH (1 mL) giving

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murracarpin (**2**, 55.0 mg).

Kimcuongin (1): 2*H*-8-(2-Seneciyoxyloxy-3-methyl-but-2-enoyl)-7-methoxy-1-benzopyran-2-one; white crystal, soluble in CHCl_3 , $\text{C}_{20}\text{H}_{20}\text{O}_6$ ($M=356$), mp 149–151°C. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ (ppm): 6.23 (1H, d, $J=1.5$, 9.5 Hz, H-3), 7.61 (1H, d, $J=9.5$ Hz, H-4), 7.42 (1H, d, $J=8.5$ Hz, H-5), 6.82 (1H, d, $J=8.5$ Hz, H-6), 3.85 (3H, s, 7-OCH₃), 1.83 (3H, s, H-4'), 2.30 (3H, s, H-5'), 5.44 (1H, s, H-2''), 1.78 (3H, s, H-4'') and 1.99 (3H, s, H-5''). $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 159.9 (s, C-2), 113.7 (d, C-3), 142.9 (d, C-4), 129.4 (d, C-5), 107.7 (d, C-6), 159.5 (s, C-7), 117.9 (s, C-8), 151.7 (s, C-9), 112.5 (s, C-10), 56.4 (s, 7-OCH₃), 186.9 (s, C-1'), 142.6 (s, C-2'), 140.4 (s, C-3'), 21.6 (q, C-4'), 20.3 (q, C-5'), 163.9 (s, C-1''), 114.2 (d, C-2''), 159.1 (s, C-3''), 27.3 (q, C-4'') and 20.1 (q, C-5''). IR (KBr) cm^{-1} : 2979 (aromatic C-H), 1729 (coumarin C=O), 1611 (aromatic ether CO). UV λ_{max} (CHCl_3) nm (log ϵ): 324 (2.45), 270 (2.04). HR-ESI-MS m/z : 357.1338 (20%) $[\text{M}+\text{H}]^+$ (Calcd for $\text{C}_{20}\text{H}_{21}\text{O}_6$: 357.13381), 379.1190 (100%) $[\text{M}+\text{Na}]^+$ (Calcd for $\text{C}_{20}\text{H}_{20}\text{O}_6\text{Na}$: 379.1152); ESI-MS m/z : 357.1 $[\text{M}+\text{H}]^+$.

Murracarpin (2): 2*H*-8-[(1*S*,2*S*)-2-Hydroxy-1-methoxy-3-methyl-3-buten-1-yl]-7-methoxy-1-benzopyran-2-one, white compound, soluble in MeOH and CHCl_3 , $\text{C}_{16}\text{H}_{18}\text{O}_5$ ($M=290$), mp 148–149°C. $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ (ppm): 6.27 (1H, d, $J=9.5$ Hz, H-3), 7.96 (1H, d, $J=9.5$ Hz, H-4), 7.62 (1H, d, $J=8.5$ Hz, H-5), 7.07 (1H, d, $J=8.5$ Hz, H-6), 3.88 (3H, s, 7-OCH₃), 4.81 (d, $J=8.5$ Hz, H-1'), 4.86 (dd, $J=3.5$, 8.5 Hz, H-2'), 5.17 (d, 1H, $J=3.5$ Hz, -OH), 4.50 (br s, H-4'a), 4.39 (br s, H-4'b), 1.48 (3H, s, H-5'), 3.15 (3H, s, 1'-OCH₃). $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO}-d_6$) δ : 159.9 (s, C-2), 112.2 (d, C-3), 144.8 (d, C-4), 129.4 (d, C-5), 108.4 (d, C-6), 160.8 (s, C-7), 113.7 (s, C-8), 153.1 (s, C-9), 112.6 (s, C-10), 56.3 (s, 7-OCH₃), 78.1 (d, C-1'), 75.9 (d, C-2'), 145.2 (s, C-3'), 112.0 (dd, C-4'), 16.7 (q, C-5'), 57.1 (1'-OCH₃). $[\alpha]_D^{25}$ -15.6° ($c=0.063$, CHCl_3).

Vasodilatory Activity Testing Aorta rings preparation and equilibration period for vasodilatory activity testing was already described by Fusi *et al.*¹⁰ Relaxation was reported as a percentage of the initial tension induced by either phenylephrine or K60, taken as 100%. Drug potency and efficacy was reported as IC_{50} and E_{max} , respectively. E_{max} represents the residual tone of the preparation, expressed as percentage of the contraction induced by either K60 or 0.3 μM phenylephrine, recorded in the presence of the maximum concentration

of the drug.

Statistical Analysis Analysis of data was accomplished by using GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, U.S.A.). Data are reported as mean $\pm$ S.E.M.; n is the number of rings processed (indicated in parentheses), isolated from at least 3 animals. Statistical analyses and significance as measured by one-way ordinary ANOVA (followed by Bonferroni post test) were obtained using GraphPad InStat version 3.06 (GraphPad Software, U.S.A.). In all comparisons, $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

The methanol extract of the dried leaves of *Murraya paniculata* was much more effective at inhibiting the contraction induced by membrane depolarization (K60) than by receptor stimulation (phenylephrine). In fact, in endothelium-denuded rat aorta rings stimulated with K60, the extract caused a concentration-dependent relaxation with an IC_{50} value of $15.6 \pm 3.3 \mu\text{g/mL}$ and an E_{max} value of $91.7 \pm 3.5\%$ ($n=5$; Fig. 1). On the contrary, in rings pre-contracted by 0.3 μM phenylephrine, the IC_{50} value was significantly increased to $38.1 \pm 7.4 \mu\text{g/mL}$ ($n=5$; $p<0.05$ vs. K60), while the E_{max} value was unchanged ($93.0 \pm 3.1\%$; Fig. 1). The presence of an intact endothelium further increased, albeit not significantly, both IC_{50} and an E_{max} values ($60.1 \pm 3.1 \mu\text{g/mL}$; $p<0.01$ vs. K60; and $82.7 \pm 7.9\%$, $n=3$; Fig. 1).

High K^+ -induced contraction is believed to be the result of Ca^{2+} influx through voltage-dependent Ca^{2+} channels; moreover, it is specifically inhibited and fully reverted by Ca^{2+} -antagonists such as nifedipine.¹¹ Since the methanol extract acted as a Ca^{2+} channel blocker, a bioactivity-guided fractionation of the extract was performed in order to identify the active principle(s) concurring to this effect. The methanol extract of the dried leaves of *Murraya paniculata* was suspended with water and subsequently fractionated with *n*-hexane, chloroform, ethyl acetate, or butanol. These fractions were assessed for their vasorelaxing effect on endothelium-denuded rat aorta rings stimulated with K60. Only the chloroform fraction caused a concentration-dependent relaxation of the preparations (data not shown).

Compounds **1** and **2** were subsequently isolated from the chloroform fraction of the leaves of *Murraya paniculata* by

Fig. 1. Effect of the Methanol Extract on Either Phenylephrine (Phe)- or 60 mM K^+ (K60)-Induced Contraction of Rat Aorta Rings

Concentration–response curves of the extract on endothelium-denuded (K60 and Phe-endo) or endothelium-intact preparations (Phe+endo). In the ordinate scale, relaxation is reported as percentage of the initial tension induced by either phenylephrine or 60 mM K^+ , taken as 100%. Data are mean $\pm$ S.E.M. ($n=3-5$).

means of column chromatography.

The distortionless enhancement by polarization transfer (DEPT) and ^{13}C -NMR spectra (CDCl_3) showed that **1** possessed 20 carbon signals assignable to three carbonyl carbons, seven quaternary carbons, one methoxy, five methines, and four methyls. From the spectroscopic data, it can be deduced that **1** comprised two parts: a 7,8-disubstituted coumarin nucleus ($\text{C}_{10}\text{H}_7\text{O}_3$) and an isoprenoid side chain ($\text{C}_{10}\text{H}_{13}\text{O}_3$). The heteronuclear multiple bond correlation (HMBC) cross-peak between proton at δ_{H} 3.85 (s, 7-OCH₃) and carbon δ_{C} 159.5 (C-7) suggested the placement of one methoxy group on C-7 of the coumarin ring. Furthermore, in the HMBC spectrum, the proton H-6 (δ_{H} 6.82) correlated not only to two coumarin carbons at δ_{C} 117.9 (C-8) and 112.5 (C-10), but also to carbonyl carbon at δ_{C} 186.9 (C-1') in a W-coupling type, clearly indicating that the terpenoid side chain was attached to the coumarin ring at C-8 position through the carbonyl carbon C-1'. The protons of two *gem*-methyls at δ_{H} 1.83 (CH₃-4') and δ_{H} 2.30 (CH₃-5') had not only HMBC cross-peak to the prenyl carbon C-1' but also to the olefinic carbons C-2' (δ_{C} 142.6), C-3' (δ_{C} 140.4), and to the carbonyl carbon C-1'' (δ_{C} 163.9) of the senecioate ester moiety. Similarly, the other *gem*-methyl protons at CH₃-4'' (δ_{H} 1.78) and CH₃-5'' (δ_{H} 1.99) had HMBC correlations with olefinic carbons at C-2'' (δ_{C} 114.2), C-3'' (δ_{C} 159.1), and C-1'' (δ_{C} 163.9) of the senecioate ester group. These spectral data revealed the presence of the prenyl-1-on moiety in the terpenoid side chain of **1** which was connected to a senecioyl ester group through an oxygen-bridge. Additionally, in the correlation spectroscopy (COSY) spectrum, the methine proton H-2'' (δ_{H} 5.44) had obvious COSY correlation with both *gem*-methyl CH₃-4'' and CH₃-5''. In the nuclear Overhauser effect spectroscopy (NOESY) spectrum, the methine proton H-2'' had only NOE correlation with methyl proton CH₃-4'' but no NOE correlation with either protons CH₃-5'' and CH₃-4' (δ_{H} 1.83), or CH₃-5' (δ_{H} 2.30). The NMR data of the senecioyl moiety and the whole isoprenoid side chain in **1** were found similar to those previously published^{12,13} and to omphamurrayin, which was isolated from *Murraya paniculata* var. *omphalocarpa*.¹⁴ Based on the spectroscopic data, compound **1** was finally identified as a new coumarin, named kimcuongin.

Compound **2** was isolated as a white powder. The similarity of ^1H - and ^{13}C -NMR patterns of **2** with those of kimcuongin (**1**) suggested that **2** had also a 7-methoxy-8-disubstituted coumarin skeleton. The ^1H -NMR spectrum of compound **2** displayed characteristic signals for olefinic protons of coumarin nucleus δ_{H} 6.23 (1H, d, H-3) and 7.96 (1H, d, H-4) with $J=9.5\text{ Hz}$, two aromatic *ortho* protons δ_{H} 7.62 (1H, d, H-5) and 7.07 (1H, d, H-6) with $J=8.5\text{ Hz}$, two methoxy groups at δ_{H} 3.88 (s, 7-OCH₃) and 3.15 (s, 1'-OCH₃), a methyl group (δ_{H} 1.48, s, 5'-CH₃), an *exo*-methylene (δ_{H} 4.50, s, H-4'a; δ_{H} 4.39, s, H-4'b), an hydroxyl group (δ_{H} 5.17, $J=3.5\text{ Hz}$) and two methines (δ_{H} 4.81, d, H-1' and δ_{H} 4.86, d, H-2') with $J=8.5\text{ Hz}$. These spectroscopic data and those previously published led to murracarpin as the structure of compound **2**.⁵ Although several publications have already illustrated the structure of murracarpin, to our knowledge this is the first report fully discussing its absolute structure.¹⁵⁻¹⁷ Furthermore, the specific rotation value $[\alpha]_{\text{D}}^{25} -15.6^\circ$ ($c=0.063$, CHCl_3) of compound **2** is similar to that of (–)-murracarpin reported by Wu *et al.*⁵

The rotational frame Overhauser effect spectroscopy (ROESY) spectrum of compound **2** showed the cross-peaks

from the methoxy 7-OCH₃ (δ_{H} 3.88) to the methoxy 1'-OCH₃ (δ_{H} 3.15). The methyl group 5'-CH₃ had an important ROESY correlation to the methoxy 1'-OCH₃ (δ_{H} 3.15) and also to the methine proton H-2' (δ_{H} 4.86). In addition, with the coupling constant $^3J_{1',2'}=8.5\text{ Hz}$, two vicinal protons H-1' and H-2' must be in *trans*-position. Recently, a murracarpin analog, 2'-*O*-ethylmurrangatin, was shown to have a configuration 1'-*S*, 2'-*R*, as to the terpenoid side chain.¹⁸

The absolute configuration of (–)-murracarpin **2** was determined on the basis of spectroscopic and physical evidences, molecular modelling as well as conformational analysis of 7-methoxycoumarin.¹⁹ The preferred stereochemistry at C-1' was found to be “*S*” while the adjacent C-2' was concluded to be “*R*” (Fig. 2).

Finally, we investigated the effect of the two coumarins (**1**, **2**) on endothelium-denuded rings pre-contracted by K60. The vasorelaxing effect of the methanol extract, in fact, was more marked in rings pre-contracted with high concentrations of KCl than with phenylephrine (see Fig. 1). Both compounds caused a concentration-dependent relaxation of the preparations. Kimcuongin was the most powerful and effective compound with an IC_{50} value of $37.7 \pm 17.7\text{ }\mu\text{M}$ and an E_{max} value of $89.6 \pm 4.5\%$ ($n=4$; Fig. 3), while murracarpin was less effective ($139.3 \pm 19.5\text{ }\mu\text{M}$ and $75.4 \pm 3.5\%$, respectively, $n=8$; Fig. 3). The reference compound diltiazem, under the same experimental conditions, relaxed ring preparations with an IC_{50} value of $0.18 \pm 0.02\text{ }\mu\text{M}$ and an E_{max} value of $98.9 \pm 1.1\%$ ($n=4$). When expressed in $\mu\text{g/mL}$, the potency of murracarpin (IC_{50} value of $40.4\text{ }\mu\text{g/mL}$) and, in particular, that of kimcuongin (IC_{50} value

Fig. 2. Structure of Kimcuongin (**1**) and Murracarpin (**2**) Isolated from *Murraya paniculata* (the Wavy Line Shows the Attachment Point to the Coumarin Ring)

Fig. 3. Effect of Compound **1** and Compound **2** on 60mM K^+ -Induced Contraction of Rat Aorta Rings

Concentration-response curves of compounds **1** and **2**. In the ordinate scale, relaxation is reported as percentage of the initial tension induced by 60mM K^+ , taken as 100%. Data represent the mean $\pm$ S.E.M. ($n=4-8$).

of 13.4 $\mu\text{g/mL}$), were comparable to that of the plant extract (see above). At present we have no explanation for this awkward result, and we can only speculate that the two coumarins have a synergistic vasodilating effect when present together in the plant extract. This point, however, deserves further investigation.

CONCLUSION

To discover novel natural leads from Vietnamese medicinal plants, we have investigated *Murraya paniculata* belonging to the Rutaceae family. The present findings suggest that the methanol extract of the leaves possesses *in vitro* vasodilating activity, which was ascribed in part to the presence of two coumarins, the novel kimcuongin (1) and the already known murracarpin. Coumarins may thus represent a class of vasodilators or at least provide a chemical scaffold for the design and synthesis of novel vasoactive agents. Further studies, however, are needed to clarify whether they act as Ca^{2+} channel blockers, as it has been recently demonstrated for the coumarin osthole.⁹⁾ Finally, these results are important for validating the traditional use of *Murraya paniculata* and developing novel antihypertensive agents.

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